Is copper hepatotoxic in primary biliary cirrhosis?

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SUMMARY In primary biliary cirrhosis (PBC) liver copper retention occurs as a complication of cholestasis. By analogy with Wilson's disease, it has been suggested that copper retention is hepatotoxic in PBC, and this has been the rationale for the use of D-penicillamine in this disease. The hypothesis that copper is hepatotoxic in PBC has not been tested and in this study we have evaluated the role of liver copper retention in the pathogenesis of PBC.

Sixty-four patients with PBC have been studied. Fifty-four had increased liver copper concentrations. Liver cell synthetic function was well preserved. All the patients had normal prothrombin times, and only two had subnormal serum albumin concentrations. There was no correlation between liver copper concentrations and the degree of liver cell damage assessed biochemically (aspartate transaminase), and histologically. Electron microscopy was performed on liver biopsies from five patients with markedly increased liver copper concentrations. The liver cell ultrastructure was compatible with cholestasis. Liver cells contained electron dense lysosomes, which were shown to contain copper and sulphur by x-ray probe microanalysis. The characteristic organelle changes associated with copper toxicity in Wilson's disease were not observed.

The biochemical, histological, and histochemical differences between PBC complicated by liver copper retention, and Wilson's disease, indicates that there are differences in the handling of copper in these disease. In this study we could find no evidence to suggest that copper plays an important role in the pathogenesis of liver dysfunction in PBC.

Primary biliary cirrhosis (PBC) is a chronic cholestatic liver disease complicated by liver copper accumulation.¹ By analogy with Wilson's disease, and acute copper sulphate poisoning, it has been suggested that copper is hepatotoxic in PBC, and this has been the rationale for clinical trials of D-penicillamine treatment.^{2,3} The analogy between copper-induced liver cell damage in Wilson's disease and PBC is superficial, and there has been no attempt to test the hypothesis that copper is hepatotoxic in PBC. The aim of this study is to evaluate the role of liver copper retention in the pathogenesis of PBC.

Patients and methods

Sixty-four patients with PBC diagnosed on the

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usual criteria⁴ have been studied. The initial diagnostic liver biopsies were performed using a copper-free Menghini needle, and liver copper concentrations were measured by neutron-activation analysis.⁵ Serum albumin and the prothrombin time (after Vitamin K) were used as markers of hepatic synthetic function, and serum aspartate transaminase activities (AST) as a marker of liver cell damage. To investigate the relation between liver copper concentrations and histological liver damage, the diagnostic liver biopsy specimens from 38 PBC patients with increased liver copper concentrations were examined by light microscopy. An equal number of control biopsies from patients with other liver diseases were also examined. Sections from each biopsy specimen were stained with haematoxylin and eosin, for reticulin, with rhodanine (for copper) and orcein (for copper-associated protein). The biopsies were viewed without prior knowledge of the diagnosis or liver copper concentration. Histological features were graded on a scale of 0 to + + + (0 = absent, + = minimal or mild, + + = mod-

erate, +++ = marked or severe). In Wilson's disease, copper cytotoxicity is associated with characteristic organelle abnormalities seen on electron microscopy.⁶⁻⁹ To determine whether these organelle abnormalities occur in PBC, electron microscopy was performed on liver biopsies from five PBC patients with markedly raised liver copper concentrations. The elemental composition of liver cell lysosomes seen on electron microscopy were studied by x-ray probe microanalysis. For this purpose, 2000 Å (200 nm) sections were cut from Epon blocks using glass knives. The sections were picked up on nylon grids supported by a carbon holder, and unstained sections were probed using a Keveex energy dispersive spectrometer, and a Jeol 100 C electron microscope provided with a Jeol ASID scanning attachment. The material was studied in the scanning transmission mode at 100 kV using a take-off angle of 30°. The counting time was 50 s, at a counting rate of 1500 counts/s. In order to detect primarily copper, sulphur, and zinc, the window was set at a width of 9 channels. There was no contribution to the spectra from the microscope.

Results

The mean liver copper concentration was 321 µg/g dry weight (range 32-1388 µg/g; normal adult less than 55 µg/g). Fifty-four patients (85%) had increased liver copper concentrations, and 29 (45%) had liver copper concentrations within the range associated with homozygous Wilson's disease (Fig. 1).

Hepatocyte function was well preserved in the 64 patients studied. All had normal prothrombin times. The mean serum albumin concentration was 42.5 ± 4.6 g/l (mean ± SD; normal range 35-50 g/l), and only two patients had subnormal albumin concentrations. In patients with increased liver copper concentrations, there was no correlation between liver copper and serum AST activities ($r = 0.29$).

Histological features evaluated on liver biopsies included focal and piecemeal necrosis, portal periportal and lobular inflammation, steatosis, nuclear vacuolation, fibrosis and overall inflammation and liver cell damage ("histological activity"). When the graded histological features were plotted against liver copper concentration, no correlation or trends were observed. In particular, there was no correlation with focal or piecemeal necrosis, nor with overall histological activity (Fig. 2). Nuclear vacuolation and steatosis (two characteristic histological features of Wilson's disease¹⁰) were not seen.

Electron microscopy was performed on five liver

Fig. 1 Liver copper concentrations in 64 patients with PBC.

biopsies (range of liver copper concentration 502-970 µg/g dry weight). The ultrastructural changes were consistent with cholestasis.^{11 12} The liver cell mitochondria were large, oval or elongated. The cristae were curled or curved, and occasional dilatation of cristae was observed. The inner and outer mitochondrial membranes were normally apposed. The characteristic mitochondrial abnormalities seen in the early phase of Wilson's disease (ballooned mitochondria with marked dilatation of cristae, inclusion bodies, and separation of inner and outer mitochondrial membranes), were not seen. The Golgi apparatus and smooth endoplasmic reticulum showed morphological evidence of hypertrophy, and membrane bound glycogen and bile was seen in the cytosol. Irregular electron dense bodies bound by a single membrane, resembling secondary lysosomes were prominent in some liver cells (Fig. 3).

The presence of copper and sulphur was demonstrated by x-ray probe analysis of these lysosomes (Fig. 3).

Discussion

In PBC, the primary pathogenic process is directed against small intrahepatic bile ducts. Copper re-

Fig. 2 Relation between liver copper concentration and hepatic parenchymal (focal) necrosis, piecemeal necrosis and overall histological activity (inflammation and necrosis) in 38 PBC liver biopsies.

Fig. 3a Representative electron photomicrograph demonstrating characteristic electron dense lysosome-like organelles observed in a liver biopsy from a patient with PBC and increased liver copper concentration (640 µg/g). L = lysosome, M = Mitochondrion.

Fig. 3b Representative electron probe microanalysis of an electron dense hepatocyte lysosome. S = Sulphur, Cu = Copper, Zn = Zinc. Energy range is 0-10 keV. The centroids for S, Cu and Zn are indicated with arrows.

Mean liver copper concentration, copper staining reaction with rubeanic acid, subcellular distribution of copper (lysosomal, cytosolic), and presence/absence of ultrastructural changes of copper toxicity on electron microscopy in PBC, early and late Wilson's disease and neonate

	Mean liver copper concentration ($\mu\text{g/g dry wt}$)	Rubeanic acid stain for copper	Lysosome copper	Cytosol copper	Mitochondrial changes of copper toxicity
Primary biliary cirrhosis	321	+	+	+	-
Early Wilson's disease ^{6,7}	983	-	-	+	+
Late Wilson's disease ^{6,7}	493	+	+	+	-
Neonate ^{14,16}	435	+	+	+	-

tention in liver cells occurs as a complication of impaired copper excretion in bile. It has been suggested that like Wilson's disease, liver copper retention in PBC is an important factor causing liver cell damage, and copper chelation may preserve liver cell function. At the time of diagnosis, 55% of patients with PBC have liver copper concentrations below the range occurring in Wilson's disease. Despite the retention of copper in hepatocytes, liver cell function is well preserved, and there is no correlation between liver copper concentrations, and the degree of liver damage judged biochemically or by histology. The histological feature of Wilson's disease¹⁰ (steatosis, nuclear vacuolation and piecemeal necrosis) are not prominent in PBC patients with increased liver copper concentrations. In PBC, liver copper and copper-associated protein can be consistently demonstrated with appropriate histochemical stains,¹³ whereas these stains are often negative in Wilson's disease.^{7,13} This suggests that copper is retained in different chemical forms in the two diseases.

In Wilson's disease, ultrastructural evidence of copper cytotoxicity in liver cells is seen in the early phase of the disease,⁶⁻⁹ when mean liver copper concentrations are approximately three times those of PBC^{6,7} (Table). At these markedly increased concentrations, copper-containing lysosomes are absent, the copper is diffusely distributed in the cytosol, and the copper does not stain with rubeanic acid.⁷ Under these conditions, the copper is cytotoxic, and this is reflected by characteristic mitochondrial abnormalities seen on electron microscopy.⁷⁻⁹ In PBC, neonatal liver,¹⁴⁻¹⁶ and the late stage of Wilson's disease⁷ (once macronodular cirrhosis has developed), mean liver copper concentrations are similar^{6,7} (Table). The copper is present in both lysosomes and the cytosol,^{6,7,16,17} and can be stained with rubeanic acid.^{6,7,13,18} The sulphur detected with copper in PBC lysosomes probably reflects the presence of the sulphur-rich copper-associated protein which stains with orcein,¹⁹ and is present in both PBC¹³ and neonatal¹¹ liver. The staining characteristics and subcellular distribution of liver copper in PBC is similar to the neonate, where copper is not thought

to be hepatotoxic.^{16,18} It is likely that at liver copper concentrations occurring in PBC, the copper is retained in a non-toxic form.

This study stresses that there is no simple analogy between liver copper retention in PBC and Wilson's disease. Wilson's disease is an inborn error of copper metabolism, whereas in PBC, cholestasis and biliary cirrhosis dominate the clinical and biochemical evolution of the disease, with liver copper retention occurring as a complication of cholestasis. In patients with PBC and increased liver copper concentrations, liver cell function is preserved and there is no positive evidence to suggest that copper retention in PBC is an important factor in the pathogenesis of the disease. Copper chelation with D-penicillamine is effective treatment in Wilson's disease. The potential value of this drug as a purely copper chelating agent in PBC is questionable.²⁰ Penicillamine causes a fall in immune complexes and immunoglobulins in PBC,²¹ and the immunological rather than the copper chelating effects of this drug may therefore be of greater importance in treatment.²²

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