Increased Serum Concentration of Apolipoprotein C-III and Its Greater Distribution to Chylomicrons than to the High-Density Lipoprotein Fraction in a Calf with Hyperlipidemia

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ABSTRACT. A calf having extremely high concentrations of triglycerides, cholesterol and phospholipids, in particular in chylomicrons (CM) and very low-density lipoprotein (VLDL) fraction was found. The purpose of the present study was to determine serum concentration and distribution of apolipoprotein (apo) C-III, a low molecular mass protein mainly distributed in high-density lipoprotein (HDL) fraction in normolipidemic cattle, in the calf with hyperlipidemia. The serum apoC-III concentration in the calf increased to more than 10-fold that of normolipidemic control calves, and apoC-III was distributed more in the CM than in the HDL. The concentration of apoA-I (a predominant apoprotein in the HDL) was also increased to nearly 4-fold that of controls in the serum from the calf, and its major distribution site was the CM. Haptoglobin was detected in the serum from the hyperlipidemic calf, and was distributed in the CM as well as in the HDL. Serum amyloid A was also induced. In contrast to apoC-III, apoA-I and haptoglobin, the majority of apoSAA was found in the HDL fraction, as observed in normolipidemic calves. Increased concentrations in the CM of apoC-III and apoA-I suggest that the two apolipoproteins may be involved in the pathogenesis of calf hyperlipidemia. The presence of haptoglobin in the CM and HDL also implies the relevance of this acute-phase protein in the regulation of lipid metabolism.

KEY WORDS: apolipoprotein C-III, bovine, chylomicron, haptoglobin, hyperlipidemia.

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Apolipoprotein (apo) C-III, the low molecular mass protein component of lipoproteins, is distributed in human plasma in chylomicrons (CM), very low-density lipoprotein (VLDL) and high-density lipoprotein (HDL) fractions [14, 16]. ApoC-III is associated with HDL in the fasting state, but is transferred to CM during absorption of dietary fat by the intestine [7]. The plasma concentration of apoC-III increases in patients with hypertriglyceridemia [5, 10], thereby suggesting the involvement of this apolipoprotein in the regulation of the metabolism of triglycerides (TG). There are two sites of synthesis of apoC-III: the liver and the small intestine, of which the liver is the major site [14].

We recently purified apoC-III from cow serum and determined its distribution in the lipoprotein fractions [22]. Cow apoC-III, in contrast to its human counterpart, is mainly detected in the HDL, not in the CM and VLDL fraction. The predominant distribution of apoC-III in HDL of normolipidemic cows is not changed by feeding [22], probably because of indistinct alimentary triglyceridemia attributable to the buffering effect of the rumen and the low fat content in the cattle diet [18]. We had an opportunity to collect serum and liver samples from a calf having extremely high concentrations of TG, cholesterol and phospholipids (PL), in particular in the CM and VLDL fraction. The analysis in the lipoprotein fractions of the calf with hyperlipidemia would seem to pro-

vide useful information on the relevance of apoC-III in lipid metabolism. The purpose of the present study was to determine the apoC-III concentration and its distribution among lipoprotein fractions in the calf with hyperlipidemia. The concentration and distribution of apoA-I (the major apoprotein in the HDL; the gene of which is reported to be linked with the *apoC-III* gene [9]) and distribution of haptoglobin (Hp), which has affinity to apoA-I [12], were also examined in the calf with hyperlipidemia.

MATERIALS AND METHODS

Samples: Sera were collected from 9 male normolipidemic (44- to 62-day-old) and 1 male hyperlipidemic Japanese black beef calves. Liver samples from 5 normolipidemic Holstein steers (2 years old) and the hyperlipidemic calf were also used.

Separation of lipoprotein fractions: All procedures were done at 20°C. CM ($d < 0.95$), VLDL ($d < 1.006$), low-density lipoprotein (LDL; $d < 1.063$) and HDL ($d < 1.21$) fractions from normolipidemic sera were prepared as described previously [20]. In the case of the hyperlipidemic serum, it was not possible to overlay the $d = 1.006$ solution onto the serum. The density solution (2 ml) was instead mixed with 4 ml of the serum that had been diluted 5.33-fold with phosphate-buffered saline (PBS), and the mixture was centrifuged at $26,000 \times g$ for 30 min. The floated lipid layer was washed twice by mixing with 4 ml of the $d = 1.006$ solution and by

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centrifuging at $26,000 \times g$ for 30 min. The washed lipid layer was used as the CM. The resulting bottom layer (4 ml) was mixed with 2 ml of the $d=1.006$ solution and centrifuged at $114,000 \times g$ for 16 hr to obtain VLDL. Separation of the LDL and HDL fractions from the hyperlipidemic serum was done as in normolipidemic sera. The final bottom layer, after the HDL was separated, was saved as the $d>1.21$ fraction (containing very high-density lipoprotein and lipoprotein-deficient fractions).

Lipid analysis: Lipids in lipoprotein fractions and in liver samples were analyzed as previously reported [15]. Briefly, lipids extracted with chloroform-methanol (2:1, vol/vol) containing 1.5 mM sulfuric acid were applied to a thin-layer plate (Silica gel 60, Merck, Darmstadt, Germany) and chromatographed using a solvent system of *n*-hexane-diethyl ether-acetic acid (80:20:1, by vol). The spots for TG, free cholesterol (FC), cholesteryl esters (CE) and nonesterified fatty acids (NEFA) were visualized by spraying 50% (vol/vol) sulfuric acid and heating at 120°C for 10 min. Their concentrations were estimated by comparing the absorbance at 590 nm, obtained by densitometer scanning, with those of authentic standards (Sigma Chemical Co., St Louis, Mo. U.S.A.). The concentration of PL in the extract was determined as reported by Bartlett [1].

Apolipoprotein analysis: Concentrations of apoC-III [23], apoA-I [17] and apoB-100 [24] were determined by enzyme-linked immunosorbent assay (ELISA). Immunoblot analysis of apoC-III, apoA-I, Hp and serum amyloid A (apoSAA) was performed as described previously [11]. ApoA-I was purified from the total lipoprotein fraction ($d<1.21$, containing CM to HDL) from a lactating cow by use of diethylaminoethyl-cellulose (DE-52, Whatman International Ltd, Maidstone, Kent, UK) chromatography. After delipidation with ethanol and diethyl ether, the lipoprotein fraction (298 mg of protein) was applied to a column (2.2×19.5 cm) equilibrated with 6 M urea and 10 mM Tris-HCl, pH 8.0. The column was washed with the equilibration buffer, after which bound proteins were eluted by use of a linear gradient of NaCl (0 to 0.25 M; total 800 ml). Fractions containing apoA-I were combined. The purity of apoA-I was assessed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and immunoblot analysis using anti-apoA-I, and was shown to be free from apparent contaminants. Purified apoA-I was used as the standard for ELISA and immunoblot analysis.

Other methods: Serum concentrations of TG, total cholesterol (TC), FC, PL, NEFA, blood urea nitrogen (BUN), total protein (TP) and albumin, and activities of lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and creatine phosphokinase (CPK) were measured using commercial kits (Wako Pure Chemicals, Osaka, Japan). The serum from the calf with hyperlipidemia was subjected to autoanalysis after the CM fraction had been removed by centrifugation. The serum CE concentration was calculated by subtracting the FC concentration from that for TC. All assays were done in duplicate or triplicate. Values are expressed as mean $\pm$ SD.

RESULTS

A calf with hyperlipidemia was found in Aomori Prefecture, Japan. Cloudy, white and turbid blood was noticed at 68 days of age during treatment for pneumonia. He died at 77 days of age. The main pathologic findings were acute mycotic esophagitis, rumenitis and reticulitis, fatty infiltration of the liver and the kidney, and catarrhal and serous pneumonia. The calf serum had higher AST, ALT and CPK activities and a higher BUN concentration than control calves (Table 1). Activity of LDH and concentrations of TP, albumin and TB were not largely different. The calf serum showed extremely high TG, FC, PL and NEFA concentrations, compared with those in the control calves (Table 2). An increased CE concentration was also observed, but it was not as distinct as for the other lipids. Lipid analysis of the lipoprotein fractions showed markedly higher TG, FC, CE and PL concentrations in the CM and VLDL fractions (Table 3). Except for CE, higher lipid concentrations were similarly observed in the LDL fraction. In the HDL fraction, all the lipid concentrations appeared to be similar to those in controls.

Excess TG was accumulated in the liver from the calf with hyperlipidemia (Table 4). Hepatic contents in FC, CE and NEFA were also higher in the calf than in controls. By comparison, PL content appeared to be lower than the control value.

The serum apoC-III concentration of the calf with hyperlipidemia was $289 \mu\text{g/ml}$ and was more than 10-fold higher

Table 1. Activities of enzymes and concentrations of urea nitrogen, protein and total bilirubin in the serum of a calf with hyperlipidemia

Variable	Control ^{a)}	Hyperlipidemia
LDH	$1,808 \pm 257$	1,233
AST	55.4 ± 11.1	237
ALT	10.6 ± 3.3	69
CPK	123 ± 48	14,375
BUN	9.10 ± 1.90	27
TP	6.12 ± 0.39	6.7
Albumin	3.47 ± 0.26	2.85
TB	0.74 ± 0.41	0.7

Units are IU/L for LDH, AST, ALT and CPK, mg/100 ml for BUN and TB, and g/100 ml for TP and albumin. a) Obtained from male Japanese black beef calves (44 to 62 days old; $n=9$).

Table 2. Serum lipid concentrations in a calf with hyperlipidemia

Lipid	Control	Hyperlipidemia
TG	33.8 ± 8.7	6,792
FC	22.3 ± 5.4	1,222
CE	110 ± 21	249
PL	194 ± 28	1,840
NEFA	0.329 ± 0.059	5.00

Units are mg/100 ml for TG, FC, CE and PL, and mEq/L for NEFA. Control serum samples were as in Table 1.

Table 3. Lipid concentrations in lipoprotein fractions from serum of a calf with hyperlipidemia

Lipoprotein/ Lipid	Control	Hyperlipidemia	Lipoprotein/ Lipid	Control	Hyperlipidemia
CM			LDL		
TG	0.488 ± 0.112 (8.1)	2,277 (78.3)	TG	1.40 ± 0.54 (23.3)	47 (1.6)
FC	0.180 ± 0.076 (0.7)	559 (71.1)	FC	9.82 ± 5.77 (40.4)	51 (6.5)
CE	0.319 ± 0.078 (0.3)	128 (56.9)	CE	26.0 ± 13.5 (24.5)	16 (7.1)
PL	0.966 ± 0.546 (2.0)	407 (58.6)	PL	12.3 ± 6.7 (26.0)	101 (14.5)
VLDL			HDL		
TG	1.50 ± 0.64 (25.0)	576 (19.8)	TG	2.62 ± 1.01 (43.6)	7 (0.3)
FC	0.413 ± 0.189 (1.7)	162 (20.6)	FC	13.9 ± 5.4 (57.2)	14 (1.8)
CE	0.281 ± 0.142 (0.3)	20 (8.9)	CE	79.4 ± 17.5 (74.9)	61 (27.1)
PL	2.40 ± 0.94 (5.1)	152 (21.9)	PL	31.6 ± 10.3 (66.9)	35 (5.0)

Unit is mg/100 ml of serum. Values in parentheses show the percent distribution in the 4 lipoprotein fractions.

Table 4. Lipid compositions in the liver of a calf with hyperlipidemia

Lipid	Control ^{a)}	Hyperlipidemia
TG	5.53 ± 2.90	49.2
FC	2.60 ± 0.87	4.83
CE	0.452 ± 0.185	2.54
PL	37.3 ± 8.8	19.5
NEFA	0.470 ± 0.212	2.55

Unit is mg of lipid/g of liver (wet weight).

^{a)} Obtained from Holstein steers (2 years old; n=5).

than those of normolipidemic Japanese black beef calves ($25.8 \pm 7.9 \mu\text{g/ml}$; n=9). The apoC-III concentration in the control calves was lower than that in lactating Holstein cows (approximately $100 \mu\text{g/ml}$) [23], but was comparable to the value for Holstein heifers ($37.1 \pm 3.1 \mu\text{g/ml}$, n=18; 7- to 10-month-old; Yamamoto *et al.*, unpublished results). Concentrations of apoC-III ($\mu\text{g/ml}$ of serum) in the lipoprotein fractions from the calf with hyperlipidemia were 250 for CM, 11.2 for VLDL, 31.3 for LDL, 40.5 for HDL; and <4 for the $d>1.21$ fraction. The recovery of apoC-III in the lipoprotein fractions was estimated to be 115% when compared to its serum concentration ($289 \mu\text{g/ml}$). The predominant distribution of apoC-III in the CM rather than in the HDL fraction in the calf with hyperlipidemia was further confirmed by immunoblot analysis (Fig. 1). The percent distributions of apoC-III were 77.4% in CM, not detected in VLDL, 4.6% in LDL, 17.9% in HDL and not in the $d>1.21$ fraction, and these values were comparable to those obtained by ELISA. Bands for apoC-III in the lipoprotein fractions, in particular in the CM and LDL, migrated more slowly than did the apoC-III standard, which had been delipidated during the purification procedures. Moreover, all the apoC-III bands, including that from whole serum, were arc-shaped, as previously observed for the apoC-III band in the HDL fraction [22]. The slow migration of the arc-shaped bands seemed to be caused by the excess presence of lipids. We did not delipidate lipoprotein fractions and serum from the hyperlipidemic calf, because delipidation results in losses of apoC-III and other apolipoproteins to some extent. In the control calves, almost all apoC-III was detected in the HDL fractions, as observed

Fig. 1. Immunoblot analysis of apoC-III in the lipoprotein fractions from serum of a calf with hyperlipidemia. CM, VLDL, LDL and HDL fractions (undiluted) and $d>1.21$ fraction and serum (diluted 20-fold with PBS) were mixed with a half volume of the SDS-sample buffer and boiled for 3 min. Volumes applied per well were $3 \mu\text{l}$ (CM to HDL) and $10 \mu\text{l}$ ($d>1.21$ fraction and serum). Purified apoC-III ($0.586 \mu\text{g}$) was run in the right lane.

in cows [22] (Fig. 2). The predominant distribution in the HDL of apoC-III in the control calves was also shown by ELISA (data not shown).

The concentration of apoA-I in serum from the calf with hyperlipidemia was $2,700 \mu\text{g/ml}$, whereas in the control calves, it was $691 \pm 169 \mu\text{g/ml}$. ApoA-I was mainly distributed in the HDL (74.0%) and the LDL (26.0%) fractions from sera of the control calves (Fig. 3). Like in apoC-III, apoA-I was distributed more in the CM than in the HDL in the calf with hyperlipidemia (68.0% in CM, not detected in VLDL, 10.3% in LDL, and 21.7% in HDL). The acute-phase protein Hp was detected in serum from the calf with hyperlipidemia. Hp in the calf with hyperlipidemia was distributed in the CM as well as in the HDL (Fig. 4). The percent distri-

Fig. 2. Immunoblot analysis of apoC-III in the lipoprotein fractions from sera of 2 normolipidemic Japanese black beef calves (A and B). Lanes: 1, 5, CM; 2, 6, VLDL; 3, 7, LDL; 4, 8, HDL; and 9, apoC-III (0.586 µg). Undiluted lipoprotein fractions were mixed with a half volume of the SDS-sample buffer and 10 µl of each of the fractions was applied to wells. Similar results were obtained when lipoprotein fractions from 7 other calves were analyzed.

Fig. 3. Immunoblot analysis of apoA-I in the lipoprotein fractions from sera of a normolipidemic calf (A) and a calf with hyperlipidemia (B). Lanes: 1, 6, CM; 2, 7, VLDL; 3, 8, LDL; 4, 9, HDL; 5, 10, serum; and 11, purified apoA-I (0.205 µg).

bution of Hp in the lipoprotein fractions could not be determined because of unclear bands of Hp in the CM. In the calf, apoSAA was also induced. As observed in normolipidemic calves with pneumonia [21], apoSAA was predominantly distributed in the HDL (29.0% in CM, not detected in VLDL and LDL, and 71.0% in HDL) (Fig. 5). The serum concentration of apoB-100 in the hyperlipidemic calf (108 µg/ml) appeared to be similar or lower than in normolipidemic cows [24] and in 6-month-old male Holstein calves (210 µg/ml, *n*=35; Oikawa *et al.*, unpublished results). We failed to detect, by immunoblot analysis, the apoB-100 band in lipoprotein fractions and serum from the hyperlipidemic calf, probably because of interference by excess lipids of the

transfer from the gel to the membrane of the large molecular mass protein apoB-100 (>500 kDa). In the control calves, the majority of apoB-100 was detected in the LDL fraction (figure not shown).

DISCUSSION

The present study indicated that the serum apoC-III concentration was increased in the calf with hyperlipidemia. The increase in this concentration resulted in the greater distribution of apoC-III to the CM than to the HDL fraction. An increased apoA-I concentration in the CM was also observed. Hp was induced in the hyperlipidemic calf and was detected

Fig. 4. Immunoblot analysis of Hp in the lipoprotein fractions from serum of a calf with hyperlipidemia. Lanes: 1, CM; 2, VLDL; 3, LDL; 4, HDL; 5, serum; and 6, purified haptoglobin (0.165 μ g).

in the CM as well as in the HDL.

The origin of apoC-III in the CM is not known. Redistribution from the HDL was initially proposed. However, this hypothesis appears to be inconsistent with the finding that the apoC-III concentration in the HDL of the hyperlipidemic calf (40.5 μ g/ml) was not largely different from that in normolipidemic control calves (25.8 μ g/ml) and also that HDL lipid concentrations were in the control ranges. ApoSAA was induced in the calf with hyperlipidemia. This acute-phase protein in plasma is associated with HDL [2, 21]. The association of apoSAA results in a decrease of HDL lipids, and lipids released from the apoSAA-enriched HDL redistribute to other lipoprotein fractions [4]. It is possible to assume that HDL lipids and apoC-III, whose concentrations are inferred to be increased by accelerated hepatic secretion, were redistributed to the CM in response to the binding of apoSAA. An alternative explanation is that apoC-III in the CM is of intestinal origin. The synthesis of apoC-III by this tissue may be greatly increased.

In mice, overexpression of the apoC-III gene results in hypertriglyceridemia [8], whereas disruption of the apoC-III gene induces hypotriglyceridemia [13]. Rat apoC-III suppresses the uptake of TG-rich CM remnants by the liver [19], by inhibiting apoC-II activation of lipoprotein lipase [3], the enzyme responsible for hydrolysis of TG in the CM and VLDL. It is conceivable from the results obtained using laboratory animals that the calf's hyperlipidemia was primarily caused by an increase of the apoC-III concentration, and that excess apoC-III in turn resulted in inhibition of the hepatic removal of the remnants, thereby increasing the TG concentration.

In addition to the TG concentration, the calf with hyperlip-

Fig. 5. Immunoblot analysis of apoSAA in the lipoprotein fractions from serum of a calf with hyperlipidemia. Lanes: 1, CM; 2, VLDL; 3, LDL; 4, HDL; 5, serum; and 6, purified apoSAA (0.309 μ g).

idemia showed higher FC, CE and PL concentrations particularly in the CM and VLDL fractions. Human heritable hyperlipidemia is classified mainly into 5 types [6]. Except for type I [3], the apoC-III concentration increases in these heritable hyperlipidemias [5, 10, 16]. Because of increases in both TG and cholesterol concentrations, the calf hyperlipidemia appeared to be similar to type V, in which CM and VLDL concentrations are elevated. Separation of the VLDL fraction free from the CM is very difficult, particularly in hyperlipidemic serum. Other than the ultracentrifugal method, separation of the VLDL from the CM was tried by gel filtration chromatography. This attempt was unsuccessful because of the turbidity of the serum. The detection of apoC-III, apoA-I and Hp in the CM, not or only faintly in the VLDL, implies that the VLDL fraction was not largely contaminated by the CM, thereby suggesting that the concentration in the VLDL, as well as that of the CM, was substantially increased in the calf with hyperlipidemia. The concentration of apoB-100, the major apolipoprotein in the VLDL and the LDL, appeared not to be largely altered in the calf with hyperlipidemia, in spite of the increases in the VLDL lipid concentrations. This discrepancy may be explained by the different metabolic pathways of VLDL lipids and apoB-100; VLDL lipids are absorbed by the liver and extrahepatic tissues after hydrolysis by lipoprotein lipase, whereas apoB-100 serves as the ligand for the receptor-mediated LDL uptake by steroidogenic tissues.

It is unclear whether calf hyperlipidemia is heritable or not, although hyperlipidemia was not found in other brothers or sisters from the same venter. The calf showed fatty infiltration of the liver and the kidney. Increases in AST and ALT activities appear to be caused by fatty liver, whereas that in the BUN concentration may be attributable to fatty infiltration of the kidney. Fatty liver, as well as fatty infiltration of the kidney, is thought to be primarily caused by an increased NEFA concentration, rather than decreases in hepatic secretions of VLDL and HDL, which are observed in lactating cows [15, 20]. Because TP, albumin and TB concentrations were in the control ranges, liver functions appeared not to be severely affected. The increase in CPK

activity seemed to be a secondary effect.

The linkage of apoC-III and apoA-I genes has been shown in human chromosome 11 [9]. As in apoC-III, an increased concentration of apoA-I, as well as the predominant distribution in the CM than in the HDL, was observed in the calf with hyperlipidemia. It is conceivable that a close linkage between the two genes also exists in cattle and that apoA-I is overexpressed in association with apoC-III. Haptoglobin is induced in cows with fatty liver [25]. It has an affinity to apoA-I [12]. We have recently shown that haptoglobin is distributed in the HDL fractions, in addition to a $d > 1.25$ lipoprotein-deficient fraction, in sera of cows with fatty liver and calves with pneumonia [11]. In the calf with hyperlipidemia, haptoglobin appeared to be induced by fatty liver or inflammation (such as pneumonia) or by both, and bound to apoA-I distributed in both the CM and HDL fractions.

The majority (>70%) of apoSAA was found in the HDL fraction in the calf with hyperlipidemia. We used apoSAA as a negative control for the preferential distribution to the CM of apoC-III, apoA-I and Hp. That apoSAA was detected mainly in the HDL suggests that the preferential distribution in the CM fraction of apoC-III, apoA-I and Hp cannot entirely be explained by artefacts caused by the excess presence of lipids in the CM (such as co-flotation of the HDL particles with CM lipids during ultracentrifugation).

In conclusion, apoC-III was suggested to be involved in the pathogenesis of calf hyperlipidemia. The linkage of apoC-III and apoA-I genes and the affinity of apoA-I to Hp were also assumed to be present in cattle. Further studies on cases of hyperlipidemia may resolve the functional relations of apoC-III, apoA-I and Hp in the lipid metabolism.

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