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Novel monoclonal antibody recognizing triglyceride-rich oxidized LDLs
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associated with severe liver disease and small oxidized LDLs in normal subjects
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10   A short title: Novel antibody to oxidized LDL
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DECLARATIONS

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ABSTRACT

Background: Triglyceride-rich low-density lipoproteins (TG-rich LDLs) in the plasma of patients with severe liver disease are reported to change macrophages into foam cells *in vitro*.

Methods: Male BALB/c mice were immunized with TG-rich LDLs isolated from the plasma of a patient with severe liver disease. The resulting monoclonal antibody (G11-6) was used in a sandwich enzyme-linked immunosorbent assay (ELISA) in combination with polyclonal anti-apolipoprotein B antibodies. The time course of copper-mediated LDL oxidation was monitored using this ELISA. The results were compared to those of the two commercial ELISAs for oxidized LDL using DLH or ML25, thiobarbituric acid reactive substances (TBARS), and the optical absorbance for the conjugated dienes generated in lipid peroxides. Further, the lipoprotein fractions separated by gel filtration were tested with this ELISA in healthy volunteers (*n*=11) and patients (*n*=3) with liver disease.

Results: G11-6 reacted with oxidized LDLs during only the early phase of copper-oxidation, being distinct from the other monoclonal antibodies and methods. G11-6 was confirmed to react with TG-rich LDLs in patients, while it reacted with small LDL particles in normal controls.

Conclusions: The monoclonal antibody G11-6 is useful for detecting oxidized small LDLs in normal controls and oxidized TG-rich LDLs in patients with severe liver disease.

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INTRODUCTION

Oxidative modification of low-density lipoproteins (LDLs) and the subsequent conversion of macrophages into foam cells in atherosclerotic lesions plays a key role in early atherogenesis.[1-3] Oxidized LDLs have been detected at elevated levels in circulating plasma from patients with coronary artery disease using enzyme-linked immunosorbent assays (ELISAs).[4-7] Small dense LDLs are more susceptible to oxidation than larger, buoyant ones.[8] Copper-mediated LDL oxidation *in vitro* decreases LDL particle size, which could contribute to the generation of small, dense LDL particles.[9] Oxidation of lipids and lipoproteins has been implicated in a growing number of diseases including diabetes mellitus,[10] chronic kidney disease,[11] Alzheimer disease,[12] autoimmune disease,[13] and cancer,[14] as well as in ageing.[15]

In our previous study, we observed abnormal triglyceride-rich LDLs (TG-rich LDLs) in patients with cholestatic liver disease. This lipoprotein species strongly promotes the conversion of macrophages into foam cells *in vitro*.[16] To further investigate its pathophysiological role, we developed a new monoclonal antibody against TG-rich LDLs. Its biochemical properties and reactivity with lipoproteins are described in the present report.

MATERIALS AND METHODS

Immunogen

TG-rich LDLs were used as immunogens to develop a new monoclonal antibody, designated G11-6. Briefly, blood was drawn from a patient with severe cholestasis due to advanced primary biliary cirrhosis, who had provided informed consent. The change in electrophoretic mobility of sera was confirmed by agarose gel electrophoresis.[17] TG-rich LDLs were isolated from the serum by ultracentrifugation and gel chromatography, as previously reported.[17,18] The chemical composition of

this lipoprotein fraction was determined. Lipids were measured by automated enzymatic methods using the following commercial kits: Cholestest CHO for total cholesterol (TC), Cholestest TG for triglycerides (TG), Pureauto S PL for phospholipids (PL), Qualigent LDL for LDL-cholesterol, and Qualigent HDL for high-density lipoprotein (HDL)-cholesterol (Sekisui Medical Co., Ltd., Tokyo, Japan), and Determiner L FC for free cholesterol (Kyowa medex Co., Ltd., Tokyo, Japan). Cholesterylester (CE) concentrations were calculated by multiplying the esterified cholesterol concentrations (obtained by subtracting FC from TC) by 1.72. Protein was determined by the method of Lowry, modified by Markwell et al.[19] Apolipoproteins were analyzed by 3–10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) after reduction with 2-mercaptoethanol.

Immunization

Male BALB/c mice (6 weeks old) were immunized with the isolated TG-rich LDLs (0.5–1.0 mg/ml, 0.1 ml/injection) and were given booster injections 2 and 6 weeks later.[20] Three days after the final injection, spleen cells were collected and fused with P3/U1 mouse myeloma cells using 50% polyethylene glycol 1500 (Roche Diagnostics, Mannheim, Germany). The fused cells were cultured in Gibco RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing fetal bovine albumin (10%), hypoxanthine, aminopterin, and thymidine in a 96-well microtiter plate.

Ten days after cell fusion, cells from antibody-containing wells were cloned by limiting dilution. The hybridoma clones were cultured in RPMI 1640 medium and injected intraperitoneally into mice primed with 0.5 ml pristine. Ascitic fluid was purified by ammonium sulfate precipitation followed by gel filtration chromatography on a Superose 6 column.

Antibody characterization

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The selected antibodies were isotyped using a commercially-available kit (IsoStrip Mouse Monoclonal Antibody Isotyping Kit; Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

ELISA

The wells of a 96-well microtiter plate (Nalge Nunc International, Reskilde, Denmark) were coated with 50 µl G11-6 solution (5 µg/ml in 10 mmol/l phosphate-buffered saline [PBS, pH 7.4]), and the plate was incubated for 2 h at 37°C. The wells were then blocked with 150 µl 1% bovine serum albumin (BSA) in PBS for 2 h at 37°C. After four washes with PBS containing 0.05% Tween 20 (PBS/T), 50 µl sample was added to each well, and the plate was incubated overnight at 4°C. After washing, 50 µl biotinylated goat polyclonal antibody against human apolipoprotein B (ApoB) (10 µg/ml; BW127; WatPa Enterprises, Auckland, New Zealand) was added to each well, and the plate was incubated for 1 h at room temperature. After washing, 50 µl alkaline phosphatase-conjugated streptavidin (1:250 in PBS/T; Zymed Laboratories, San Francisco, CA) was added to each well, and the plate was incubated for 30 min at room temperature. After washing, 100 µl p-nitrophenyl phosphate (1 mg/ml) in diethanolamine solution was added to each well and allowed to react with the enzyme for 60 min at room temperature. The absorbance at 405 nm was then measured against a reference absorbance at 620 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA).

Apolipoproteins and oxidative stress markers

ApoB was measured by sandwich ELISA using the BW127 anti-ApoB antibody using the same procedures as those used in the ELISA with G11-6. Two commercial ELISA kits for oxidized LDL measurement were used. The MX kit (Kyowa Medex Co., Ltd., Tokyo, Japan) using the DLH monoclonal antibody detects oxidation-induced short-chain phosphatidylcholine (oxPC).[21] The

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MDA-LDL ELISA kit (Sekisui Medical Co., Ltd., Tokyo, Japan) using the ML25 monoclonal antibody detects malondialdehyde (MDA)-modified ApoB.[22] The lipoproteins detected by the MX and MDA-LDL ELISA kits were designated oxPC-LDL and MDA-LDL, respectively.

Thiobarbituric acid reactive substances (TBARS) were measured using a commercial colorimetric kit (Cayman Chemical Co., Ann Arbor, MI, USA), and levels of conjugated dienes were monitored by following the absorbance at 234 nm.[23]

Copper-mediated oxidation of LDLs purified from serum

LDLs were purified from serum by sequential ultracentrifugation in a near-vertical tube rotor (MLN-80; Beckman Coulter, Fullerton, CA) in a model Optima MAX ultracentrifuge (Beckman Coulter).[18,24,25] Each serum sample (2.0 ml) was adjusted with KBr solution to a density of 1.019 kg/l and then centrifuged at 40,000 rpm for 20 h at 15°C. The upper fraction (2.5 ml) containing chylomicron (CM), very low-density lipoprotein (VLDL), and intermediate-density lipoprotein (IDL) was removed. The lower fraction was adjusted with KBr solution to a density of 1.063 kg/l and centrifuged at 50,000 rpm for 18 h at 15°C. The resulting LDL-containing upper fraction (2.5 ml) and the resulting lower fraction, which contained HDL and other serum proteins, were both recovered and assayed for their protein concentrations using a modified Lowry method.[19] Excellent separation of the lipoproteins was confirmed by polyacrylamide gel electrophoresis (LipoPhor; Jokoh Co., Ltd., Tokyo, Japan) (data not shown).

To prepare copper-oxidized LDL, the purified LDL fraction was dialyzed against PBS for 16 h at 4°C and diluted with PBS to a protein concentration of 0.5 mg/ml. Aliquots of diluted LDL were then incubated in the presence of 3.3 µmol/l $CuSO_4$ at 37°C. After various lengths of time (0–8 h), the

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reaction mixtures were immediately analyzed after dilution to the specified protein concentrations using the following assays: G11-6 ELISA (50 µg protein/ml), ApoB (0.1 µg/ml), oxPC-LDL (0.2 µg/ml), MDA-LDL (0.5 µg/ml), TBARS (not diluted), and conjugated diene (40 µg/ml). All data are presented as a ratio to the baseline value (0 h). The change in the electrophoretic mobility of LDLs was confirmed by agarose gel electrophoresis.[17]

Preparation of malondialdehyde-modified LDLs

Malondialdehyde solution (0.02 mol/l) in 0.1 mol/l phosphate buffer (pH 6.4) was prepared from malondialdehyde tetrabutylammonium salt (Sigma, St Louis, MO, USA). LDL (0.5 mg/ml protein) were dialyzed against 10 mmol/l phosphate buffer (pH 7.4) containing 150 mmol/l NaCl and 0.268 mmol/l EDTA, and modified by mixing equal volumes of 0.02 mol/l malondialdehyde solution followed by incubation for 3 or 18 h at 4°C. To remove excess malondialdehyde, the mixture was dialyzed against 10 mmol/l phosphate buffer (pH 7.4) containing 150 mmol/l NaCl and 0.268 mmol/l EDTA. The reaction mixtures were analyzed by the following assays after dilution to the specified protein concentrations: G11-6 ELISA (50 µg protein/ml), MDA-LDL (0.5 µg/ml), TBARS (not diluted). All data are presented as ratios of the baseline value (0 h).

Gel filtration chromatography

Serum samples were fractionated by gel filtration chromatography on a Superose 6 column (GE Healthcare, Little Chalfont, England) in a liquid chromatography apparatus (Shimadzu, Kyoto, Japan) using a procedure modified from a previous report.[26] The column was eluted with 50 mmol/l phosphate buffer (pH 7.4) containing 150 mmol/l NaCl and 1 mmol/l EDTA at a flow rate of 0.5 ml/min. Eluted fractions (0.5 ml each) were subjected to lipid analyses and immunoassays.

Serum samples

Blood was drawn from 11 healthy volunteers (7 male and 4 female; mean age ± SD, 23.1 ± 1.0 years; range, 21–24 years) and 3 patients with severe liver disease (2 male and 1 female; 52.7 ± 5.8 years; range, 46–56 years) after an overnight fast. In agarose gel electrophoresis, presence of defective alphalipoproteins was classified as severe liver disease (HCV, n=2; alcoholic hepatitis, n=1). Clinical data are shown in Table 1. It is noteworthy that serum lipid levels were low, particularly HDL-cholesterol, in patients with severe liver disease. Serum samples were obtained by centrifugation at $2000 \times g$ for 10 min at room temperature and were stored at 4°C until use.

Procedural ethics

All individuals provided written informed consent to participate in the study. The study was approved by the ethics review board of the Faculty of Health Sciences, Hokkaido University (approval number 08-57).

Statistical analysis

All clinical parameters except sex between the groups were compared using a non-parametric Mann-Whitney U-test. The influence of sex among groups was assessed by Fisher's exact probability test. Differences were considered to be statistically significant at $P<0.05$.

RESULTS

Chemical composition of the immunogen

The serum from the immunogen donor showed only a β-band in an agarose gel (Fig. 1). This β-band migrated faster and broader than did normal examples. The chemical composition (weight%) of the β-migrating lipoproteins isolated by ultracentrifugation and gel filtration demonstrated the abundance of LDL with triglyceride: CE, 9.4%; FC, 13.8%; TG 28.7%; PL, 29.1%; proteins, 19.0% (normal: CE,

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39.1%; FC, 7.2%; TG 8.4%; PL, 22.4%; proteins, 21.3%[16]). SDS-PAGE showed that the TG-rich LDLs contained a 550 kDa protein as the major apolipoprotein (data not shown).

Biochemical characteristics

Hybridomas more reactive to copper-oxidized LDL and less reactive to native LDL were screened. Of a number of positive clones, G11-6 was selected because it showed a significant reaction with copper-oxidized LDLs. The isotype of G11-6 was IgM, with a κ light chain.

Copper-mediated oxidation of LDL

LDL oxidation was confirmed by mobility shift in agarose gel electrophoresis (Fig. 2). The reactivity of LDLs with the ELISA using G11-6 increased promptly and linearly from the beginning of copper oxidation, peaking at 3 h, and then declined to a basal level at 8 h (Fig. 3). TBARS also showed a prompt elevation and reached maximum at 3 h, but remained elevated at 8 h. Lag occurred in MDA-LDLs and conjugated diene until 1 h, and in oxPC-LDLs until 2 h. MDA-LDLs reached maximum at 3 h and declined after 4 h, but remained elevated at 8 h. The conjugated diene and oxPC-LDLs did not decline at any time point. The ApoB ELISA remained constant for 8 h.

Interaction between the G11-6 and MDA-modified LDL

The G11-6 ELISA did not react with MDA-modified LDLs, while the MDA-LDL ELISA and TBARS exhibited clear responses (Fig. 4).

Gel filtration chromatography

The serum sample obtained from a male patient with severe liver disease (TC 66 mg/dl, TG 53 mg/dl, PL 89 mg/dl, HDL-C 4 mg/dl, and LDL-C 35 mg/dl) was separated by gel filtration HPLC (Fig. 5A). The major lipoproteins eluted in fractions 11–15 were characterized by a high TG content,

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demonstrating the characteristics of the TG-rich LDLs associated with severe liver disease. The

reactivity of G11-6 with the TG-rich LDLs was confirmed by the overlapping profiles. Essentially

identical elution profiles were observed in all of the three patients with severe liver disease examined

in this study. In contrast, as shown in Figure 5B, serum from a normal male (TC 219 mg/dl, TG 116

mg/dl, PL 236 mg/dl, HDL-C 66 mg/dl, and LDL-C 134 mg/dl) was separated using an identical

method. Normal LDLs eluted in identical fractions as the TG-rich LDLs (Fig. 5A). Lipoproteins

reactive with G11-6 eluted slower than the peak of LDL cholesterol, indicating that G11-6 reacted

with small, dense LDLs. All healthy volunteers (*n*=11) yielded essentially the same results.

DISCUSSION

Previous attempts to develop monoclonal antibodies against oxidized LDLs have used as

immunogens the homogenates of human atheromatous plaques or LDLs modified *in vitro*, such as by

MDA treatment or metal-induced oxidation.[3,22,27,28] In this study, naturally oxidized lipoproteins,

TG-rich LDLs, circulating in a patient with severe liver disease were employed as the immunogen.

To the best of our knowledge, G11-6 represents the only monoclonal antibody against circulating

oxidized LDLs.

TG-rich LDLs used as immunogens were strikingly rich in TG content, but poor in CE, as previously

reported.[29,30] Their major apolipoprotein was apoB-100, which originated in the liver. Our previous

study reported that serum MDA-LDL level was high in patients with severe liver disease and that the

TG-rich LDL isolated from plasma of a patient with severe liver disease promoted the conversion of

macrophages into foam cells *in vitro* as efficiently as copper-oxidized LDL.[16] Furthermore, the

striking increase of the hydroperoxides of cholesterylesters and triglycerides (TG) in plasma from the

patients with liver failure has been reported. [31] Although the immunogens used in this study were not

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analyzed for any oxidized materials, it is very likely that the immunogens used here are oxidized LDL.

It is often the case that the extent of lipoprotein oxidation is defined by the change in the electrophoretic mobility of LDLs in agarose gel electrophoresis, and by the loss of recognition for LDL by the LDL receptor, and by strength of the oxidation-related response in cells to oxidized LDL. [32] In this study, the relative mobility, which was measured by dividing the electromobility of oxidized LDLs by that of native LDL, for our copper-oxidized LDL (oxidized for 3-4 h) was similar with that for mildly modified LDLs reported by Itabe et al. [33].As shown in Figure 3, G11-6 did not recognize heavily oxidized LDLs, but did detect oxidized lipoproteins during the early process of oxidation. The rapid decline in the reactivity of oxidized LDL with G11-6 after 3 h was not due to the deterioration of ApoB-100, because the reactivity of the ApoB ELISA was constant.

This delayed and prolonged time course for oxPC-LDL detection clearly demonstrates that the DLH antibody recognizes more oxidized LDLs than do G11-6 or ML25. The G11-6 epitope remains unknown, but is different than that for ML25, as evidenced by the diverse time-courses during copper oxidation (Fig. 3), and by the lack of G11-6 reactivity with MDA-modified LDLs (Fig. 4). In addition, ML25 requires sodium dodecyl sulfate in the reaction buffer to expose the epitope, while G11-6 does not.

It is of interest that DLH and ML25 both exhibited a lag-time, but G11-6 did not (Fig. 3). DLH and ML25 recognize short acyl-chained phosphatidylcholines and MDA-modified ApoB, respectively. Both structures are thought to be produced during lipid oxidation later than lipid hydroperoxide formation. [34] The identity of the G11-6 epitope is a focus of research in our laboratory.

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Our gel filtration study confirmed that G11-6 reacts with TG-rich LDLs in the blood of patients with severe liver disease (Fig. 5A), which was expected, because TG-rich LDLs were used to raise the G11-6. It is noteworthy that G11-6 can bind to small, dense LDLs in normolipidemic sera (Fig. 5B), indicating that identical or very similar structures relevant to oxidation exist on the surface of both lipoproteins. Accordingly, small, dense LDLs have been reported to be oxidized,[22] or more easily oxidizable than large, buoyant ones.[8] Hence, our observations provide support for the role of small, dense LDLs in atherosclerosis.

The biological effect of LDL oxidation is reported to be diverse and to depend on oxidation level.[32] Mildly oxidized LDLs induce overall cell death, such as necrosis and apoptosis, more than moderately oxidized LDLs in human monocyte/macrophage systems, where the former are defined as containing more lipid hydroperoxides, but less oxysterol, malondialdehyde, and negative charge than the latter.[35,36] Further, mildly oxidized LDLs are reported to exhibit cytotoxic and pro-inflammatory activities.[37] In this context, the oxidized lipoproteins reactive with G11-6 may be more important for clinical and research purposes than heavily oxidized LDLs.

In terms of liver disease, the G11-6 might serve as a tool for evaluating oxidation and inflammation in the liver.[38] Recent studies have suggested that antioxidant therapy improves liver function in patients with chronic liver disease, such as chronic hepatitis C and non-alcoholic steatohepatitis.[37,39] The potential utility of G11-6 in such antioxidant therapies for liver disease should be tested. We are now preparing for a large-scale clinical study for this purpose.

The generation mechanism for the TG-rich LDLs in liver disease also remains to be addressed in the future. It has been reported that the MDR2transporter, which is involved in toxicant and drug metabolism, is involved in LpX secretion.[40] The interactions between lipoprotein metabolism with

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such detoxification systems remain to be explored. G11-6 may prove useful for such studies.

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Figure legends

Fig. 1. Typical electrophoretic patterns of serum lipoproteins on agarose gel electrophoresis: a normal control (A) and a patient with severe liver disease (B). Fat Red staining.

Fig. 2. The change in electrophoretic mobility on agarose gel during copper oxidation of normal LDL.

Fig. 3. The time course of copper-mediated LDL oxidation as determined using the ELISA using the G11-6 (○, left axis) and the MDA-LDL (▲, left axis), the TBARS (×, left axis), apoB (●, left axis), the oxPC-LDL (■, right axis), and the conjugated dienes (△, right axis). All data were presented as the ratio of absorbance at each time-point to that for the zero-time.

Fig. 4. The reactivity of the MDA-modified LDL with the ELISA using the G11-6 (○, left axis), the MDA-LDL ELISA (▲, left axis) and TBARS assay (×, right axis). All data were presented as the ratio of absorbance at each time-point to that for the zero-time.

Fig. 5. Elution profile in gel filtration HPLC for (A) a serum sample from a representative patient with severe liver disease, and (B) a serum sample from a representative healthy volunteer. Total cholesterol (●, left axis), triglycerides (▲, left axis), phospholipids (×, left axis), and the ELISA using the G11-6 (○, right axis), a, void volume; b, large, buoyant LDLs; c, small dense LDLs; d, HDLs; e, TG-rich LDLs.
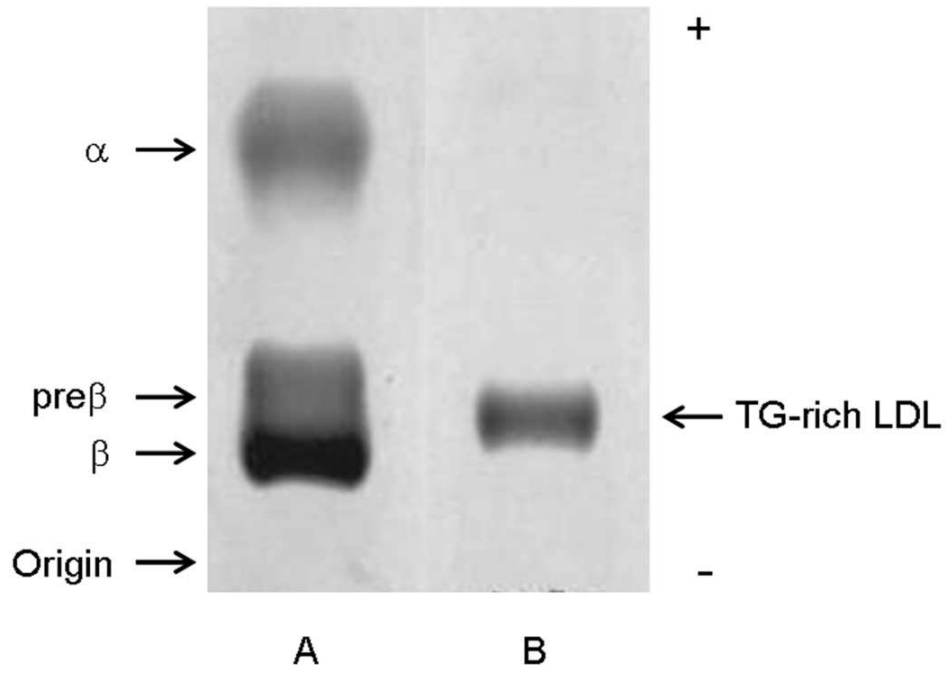
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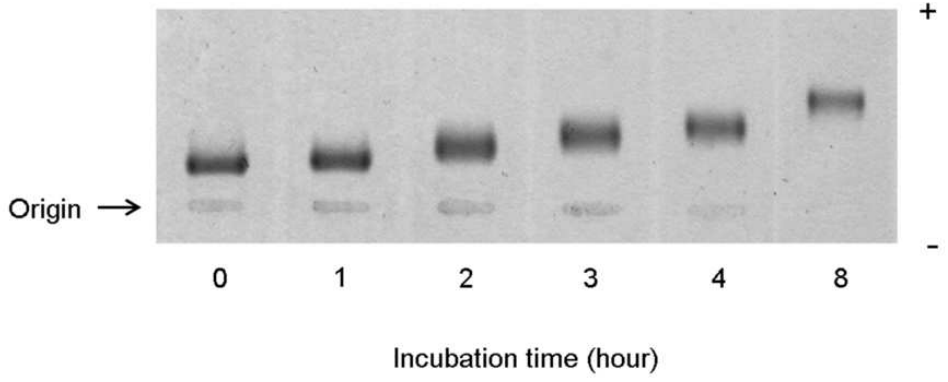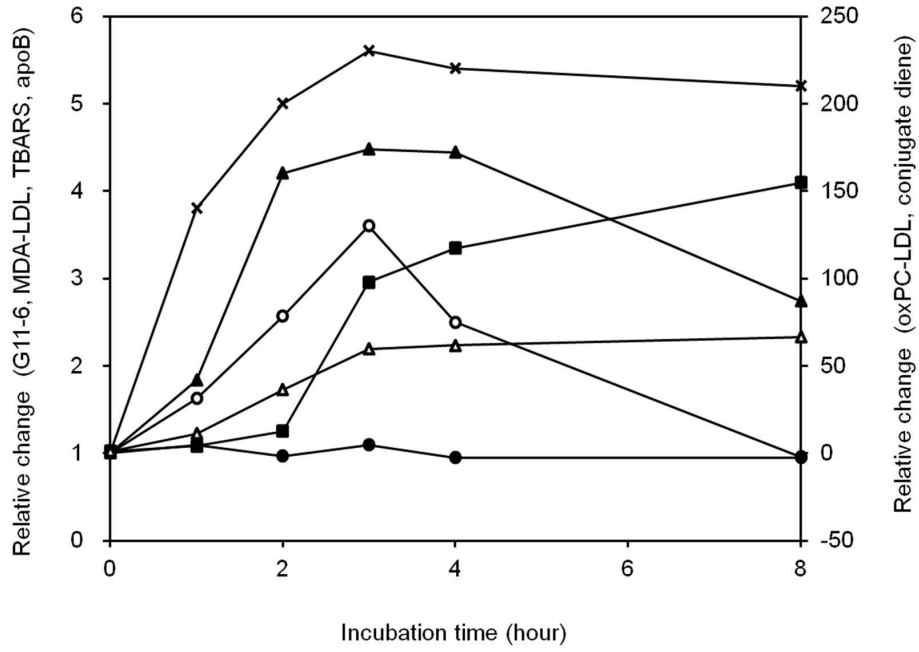
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Table 1. Clinical parameters and serum lipids in studied groups

Traits	Healthy volunteers	Severe liver disease
Age (years), mean ± SD (range)	23.1 ± 1.0 (21-24)	52.7 ± 5.8 (46-56) *
Male/Female	7/4	2/1
Total cholesterol, mg/dl	190 ± 33	84 ± 38 *
Triglyceride, mg/dl	74 ± 37	53 ± 10
Phospholipid, mg/dl	213 ± 36	105 ± 36 *
HDL-cholesterol, mg/dl	70 ± 19	8 ± 8 *
LDL-cholesterol, mg/dl	107 ± 28	47 ± 30 *

* P <0.05 vs. healthy volunteers.

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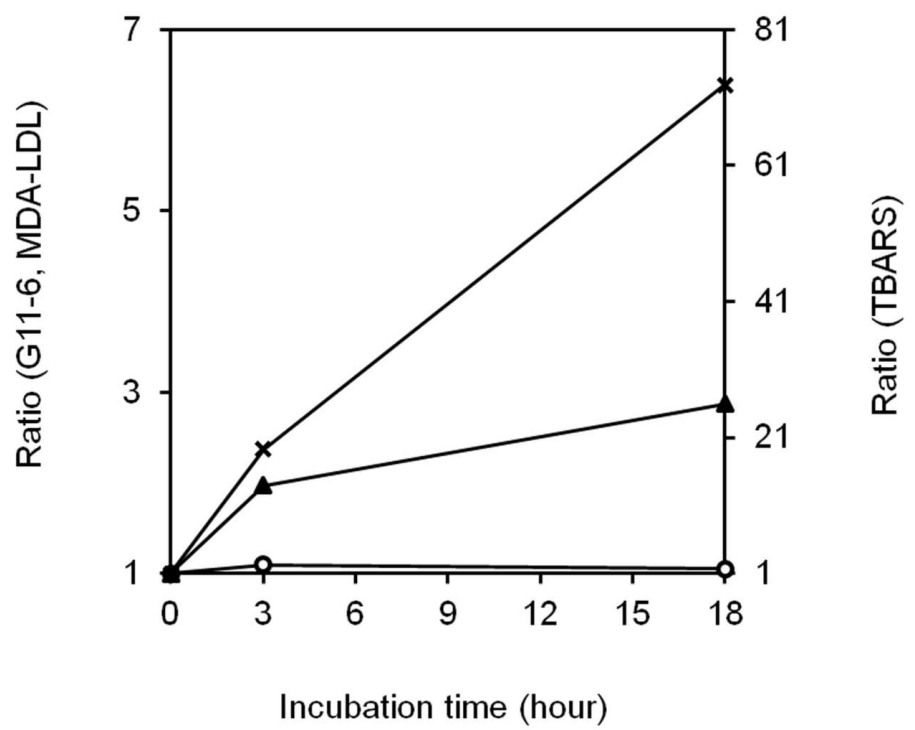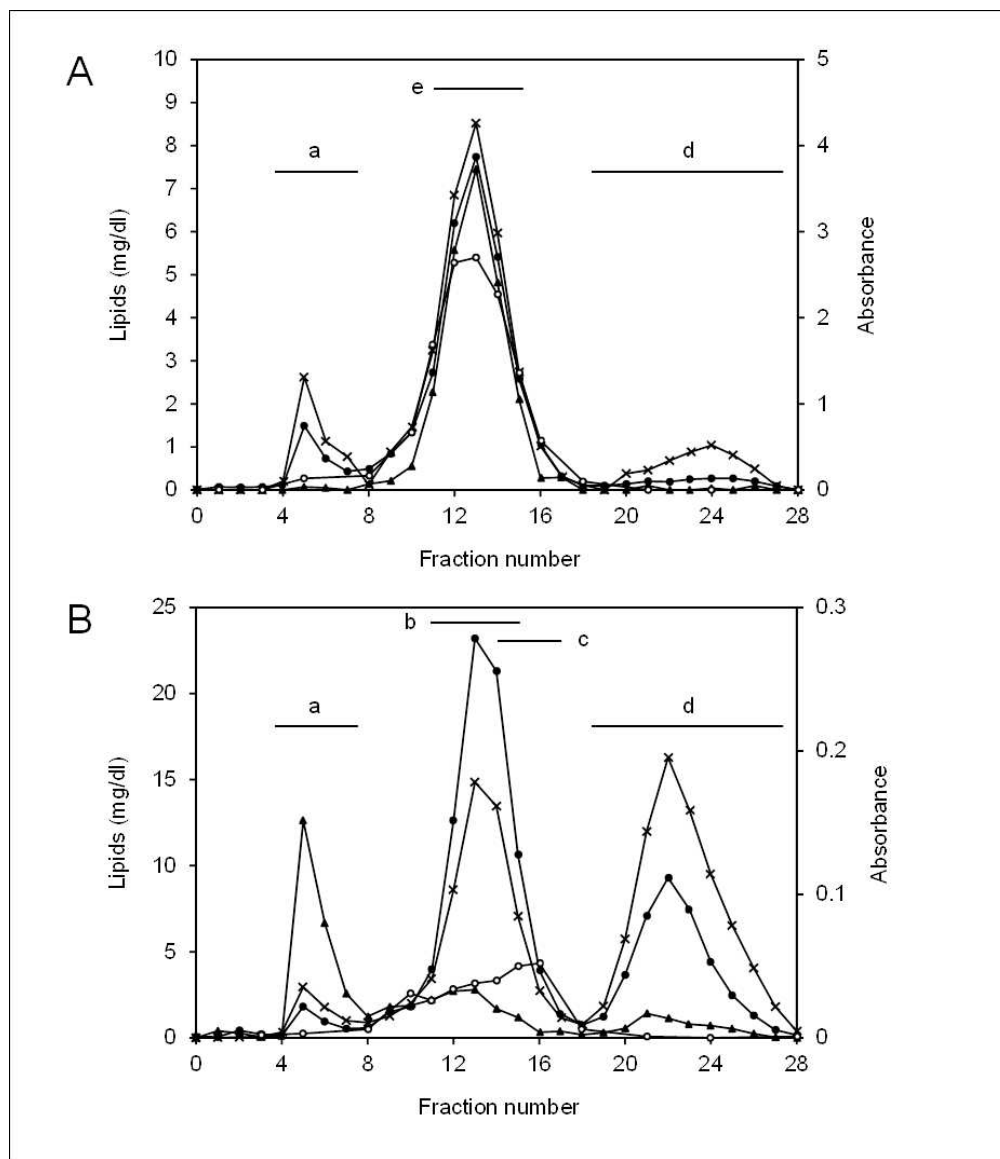148x104mm (300 x 300 DPI)

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