Original

Alterations in Lipoprotein Composition Associated with Carbon Tetrachloride-Induced Rat Liver Injury

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Abstract: To investigate alterations in lipoprotein composition associated with carbon tetrachloride-induced rat liver injury, electrophoretic patterns of rat apoproteins were investigated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and compared with those in man. Rat apoprotein (apo) A-I and apo E were shown to migrate the same distances as those of man, though rat apo A-II + apo C-II and apo C-III migrated farther. Administration of 1.0 ml/kg CCl₄ decreased the lipid contents, the very low density lipoprotein (VLDL), low density lipoprotein (LDL), and high density lipoprotein (HDL) fractions in the serum. Transaminase activity and total protein (TP) content increased with increased CCl₄ administration. The percentage of albumin non-esterified fatty acid (Alb-NEFA) increased and HDL₂ fraction decreased. Administration of 1.0 ml/kg CCl₄ decreased apo C-I and apo E in the HDL fraction. The bands of apo A-I, apo A-II, apo A-II + apo C-II and apo E in the HDL fraction declined after 2.0 ml/kg CCl₄ administration. Apoproteins in the VLDL + LDL fraction appeared to decrease after CCl₄ treatment. It was suggested that lipoprotein abnormalities induced by CCl₄ application may be related to inhibition of apoprotein synthesis, suppression of lipoprotein lipase activity, and the decline of HDL₂.

Key words: apoproteins, lipoproteins, CCl₄-induced liver injury, SDS-PAGE, rat

Introduction

Recent pharmacological studies of lipid metabolism have focused on determination of lipoprotein composition and apoprotein levels. Among many reports of lipoproteins, investigation of high density lipoprotein (HDL) seems to be of great interest, since HDL is a protective lipoprotein against premature development of atherosclerotic diseases.^{1,2)} Although lipoprotein fractions, such as very low density lipoprotein (VLDL), low density lipoprotein (LDL), and HDL are separated by ultracentrifugation or chemical procedures, apoprotein analysis is performed mainly by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) or isoelectric focusing (IEF).^{3,4)} Both the composition and function of apoproteins obtained from normal human serum have been studied.^{5,6)} It is also well known that various disease-induced hyperlipemia and liver diseases frequently develop abnormalities in lipid levels and lipoprotein compositions in human plasma or serum.^{7,8)} It is generally accepted that the composition and functions of human serum can be extrapolated from data obtained in experimental animals, yet few studies of lipoprotein and apoprotein patterns of

normal and disease animal models have been available.^{9,10)} Accumulating detailed data on apoproteins in animals is required to develop a disease model of human hepatic disorder. To do this we compared electrophoretic patterns of apoproteins in rat serum with those in human serum in the present study. In addition, changes in lipid levels and apoprotein patterns were examined in rats with liver injury induced by carbon tetrachloride (CCl₄). Such data could eventually lead to establishing an experimental animal model in which lipoprotein synthesis and metabolism can be studied in the light of updated lipid science.

Materials and Methods

Animals and reagents: Male, 8-week-old Sprague-Dawley rats weighing 250–280 g were used. Sodium chloride and sodium bromide were employed for lipoprotein separation. The chemicals used for lipoprotein analysis were as follows: hydrochloric acid, Tris-hydroxymethylaminoethane, N,N,N',N'-tetramethylethylenediamine, acrylamide, N,N'-methylene bisacrylamide, riboflavin, glycine, Sudan Black B. The chemicals used for apoprotein analysis by SDS-PAGE were as follows: acrylamide, sodium dodecyl sulfate, Tris-hydroxymethylaminoethane, N,N,N',N'-tetramethylethylenediamine, ammonium persulfate, Coomassie Brilliant Blue R, acetic acid, trichloroacetic acid, and methanol. Determinations of lipid, protein, and transaminase were carried out with commercial kits (Cholesterol B-Test, Phospholipid B-Test, Triglyceride G-Test, A/G B-Test, and Transaminase C II-Test were purchased from Wako Pure Chemical Industries Ltd., Japan, and BIO-RAD protein assay was obtained from BIO-RAD U.S.A.).

CCl₄-induced liver injury: CCl₄ was dissolved in olive oil, and its concentration was adjusted to 20% or 50%. Three doses, 0.2, 1.0, and 2.0 ml/kg body weight, were given forcibly via stomach tube. The control animals were given olive oil alone at 2.0 ml/kg body weight. All animals were sacrificed 24 hr after administration of the olive oil or CCl₄.

Separation of lipoproteins: After fasting for 18 hr, animals were anesthetized with sodium pentobarbital (40 mg/kg, i.p.). Subsequently, the abdomen was opened, and 5 to 6 ml of blood was collected from the inferior vena cava with a disposable syringe. Serum was separated by centrifugation at 2,500 r.p.m. for 15 min. Lipoprotein fractions were obtained from the serum of 2 animals by ultracentrifugation with a HIMAC ultracentrifuge (Hitachi, Japan) using an RP-40 rotor; for the isolation of VLDL and LDL fraction ($d < 1.063$ g/ml), serum was overlaid with a NaCl solution ($d = 1.21$ g/ml, containing 95.4 mg/ml NaCl and 197 mg/ml NaBr) at a volume ratio of 1:6.4 (saline solution to serum). Centrifugation was then carried out at $105,000 \times g$ for 22 hr. The upper lipoprotein layer of 3.5 ml was used as the VLDL+LDL fraction, and 3.5 ml of the bottom fraction was used as the HDL fraction.

Separation of apoproteins: The VLDL+LDL fraction and HDL fraction were dialyzed for 24 hr with saline containing 0.05% Na₂EDTA. Apoproteins were isolated by delipidation using ethanol/diethyl ether (3:1 for VLDL+LDL, 3:2 for HDL) and anhydrous diethyl ether at 0°C, and the products were dried under N₂ gas flow.

Lipoproteins and albumin nonesterified fatty acids (Alb-NEFA) electrophoresis: Serum lipoproteins were assayed by the prestaining disk PAGE method.¹¹⁾ Serum was stained with saturated-Sudan Black B for 20 hr. Three layers of acrylamide were piled; acrylamide concentrations were 1.875% for the stacking gel and 3% and 5% for the running gels. Prestained serum (100 μ l) was added onto the stacking gel. Electrophoresis was carried out with Tris/glycine buffer (0.05 mM, pH 8.4) at 1.5 mA/disk,

Apoprotein electrophoresis: SDS-PAGE of apoproteins was carried out according to a modified method of Weber and Osborn.¹²⁾ The concentrations of acrylamide were 7% for the stacking gel and 12% for the running gel. Two mg of apoprotein was dissolved in 500 μ l of 0.5 M Tris/HCl (pH 6.8, containing 1% SDS), 0.002% bromophenol blue, and 6% sucrose. This solution (25 μ l) was added onto the stacking gel. Monomeric, dimeric, trimeric, tetrameric, and hexameric forms of cytochrome c were mixed (5 μ l each, 25 μ l total volume) and introduced into a hole as a molecular weight marker for migration. Electrophoresis was carried out with Tris/glycine buffer (0.05 M/0.28 M, pH 8.3) containing 0.1% SDS at a current of 5 mA followed by 30 mA. Subsequently, the gel was fixed for 2 hr with 10% trichloroacetic acid and 25% methanol at room temperature, then stained for 2 hr with a solution containing 0.05% Coomassie Brilliant Blue R, 10% acetic acid and 10% methanol, followed by overnight destaining with 10% methanol and 10% acetic acid. Apoprotein bands were identified by comparing with the mobilities of the reference proteins and rat HDL apoproteins whose molecular weights were already known.^{3,13)}

Densitometry: The densities of lipoprotein and apoprotein bands were measured with a densitometer (F-808, Cosmo Co. Ltd., Japan).

Analysis of lipid, protein, and enzyme: Chemical analysis of serum and lipoprotein fractions was performed as follows: total cholesterol (TC) was determined by the colorimetric method;¹⁴⁾ phospholipid (PL) and triglyceride (TG), by the enzymatic method;^{15,16)} activity of transaminase (GOT, GPT) in serum, by the colorimetric method;¹⁷⁾ total protein (TP) in serum, by the method for Buret;¹⁸⁾ and protein in lipoprotein fractions, by the method of Bradford.¹⁹⁾

Statistical analysis: Statistical significance was evaluated using Student's *t*-test. A *p*-value of less than 0.05 was considered statistically significant.

Results

1. Comparison of electrophoretic patterns of apoproteins in HDL fraction derived from human and rat

Stained bands of apo A-I, apo A-II+apo C-II, apo C-I, and apo E obtained from rat HDL were separated by SDS-PAGE. Apo A-I and apo E in rat HDL fraction migrated the same distances as those of man (Fig. 1). The migrations of apo A-II+apo C-II and apo C-I of rats were slightly farther of those of human. Since the amounts of apo A-II+C-II and apo C-I in rat samples were very small, it was difficult to recognize the bands in the left-most column which was produced from an application of 25 μ l of rat sample. For confirmation, electrophoresis was repeated with an application of 100 μ l of the rat sample; the result of which is shown in the second column from the left. The position of all rat apoprotein bands was faintly but positively recognizable on the photograph. In quantitative densitometrical measurement, only electrophoretic results obtained with applications of 25 μ l of the sample were compared. Stained bands of human apoproteins in Fig. 1 were not clear.

2. CCl₄-induced liver injury in rats

Changes in serum transaminase: GOT and GPT levels in serum were increased significantly by CCl₄ administrations at 1.0 and 2.0 ml/kg. Administration of CCl₄ at 0.2 ml/kg caused no change in transaminase levels (Table 1).

Changes in lipids and proteins in serum and lipoprotein fractions: Serum TC content was significantly decreased by CCl₄ administration at 1.0 ml/kg, mainly due to the decline of TC

Fig. 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) patterns of apoproteins in high density lipoprotein (HDL) obtained from rats and humans. R: rat, H: human. SDS-PAGE was conducted with application of 25 μ l of sample except for the second column from the left, in which 100 μ l of the rat sample was applied.

Table 1. Changes in transaminase activity, serum lipid and protein levels in CCl₄-liver-damage rats.

		Control	Doses of CCl ₄ (ml/kg, p.o.)		
			0.2	1.0	2.0
GOT	K.U.	85 ± 3	84 ± 12	900 ± 618**	3013 ± 671**
GOT	K.U.	20 ± 2	22 ± 3	529 ± 377**	1712 ± 310**
TC	mg/dl	75.9 ± 13.7	62.9 ± 14.0	49.4 ± 5.9**	67.5 ± 18.3
PL	mg/dl	92.8 ± 12.6	87.0 ± 16.4	67.2 ± 9.1**	93.2 ± 26.5
TG	mg/dl	43.5 ± 5.0	32.8 ± 11.1	14.8 ± 7.5**	61.6 ± 42.1
TP	g/dl	5.1 ± 0.2	5.3 ± 0.2*	5.5 ± 0.2**	5.6 ± 0.4*

Each value represents mean $\pm$ S.D. *: $p < 0.05$, **: $p < 0.01$, significantly different from control.

content of the HDL fraction (Tables 1 and 2). After CCl₄ administered at 1.0 ml/kg, PL contents decreased significantly in serum and VLDL+LDL fraction. In contrast, administration of 2.0 ml/kg CCl₄ produced TC and PL contents in the serum comparable to control levels. The TG content of VLDL+LDL and HDL fractions was decreased by administration of 0.2 ml/kg CCl₄. This decline became greater after administration of 1.0 ml/kg CCl₄. Administration at 2.0 ml/kg CCl₄ tended to increase the TG contents of every fraction, but not significantly. All three doses of CCl₄ increased serum TP levels. The protein content of VLDL+LDL fraction was significantly decreased by 1.0 ml/kg CCl₄, while at 2.0 ml/kg CCl₄ did not cause significant change. Administrations of CCl₄ markedly decreased the pro-

Table 2. Changes in lipid and protein contents of VLDL+LDL and HDL fractions in CCl₄-liver-damage rats.

			Doses of CCl ₄ (ml/kg, p.o.)			
			Control	0.2	1.0	2.0
TC	mg/dl	V + L	8.2±0.1	5.7±1.8	5.7±2.8	30.8±16.5
		H	66.4±14.1	52.6±7.6	40.2±2.9*	42.1±11.0
PL	mg/dl	V + L	6.6±0.5	4.5±1.7	3.2±1.7*	20.6±11.2
		H	59.4±7.9	57.7±8.5	45.8±4.3	48.0±11.7
TG	mg/dl	V + L	28.0±2.0	18.9±4.3*	6.5±2.4**	38.6±31.3
		H	5.6±0.6	3.1±1.5*	1.2±0.8**	7.6±5.4
Protein	mg/dl	V + L	9.6±0.7	8.9±1.0	5.4±1.4**	13.2±7.8
	g/dl	H	2.79±0.14	1.56±0.07**	1.93±0.19**	0.35±0.02**

Each value represents mean ± S.D. *: $p < 0.05$, **: $p < 0.01$, significantly different from control. V+L: VLDL+LDL fraction, H: HDL fraction.

Fig. 2. Effects of CCl₄ on rat lipoprotein composition and Alb-NEFA. Each column represents the mean ± S.D. from 5 or 6 experiments and is expressed as percentage content at each concentration of CCl₄. * $p < 0.05$ and ** $p < 0.01$: Significant differences.

tein content of HDL fraction (Tables 1 and 2).

Changes in lipoprotein population: Densitometrical analysis of polyacrylamide gel disk electrophoresis revealed rat serum lipoprotein migration bands of VLDL (pre beta-lipoprotein), LDL (beta-lipoprotein), HDL₁, HDL₂, HDL₃ (alpha-lipoproteins) and Alb-NEFA (Fig. 2). Percentages of VLDL and LDL fractions were not changed by administrations of 0.2 and 1.0 ml/kg CCl₄. Administration of CCl₄ at 2.0 ml/kg slightly increased VLDL and LDL percentages, but not significantly. The percentage of Alb-NEFA increased significantly after CCl₄ treatment at all three doses. Administration of CCl₄ decreased HDL₃ percentage. Administration of 2.0 ml/kg CCl₄ significantly increased in HDL₁ percentage and markedly decreased HDL₂ and HDL₃ (Fig. 2).

Changes in apoprotein contents of HDL fraction: Changes in HDL apoproteins are

Fig. 3. Effects of CCl_4 on SDS-PAGE patterns of rat apoproteins. M.W.: molecular weight marker gave stained bands of cytochrome c's (numbers in parenthesis are molecular weights, the order is from the bottom): monomer (12300), dimer (24600), trimer (36900), tetramer (49200), pentamer (61500), and hexamer (73800).

Fig. 4. Effects of CCl_4 on rat HDL apoproteins. Each point represents the mean $\pm$ S.D. of densitometrical analysis in SDS-PAGE from 3 experiments. Each value is expressed as a percentage of the control. * $p < 0.05$ and ** $p < 0.01$: Significant differences.

shown in Fig. 3. The stained bands of cytochrome c mixture, a molecular weight marker for SDS-PAGE, were in order from the bottom: figures in parenthesis are molecular weight: monomer (12300), dimer (24600), trimer (36900), tetramer (49200), pentamer (61500), and hexamer (73800) (Fig. 3).

Changes in HDL apoproteins were measured with densitometry of the electrophoretic bands and expressed as percentages of control values in Fig. 4. Apo C-I and apo E significantly decreased with administration of 1.0 ml/kg CCl_4 . Significant declines of apo A-I, apo

Fig. 5. Effects of CCl_4 on SDS-PAGE patterns of rat VLDL and LDL apoproteins. Other explanations as for Fig. 4.

A-II, apo A-II+apo C-II, and apo E were induced by 2.0 ml/kg CCl_4 . The apo C-I level, at 2.0 ml/kg, was similar to the control level, although the value at 2.0 ml/kg had a large standard deviation.

Changes in VLDL+LDL apoproteins: Electrophoretic patterns of VLDL+LDL apoproteins separated by SDS-PAGE are shown in Fig. 5. A control electrophoretic pattern showed clearly stained bands of apo A-I, apo C-I, apo A-II+C-II, apo C-III and apo E. Administrations of 0.2 and 1.0 ml/kg CCl_4 weakened the apo A-I, apo A-II+C-II and apo E of bands. Administration of 2.0 ml/kg CCl_4 induced an increase in apo C-I, and decreases in apo A-I, apo A-II+C-II, and apo E.

Apo B, which is not water soluble and has a high molecular weight, does not migrate in the gel employed in this study. Therefore, densitometrical analysis of apo B could not be performed. When VLDL+LDL apoprotein contents were expressed in comparison with the control value, all apoproteins except apo C-I tended to decrease after CCl_4 treatments at all three doses. Administration of 2.0 ml/kg CCl_4 produced an apo C-I level comparable to the control level.

Discussion

Our results demonstrated that rat apoprotein patterns in HDL fraction produced in SDS-PAGE were similar to those of humans since the migration distances were almost the same.

The respective molecular weights of human and rat apoproteins are as follows: apo A-I (28300, 27000), apo A-II (8690, 8000), apo C-I (6631, 7000), apo C-II (8837, 8000), apo C-III (8764, 10000), apo E (34000, 35000).²⁰⁾ The anhydrous molecular weights of apo C-I and apo C-III are 6631 and 8837, respectively.^{21,22)} The migration ranges of human apo A-II+apo C-II and apo C-III differed from those of rats. The difference between the molecular weights of these proteins of rats and of man might account for the difference in migration distances since a heavier molecule moves less. Although rat apo A-II had been thought to exist as a monomer, the band of rat apo A-II in the present study migrated the same distance as the human apo A-II, which is identified as a dimer (molecular weight 17414).²⁰⁾ Thus, from our data we could not deny the possibility that rat apo A-II may be present in a dimeric form. The present study proved that SDS-PAGE and isoelectric focusing, the methods generally employed for human apoprotein determination, could be adapted for the determination of rat apoproteins. Accumulation of detailed data on apoproteins in animals, i.e., their normal levels and changes due to CCl₄-induced liver damage, should be indispensable for developing a disease model of human hepatic disorder.

CCl₄-induced liver injury is a useful experimental model.²³⁾ Subcutaneous injection of 1.0 ml/kg CCl₄ is supposed to trigger centrilobular steatosis and necrosis in the liver.²⁴⁾ It decreases serum lipid and TP levels, and increases serum transaminases. In the present study, CCl₄ treatment induced liver damage dose-dependently, and increased remarkably serum transaminase activity. The lipid and protein contents of lipoprotein fractions were decreased from the control level at the lower two doses of 0.2 ml/kg and 1.0 ml/kg, and were comparable to the control level at the highest dose, 2.0 ml/kg. This might reflect the process of necrosis in the liver.

CCl₄ increased Alb-NEFA and decreased HDL₂ observed in electrophoretic analysis of serum lipoproteins. The increase in Alb-NEFA may reflect an increase in the amount of release of TG decomposition products from the liver, where TG accumulated. The decrease in HDL₂ percentage indicates inhibition of the conversion of HDL₃ to HDL₂. Especially, the decrease in apo E content of the HDL fraction by CCl₄ administrations at 0.2 ml/kg and 1.0 ml/kg correlated with the decrease in conversion from HDL₃ to HDL₂. Felker *et al.* reported that apo E was a major protein component of both VLDL and HDL in rat liver perfusates.²⁵⁾ The apo E content of HDL₃ may control the conversion. Furthermore, the decline of the HDL₃ percentage induced by 2.0 ml/kg CCl₄ suggests inhibition of HDL₃ synthesis. The decreases of apo A-I, apo A-II, apo A-II+apo C-II, and apo E contents of the HDL fraction by 2.0 ml/kg CCl₄ correlated with the decreases of protein and lipid contents of the HDL fraction. These decreases also indicate suppression of apoprotein synthesis in the liver. In the VLDL+LDL fraction, the administration of CCl₄ at 2.0 ml/kg increased lipids and apoproteins which had been decreased by 1.0 ml/kg of CCl₄. This might reflect VLDL+LDL metabolism alterations, such as reduction of lipoprotein lipase activity in serum due to the decrease of apo C-II.

Galactosamine induces diffusing hepatocellular injury which closely resembles human hepatitis. Galactosamine is widely used to produce an experimental liver damage model,²⁶⁾ but its injury form and resulting changes in serum lipid levels differ from those induced by CCl₄. Sirowej *et al.*¹⁰⁾ reported that apo A-II and apo C's in VLDL and HDL diminished with galactosamine treatment. Cartwright *et al.*²⁷⁾ also reported that galactosamine-induced hepatitis in rats was associated with various alterations in plasma; increased contents of unesteri-

fied cholesterol, PL, and TG, severe deficiency of lecithin-cholesterol acyl transferase (LCAT), and the appearance of discoidal lipoproteins. In addition, decline of apo C-III in the VLDL fraction, and increase of apo E as well as decrease of apo C's in the HDL fraction were triggered by galactosamine treatment. Investigators who employed lower doses of galactosamine detected no changes in apo A-I or apo E content of the HDL fraction after galactosamine treatment.

In the present study, we demonstrated that apoprotein changes in CCl₄-induced liver injury differed from those in galactosamine-induced liver injury. Administration of 1.0 ml/kg CCl₄ decreased the apo E content of the HDL fraction, which was opposite to the effect of galactosamine treatment. HDL₁, an apo E-rich HDL fraction, proved to be a better LCAT substrate than any other HDL fraction in serum. Lipoprotein abnormalities in the galactosamine-induced LCAT deficiency, familial LCAT deficiency, and patients with liver disease, emphasize the central role of LCAT in plasma lipoprotein metabolism.¹⁰⁾ CCl₄ treatment suppressed protein synthesis, decreased protein content in HDL fractions and reduced HDL₂ percentages. Especially, the decline of HDL₃ percentage upon administration of 2.0 ml/kg CCl₄ might be caused by the inhibition of LCAT activity and the decrease of Apo A-I level.

In summary, our data suggest that lipoprotein abnormalities induced by CCl₄ may be related to inhibition of apoprotein synthesis, suppression of lipoprotein lipase activity, and the decline of HDL₂ percentage. Also, our data revealed differences between changes due to CCl₄- and galactosamine-induced liver injuries in rat. The understanding of such detailed differences in experimental animals will be important in developing an appropriate disease model of human hepatic disorder.

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