Changes in Plasma Lipoproteins as Toxicity Markers for Carbon Tetrachloride, Chloroform, and Dichloromethane

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Abstract: Effects of single intraperitoneal (i.p.) administration of carbon tetrachloride (CCl_4), chloroform (CHCl_3), and dichloromethane (CH_2Cl_2) on lipoproteins in plasma and liver were investigated in rats. Changes in lipoproteins caused by these solvents were compared with changes in traditional hepatotoxicity markers such as GPT (ALT). Following the administration, concentrations of lipoproteins (VLDL, LDL, HDL), triglyceride, cholesterol, and GPT activity in plasma were determined through changes in liver weight, liver content of triglyceride, malon dialdehyde (MDA), and glutathione (GSH). Time-course study revealed that changes in plasma and liver reached their peaks at 19 or 32 hr following the administration of CCl_4 or CHCl_3 . Peaks of changes were observed at 8 or 19 hr following the administration of CH_2Cl_2 . Dose dependency of these changes was investigated at dosages of 3, 30, and 300 mg/kg of CCl_4 or CHCl_3 , and 300, and 1,000 mg/kg of CH_2Cl_2 . Significant decreases in triglyceride and apolipoproteins in VLDL fraction were observed at 3 mg/kg of CCl_4 . Such VLDL components decreased at 30 mg/kg of CHCl_3 . HDL decreased significantly at 300 mg/kg of CH_2Cl_2 and marked increase in LDL occurred at 1,000 mg/kg of the solvent. Liver weight and liver content of triglyceride and MDA significantly increased at 30 mg/kg of CCl_4 , while significant increase in GPT activity was observed at 300 mg/kg of CCl_4 and CHCl_3 . GPT increased significantly at 1,000 mg/kg of CH_2Cl_2 . These results revealed that changes in plasma lipoproteins can serve as sensitive and simple markers for liver disorders caused by chlorinated hydrocarbon solvents such as CCl_4 , CHCl_3 , or CH_2Cl_2 .

Key words: Rat, Plasma, Lipoproteins, Toxicity markers, Carbon tetrachloride, Chloroform, Dichloromethane, Liver disorders

Introduction

Many chlorinated organic chemicals have been widely used

for purposes such as industrial materials and degreasers^{1–3)}. Since the use of chemicals such as chlorofluorocarbons and 1,1,1-trichloroethane is limited due to concerns about the ozone layer, the demand for dichloromethane (CH_2Cl_2) as a cleaning solvent is increasing. For the same reason, the use of carbon tetrachloride (CCl_4) is limited and chloroform (CHCl_3) is being considered as an alternative solvent. CHCl_3 is widely used in manufacturing plastics, drugs, and cleaning agents, while drinking water is frequently contaminated with

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Abbreviations: VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; d, density; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodesyl sulfate; EDTA, ethylenediaminetetraacetic acid; DTNB, 5,5'-dithiobis (2-nitrobenzoic acid); GOT, Glutamic oxaloacetic transaminase; GPT, Glutamic pyruvic transaminase; MDA, malon dialdehyde; GSH, glutathione.

CHCl_3 . Consequently, the need for toxicity evaluation of CH_2Cl_2 and CHCl_3 has become more pronounced.

Many chlorinated hydrocarbon solvents, such as CCl_4 and CHCl_3 , cause liver and kidney damage and brain disorders⁴⁻⁹. Although CH_2Cl_2 is regarded as less toxic than CCl_4 and CHCl_3 , it exhibits hepatotoxicity and neurotoxicity^{10, 11}. While many toxicity studies have been performed on CCl_4 , the toxicity of CHCl_3 and CH_2Cl_2 has not been studied as extensively.

Liver damages from CCl_4 is thought to be due to the breakdown of membrane structure caused by lipid peroxide, or the covalent binding of CCl_4 metabolites with macromolecules in the liver^{9, 12}. CHCl_3 -induced liver damage is considered the result of phosgene formation¹³.

With exposure to highly toxic compounds such as CCl_4 , the concentrations of plasma lipoproteins decrease^{14, 15}, likely the result of inhibition of synthesis or secretion of lipoproteins. These changes in lipoprotein metabolism appear to be produced by liver damage from hepatotoxins¹⁶⁻¹⁸.

In previous reports, the authors described that changes in lipoproteins were observed following a single intraperitoneal (i.p.) administration of CCl_4 and trichloroethylene¹⁹⁻²¹. The doses at which these changes occurred were much lower than the doses which elevated the level of plasma transaminase, GOT (AST) and GPT (ALT) activities. These results suggest that changes in plasma lipoproteins can serve as sensitive markers for exposure to these chlorinated hydrocarbon solvents. Plasma concentration of VLDL was significantly reduced by CCl_4 at a dosage of 30 mg/kg, and significant liver enlargement was observed at the same dose of CCl_4 . In contrast to these findings, significant elevation of transaminase activity was not observed at 30 and 100 mg/kg. These facts suggest that changes in plasma lipoproteins can serve as markers for hepatotoxicity caused by inhalation exposure to chlorinated hydrocarbon solvents.

This paper aims to evaluate the toxicity of CHCl_3 and CH_2Cl_2 relative to CCl_4 , and to pursue the possibility that plasma lipoproteins might be able to serve as hepatotoxicity markers for these chlorinated methane solvents, including CCl_4 .

Materials and Methods

Animals

Male Fischer 344 rats, between 12 to 16 weeks-old (Japan Charles River) were used in this study. All subjects were group housed in cages made with stainless steel wire net and provided with ready access to food (CE-2, Nihon Clea,

Japan) and water under a 12:12 hr light:dark cycle with lights being turned on at 8:00 a.m. prior to testing. The room was maintained at $23 \pm 1^\circ\text{C}$ and $55 \pm 5\%$ relative humidity. In each experiment, the ages of the rats were within 3 days of each other. This investigation was performed in accordance with the principles of the Declaration of Helsinki, and followed a guideline approved by the Institutional Animal Care and Use Committee of the National Institute of Industrial Health.

Solvent administration

The solvents used were CCl_4 (specially prepared for spectroscopy, Nacalai tesque Inc., Japan), CHCl_3 (ultrafine grade, Nacalai), and CH_2Cl_2 (pesticide residue grade, Wako Pure Chemicals, Japan). These organic solvents were dissolved in 1 ml of olive oil/kg body weight, and injected intraperitoneally (i.p.). Control animals were treated with the same volume of olive oil. Chemicals were administered between 2:00 p.m. and 4:00 p.m., and rats were starved from 10:00 a.m. on the day of administration. However, tap water was allowed *ad libitum*.

Preparation of lipoprotein fractions

Under light anesthesia with diethylether, the rats were exsanguinated via the abdominal vein. Blood plasmas containing 1 mg of EDTA/ml were separated by centrifugation. To each plasma sample used for separation of lipoprotein fractions were added 0.1 mg of sodium azide/ml, and 0.01 mg of DTNB/ml. Sodium azide and DTNB were used as fungicide and lipase inhibitor, respectively.

The various lipoprotein fractions in plasmas were separated by preparative ultracentrifugation using a RP-50AT4 rotor (Hitachi, Japan) at 48,000 rpm (270,000 g) in a SCP-70H ultracentrifuge (Hitachi) at 10°C . The ultracentrifugation was performed immediately after the plasma sampling. The density of the plasma samples was adjusted by adding NaBr solutions. The plasmas were pipetted into ultracentrifuge tubes, and filled up to 3.3-ml with a d 1.006 solution containing 0.15 M NaCl, 0.1% EDTA, 0.01% sodium azide, and 0.001% DTNB (END mixture), and centrifuged for 16 hr. The top 1.0 ml containing VLDL was carefully removed as VLDL fraction. The density in the tube was adjusted to 1.063 by adding high density NaBr solution containing END mixture. After centrifuging for 16 hr, the top 1.0 ml was removed as LDL fraction. The LDL fraction that was obtained by ultracentrifugation of rat plasma at d 1.063 contains LDL and HDL₁, and these two lipoproteins could not be separated in any density range. To isolate HDL

fraction, the density was raised to 1.210, and the top 1.0 ml was removed after centrifuging for 22 hr. The HDL fraction thus obtained contained HDL₂. Each lipoprotein fraction was stored at -50°C until analysis, except the plasma and LDL fraction used for native PAGE (polyacrylamide gel electrophoresis).

Electrophoresis of lipoproteins

Plasmas and LDL fraction were applied to native 2–15% gradient PAGE immediately after the preparation, and lipoproteins in the electrophoresis gel were stained with Oil Red O (BHD Chemicals, England). Lipid staining density was measured at 530 nm using a TLC scanner (CS-910, Shimadzu, Kyoto). In time-course study, plasmas were applied to native PAGE, and three broad bands of VLDL, LDL, and HDL were obtained. In this PAGE, LDL band was composed of LDL, and HDL band was composed of HDL₁ and HDL₂ because these two HDL subclasses could not be separated. In dose-effect relationship study, LDL fraction obtained at d 1.063 through ultracentrifugation was applied to native PAGE. By the application of LDL fraction, two bands of LDL and HDL₁ were obtained. Concentrations of these two lipoproteins thus separated were determined by measuring the lipid staining density. Apolipoproteins in each lipoprotein fraction were analyzed by SDS 4–28% gradient PAGE. Each apolipoprotein in the electrophoresis gel was stained with Coomassie Brilliant blue R 250, and the staining density was determined at 555 nm using a flying-spot scanner (CS-9000, Shimadzu). By application of VLDL fraction to SDS PAGE, apolipoprotein bands B1, B_s, E's, and C's were obtained. Application of LDL fraction to SDS PAGE produced three apolipoprotein bands. Of these bands, B1 and B_s are components of LDL and E's are components of HDL₁. SDS PAGE of HDL fraction offered many apolipoprotein bands, and the main components were A-I, E's, C's, and A-IV.

Biochemical analysis

The plasma, each lipoprotein fraction, and liver were analyzed for lipids by enzymatic procedures, as early on in the process as possible. Triglyceride, total cholesterol, and phospholipid were measured using Determiner TG-S555® (Kyowa Medex, Japan), Monotest cholesterol® (Boeringer mannheim, Germany), and PL Kainos® (Kainos Laboratories, Japan) kits, respectively. Liver lipids were extracted with methanol-chloroform mixture by the method reported by Folch²². Plasma GPT activity was determined using a Monotest GPT® (Boeringer mannheim) kit. Liver

malondialdehyde (MDA) was confirmed as an indicator of lipid peroxide formation with a colorimetric assay developed by Yagi²³. Liver total, reduced + oxidized, glutathione (GSH) was measured using glutathione reductase²⁴. The protein contents of each lipoprotein fraction were estimated using a modified Lowry method²⁵.

Statistical analysis

Differences among the three or four groups were analyzed by analysis of variance (F-test). Dunnett's multiple *t*-test was also used to compare the control and the solvent-treated groups. A *p*-value of <0.05 was chosen to indicate significance.

Results

Time-course study

The time-course of the effects of solvent administration were investigated with each of three chlorinated organic compounds. Figures 1A, 1B, and 1C show the time-course changes in lipoproteins (VLDL, LDL, and HDL), plasma lipids (triglyceride and cholesterol), GPT activity, and liver (liver weight, triglyceride, MDA, and GSH) after an i.p. administration of CCl₄ at a dose of 10 mg/kg. Rats were sacrificed 8, 19, and 32 hr following the administration. Figure 1A shows the density of lipid staining of lipoproteins, which was obtained by applying rat plasmas to native PAGE. Plasma concentrations of VLDL, LDL, and HDL decreased in administered rats and statistical significance between control and dosed groups was obtained in all 3 lipoproteins, as observed in Figure 1A. Differences between control and dosed groups were largest 19 or 32 hr after the administration. Figure 1B shows plasma concentrations of triglyceride and cholesterol, and GPT activity. Triglyceride and cholesterol decreased in dosed rats, but no change in GPT activity was observed. The largest difference between control and dosed group in triglyceride and cholesterol was obtained at 19 or 32 hr. Figure 1C shows liver-to-body weight ratios, triglyceride contents of the liver, MDA and GSH concentrations in the liver. Liver weight, triglyceride, and MDA increased in administered rats, although the increases in triglyceride was not significant. Differences between the control and dosed groups in these items were largest 19 or 32 hr after the administration.

Figures 2A, 2B, and 2C demonstrate the time-course changes in plasma substances and liver of rats received i.p. administration of CHCl₃ at 100 mg/kg. Measurements were performed 8, 19, and 32 hr after the administration. Plasma

Fig. 1A. Time-course changes in the plasma concentrations of VLDL, LDL, and HDL fractions following 10 mg/kg i.p. CCl_4 injections to rats

Open circles: control groups; closed circles: injected groups. Each point is the mean $\pm$ SEM (vertical bar) for 4 rats. * and ** represent statistically significant differences from correspondent control at $p < 0.05$ and $p < 0.01$, respectively, by Student's t -test. A.U.: arbitrary units for the staining density of the lipid staining of the electrophoresis gel.

Fig. 1B. Time-course changes in the plasma concentrations of triglyceride, cholesterol, and GPT following 10 mg/kg i.p. CCl_4 injections to rats

TG: triglyceride; chol: cholesterol. Open triangles: control groups; closed triangles: injected groups. Other legends are as in Fig. 1A.

Fig. 1C. Time-course changes in liver weight, liver triglyceride, liver malondialdehyde, and liver glutathione following 10 mg/kg i.p. CCl_4 injections to rats

TG: triglyceride; MDA: malondialdehyde; GSH: reduced and oxidized glutathione. Open squares: control groups; closed squares: injected groups. Liver weight was obtained as percent value of the body weight. TG contents are mg per whole liver. MDA and GSH contents were obtained as the amount per g tissue weight of the liver. Other legends are as in Fig. 1A.

VLDL was reduced by the CHCl_3 administration at all measurement point (Fig. 2A). The largest difference of VLDL between the control and dosed groups was observed 19 hr after the administration. LDL was reduced by CHCl_3 at 8 hr but unchanged at 19 and 32 hr. HDL was reduced in the administrated group but statistical significance was not obtained. Triglyceride and cholesterol in plasma was reduced and statistical significance was obtained at 19 hr after (Fig. 2B). Plasma GPT activity was raised at 19 and 32 hr and the most marked increase was observed at 19 hr. Liver weight increased at 19 and 32 hr and the increase at 32 hr was significant (Fig. 2C). Hepatic triglyceride content, and MDA and GSH concentrations in the liver increased at 19 and 32 hr; however, these increases were not significant. The largest differences in liver weight, triglyceride, and GSH between the control and administered groups were observed at 19 hr. The largest difference of MDA was observed at 32 hr.

Figures 3A, 3B, and 3C show the effects of the administration of CH_2Cl_2 at 600 mg/kg. The measurement was performed 4, 8, and 19 hr after the solvent administration. Plasma VLDL levels were lower in injected rats than in control subjects (Fig. 3A). The lowest VLDL level was obtained 8 hr after the injection. LDL was increased in injected groups, and the maximum increase was obtained at 8 hr. HDL was decreased by CH_2Cl_2 and significant differences in plasma HDL concentrations between the control and injected groups was observed 19 hr after the administration. Plasma triglyceride and cholesterol levels in the injected groups were lower than those in the control groups at 8 and 19 hr, but these differences were not significant (Fig. 3B). GPT activity was raised by the CH_2Cl_2 injection, and maximum increase was observed at 8 hr. Liver weight was reduced by the injection, and was minimal at 8 hr (Fig. 3C). No significant change in triglyceride content and MDA concentration was observed. GSH was reduced significantly at 8 hr.

Dose-effect relationships

Effects of different doses of CCl_4 , CHCl_3 , and CH_2Cl_2 on plasma lipoprotein and lipid concentrations, and GPT activity were measured. Changes in liver weight, and triglyceride, MDA, and GSH in the liver were investigated. Rats were administered with CCl_4 at dosages of 3, 30 or 300 mg/kg. Changes in plasma and liver were measured 19 hr after the administration and results are summarized in Tables 1A, 1B, 1C, and 1D. Table 1A shows changes in lipid and apolipoprotein components of VLDL fraction. Most of the components of VLDL was triglyceride, and the ratios of protein, cholesterol, and phospholipid were very small.

Triglyceride, total protein, and apolipoprotein C's in VLDL fraction decreased significantly at 3 mg/kg. VLDL-triglyceride decreased to 77% of control level. Apolipoprotein B_I, B_S, and E's also decreased, although no statistical significance was obtained. All components of VLDL were reduced at 30 and 300 mg/kg with statistical significance. At 300 mg/kg, levels of all components were 31% of the control or less. Table 1B shows the changes in LDL fraction. In lipoprotein separation from rat plasma using ultracentrifugation, LDL were always accompanied with HDL₁ and it was impossible to obtain pure LDL in any density range. Therefore, LDL fraction prepared in ultracentrifugation contained both LDL and HDL₁, which were separated on electrophoresis gel. Lipids in these lipoproteins were stained with Oil Red O and the staining density is shown in Table 1B. LDL contains only B_I and B_S as apolipoprotein components. HDL₁ contains only E's as apolipoproteins. Therefore, apolipoprotein components of LDL and HDL₁ could be quantitated separately on SDS PAGE gel, although LDL fraction contained both LDL and HDL₁. As shown in Table 1B, LDL lipid, apolipoproteins B_I and B_S increased with no significance at 3 mg/kg. At 30 mg/kg, levels of LDL components were between 51 and 60% of the control, while at 300 mg/kg, levels of these components were between 46 and 55% of the control. Three mg/kg of CCl_4 produced no change in HDL₁ lipid and apolipoprotein E's. Thirty mg/kg of the solvent decreased these HDL₁ components to 58, or 69% of the control. At 300 mg/kg, these components decreased to 30, or 43% of the control. Total protein of LDL fraction (LDL + HDL₁) was reduced significantly at 30 and 300 mg/kg. Table 1C shows the effects of CCl_4 on HDL fraction (HDL₂). Among the components of HDL fraction, the ratio of triglyceride was very small. Components of HDL fraction except cholesterol increased slightly at 3 mg/kg, while they were reduced to 51, or 88% of the control at 30 mg/kg, and reduced to 41, or 55% of the control at 300 mg/kg (Table 1C). Table 1D summarizes changes in plasma components and the liver. Plasma triglyceride and cholesterol concentrations decreased at 30 mg/kg or more. Significant increase in GPT was observed at 300 mg/kg. Liver weight, triglyceride content, and MDA concentration in the liver increased significantly at 30 mg/kg or more, while increases in cholesterol, phospholipid, and GSH were not significant. Phospholipid content and GSH concentration increased significantly at 300 mg/kg. To ascertain the effects of olive oil administration on plasma lipids, two rats were injected i.p. with olive oil at a dosage of 1 ml/kg, and the other two rats received no treatment

A

Fig. 2A. Time-course changes in the plasma concentrations of VLDL, LDL, and HDL fractions following 100 mg/kg i.p. CHCl_3 injections to rats. Legends are as in Fig. 1A.

Fig. 2B. Time-course changes in the plasma concentrations of triglyceride, cholesterol, and GPT following 100 mg/kg i.p. CHCl_3 injections to rats. Legends are as in Fig. 1B.

Fig. 2C. Time-course changes in the liver weight, liver triglyceride, liver malondialdehyde, and liver glutathione following 100 mg/kg i.p. CHCl_3 injections to rats. Legends are as in Fig. 1C.

B

C

A

Fig. 3A. Time-course changes in the plasma concentrations of VLDL, LDL, and HDL fractions following 600 mg/kg i.p. CH_2Cl_2 injections to rats. Legends are as in Fig. 1A.

Fig. 3B. Time-course changes in the plasma concentrations of triglyceride, cholesterol, and GPT following 600 mg/kg i.p. CH_2Cl_2 injections to rats. Legends are as in Fig. 1B.

Fig. 3C. Time-course changes in the liver weight, liver triglyceride, liver malondialdehyde, and liver glutathione following 600 mg/kg i.p. CH_2Cl_2 injections to rats. Legends are as in Fig. 1C.

B **C**

Table 1A. Effects of a single administration of CCl₄ on the plasma VLDL fraction of rats

Dose (mg/kg)	F-test	0	3	30	300
TG (mg/dl plasma)	p<0.01	63.3 ± 4.4	49.0 ± 3.0 (77)*	32.0 ± 3.9 (51) [#]	19.5 ± 1.8 (31) [#]
PR (mg/dl plasma)	p<0.01	12.5 ± 0.6	10.4 ± 0.5 (83) [#]	4.6 ± 0.2 (37) [#]	1.5 ± 0.2 (12) [#]
Apo B1 (A.U.)	p<0.01	26.0 ± 1.2	24.5 ± 2.3 (94)	11.5 ± 1.4 (44) [#]	7.8 ± 1.7 (30) [#]
Apo Bs (A.U.)	p<0.01	30.3 ± 2.2	29.0 ± 0.8 (96)	16.5 ± 1.2 (54) [#]	6.0 ± 1.1 (20) [#]
Apo E's (A.U.)	p<0.01	209.3 ± 13.3	190.8 ± 16.1 (91)	124.3 ± 10.8 (59) [#]	17.3 ± 3.6 (8) [#]
Apo C's (A.U.)	p<0.01	105.5 ± 4.2	91.0 ± 4.1 (86)*	37.3 ± 2.3 (35) [#]	12.0 ± 3.0 (11) [#]

TG, triglyceride. PR, protein. Apo, apolipoprotein. A.U., arbitrary units for staining density of protein staining in electrophoresis gel. Each value represents the mean ± SEM (N=6). * and [#] represent significant differences at 0.05>p and 0.01>p, respectively, between the control and solvent-treated groups by Dunnett's test. n.s., p<0.05, and p<0.01 in the table represent not significant, significant at 0.05>p, and significant at 0.01>p, respectively, by the analysis of variance (F-test). Percent values of the exposed group when control value was set at 100% are shown in parenthesis.

Table 1B. Effects of a single administration of carbon tetrachloride on the plasma LDL fraction of rats

Dose (mg/kg)	F-test	0	3	30	300
LDL					
Lipid (A.U.)	p<0.01	184.3 ± 7.4	206.3 ± 9.4 (112)	110.8 ± 12.0 (60) [#]	102.0 ± 3.5 (55) [#]
Apo B1 (A.U.)	p<0.01	110.3 ± 6.5	137.5 ± 12.0 (125)	56.5 ± 11.4 (51) [#]	54.8 ± 6.7 (50) [#]
Apo Bs (A.U.)	p<0.01	21.3 ± 2.2	27.0 ± 2.1 (127)	12.8 ± 0.9 (60) [#]	9.8 ± 1.0 (46) [#]
HDL₁					
Lipid (A.U.)	p<0.01	91.3 ± 4.7	86.5 ± 3.5 (95)	53.3 ± 5.3 (58) [#]	27.5 ± 2.1 (30) [#]
Apo E's (A.U.)	p<0.01	148.0 ± 6.3	138.5 ± 6.0 (94)	102.5 ± 9.7 (69) [#]	63.8 ± 5.7 (43) [#]
LDL + HDL₁					
PR (mg/dl plasma)	p<0.01	2.3 ± 0.1	2.5 ± 0.2 (109)	1.6 ± 0.1 (70) [#]	1.4 ± 0.1 (61) [#]

A.U., Arbitrary units for staining density of lipid staining for LDL and HDL₁, or protein staining for apolipoproteins in electrophoresis gel. Other legends are as in Table 1A.

Table 1C. Effects of a single administration of carbon tetrachloride on the plasma HDL fraction of rats

Dose (mg/kg)	F-test	0	3	30	300
CH (mg/dl plasma)	p<0.01	19.8 ± 1.4	17.8 ± 1.8 (90)	10.5 ± 1.7 (53) [#]	8.5 ± 1.0 (43) [#]
PL (mg/dl plasma)	p<0.01	21.5 ± 1.8	23.3 ± 2.9 (108)	15.3 ± 1.4 (71)	11.8 ± 0.9 (55) [#]
PR (mg/dl plasma)	p<0.01	61.8 ± 4.9	68.3 ± 4.8 (111)	46.8 ± 3.8 (76)	30.8 ± 1.7 (50) [#]
Apo A-IV (A.U.)	p<0.01	218 ± 11	248 ± 11 (114)	111 ± 8 (51) [#]	104 ± 13 (48) [#]
Apo E's (A.U.)	p<0.01	966 ± 63	1,122 ± 45 (116)	630 ± 50 (65) [#]	398 ± 22 (41) [#]
Apo A-I (A.U.)	p<0.01	1,903 ± 109	2,260 ± 152 (119)	1,670 ± 114 (88)	1,052 ± 61 (55) [#]
Apo C's (A.U.)	p<0.01	452 ± 49	509 ± 32 (113)	311 ± 25 (69)*	200 ± 12 (44) [#]

CH, cholesterol; PL, phospholipid. Other legends are as in Table 1A.

Table 1D. Effects of a single administration of carbon tetrachloride on plasma lipid contents, GPT activity, and liver of rats

Dose (mg/kg)	F-test	0	3	30	300
Plasma					
TG (mg/dl plasma)	p<0.01	89.5 ± 6.0	85.3 ± 4.9 (95)	55.3 ± 5.4 (62) [#]	35.5 ± 4.2 (40) [#]
CH (mg/dl plasma)	p<0.01	47.5 ± 1.8	48.0 ± 1.5 (101)	31.5 ± 2.5 (66) [#]	25.5 ± 0.6 (54) [#]
GPT (IU/L)	p<0.01	34.8 ± 2.4	34.3 ± 2.9 (99)	56.8 ± 6.5 (163)	745.8 ± 103.5 (2143) [#]
Liver					
Weight (%)	p<0.01	2.64 ± 0.06	2.74 ± 0.01 (104)	3.01 ± 0.04 (114) [#]	3.25 ± 0.02 (123) [#]
TG (mg/liver)	p<0.01	66.0 ± 2.7	76.0 ± 3.6 (115)	109.0 ± 5.6 (165)*	145.8 ± 15.6 (221) [#]
CH (mg/liver)	p<0.05	34.3 ± 1.9	32.8 ± 0.9 (96)	38.5 ± 2.0 (112)	41.5 ± 2.7 (121)
PL (mg/liver)	p<0.05	69.0 ± 3.8	70.0 ± 1.8 (101)	73.5 ± 1.2 (107)	82.3 ± 2.5 (119) [#]
MDA (nmoles/g liver)	p<0.01	100.8 ± 4.5	120.5 ± 2.5 (120)	141.3 ± 6.0 (140)*	170.8 ± 14.5 (169) [#]
GSH (umoles/g liver)	p<0.01	3.04 ± 0.24	3.28 ± 0.02 (108)	4.68 ± 0.31 (154)	5.15 ± 0.54 (169) [#]

Liver weight was calculated as percent value of body weight. MDA, malondialdehyde. GSH, glutathione. Other legends are as in Tables 1A and 1C.

with the oil. Rats were starved, and 19 hr later were exsanguinated via the abdominal vein. Plasma contents of triglyceride were 98 and 71 mg/dl in no-treatment rats and 96 and 79 mg/dl in oil-treated rats. No difference existed between these two groups.

Rats were injected with CHCl_3 in dosages of 3, 30 or 300 mg/kg. Changes in plasma substances and liver 19 hr after the injection are summarized in Tables 2A, 2B, 2C, and 2D. Lipid and apolipoprotein components of VLDL were reduced at 30 mg/kg, and changes in triglyceride and apolipoprotein B₁ were significant (Table 2A). Components of VLDL were reduced to 20, or 36% of the control value at 300 mg/kg. CHCl_3 at 3 mg/kg increased LDL components slightly, but at 30 mg/kg exerted no effect on LDL, HDL₁, and HDL₂ (Tables 2B and 2C). CHCl_3 reduced these lipoprotein components by 26 to 48% at 300 mg/kg. No significant change occurred in plasma components and liver at 3 and 30 mg/kg except plasma triglyceride (Table 2D). At a dosage of 300 mg/kg significant decreases in plasma triglyceride and cholesterol were observed. Plasma GPT increased significantly at the same dosage. Increases in liver weight, and in triglyceride, MDA, and GSH in the liver also were significant at the same dosage.

Rats were treated i.p. with CH_2Cl_2 in dosages of 300 or 1,000 mg/kg. Changes in plasma substances and liver 8 hr after the treatment are summarized in Tables 3A, 3B, 3C, and 3D. CH_2Cl_2 at 300 mg/kg exerted a little effect on each concentration of VLDL components, e.g., triglyceride, protein, apolipoproteins B₁, B_s, E's, and C's (Table 3A). These components were reduced with no statistical significance. At a dosage of 1,000 mg/kg, CH_2Cl_2 reduced concentrations of triglyceride, total protein, and 4 apolipoprotein components of VLDL. Statistical significance was obtained in all items except triglyceride. CH_2Cl_2 exerted no effect on LDL and HDL₁ components at 300 mg/kg (Table 3B). At 1,000 mg/kg, however, LDL lipids, apolipoproteins B₁ and B_s increased 511 to 658% of control values. HDL₁ lipids and apolipoprotein E's increased 244 to 313% of control values. Total protein concentration of LDL fraction (LDL + HDL₁) was increased to 361% of control. As Table 3C shows, lipid and apolipoprotein components of HDL fraction were reduced to the same degree at both 300 and 1,000 mg/kg. No significant changes were observed in any determination of plasma components and liver at 300 mg/kg (Table 3D). Plasma GPT activity increased by 286% at 1,000 mg/kg. Liver weight and liver contents of triglyceride, cholesterol, and phospholipid were reduced. GSH concentration in the liver was reduced to almost half of the

control value. No significant changes occurred in MDA concentration.

Discussion

In their previous report, the authors described changes in lipoproteins of rat plasma following a single i.p. administration of CCl_4 , trichloroethylene, and 1,1,1-trichloroethane²¹). In the present experiments, plasma concentrations of lipoproteins were found to be affected by an i.p. administration of CHCl_3 and CH_2Cl_2 as well as CCl_4 .

Present time-course studies revealed that the maximum changes in plasma components and liver substances occurred between 19 and 32 hr following the injection of CCl_4 or CHCl_3 at 10 or 100 mg/kg, respectively. With CH_2Cl_2 at 600 mg/kg, maximal changes in these parameters were produced between 8 and 19 hr following the injection. From these data, 19 hr was chosen for CCl_4 and CHCl_3 as an interval between the administration of solvents and the sacrifice of rats, and 8 hr was chosen for CH_2Cl_2 .

Effects of CCl_4 on VLDL were almost the same at both 19 and 32 hr. Similar tendencies were observed in effects of CCl_4 on LDL, plasma triglyceride, liver weight, and liver MDA. CHCl_3 exerted almost the same effects at both 19 and 32 hr on liver weight. The effects of CCl_4 seemed to be long-lasting compared to those of CHCl_3 , even though the dose of CHCl_3 was ten times higher than that of CCl_4 . Though statistical significance was not obtained, increases in liver triglyceride induced by CCl_4 and CHCl_3 reached maximum at 19 to 32 hr. This increase indicates a lipid accumulation due to an inhibition of lipid metabolism by CCl_4 and CHCl_3 ^{16,17}). Effects of CH_2Cl_2 reached their peak at 8 hr in almost all parameters, while changes in VLDL and liver weight were almost the same at both 8 and 19 hr. Changes in HDL induced by CH_2Cl_2 were maximal at 19 hr rather than 8 hr. The effects of CH_2Cl_2 seemed to be short-lasting compared with those of CHCl_3 and CH_2Cl_2 , while a higher dosage of CH_2Cl_2 compared to the other two solvents was needed to exert effects on plasma and liver.

In dose-effect relationship study, CCl_4 increased liver-to-body weight ratio, liver content of triglyceride, and MDA concentration in the liver at a dosage of 30 mg/kg. Slight increases in these items were observed at a very low dosage of 3 mg/kg. Accumulation of hepatic triglyceride by CCl_4 is considered to be a result of the inhibition of lipoprotein excretion from the liver to the blood stream^{16,17,21}). This scheme clearly explains the decrease in plasma lipids and lipoproteins, as well as the increase in hepatic triglyceride

Table 2A. Effects of a single administration of chloroform on the plasma VLDL fraction of rats

Dose (mg/kg)	F-test	0	3	30	300
TG (mg/dl plasma)	p<0.01	84.8 ± 3.6	78.0 ± 2.1 (92)	54.8 ± 2.9 (65) [#]	30.8 ± 5.4 (36) [#]
PR (mg/dl plasma)	p<0.01	13.5 ± 0.4	13.4 ± 0.5 (99)	12.4 ± 0.6 (92)	3.4 ± 0.5 (25) [#]
Apo B1 (A.U.)	p<0.01	23.8 ± 1.3	20.3 ± 1.5 (85)	18.3 ± 1.4 (77) [*]	8.3 ± 1.1 (35) [#]
Apo Bs (A.U.)	p<0.01	58.5 ± 3.0	60.3 ± 3.0 (103)	49.3 ± 2.4 (84)	15.0 ± 1.4 (26) [#]
Apo E's (A.U.)	p<0.01	195.5 ± 12.5	190.3 ± 8.5 (97)	183.3 ± 11.1 (94)	39.0 ± 5.1 (20) [#]
Apo C's (A.U.)	p<0.01	127.5 ± 6.0	125.5 ± 5.7 (98)	119.3 ± 7.9 (94)	39.3 ± 5.1 (31) [#]

Legends are as in Table 1A.

Table 2B. Effects of a single administration of chloroform on the plasma LDL fraction of rats

Dose (mg/kg)	F-test	0	3	30	300
LDL					
Lipid (A.U.)	p<0.01	167.0 ± 5.8	193.8 ± 15.5 (116)	164.5 ± 10.5 (99)	115.8 ± 9.0 (69) [*]
Apo B1 (A.U.)	p<0.01	121.8 ± 4.5	146.0 ± 6.5 (120)	128.3 ± 10.0 (105)	90.3 ± 8.7 (74) [*]
Apo Bs (A.U.)	p<0.01	23.0 ± 1.9	32.0 ± 3.8 (139)	23.0 ± 0.4 (100)	12.0 ± 1.7 (52) [*]
HDL₁					
Lipid (A.U.)	p<0.01	106.0 ± 6.6	97.5 ± 5.6 (92)	94.5 ± 4.9 (89)	59.5 ± 3.3 (56) [#]
Apo E's (A.U.)	p<0.01	173.3 ± 7.7	170.3 ± 4.0 (98)	165.8 ± 6.4 (96)	111.3 ± 4.0 (64) [#]
LDL + HDL₁					
PR (mg/dl plasma)	p<0.01	2.68 ± 0.13	2.68 ± 0.10 (100)	2.55 ± 0.21 (95)	1.73 ± 0.17 (65) [#]

Legends are as in Table 1B.

Table 2C. Effects of a single administration of chloroform on the plasma HDL fraction of rats

Dose (mg/kg)	F-test	0	3	30	300
CH (mg/dl plasma)	p<0.01	25.3 ± 1.3	26.0 ± 1.7 (103)	22.8 ± 0.6 (90)	15.0 ± 1.1 (59) [#]
PL (mg/dl plasma)	p<0.01	24.8 ± 0.6	24.5 ± 0.9 (99)	23.3 ± 0.5 (94)	14.3 ± 0.5 (58) [#]
PR (mg/dl plasma)	p<0.01	74.0 ± 4.6	73.8 ± 4.2 (100)	74.0 ± 3.9 (100)	47.0 ± 4.2 (64) [#]
Apo A-IV (A.U.)	p<0.01	257 ± 8	252 ± 7 (98)	249 ± 5 (97)	139 ± 11 (54) [#]
Apo E's (A.U.)	p<0.01	1,174 ± 67	1,192 ± 53 (102)	1,245 ± 66 (106)	646 ± 65 (55) [#]
Apo A-I (A.U.)	p<0.01	2,324 ± 87	2,346 ± 97 (101)	2,359 ± 71 (102)	1,238 ± 152 (53) [#]
Apo C's (A.U.)	p<0.01	637 ± 32	700 ± 32 (110)	717 ± 55 (113)	376 ± 18 (59) [#]

Legends are as in Table 1C.

Table 2D. Effects of a single administration of chloroform on the plasma lipid contents, GPT activity, and liver of rats

Dose (mg/kg)	F-test	0	3	30	300
Plasma					
TG (mg/dl plasma)	p<0.01	123.5 ± 6.0	117.0 ± 4.3 (95)	95.3 ± 5.4 (77) [*]	53.0 ± 11.4 (43) [#]
CH (mg/dl plasma)	p<0.01	63.3 ± 3.8	65.3 ± 4.4 (103)	61.5 ± 2.6 (97)	38.8 ± 1.7 (61) [#]
GPT (IU/L)	p<0.01	44.5 ± 2.9	43.5 ± 2.6 (98)	52.8 ± 2.9 (119)	388.5 ± 66.9 (873) [#]
Liver					
Weight (%)	p<0.05	2.64 ± 0.06	2.64 ± 0.04 (100)	2.70 ± 0.07 (102)	2.96 ± 0.07 (110) [*]
TG (mg/liver)	p<0.01	63.5 ± 3.7	63.5 ± 3.2 (100)	66.5 ± 1.7 (105)	103.8 ± 7.6 (164) [#]
CH (mg/liver)	n.s.	46.8 ± 3.1	47.8 ± 2.1 (102)	47.3 ± 0.9 (101)	51.5 ± 2.1 (110)
PL (mg/liver)	n.s.	68.0 ± 4.9	69.8 ± 3.0 (103)	69.3 ± 1.1 (102)	72.3 ± 3.0 (106)
MDA (nmoles/g liver)	p<0.05	124.8 ± 10.0	125.0 ± 6.0 (100)	137.8 ± 7.8 (110)	164.5 ± 7.5 (132) [*]
GSH (umoles/g liver)	p<0.01	4.43 ± 0.22	4.30 ± 0.18 (97)	4.60 ± 0.27 (104)	6.78 ± 0.58 (153) [#]

Legends are as in Table 1D.

Table 3A. Effects of a single administration of dichloromethane on the plasma VLDL fraction of rats

Dose (mg/kg)	F-test	0	300	1,000
TG (mg/dl plasma)	n.s.	64.0 ± 3.3	54.0 ± 5.2 (84)	46.5 ± 8.8 (73)
PR (mg/dl plasma)	p<0.01	9.8 ± 0.6	9.3 ± 0.8 (95)	5.5 ± 0.4 (56)*
Apo B1 (A.U.)	p<0.05	19.5 ± 1.3	15.5 ± 2.0 (80)	12.0 ± 0.4 (62)*
Apo Bs (A.U.)	p<0.01	46.5 ± 1.8	39.5 ± 3.8 (85)	26.0 ± 2.2 (56)*
Apo E's (A.U.)	p<0.01	170.3 ± 8.2	156.3 ± 23.7 (92)	82.5 ± 11.4 (48)*
Apo C's (A.U.)	p<0.05	91.8 ± 5.2	70.3 ± 6.9 (77)	59.0 ± 5.9 (64)*

Legends are as in Table 1A.

Table 3B. Effects of a single administration of dichloromethane on the plasma LDL fraction of rats

Dose (mg/kg)	F-test	0	300	1,000
LDL				
Lipid (A.U.)	p<0.01	137.8 ± 7.4	143.0 ± 12.7 (104)	771.5 ± 81.7 (560)*
Apo B1 (A.U.)	p<0.01	90.8 ± 6.1	84.5 ± 6.0 (93)	464.0 ± 30.9 (511)*
Apo Bs (A.U.)	p<0.01	12.5 ± 0.6	12.5 ± 1.3 (100)	82.3 ± 3.9 (658)*
HDL₁				
Lipid (A.U.)	p<0.01	73.5 ± 4.2	86.5 ± 5.0 (118)	230.0 ± 24.1 (313)*
Apo E's (A.U.)	p<0.01	182.8 ± 12.3	184.0 ± 9.1 (101)	446.8 ± 51.4 (244)*
LDL + HDL₁				
PR (mg/dl plasma)	p<0.01	3.1 ± 0.1	3.0 ± 0.2 (97)	11.2 ± 1.0 (361)*

Legends are as in Table 1B.

Table 3C. Effects of a single administration of dichloromethane on the plasma HDL fraction of rats

Dose (mg/kg)	F-test	0	300	1,000
CH (mg/dl plasma)	p<0.01	25.8 ± 0.9	18.3 ± 0.9 (71)*	19.8 ± 1.9 (77)*
PL (mg/dl plasma)	p<0.01	26.5 ± 1.7	20.0 ± 2.0 (76)	23.0 ± 2.7 (87)
PR (mg/dl plasma)	n.s.	61.0 ± 3.0	47.8 ± 1.5 (78)*	45.8 ± 2.7 (75)*
Apo A-IV (A.U.)	n.s.	209 ± 14	149 ± 20 (71)	174 ± 14 (83)
Apo E's (A.U.)	n.s.	1,290 ± 68	1,079 ± 49 (84)	1,117 ± 67 (87)
Apo A-I (A.U.)	p<0.01	1,866 ± 88	1,148 ± 66 (62)*	1,288 ± 184 (69)*
Apo C's (A.U.)	n.s.	466 ± 38	388 ± 66 (83)	403 ± 32 (87)

Legends are as in Table 1C.

Table 3D. Effects of a single administration of dichloromethane on plasma lipid contents, GPT activity, and liver of rats

Dose (mg/kg)	F-test	0	300	1,000
Plasma				
TG (mg/dl plasma)	p<0.01	85.0 ± 2.9	70.8 ± 11.6 (83)	66.3 ± 4.2 (78)
CH (mg/dl plasma)	n.s.	64.3 ± 5.3	54.3 ± 4.1 (84)	57.8 ± 8.5 (90)
GPT (IU/L)	p<0.01	30.8 ± 3.1	39.3 ± 2.5 (128)	119.0 ± 8.9 (386)*
Liver				
Weight (%)	p<0.05	2.52 ± 0.05	2.31 ± 0.08 (92)	2.25 ± 0.05 (89)*
TG (mg/liver)	p<0.05	44.0 ± 2.7	39.5 ± 2.6 (90)	31.8 ± 1.5 (72)*
CH (mg/liver)	p<0.05	28.0 ± 1.9	27.5 ± 1.3 (98)	22.0 ± 1.6 (79)*
PL (mg/liver)	p<0.01	94.0 ± 5.2	83.8 ± 2.9 (89)	74.5 ± 2.7 (79)*
MDA (nmoles/g liver)	n.s.	104.5 ± 6.5	107.3 ± 13.6 (103)	90.3 ± 4.3 (86)
GSH (umoles/g liver)	p<0.01	4.28 ± 0.36	3.85 ± 0.41 (90)	2.53 ± 0.15 (59)*

Legends are as in Table 1D.

by CCl_4 observed in the current study. MDA is thought to be a good indicator of lipid peroxide production²³. Lipid peroxide is one of main causes of liver injury produced by CCl_4 ¹². In the current study, MDA was increased by CCl_4 at 30 mg/kg, while changes in plasma GPT were not significant at this dosage. A dosage of 300 mg/kg of CCl_4 was needed to increase GPT activity significantly. Distinct decreases in plasma lipids and lipoproteins induced by CCl_4 at 30 mg/kg suggest that decreases in these items are more sensitive indicators of liver damage due to CCl_4 than traditional markers such as GPT.

Among various plasma lipoproteins, components of VLDL, e.g., triglyceride, protein, and apolipoprotein C's, decreased significantly with an i.p. administration of CCl_4 at 3 mg/kg or more. All components of LDL fraction and some components of HDL fraction decreased significantly with CCl_4 at 30 mg/kg or more. Total triglyceride and cholesterol concentrations in plasma decreased significantly with CCl_4 at 30 mg/kg or more, while these lipids in plasma exerted no change at 3 mg/kg of CCl_4 . On the other hand, components of VLDL decreased significantly at 3 mg/kg, which indicates that an analysis of lipoprotein components, particularly of VLDL, can reveal sensitive markers regarding CCl_4 toxicity.

An administration of CHCl_3 at 30 mg/kg produced significant decreases in plasma triglyceride and apolipoprotein B1 of VLDL fraction. Significant increase in GPT activity was observed at 300 mg/kg and not at 30 mg/kg. These results mean that plasma triglyceride and VLDL are more sensitive markers than GPT for CHCl_3 exposure.

CH_2Cl_2 reduced VLDL components slightly at 300 mg/kg and significantly at 1,000 mg/kg, and significant decreases in HDL components occurred at 300 mg/kg. As an increase in GPT activity was observed at 1,000 mg/kg, the decrease in HDL was a more sensitive marker for exposure to CH_2Cl_2 than plasma GPT. A large increase in LDL was observed following exposure to 1,000 mg/kg of CH_2Cl_2 . Plasma LDL was increased following i.p. administration of trichloroethylene and 1,1,1-trichloroethane²¹. In this study, a slight increase in LDL was induced by CCl_4 and CHCl_3 at 3 mg/kg. LDL increases induced by these chemicals, however, were not so marked as those observed after an injection of CH_2Cl_2 . A marked increase in LDL seems to be characteristic for injections of high dosages of CH_2Cl_2 .

Hepatic triglyceride is excreted to the blood stream as a component of VLDL²⁶. VLDL particles in the blood lose triglyceride under the action of lipase, etc., and is converted

into LDL, which is then absorbed into the liver²⁷. Details of the synthesis of HDL is not known but most HDL is thought to be synthesized in the liver. According to Lombardi and Ugazio¹⁷, CCl_4 inhibits the synthesis of apolipoproteins in the liver. Due to this lack of apolipoprotein components, the synthesis of lipoproteins is disturbed, and excretion of lipoproteins from the liver to the blood stream is inhibited. This is the main scheme of the decrease in plasma lipoproteins and the increase in hepatic triglyceride. The results obtained in the current study that the decrease in VLDL and LDL by CCl_4 is accompanied by the increase in hepatic triglyceride, are well consistent with this scheme. The results of the current study show that changes in plasma lipoproteins serve as markers for liver dysfunction in addition to playing the role of exposure markers. The present results also indicate that the synthesis inhibition of lipoproteins probably occurs at much lower dosages of CCl_4 , CHCl_3 , and CH_2Cl_2 than the dosages at which the leaking of GPT molecule from hepatic cells occurs, which indicates that changes in plasma lipoproteins are sensitive as hepatotoxicity markers.

With the administration of CHCl_3 , all lipoprotein fractions decreased. CHCl_3 induced these effects at higher dosages than those where CCl_4 decreased lipoproteins. While CHCl_3 appears to inhibit lipoprotein metabolism as CCl_4 does, CHCl_3 is less potent than CCl_4 .

CH_2Cl_2 decreased plasma VLDL and HDL, whereas it increased LDL at 1,000 mg/kg. It is not known what chemicals produce such phenomena. Plasma LDL disappears from the blood stream by an uptake of LDL into the liver via LDL receptors on the liver's surface²⁷. The uptake of LDL into the liver may be strongly inhibited by CH_2Cl_2 . However, the mechanism of the LDL increase observed in this study remains unclear. High dosages of CH_2Cl_2 increased HDL_1 , although the degree of the increase was less than that of LDL. As HDL_1 does not exist in normal human blood, the study on the metabolism of HDL_1 did not make any significant discoveries. At present, it is difficult to explain the mechanism of increase in HDL_1 by CH_2Cl_2 .

Our present study showed that i.p. administration of chlorinated organic solvents produced different changes in the lipoproteins in the blood. Many of these changes occurred at lower dosages than those where GPT activity was elevated. Different changes in the liver occurred at the dosages where changes in lipoproteins occurred than those dosages where GPT activity was elevated. These findings indicate that changes in plasma lipoproteins are closely related with liver dysfunction. Study on the mechanism of this relationship is in progress.

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