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Hepatic and Serum Bile Acid Compositions in Patients with Biliary Atresia: A Microanalysis Using Gas Chromatography-Mass Spectrometry with Negative Ion Chemical Ionization Detection

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Abukawa, D., Nakagawa, M., Iinuma, K., Nio, M., Ohi, R. and Goto, J. *Hepatic and Serum Bile Acid Compositions in Patients with Biliary Atresia: A Microanalysis Using Gas Chromatography-Mass Spectrometry with Negative Ion Chemical Ionization Detection.* Tohoku J. Exp. Med., 1998, **185** (4), 227-237 —— Hepatic and serum bile acids in five patients with biliary atresia were preoperatively determined by microanalysis using gas chromatography-mass spectrometry with negative ion chemical ionization detection. The hepatic content of total bile acids was markedly elevated (3079±711 nmol/g protein), most of which were primary bile acids. Accumulation of unconjugated bile acids (2.93% to 4.62% of the total) was observed in the liver tissue of these patients, although only trace amounts were detected in their sera. The ratio of glycine-conjugated to taurine-conjugated bile acids was 0.44±0.18 in liver tissue and 0.79±0.52 in serum and these values were significantly lower than those of controls. This study has shown that the composition of bile acids in serum does not reflect that in liver tissue faithfully. The accumulation of these hydrophobic bile acids may contribute to initiating or exacerbating liver injury in infants with cholestatic liver diseases. ———— hepatic bile acids; biliary atresia; gas chromatography-mass spectrometry

Biliary atresia (BA) is one of the most common cholestatic liver diseases of infancy, and is a major cause of fatal childhood cirrhosis. The most striking manifestation of bile acid metabolism in BA is an impairment of the enterohepatic circulation due to partial or complete absence of the bile conduit system. Though the main function of the enterohepatic circulation is appropriate cycling of the

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bile acid pool through the liver and intestine, in BA most of the bile acid pool is stored in the liver. Hence the composition of hepatic bile acids in a patient with BA reflects the dynamics of the bile acid pool, and analysis of bile acids in liver tissue could provide useful information on the state of bile acid metabolism in these patients.

While many investigators have analyzed serum and urinary bile acid profiles in patients with BA (Délèze and Paumgartner 1977; Javitt et al. 1977; Tazawa et al. 1984; Nittono et al. 1986), to our knowledge there has been no report on the bile acids in the liver tissue of these patients. In this study, we determined the profiles of hepatic and serum bile acids in patients with BA by gas chromatography-mass spectrometry with negative ion chemical ionization detection (GC-NICI-MS), an excellent derivatization method for bile acid microanalysis. We have focused on the differences in composition and conjugation comparing the main bile acids in the liver to those in the serum of these patients.

Materials and Methods

Patients and control subjects

Five female patients with BA were studied. Clinical manifestations and serum biochemical findings are shown in Table 1. All of the patients were fed artificial formula and underwent the Kasai procedure between 50 and 67 days of age. Two of the five cases of BA were classified as correctable forms (type I and II), and three as noncorrectable forms (type III) as determined by operative cholangiography (Kasai 1974). From each patient, liver tissue was obtained at the time of initial laparotomy, and serum was obtained from blood collected prior to the operation.

As control subjects, we obtained liver tissue specimens from two infants, ages 3 months and 7 months, who had extrahepatic malignant neoplasms. Their serum biochemical data were as follows; total bilirubin, 0.9 and 0.2 mg/100 ml; direct bilirubin, 0.5 and 0.1 mg/100 ml; aspartate aminotransferase (AST), 69 and 80 IU/liter; alanine aminotransferase (ALT), 40 and 34 IU/liter; lactate dehydrogenase (LDH), 542 and 1080 IU/liter, respectively. Sera were obtained from five outpatients without hepatobiliary disease as control subjects at the age of 1–3 months.

All of the parents of the patients and control subjects were informed of this study and consented.

Preparation of bile acids in liver and serum

Hepatic bile acids were determined as described by Goto et al. (1988). Five to 10 mg of liver tissue was washed with ice-cold saline, and then 10–100 ng of unconjugated, glycine-conjugated and taurine-conjugated $^{18}O,^{2}H$-labelled lithocholic acid (LCA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA) and cholic acid (CA) were added as internal standards. The liver tissue was then

TABLE 1. *Clinical manifestations observed and serum biochemical findings for five patients with biliary atresia*

Patient	Sex	Age at surgery (days)	Type	T-Bil (mg/100 ml)	D-Bil (mg/100 ml)	ALP (IU/liter)	γ-GTP (IU/liter)	AST (IU/liter)	ALT (IU/liter)	LDH (IU/liter)	T-Chol (mg/100 ml)	Lp-x (mg/100 ml)	BUN (mg/100 ml)	Creatinine (mg/100 ml)
M.Y.	F	50	I	12.4	8.7	1965	517	345	194	1048	319	positive[a]	7	0.2
A.M.	F	55	III	6.4	4.4	705	91	118	73	541	113	10.8	12	0.2
K.O.	F	63	III	12.8	8.0	711	143	182	84	805	125	47.2	12	0.2
K.I.	F	64	III	5.3	4.0	739	127	88	64	630	121	17.4	13	0.2
S.S.	F	67	II	7.8	5.8	948	442	166	108	638	123	72.7	9	0.3

T-Bil, total bilirubin; D-Bil, direct bilirubin; ALP, alkaline phosphatase; γ-GTP, γ-glutamyltranspeptidase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; T-Chol, total cholesterol; Lp-X, lipoprotin-X; BUN, blood urea nitrogen.

[a] >10 mg/100 ml

suspended in 1 ml of 5% sodium hydrochloride and solubilized at 80°C for 30 minutes. A 100 μl aliquot was used to determine the concentration of protein according to the method described by Bradford (1976). The remaining solution was neutralized with 10% hydrochloric acid and then passed through a Sep-Pak C_{18} cartridge (Waters Assoc., Milford, MA, USA). Bile acids were eluted with 90% ethanol from the cartridge, and applied to a column of piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20 see the "Materials" below) for stepwise elution of unconjugated, glycine-conjugated and taurine-conjugated bile acids. The same was done to extract serum bile acids from a 100 μl sample of serum. After hydrolysis of glycine and taurine conjugates with cholylglycine hydrolase, deconjugated bile acids were extracted through a Sep-Pak C_{18} cartridge for removal of proteins and inorganic salts. The unconjugated fraction and the individual deconjugated fractions were converted to the pentafluorobenzyl (PFB) ester-dimethylethylsilyl (DMES) ether derivatives.

Gas chromatography-mass spectrometry

Capillary GC-MS was carried out using MM12030 quadrupole mass spectrometer (VG Analytical, Manchester, U.K.) equipped with HP 5790A gas chromatograph (Hewlett-Packard, Avondale, PA, USA). A cross-linked 5% phenylmethyl silicone fused-silica capillary column (20 m$\times$0.3 mm I.D., J & W Scientific, Folsom, CA, USA) was inserted into the ion source through a direct inlet. The temperature of the column oven was programmed to 200°C–285°C after a few minutes' delay from the starting time. The injection port and ion source were maintained at 280°C and 270°C, respectively. Helium was used as a carrier gas, and isobutane as a reagent gas. The ionization energy was set at 70 eV and the emission current was 400 μA.

Principal bile acids were analyzed by selected ion monitoring (SIM) GC-MS using a characteristic carboxylate anion $[M-181]^-$ formed by elimination of the PFB group. The monitoring ions of mono-, di- and trihydroxy bile acids were m/z 461, 563, 665 and their [^{18}O,^{2}H] analogs were m/z 464, 569, 674, respectively. The amount of each bile acid was determined from the ratio of its peak area to that of the corresponding internal standard.

Statistical analysis

Results were expressed as mean$\pm$standard deviation (S.D.) for each group, except for liver tissue controls as there were only two subjects in this group. Data of the patients in the BA group were compared to those of control subjects by Mann-Whitney's U test, and a value of $p<0.05$ was considered statistically significant.

Materials

Bile acids and cholylglycine hydrolase were supplied by Sigma (St. Louis,

MO, USA) and DMES-imidazole was obtained from Tokyo Kasei Kogyo (Tokyo). Glycine- and taurine-conjugated bile acids were prepared in our laboratories (Goto et al. 1979). PHP-LH-20 (acetate form, 0.6 mEq/g) was prepared in the manner previously described (Goto et al. 1978).

Results

The hepatic content of total bile acids was markedly elevated (3079±711 nmol/g protein, range 1895–3712) in all patients with BA, while that of control subjects at the age of 3 months and 7 months was 823 and 589 nmol/g protein, respectively (Table 2). Primary bile acids (CA plus CDCA) accounted for most of the total bile acids in liver tissue, and only trace amounts of secondary bile acids (LCA, DCA and ursodeoxycholic acid [UDCA]) were detected. The ratio of CA to CDCA (C/CDC) in the five patients varied (1.59±0.75, range 0.75–2.63); higher than 1.0 in three, lower than 1.0 in one, and nearly 1.0 in one patient.

Total serum bile acid levels in the BA group were also strikingly increased (122.5±58.0 nmol/ml, range 65.8–218.8 nmol/ml) as shown in Table 3. Also, in serum, primary bile acids accounted for most of the total bile acids. Although the C/CDC ratio in the serum of the patients varied similar to that in liver tissue, the mean C/CDC ratio (1.30±0.96) was significantly higher than that of control subjects (0.40±0.25; $p<0.025$). The C/CDC ratio in the serum of each patient did not correspond with that in liver tissue.

In the BA patients, the mean ratio of glycine to taurine conjugates (G/T) was 0.44±0.18 in liver tissue and 0.79±0.52 in serum, and these values were significantly lower than those of controls (liver tissue: 1.32 as the mean value, $p<0.05$; serum: 1.84±0.92, $p<0.05$). The unconjugated fraction accounted for 2.93% to 4.62% of all hepatic bile acids in the BA patients, while only trace amounts (0–0.25%) were detected in their sera ($p<0.005$; Fig. 1). The percentage of serum unconjugated bile acids in the BA patients was significantly lower than that of control subjects (4.60±2.60%; $p<0.005$).

Discussion

Bile acids present in urine and systemic blood are the result of spillover from the enterohepatic circulation and their composition is, therefore, similar but not the same as that in the enterohepatic circulation (Akashi et al. 1983). To elucidate the state of the enterohepatic circulation and the biosynthesis of bile acids, several authors have reported the composition of hepatic bile acids in healthy adults and adult patients with hepatobiliary disease (Greim et al. 1972, 1973; Yanagisawa et al. 1980; Akashi et al. 1983, 1987; Nagakura et al. 1989; Honda et al. 1995; Fischer et al. 1996; Setchell et al. 1997). However, bile acids in liver tissue of cholestatic infants have not been determined previously due to methodologic limitations in analyzing a small amount of sample material. Goto et al. (1987) established a new derivatization method for microanalysis of bile

TABLE 2. *Hepatic bile acid profiles of five patients with biliary atresia*

	Total bile acids (nmol/g protein)	Composition							Conjugation				
		LCA (%)	DCA (%)	CDCA (%)	UCDA (%)	CA (%)	CA+CDCA (%)	C/CDC	Unconjugated (%)	Glycine (%)	Taurine (%)	G/T	
Patient ($n=5$)													
M.Y.	3210	0	0.04	50.28	0	49.67	99.96	0.99	2.93	29.89	67.19	0.44	
A.M.	3712	0.05	0	27.51	0	72.43	99.95	2.63	4.28	40.02	55.71	0.72	
K.O.	1895	0	0	36.22	0	63.78	100	1.76	3.03	29.61	67.36	0.44	
K.I.	3528	0.20	0.03	35.20	0.14	64.42	99.62	1.83	4.54	23.36	72.10	0.32	
S.S.	3049	0	0	57.18	0	42.83	100	0.75	4.62	19.48	75.90	0.26	
Mean ±S.D.	3079 ±711			41.28 ±12.10		58.63 ±12.05	99.91 ±0.15	1.59 ±0.75	3.88 ±0.83	28.47 ±7.80	67.66 ±7.60	0.44 ±0.18	*
Control ($n=2$)													
1	823	0.17	0	32.84	0	67.00	99.89	2.04	0.84	52.95	46.21	1.15	
2	589	0.19	0	79.01	0	20.76	99.77	0.26	3.08	58.05	38.88	1.49	
Mean	706	0.18	0	55.92	0	43.88	99.80	1.15	1.96	55.50	42.54	1.32	

LCA, lithocholic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; UDCA, ursodeoxycholic acid; CA, cholic acid; C/CDC, the ratio of CA to CDCA; G/T, the ratio of glycine to taurine conjugates.

*$p<0.05$.

TABLE 3. *Serum bile acid profiles of five patients with biliary atresia*

	Total bile acids (nmol/ml)	Composition							Conjugation			
		LCA (%)	DCA (%)	CDCA (%)	UDCA (%)	CA (%)	CA + CDCA (%)	C/CDC	Unconjugated (%)	Glycine (%)	Taurine (%)	G/T
Patient ($n=5$)												
M.Y.	218.8	0	0.16	41.32	0	58.53	99.84	1.42	0	39.34	60.66	0.65
A.M.	65.8	0	0	25.81	0	74.20	100	2.88	0	60.76	39.24	1.55
K.O.	110.3	0.14	0.32	48.79	0	50.75	99.54	1.04	0.25	51.44	48.31	1.06
K.I.	123.5	0.18	0	55.57	0	44.25	99.82	0.80	0	28.26	71.74	0.39
S.S.	94.0	0.15	0	72.00	0	27.85	99.85	0.39	0	23.42	76.58	0.31
Mean	122.5			48.70		51.11	99.81	1.30		40.65	59.31	0.79
±S.D.	±58.0			±17.10		±17.15	±0.49	±0.96		±15.60	±15.64	±0.52
Control ($n=5$)												
Mean	12.3	0.35	0.41	72.66	0.12	26.47	99.13	0.40	4.60	58.60	36.81	1.84
±S.D.	±7.9	±0.24	±0.30	±11.60	±0.28	±11.76	±0.49	±0.25	±2.60	±11.94	±12.35	±0.92

LCA, lithocholic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; UDCA, ursodeoxycholic acid; CA, cholic acid; C/CDC, the ratio of CA to CDCA; G/T, the ratio of glycinye to taurine conjugates.

*$p<0.05$.

**$p<0.01$

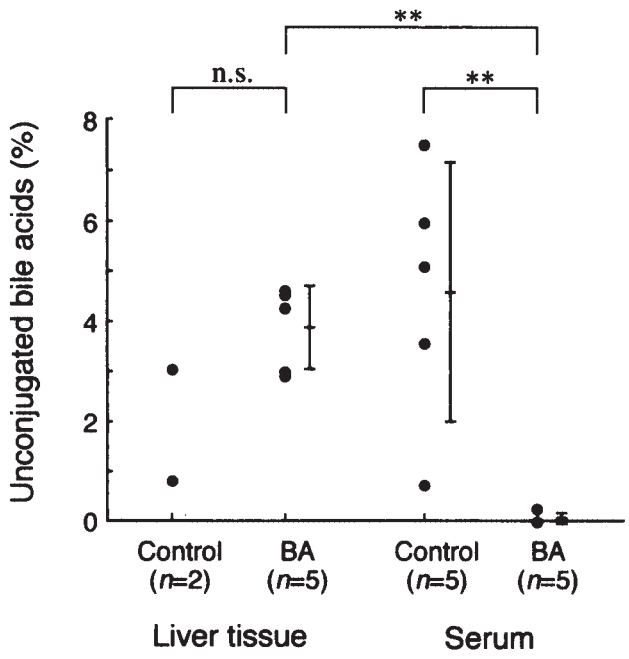

Fig. 1. Percentage of unconjugated bile acids in liver tissue and serum. BA, biliary atresia; n.s., not significant; $^{**}p<0.01$; ⊥, Mean and S.D.

acids using GC-NICI-MS. By this method, it became possible to analyze the complete profile of bile acids in 2-10 mg of liver tissue, with the detection limit for unconjugated bile acids being 10 pg/mg liver tissue (Goto et al. 1988). Thus we were now able to determine the profile of bile acids in liver tissue obtained from patients with BA.

The evaluation of bile acids metabolism in infancy should be considered with the effects of aging, particularly with regard to the activities of hydroxylation and conjugation. Because of difficulty in obtaining informed consent, we were able to obtain liver tissue specimens for controls from only two infants at the age of 3 months and 7 months, older than the patients with BA at the age of 50-67 days. However, CA and CDCA pool sizes, when corrected for body surface area, do not change with age between 7 weeks and 10 months (Heubi et al. 1982). In healthy infants, the G/T ratio in serum shows a lower value during the first month of life than that after the neonatal period with little further change at any age (Niijima 1985). In addition, the ratio of levels of conjugated bile acids to total bile acids in serum is more than 90% constantly during the first year of life (Niijima 1985). Hence we consider that it is appropriate to compare the absolute content, the G/T ratio and the proportion of unconjugated bile acids in liver tissue between the patients and the control subjects.

We found that bile acids had markedly accumulated in the liver tissue of BA patients, and that the unconjugated fraction comprised about 4% of total hepatic bile acids, whereas only trace amounts were detected in their sera. Thus, the absolute content of unconjugated bile acids in the liver of these patients is considered to be much higher than that in normal infants. In vitro, the cytotox-

icity of bile salts is known to be proportional to their detergent effect, which is related to the hydrophobic-hydrophilic proportion dependent upon the degree of hydroxylation and conjugation (Schölmerich et al. 1984; Attili et al. 1986). Also, in vivo, liver injury can occur when the liver is perfused with fluid containing a high proportion of strongly detergent bile acids. The detergent effect of unconjugated or glycine-conjugated bile acids is stronger than that of taurine-conjugated or sulfated bile acids (Attili et al. 1986). Unconjugated bile acids may not be easily excreted from the liver because of their high hydrophobicity, leading to their accumulation in liver tissue. The accumulation of these hydrophobic, cholestatic bile acids may contribute to initiating or exacerbating liver injury.

The G/T ratios both in liver tissue and in sera of these patients were significantly lower than those of controls. This result is in agreement with a report by Nittono et al. (1986). In cholestasis, taurine conjugation increases because required bile acid synthesis and conjugation decrease (Hardison 1978). Taurine conjugates have a higher polarity than glycine conjugates (Schölmerich et al. 1984) and taurine conjugation leads to enhanced excretion from liver tissue. These changes in hepatic bile acid metabolism may serve to protect the liver from accumulation of hydrophobic bile acids which are potentially more cytotoxic.

The predominance of CDCA in cholestatic infants has been reported (Délèze and Paumgartner 1977; Javitt et al. 1977; Nittono et al. 1986), and has been ascribed to diminished activity of 12α-hydroxylase, a key enzyme for synthesis of CA. However, the C/CDC ratios of our five patients varied both in liver tissue and in serum, similar to the findings in serum reported by Tazawa et al. (1984). Since CA predominates in the serum of normal infants in this period (Niijima 1985), hepatocytes in our patients with high C/CDC ratios may not have been seriously impaired and 12α-hydroxylation in these hepatocytes may still occur.

In conclusion, this study has shown that the profile of hepatic bile acids in preoperative patients with BA has characteristics as follows; marked accumulation of bile acids particularly the unconjugated fraction, and decreased G/T ratio. The discrepancy with respect to the proportion of unconjugated bile acids between the value for liver tissue and that for serum in each patient indicates that the composition of bile acids in serum does not reflect that in liver faithfully. Since hepatic bile acid metabolism reflects the dynamics of the bile acid pool when the enterohepatic circulation is significantly impaired, analysis of bile acids in liver tissue must be available in order to evaluate the possible mechanisms of tissue damage in cholestatic liver diseases in children.

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