Apolipoprotein B (apoB) Concentrations in Lipoproteins in Cows

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ABSTRACT. The concentrations of apolipoprotein B (apoB)-48 and apoB-100 in triglyceride-rich lipoproteins (TRL), intermediate density lipoprotein (IDL), and low density lipoprotein (LDL) fractions separated by gel permeation chromatography were determined in Holstein and Japanese black cows by enzyme-linked immunosorbent assay (ELISA). A significant correlation ($p<0.01$) was observed between apoB-48 in TRL and plasma triglyceride (TG) levels in both Holstein and Japanese black cows. Additionally, apoB-48 in TRL and plasma TG levels in Holstein cows were significantly lower ($p<0.01$) than those in Japanese black cows. These results suggested that TG derived from intestinal (exogenous) TRL rather than from liver (endogenous) TRL was the major source of milk fat. — KEY WORDS: apolipoprotein B, bovine, triglyceride.

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Apolipoproteins, serve as a cofactor of enzymatic lipoprotein catabolism as well as ligands to receptors that regulate cellular lipoprotein uptake [13]. While, apolipoprotein B (apoB) is known as an available marker for apoB-containing lipoproteins such as chylomicron (CM), intermediate density lipoprotein (IDL), very low density lipoprotein (VLDL), and low density lipoprotein (LDL), since apoB is a limiting factor for the structures of those lipoproteins [19, 20]. The bovine apoB has two isoforms of apoB-100 and apoB-48 [1, 2, 10], of which the former is synthesized and secreted by liver, while the latter by intestine [7].

On the other hand, the mammary gland in dairy cows synthesizes and secretes a large quantity of milk fat, which originates from two different sources of fatty acid groups. The short and middle chain fatty acids (below C_{16}) is synthesized *de novo* from volatile fatty acids (VFA) in mammary gland [12, 16] and long chain fatty acids (above C_{16}) are derived from plasma triglycerides (TG) in apoB-containing lipoproteins [6, 11, 18].

For understanding TG metabolism in cows, it is necessary to analyze separately intestine-derived (apoB-48-containing) and liver-derived (apoB-100-containing) lipoproteins. However, it was quite difficult to analyze separately intestine- and liver-derived lipoproteins in cows by a common method of ultracentrifugation.

This note deals with apoB concentrations in lipoproteins by monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) in Holstein and Japanese black cows.

Animals: Twenty four Holstein cows kept in Animal Husbandry Experiment Station of Tokyo University and 17 Japanese Black cows grazing in Stock Farm of Tohoku University were used.

Samples: A total of 78 and 75 blood samples were collected from Holstein cows over 10 months and Japanese black cows over 5 months, respectively.

Chemicals: All chemical reagents were obtained from Wako Pure Chemical (Osaka, Japan) unless indicated.

Preparation of lipoproteins: Blood was obtained from 45 clinically healthy cows using 1.5 mg/m*l* disodium ethylenediamine-tetraacetic acid (Na_2EDTA) as anticoagulant. Plasma collected was added with 100 μg/m*l* sodium azide, 50 μg/m*l* gentamycin, 10 U/m*l* aprotinin, 1 mM benzamidine, and 10 μM phenylmethylsulfonyl fluoride. Lipoproteins, triglyceride-rich lipoproteins (TRL, d<1.006 g/m*l*) consisted of CM and VLDL, LDL (d=1.006–1.063 g/m*l*), and HDL (d=1.063–1.21 g/m*l*) were separated from the plasma by sequential ultracentrifugation according to the method of Hatch and Lees [8]. For total lipoproteins (TLP, d<1.21 g/m*l*) separation, the density was adjusted to 1.21 g/m*l* with sodium bromide solution.

Monoclonal antibodies for bovine apoB: For immunization of mice, the apoB-100 was separated from LDL by 5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and collected from the gel corresponding to the mobility of human apoB-100. Eight female 4-week-old BALB/c mice (Nippon SLC, Shizuoka, Japan) were intraperitoneally injected 3 times with 100 μg purified apoB-100 per head every 2 weeks. Freund complete adjuvand (Nacalai Tesque, Kyoto, Japan) was used in the 1st immunization, and Freund incomplete adjuvand (Nacalai Tesque, Kyoto, Japan) was used in the 2nd and 3rd immunization. Spleen cells were collected at 3 days after the final immunization and fused with P3U1 mouse myeloma cells by the procedure previously described [4]. The produced hybridoma cells were maintained in an Iscove's modified Dulbecco's medium (GIBCO, Grand Island, NY, U.S.A.) supplemented with 20% of fetal bovine serum (GIBCO, Grand Island, NY, U.S.A.), 2 mM L-glutamine, 50 μM 2-mercaptoethanol (GIBCO, Grand Island, NY, U.S.A.), 100 U/m*l*, penicillin, 100 μg/m*l* streptomycin, 36 mM sodium bicarbonate, 5 mg/*l* transferrin (Behringer Mannheim, Mannheim, Germany) and 8 mg/*l* insulin (Sigma, St. Louis, MO, U.S.A.). Hybridoma cells were screened for production of the antibodies against apoB by ELISA using LDL or purified apoB-100 as standard antigens. Isotype of monoclonal antibodies obtained were examined by the commercial typing kit (Mouse Monoclonal Typing Kit, Binding Site Limited, Birmingham, UK). Eleven clones of hybridoma cells were isolated and their isotypes are listed in Table 1. Figure 1 shows

Table 1. Isotypes of monoclonal antibodies obtained

MAb	Isotype
Mb6F4	IgG1
Mb8A8	IgG1
Mb8G8	IgG1
Mb8H11	IgG1
Mb9B8	IgG2a
Mb9C12	IgG1
Mb9D1	IgM
Mb9G3	IgG1
Mb10A6	IgM
Mb10B12	IgG1
Mb11B5	IgG2b

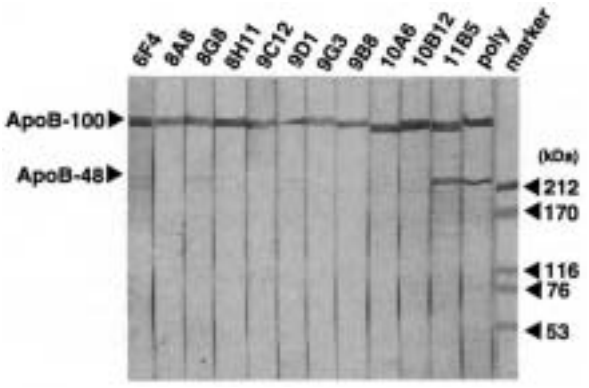

Fig. 1. Immunoblotting analysis of various monoclonal antibodies obtained to bovine apoB. ApoB-48 and apoB-100 were separated from TRL and LDL by SDS-gel (5%) and transferred onto a polyvinylidene difluoride membrane. After blocking free sites of membrane, strips were incubated overnight at 37°C with monoclonal antibodies. Lane Poly represents antiserum obtained from mice immunized with the purified apoB-100.

immunoreactivities of the monoclonal antibodies obtained against apoB-48 and apoB-100 by immunoblotting. One clone, Mb11B5 reacted with both apoB-100 and apoB-48, while the other 10 clones reacted with only apoB-100. No apoB-48-specific monoclonal antibody was obtained. Several researchers [5, 14] reported that the monoclonal antibodies against half of the N-terminal of human apoB-100 reacted with apoB-48, while those against half of the C-terminal did not. Powell *et al.* [17] demonstrated that both human apoB-48 and apoB-100 were coded on a single apoB gene, and apoB-48 was generated by the post-transcriptional editing of apoB-100 mRNA, i.e., from glutamine codon to a translational stop codon. Moreover, the presence of the editing of apoB-100 mRNA is reported in bovine intestine [7]. Therefore, bovine apoB-48 was considered to be immunochemically analogous to half of the N-terminal of apoB-100 as human apoB-48.

Gel filtration for separating TRL, IDL, LDL and HDL: Superose 6 HR 10/30 column (Pharmacia, Uppsala, Sweden) was equilibrated with 1 mM Na_2EDTA in phosphate-buffered saline (PBS, pH 7.4) at 20°C. Five hundred

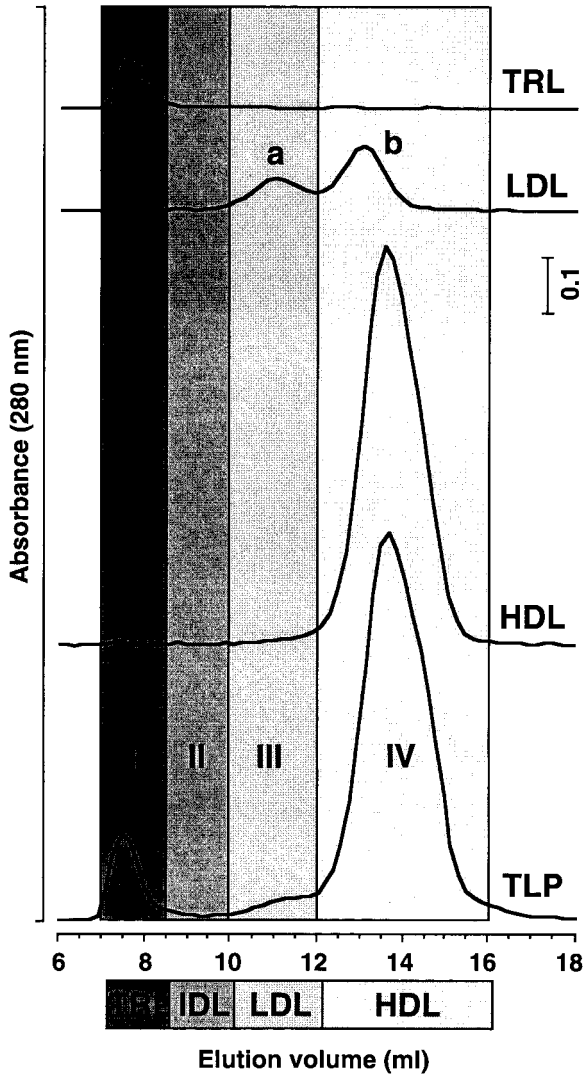

Fig. 2. Elution profiles of lipoproteins. TRL (d<1.006 g/m*l*), LDL (d=1.006–1.063 g/m*l*), HDL (d=1.063–1.21 g/m*l*), and TLP (d<1.21 g/m*l*) were separated by ultracentrifugation. Each lipoprotein was applied to Superose 6 HR 10/30 column and eluted at the rate of 0.5 m*l*/min. Eluent was collected into I, II, III, and IV fraction.

microliter of TLP was applied to the column and eluted at the rate of 500 μl/min. The eluate of lipoproteins was monitored by the absorbance at 280 nm and was collected for determination of apoB concentration by ELISA. Plasma TG levels were also determined by automatic analyzer (model 736–20, Hitachi, Tokyo, Japan). The Fractions I, II, and III of TLP corresponded to TRL, IDL, and LDL, respectively according to the elution profile of lipoproteins separated by ultracentrifugation (Fig. 2). Fraction IV included HDL and one peak (indicated as b) of LDL. Since neither apoB-48 nor apoB-100 were detected by ELISA in this fraction (data not shown), Fraction IV was considered to correspond to HDL.

Measurement of total apoB and apoB100 concentrations

by ELISA: Two monoclonal antibodies, Mb8A8 and Mb11B5 were used in apoB assay. The assay limits of apoB-100 and total apoB concentrations were 0.32 μg/m*l* and 0.21 μg/m*l*, respectively. The protein content of the standard LDL was measured by Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, U.S.A.) using bovine serum albumin (BSA) as a standard.

ELISA was carried out as follows: (1) One hundred microliter of the diluted standard LDL and samples in PBS (pH 7.4) were added to polystyrene microtiter plates (Nunc Immuno-Plate II, Nunc, Roskilde, Denmark) in tripricate, and incubated overnight at 4°C. (2) After washing the plates with PBS, remaining binding sites on the plates were blocked by the 200 μl/well of 3% BSA in PBS for 1 hr at room temperature. (3) The plates were washed 3 times with PBS-TB (0.05% Tween 20 and 0.1% BSA in PBS). Each mAb was diluted with PBS-TB, i.e., 1:2,000 for Mb8A8 and 1:100,000 for Mb11B5, and 100 μ*l* aliquot was added to each well and incubated for 1 hr at room temperature. (4) After washing with PBS-TB, 100 μ*l*/m*l* of peroxidase-conjugated goat anti-mouse IgG (Bio-Rad, Hercules, CA, U.S.A.) diluted 1:3,000 in PBS-TB was added and incubated for 1 hr at room temperature. (5) After washing, 100 μ*l* of a substrate solution containing 0.5 mM of 2,2'-azino-bis-(3-ethylbenzthiazolin-6-sulfonic acid) (Boehringer Mannheim, Mannheim, Germany) and 0.01% hydrogen peroxide in 0.1 M citrate buffer (pH 4.0) was added to each well and incubated for 1 hr at room temperature. The OD was measured at 415 nm by microplate reader (model 3550, Bio-Rad, Hercules, CA, U.S.A.). The concentrations of apoB-48 was calculated by subtracting apoB-100 from total apoB.

The concentrations of apoB-48 and apoB-100 in Fraction I (TRL), Fraction II (IDL), and Fraction III (LDL) in Holstein and Japanese black cows are shown in Table 2. The concentrations of apoB-100 in TRL in both Holstein and Japanese black cows were below the assay limit. Kleppe *et al*. [9] reported that VLDL secretion from cultured caprine hepatocytes was approximately 4% of that from rat hepatocytes and suggested hepatic VLDL synthesis in ruminants was remarkably smaller than that in non-ruminants. Therefore, apoB-48-containing lipoproteins (intestine-derived lipoproteins), CM, was considered to be the major constituent of TRL in cows. Figure 3 shows the correlations between plasma TG levels and apoB-48 or apoB-100 concentrations in the three lipoprotein fractions. The significant correlation was observed only between the concentrations of apoB-48 in TRL and plasma TG levels in both Holstein and Japanese black cows. These results suggested that plasma TG was mainly derived from intestine in cows. Additionally, the bovine and caprine lactating mammary gland were reported to utilize TG in TRL for milk fat formation [15], and high milk-yield lactating Holstein cows were considered to synthesize and secrete approximately 1.5 kg of milk fat consisted predominantly of TG per day [3]. On this basis, significantly lower levels ($p<0.01$) of plasma TG and apoB-48 in TRL in Holstein cows in comparison with Japanese black cows observed in this study suggested that apoB-48-containing lipoprotein (CM) derived from intestine is the major lipoprotein related to milk fat synthesis.

Table 2. ApoB-48 and apoB-100 concentrations (μg/m*l*) in 3 lipoprotein fractions, and plasma TG levels (mg/d*l*) in cows

Fraction	ApoB	Holstein	Japanese black
I (TRL)	48	0.82 ± 0.71	1.72 ± 1.18*
	100	–	–
II (IDL)	48	0.06 ± 0.15	0.18 ± 0.41
	100	3.02 ± 1.04	2.55 ± 1.19*
III (LDL)	48	ND	ND
	100	25.66 ± 5.74	23.77 ± 7.25
TG		9.3 ± 7.1	17.5 ±7.5*

Each value represents the mean ± SD. *: Significant difference between Holstein and Japanese black cows ($p<0.01$) was analyzed by Student's *t*-test, –: below assay limit (<0.32 μg/m*l*), ND: not determined.

The significantly higher ($p<0.01$) concentration of apoB-100 in IDL in Holstein cows compared with Japanese black cows was also observed, however, the cause of this is unknown. Further investigations will be necessary for elucidating the role of apoB-containing lipoproteins in milk fat formation.

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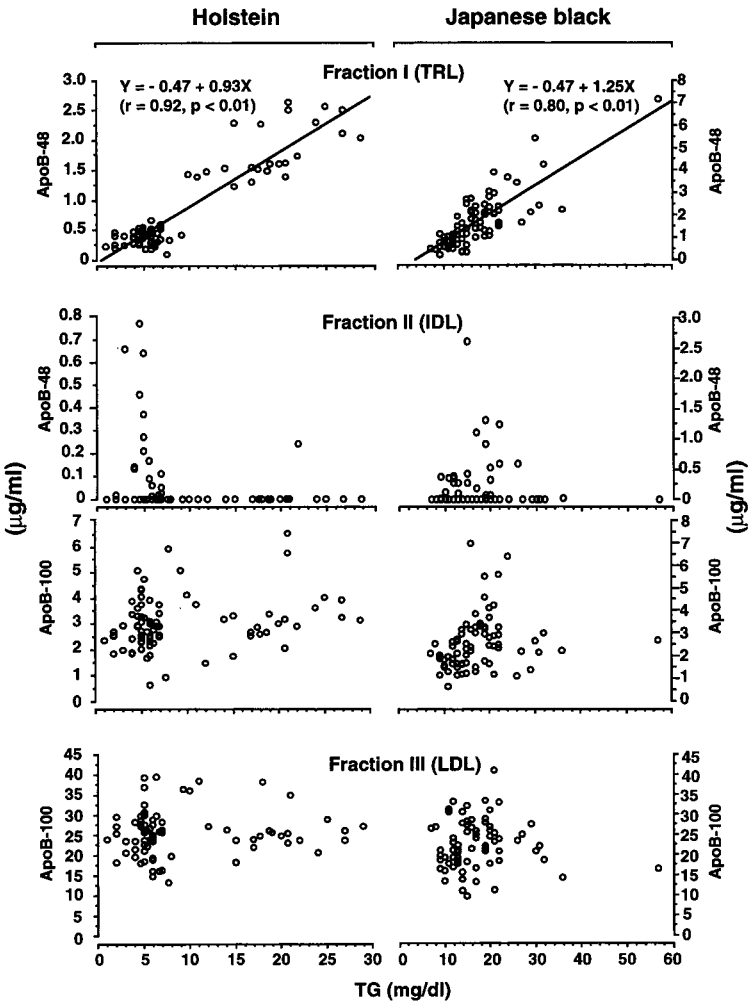

Fig. 3. Correlations between plasma TG and apoB concentrations in three lipoprotein fractions from Holstein and Japanese black cows. Significant correlation between apoB-48 in TRL and plasma TG ($p<0.01$) was alalyzed by Pearson's test.

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