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HEPATIC COPPER ACCUMULATION IN PATIENTS WITH PRIMARY BILIARY CIRRHOSIS

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ABSTRACT

Liver biopsy specimens from 18 patients with primary biliary cirrhosis were examined histochemically and by energy-dispersive x-ray microanalysis. Using two indices, we classified hepatic copper accumulation into three stages based on the Cu x-ray intensity of cuproproteins that had accumulated in hepatocyte lysosomes and on the binding ratio of postulated copper transfer proteins between the cytosol and lysosomes. Eight patients were in stage 1 with an initial accumulation of lysosomal cuproproteins, mediated by transfer proteins not saturated with copper. Two patients were in stage 2, in which transfer proteins were saturated with copper. The first two stages gave negative results for histochemical copper. The remaining eight patients were in stage 3, in which copper accumulation detected by histochemical included transfer proteins saturated with copper and large amounts of lysosomal cuproproteins. Five patients (one each in stages 1 and 2, and three in stage 3) underwent a second liver biopsy after treatment with 600 mg of ursodeoxycholic acid daily for 14 to 39 months. Results of blood chemistry tests improved, but liver histologic findings and copper accumulation were unchanged in all five patients. It seems likely that ursodeoxycholic acid does not affect the copper accumulation in hepatocyte lysosomes that reflects the state of cholestasis in patients with primary biliary cirrhosis.

Key Words: Primary biliary cirrhosis, Copper, Cuproprotein, Ursodeoxycholic acid

INTRODUCTION

In healthy subjects, excess copper, most of which is first incorporated into cuproproteins in the liver, is later eliminated into the bile[1, 2]. When the biliary tract is obstructed, cuproproteins are reabsorbed from the bile and retained within the hepatocytes. Increased hepatic copper is an index of chronic cholestasis. Histochemically detectable cuproproteins are found only in the periportal hepatocytes of patients with advanced primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC)[3]. Energy-dispersive x-ray microanalysis, a more sensitive method than histochemical analysis, can disclose small amounts of cuproproteins in hepatocyte lysosomes[3–5]. In a previous study[6], golden hamsters were loaded with copper by being provided with drinking water containing 0.5% cupric acetate, and specimens of their liver were examined by x-ray microanalysis. Cu x-ray intensity was found to reflect the amounts of cuproproteins that had accumulated in hepatocyte lysosomes, and it was postulated that proteins with a binding ratio of $\Delta(Cu/P)/\Delta(S/P)$ transferred copper between the cytosol and the lysosomes when

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correlation was good between the Cu/P and S/P of lysosomal cuproproteins. We classified hepatic copper accumulation into three stages on the basis of two indices: the Cu x-ray intensity and the binding ratio. In stage 1, copper accumulation is mediated by transfer proteins not saturated with copper. Stage 2 involves transfer proteins saturated with copper. In stage 3, copper accumulation detected by histochemical analysis includes transfer proteins saturated with copper and large amounts of lysosomal cuproproteins.

Poupon *et al.*[7] first reported that treatment with ursodeoxycholic acid (UDCA) improves results of biochemical tests of patients with PBC. In a controlled double-blind trial[8], hepatic histopathological findings were improved in six of the ten patients treated for nine months with UDCA but worsened in four of the eight patients receiving a placebo. Another controlled trial of 146 patients[9] showed that all of the characteristic histopathological features except for fibrosis improved with two years of treatment with UDCA. Progression from a noncirrhotic state to cirrhosis was found in all three patients treated with UDCA in another study[10]. To predict the long-term effects of UDCA on patients with PBC, it is important to know the target cells of this drug and its effect on chronic cholestasis. If the drug is choleretic or protective of the biliary tract, increased secretion of bile would improve the copper metabolism. In this study, we first tested whether the staging of copper accumulation obtained from the animal experiment described above[6] was applicable to human livers with copper accumulation because of chronic cholestasis. Then the staging was used to evaluate the extent of cholestasis and the therapeutic effect of UDCA on patients with PBC.

MATERIALS AND METHODS

Clinical features of the 18 patients with PBC are summarized in Table 1. All of the patients were women, and ages were from 36 to 70 years. Their informed consent was obtained. Three patients suffered from itching. In all patients, anti-mitochondrial antibodies were present, and immunoglobulin M was high. Liver biopsy was done to check the diagnosis of PBC for all patients. A second liver biopsy was done in five patients after 14 to 39 months of treatment with UDCA at 600 mg/day. The serum levels of γ-glutamyl transpeptidase and alanine aminotransferase were measured every month during treatment. Serum levels of immunoglobulin M, bile acids, and copper were measured at the time of each biopsy. Serm bile acids were measured by the method of Okuyama *et al.*[11].

All liver specimens were divided into two portions. The first portion was fixed with formalin for routine histochemical testing including copper staining with *p*-dimethyl-aminobenzylidine rhodanine. The histopathological stage was decided by Scheuer's classification[12]. Copper staining was scored as 0 for negative, 1 for the occasional appearance of hepatocytes stained for copper in periportal areas, and 2 for the frequent appearance of such hepatocytes in periportal areas. The second portion was fixed in a chilled solution of 0.5% glutaraldehyde and 0.2 M cacodylate buffer (pH 7.4) as reported previously[4]. After being rinsed in distilled water, 1-mm cubes were treated with 1.5 M sucrose, mounted on aluminum holders, and frozen in liquid nitrogen (−180°C) until being sectioned. A glass knife and the specimen holder holding a frozen specimen were cooled at −100°C with use of an LKB cryokit, and the frozen specimen was cut into 80-nm sections with an LKB ultramicrotome. The sections were mounted on Maxtaform gold grids coated with carbon (Gluticulus, London, England) with 1.5 M sucrose solution as the carrier and dried at room temperature. Electron staining was omitted. The x-ray analysis was done in a Hitachi H-800 electron microscope with a Kevex 7000-Q energy-dispersive x-ray analyzer. The specimen chamber was cooled with liquid nitrogen to reduce contamination with

Table 1. Clinical Features of Patients[a)]

Patient No.	Age	Symptoms	Remarks
1[b)]	43	–	–
2	51	–	Obesity
3	49	Itching	–
4	65	–	–
5	42	–	–
6[b)]	37	–	–
7	52	–	–
9	53	Itching	–
10	43	–	–
11	61	–	–
12	43	–	Diabetes mellitus
13	36	Itching	–
14[b)]	52	–	–
15	62	–	–
16	52	–	–
17[b)]	56	–	Gallstones
18[b)]	70	–	Polyarthritis

[a)] All patients were women.

[b)] Patient underwent a second biopsy after UDCA treatment.

silicon from the detector window. The accelerating voltage was 100 kV and the current beam was 10^{-10}A. Au Mα appears between P Kα and S Kα, so central areas as far from the gold wires as possible were examined to minimize overlapping.

To identify the location of copper, sulfur, and phosphorus in the cells, the hepatocytes were mapped with Kα x-rays of these elements. Hepatocyte lysosomes were first identified by their location, ultrastructure, and electron density by scanning transmission electron microscopy, and then their identification was confirmed by the content of elements measured by x-ray microanalysis. In selected area analysis, ten or more lysosomes with different densities were analyzed for each patient. An area of the lysosomal matrix 0.45 μm square was scanned for 200 sec. The Kα x-rays for phosphorus, sulfur, calcium, iron, and copper were recorded as the x-ray counts and as a molar percentage of all of the elements detected. Cu x-ray intensity was defined as the mean of the three largest Cu Kα x-ray counts. The molar percentage of copper and that of sulfur were divided by the molar percentage of phosphorus to standardize the thickness of ultrathin sections, and the correlation between copper (Cu/P) and sulfur (S/P) in each patient was examined. When correlation was good, $\Delta(Cu/P)/\Delta(S/P)$ was used to express the copper binding ratio of a postulated copper-transfer protein between the cytosol and lysosomes. The Cu x-ray intensity and the copper binding ratio for individual patients were combined with the histochemical Cu staining for that patient, and copper accumulation of the 18 patients was classified into three stages as previously described[6)]. The effects of UDCA treatment for the five patients who underwent a second liver biopsy was evaluated by evaluation of changes in the stages of both liver histopathology and copper accumulation.

RESULTS

The location of copper and that of sulfur was the same in the hepatocyte lysosomes, and phosphorus was found diffusely throughout the cytoplasm (Fig. 1). Histopathological staging and indices of altered copper metabolism at the start of the study are summarized in Table 2. Seven patients had hypercupremia. The coexistence of copper and sulfur, which are major elements of cuproproteins of hepatocyte lysosomes, was found in all 18 patients. The Cu x-ray intensity, which reflects the amount of lysosomal cuproproteins, ranged between 800 and 40,200.

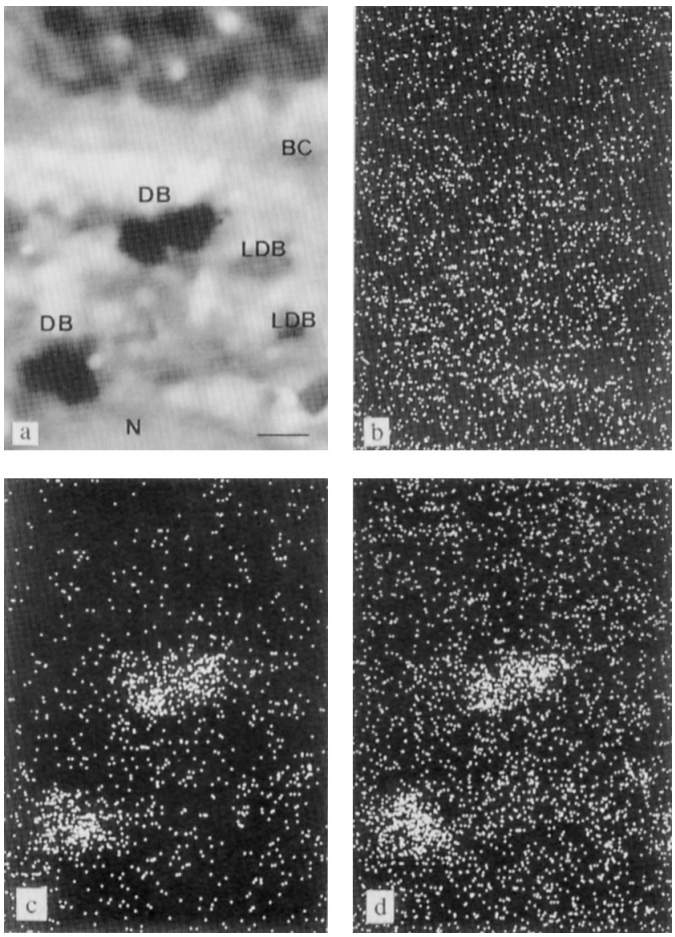

Fig. 1. X-ray analysis of hepatocyte lysosomes before treatment of a patient with PBC.

a: Scanning transmission electron microscopy shows subcellular structures of the hepatocytes, such as electron-dense bodies (DB), bodies with less electron density (LDB), a bile canaliculus (BC), and a nucleus (N). The bar indicates 1 micron. The same structures were examined by x-rays of P, Cu, and S. b: Mapping with P x-rays does not show hot areas in any hepatocyte. Mapping with Cu (c) and S (d) x-rays respetively indicates abundant DB, i.e., the electron-dense lysosomes contain large amounts of copper and sulfur.

Table 2. Copper Indices and Histopathological Findings

Patient No.	Serum Cu (80–150 μg/dl)	Histopathological Stage	Stage of Cu Accumulation	Cu Stain	ΔCu/ΔS	X-ray Intensity ×10²
1[a]	149	1	1	0	−0.01[b]	13
2	[c]	2	1	0	−0.03[b]	8
3	109	2	1	0	0.03	7
4	116	2	1	0	0.07	17
5	144	2	1	0	0.17	25
6[a]	134	2	1	0	0.19	18
7	169	2	1	0	0.19	32
8	132	2	2	0	0.59	152
9	125	2	2	0	0.75	80
10	143	2	3	1	0.61	204
11	120	2	3	1	0.69	267
12	144	3	1	0	0.39	34
13	182	3	3	1	0.66	298
14[a]	207	3	3	1	0.71	402
15	155	3	3	1	0.80	245
16	192	3	3	1	0.74	314
17[a]	180	3	3	2	0.53	246
18[a]	161	3	3	2	0.87	233

[a] Patient underwent a second biopsy after UDCA treatment.

[b] No correlation between Cu/P and S/P because of low cuproprotein concentration.

[c] Not assayed.

Figure 2 shows the correlation between S/P and Cu/P in a patient, indicating that cuproproteins were major components in lysosomal proteins and that copper transfer between the cytosol and lysosomes in this patient was mediated by a cuproprotein. The binding ratio of copper to sulfur in this postulated transfer cuproprotein, $\Delta(Cu/P)/\Delta(S/P)$, was estimated to be 0.75. Transfer proteins were identified in 16 of the 18 patients. The highest copper binding ratio was 0.87. In two patients, however, there was no correlation between Cu/P and S/P even though copper and sulfur were detected in hepatocyte lysosomes. Both patients had low x-ray intensity, suggesting that cuproproteins were minor components of lysosomal proteins in their hepatocytes, and not enough for estimation of a transfer protein.

By a modified method of Yagi et al.[6], copper accumulation of human livers were classified into three stages (Fig. 3). Eight patients were in stage 1 of copper accumulation, marked by a low x-ray intensity. In six of these eight patients, copper transfer proteins were identified; their copper binding ratio was correlated with the x-ray intensity. Their liver specimens were histochemically negative for copper. The two other patients in whom a transfer protein could not be identified were in this stage because their x-ray intensity was low. Two patients were in stage 2 of copper accumulation. This stage, in which copper was histochemically undetectable, was characterized by a high copper binding ratio (more than 0.5), and a medium Cu x-ray intensity, between 6,000 and 18,000. Eight patients with histochemically detectable copper were in stage 3 of copper accumulation. Their Cu x-ray intensity was more than 18,000. The copper binding ratio, however, was between 0.5 and 0.9.

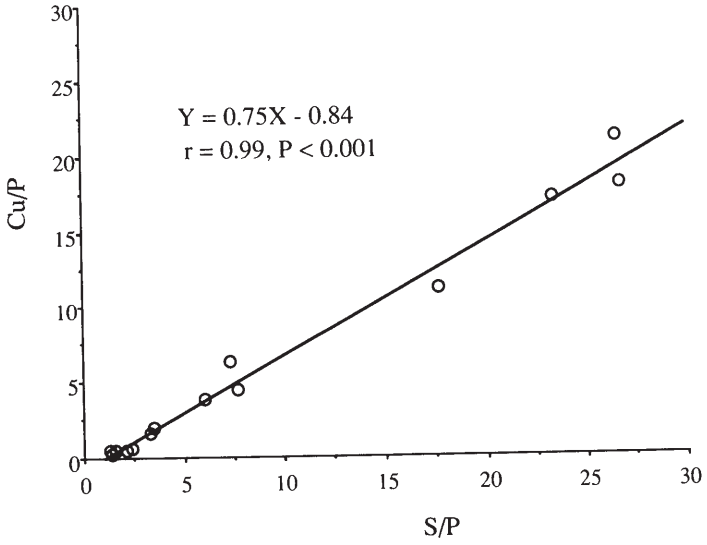

Fig. 2 Correlation between Cu/P and S/P in the hepatocyte lysosomes before treatment of a patient (No. 9) with PBC.

The correlation suggests that copper transfer between the cytosol and lysosomes is mediated by a cuproprotein with a binding ratio of copper to sulfur expressed by $\Delta(Cu/P)/\Delta(S/P)$. The binding ratio was estimated as 0.75 here. The patient had no copper detectable by histochemical analysis and her Cu x-ray intensity was 8,000. Selected area analysis was done of 14 lysosomes.

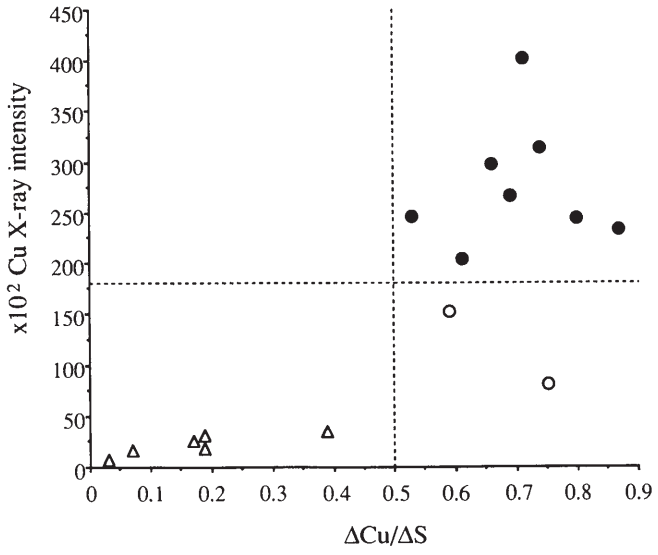

Fig. 3. Three stages of copper accumulation in hepatocyte lysosomes of patients with PBC.

The lower left area is for stage 1 of copper accumulation, in which both indices of Cu x-rays are low and correlated (triangles). These patients all lacked copper detectable histochemically. When the copper binding ratio increases to more than 0.5, the Cu x-ray intensity is not related to the binding ratio. The lower right area is for stage 2. The patients lacked copper detectable histochemically, and had moderate Cu x-ray intensities (open circles). The upper right area is for stage 3, in which patients with copper detectable histochemically had high Cu x-ray intensities with a fixed copper binding ratio between 0.5 and 0.9 (closed circles). We suggest that a Cu x-ray intensity of 18,000 is a suitable borderline between stages 2 and 3 of copper accumulation in human livers.

The effects of UDCA on blood chemistry results and liver histopathological findings of the five patients who underwent a second liver biopsy are summarized in Table 3. Administration of UDCA improved the results of biochemical tests including γ-glutamyl transpeptidase and alanine aminotransferase. Immunoglobulin M and total bile acids showed nonspecific changes. Ursodeoxycholic acid increased from 5.4% ± 5.5% to 44.3% ± 19.7% of total bile acids. Serum levels of copper decreased to the normal range in three patients. Ursodeoxycholic acid did not change the pathological features of portal tracts characteristic of PBC, and all five patients were in the same stage of liver histopathology before and after treatment. Their stage of copper accumulation was also the same as before treatment.

DISCUSSION

Chronic cholestasis of patients with PBC was evaluated by hepatocellular cuproproteins for the first time here, to the best of our knowledge. In a preliminary classification published earlier[6], based on results of copper being fed to hamsters for 12 weeks, the Cu x-ray intensity of 50,000 was used as a boundary between stages 2 and 3. In liver specimens from patients with PBC, 18,000 was chosen instead. Differences in the species and the mechanism of copper accumulation may account for the different boundary. Except for this boundary, classification of hepatic copper accumulation obtained from copper-loaded hamsters was found to be applicable to livers of human patients with chronic cholestasis. The coexistence of copper and sulfur in hepatocyte lysosomes and correlation between Cu/P and S/P of lysosomal copper-sulfur complexes were found in most patients. These observations suggest that hepatocyte lysosomes contained a large amount of cuproproteins and that copper was transferred by a protein with a binding ratio of $\Delta(Cu/P)/\Delta(S/P)$ when a cholestatic liver had excess copper.

A modified classification of copper accumulation was used to evaluate the extent of cholestasis in patients with PBC, and three stages could be defined for cuproproteins present in human hepatocytes. The first two stages, in which copper was not detectable histochemically, are important clinically. Stage 2 of copper accumulation, in which slightly more accumulation will make the copper detectable histochemically, should be differentiated from stage 1 with an initial accumulation of lysosomal cuproproteins.

Ursodeoxycholic acid has a stronger choleretic action than other bile acids[13]. The main pathway of copper excretion is biliary, but it is not known whether copper excretion into the bile is increased when bile secretion is increased by UDCA treatment. When the indices of Cu x-rays were used to evaluate the therapeutic effect of UDCA, the daily dose of 600 mg did not cause excretion of excess hepatic copper into the bile in patients with PBC. Cuproproteins remained within the hepatocyte lysosomes of all of the patients even when UDCA was effective as judged by the biochemical test results. The large amount of lysosomal cuproproteins does not necessarily require chelation therapy because cuproproteins are not hepatotoxic but are detoxified products in scavenger organelles.

The main target of UDCA may be hepatocytes rather than the epithelial cells of bile ducts. When patients with gallstones and chronic active hepatitis were treated with UDCA, serum transaminase levels decreased[14]. In rats, UDCA prevented chenodeoxycholic acid from causing biliary protein secretion[15]. These observations suggest that the main effect of UDCA is protection of hepatocytes. In fact, UDCA improved the biochemical indices of our patients, but not the histopathological findings of their livers. Ursodeoxycholic acid did not change hepatic copper metabolism, an index of chronic cholestasis, probably because UDCA affected chronic nonsuppurative destructive cholangitis little. Pulsed doses of methotrexate, which were first found to

Table 3. Effect of UDCA

Patient No.	Interval[a] (months)	Serum Cu (80–150 μg/dl)	ALT (0–45 IU/L)	γ-GTP[b] (0–40 IU/L)	IgM (100–215 mg/dl)	TBA[c] (0–10nmol/ml)	UDCA (%)	Histopathological Stage	Stage of Cu Accumulation
1	22	149–145	155–58	464–302	414–491	6.5–6.3	0–32.6	1–1	1–1
6	39	134–126	193–56	615–72	960–608	12.8–33.6	5.5–49.1	2–2	1–1
14	22	207–133	223–97	378–183	509–417	52.4–61.2	12.9–27.1	3–3	3–3
17	27	180–136	198–86	656–375	470–498	[d]–135.1	[d]–36.3	3–3	3–3
18	14	161–145	61–25	195–46	1794–1920	5.7–32.2	3.2–76.5	3–3	3–3

[a] Interval between the first and second biopsies.

[b] γ-Glutamyl transpeptidase.

[c] Total bile acid.

[d] Not assayed.

Left sides of columns show before treatment, and right sides show after treatment ended.

Serum levels of copper became normal in all three patients who had hypercupremia before UDCA treatment.

Serum activities of ALT and γ-GTP decreased. Total bile acids changed to various extents with UDCA, but the percentage of UDCA increased to more than 25 % of total bile acids.

be effective in primary sclerosing cholangitis, have been tried in patients with PBC[16]. Patients with PBC treated by UDCA may need an additional medicine such as methotrexate that acts on the primary lesions in the biliary tract.

In conclusion, the classification of hepatic copper accumulation brought about in hamsters by oral loading was tested in livers of human patients with chronic cholestasis. Modified criteria were used to divide copper accumulation into three stages, as in the animal experiment. When the new classification was used to evaluate UDCA therapy, results suggested that the therapy does not improve the primary lesions of chronic cholestasis.

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