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# INTRAHEPATOCELLULAR LOCALIZATION OF COPPER IN WILSON'S DISEASE

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## ABSTRACT

It is well known that excess copper plays a role in the pathogenesis of Wilson's disease; however, the hepatic copper contents, determined either histochemically or biochemically, are not always correlated with the activity of Wilson's disease. To better understand copper-induced cytotoxicity, intrahepatocellular localization of copper was studied in five patients with Wilson's disease. The liver specimens were obtained by biopsy to confirm the clinical diagnosis of Wilson's disease before treatment. Neither hepatic copper content nor histochemical copper deposits were correlated with any of the biochemical indices studied. Energy-dispersion X-ray microanalysis was done on the nuclei, lysosomes and lysosome-free cytoplasm of hepatocytes. Lysosomes had the highest Cu X-ray intensity but no correlation was shown between the lysosomal copper content and biochemical indices. The nucleus/lysosome-free cytoplasm ratio of the copper content was correlated with the serum levels of aminotransferases. These results suggest that the copper increasing gradient from the lysosome-free cytoplasm to the nucleus is associated with hepatocyte necrosis, and probably causes irreversible nuclear damage.

Key Words: Wilson's disease, Copper, Hepatocytes, Nucleus, Cytoplasm, X-ray analysis

## INTRODUCTION

Wilson's disease is a rare disease of genetic origin in which there is excessive accumulation of copper in the liver. With the passage of time copper causes pathologic changes in the liver. Later, the patients develop complications of the central nervous system that are always associated with cirrhosis of the liver. Thus, elevated concentrations of hepatic copper are pathognomonic of this disease, but they are not necessarily correlated with the severity of hepatocellular damage, which is shown by hyperaminotransferasemia or hepatocyte necrosis. In the early stages of this disease the hepatic copper concentration is high and copper distribution in the cytoplasm is diffuse, while in the late stages lysosomal copper deposits increase but hepatic copper content decreases[1]. To reduce copper cytotoxicity, copper is incorporated into sulphur-rich cuproproteins and stored in hepatocyte lysosomes. These cuproproteins, which are detected by histochemical studies for the copper element or by orcein staining for thiol residues, are thought to be less cytotoxic than cuproproteins in the nuclei and cytoplasm. The distribution of more cytotoxic cuproproteins in the nuclei and lysosome-free cytoplasm has not been known, and it is possible that it has some relation with the severity of hepatocellular damage. Energy-dispersion

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X-ray microanalysis (EDX) is highly sensitive and allows the detection of trace elements[2,3]; besides, the introduction of an internal standard for frozen ultrathin sections has opened the way for semi-quantitative measurement of copper element and its protein complexes[4,5]. By this method, cuproproteins in the nuclei and lysosome-free cytoplasm can be detected. In this study EDX was used to determine intracellular accumulation of copper in the liver of patients who have Wison's disease, and to clarify its role in the pathophysiology of this disease.

## MATERIALS AND METHODS

Before treatment with D-penicillamine, a liver biopsy was performed to confirm the clinical diagnosis of Wilson's disease in five patients. The mean values of alanine aminotransferase and aspartate aminotransferase were regarded as the indices of the disease activity.

Each liver specimen was divided into three portions. One portion was fixed in a 3.7% formaldehyde solution overnight and stained with hematoxylin and eosin for routine histologic analysis, and with p-dimethylaminobenzylidene rhodanine for copper histochemical analysis[6]. Another portion was dried and incinerated for copper determination by atomic absorption spectrometry. The last portion was fixed in a chilled solution of 0.5% glutaraldehyde and 0.2 M cacodylate buffer (pH 7.4), as reported previously[7]. After rinsing in distilled water, 1-mm cubes were treated with 1.5 M sucrose solution, mounted on aluminium holders, and frozen in liquid nitrogen. Ultrathin frozen sections 80-nm thick were cut with an LKB microtome and were transferred to a carbon-coated gold grid using 1.5 M sucrose solution as the carrier. All procedures for electron staining were omitted. EDX of the ultrathin sections was performed with a Hitachi H-800 electron microscope provided with a Kevex 7000-Q energy-dispersion X-ray analyser. The accelerating voltage was 100 kV and the beam current was $10^{-10}$A.

To determine the intracellular localization of the elements, each of the three ultrathin sections obtained from two patients were mapped using Kα of copper, sulphur, and phosphorus. Because the concentration of phosphorus was even throughout the hepatocytes, we assessed the possibility of using phosphorus as an internal standard as described previously[5]. First, the thickness of the ultrathin sections, which was mechanically pre-set, was measured directly by tilting the sections from 0 to 30 degrees at a magnification of 10,000. Then, areas of 10 $\mu m^2$ on the nuclei, lysosomes and lysosome-free cytoplasm of the hepatocytes were analyzed at random for 200 sec using selected area analysis (SAA). Mathematical analysis was done to determine whether the intracellular concentration of phosphorus is different in the nucleus from that in the lysosome-free cytoplasm and whether it is independent from the amount of cuproproteins accumulated.

To assess the copper content in the nucleus and lysosome-free cytoplasm, SAA without determination of section thickness was done in twenty or more areas of the nuclei and lysosome-free cytoplasm per patient. Elements commonly detected in hepatocellular nuclei and lysosome-free cytoplasm were sodium, phosphorus, sulphur, chlorine, potassium, calcium, iron, and copper. First, individual elements were expressed by Kα X-ray counts/200 sec. Second, the atomic percentages of these elements were calculated with an autoprocessor using programs based on the method of Cliff and Lorimer[8]. Each SAA thus gave us directly two figures for each element: the Kα X-ray count and its atomic percentage.

Table 1. Clinical Features and Laboratory Data of Patients

| Case No. | Age (yr) | Sex | AST (IU/L) | ALT (IU/L) | Hepatic Cu (μg/g) | Cu Stain[a)] |
|---|---|---|---|---|---|---|
| 1 | 14 | M | 40 | 64 | 262.6 | ± |
| 2 | 16 | M | 46 | 70 | 262.0 | ± |
| 3 | 18 | F | 82 | 70 | 1392.5 | + |
| 4 | 23 | M | 16 | 13 | 848.3 | ++ |
| 5 | 26 | M | 32 | 17 | 1168.8 | ++ |

[a)] Cu staining was expressed as ± for occasional appearance and + for frequent appearance of hepatocytes stained for copper in periportal area, and ++ for frequent appearance of such hepatocytes throughout hepatic lobules.

## RESULTS

As shown in Table 1, liver biopsy specimens were obtained from four male patients and one female patient to confirm the clinical diagnosis of Wilson's disease. The serum levels of alanine aminotransferase before treatment ranged from 13 to 70 IU/L, and those of aspartate aminotransferase ranged from 16 to 82 IU/L. Three of the five patients had mildly elevated serum levels of aminotransferases. Histologically, all the liver specimens were cirrhotic and two of them were in active stage with piecemeal necrosis. In two patients, peri-portal hepatocytes contained abundant amounts of copper granules, while in three patients, some of the hepatocytes were positive for copper. Copper content ranged between 260 and 1400 μg/g dry weight. Neither histochemical copper deposits nor hepatic copper content were correlated with the serum levels of aminotransferases.

Mapping of hepatocytes showed that copper and sulphur were highly concentrated in the hepatocellular lysosomes, while phosphorus was diffusely distributed in the cytoplasm except for the nuclei (Fig.1). Slightly concentrated X-ray intensity was seen in the nuclei. As for mapping by Cu Kα X-rays, no difference in the copper content was detected between the nuclei and the lysosome-free cytoplasm.

X-ray intensities of copper and sulphur were high in the lysosomes as expected from the coexistence of the two elements in mapping hepatocytes. There was no difference in the X-ray intensities of copper between the mitochondria and other lysosome-free cytoplasm. When the X-ray counts of phosphorus in the nuclei and the lysosome-free cytoplasm were adjusted for the thickness of the ultrathin section directly measured, distinct differences in the phosphorus content between the nuclei and lysosome-free cytoplasm were obtained. Ultrathin sections ranged from 0.11 to 0.42 μm in thickness. X-ray counts of phosphorus were from 2840 to 5010 per 200 sec in the nuclei and from 2120 to 6780 in the cytoplasm. When the phosphorus content was adjusted according to the thickness of ultrathin sections, the nuclear phosphorus was 1.9 times the cytoplasmic phosphorus (2160 vs. 1140 counts/200 sec/0.1 μm). This value was used to adjust the specified thickness of ultrathin sections, because the different X-ray intensity of phosphorus, which is independent from any indices of Cu Kα X-rays, depends largely on the phosphorus-rich matrix of the nuclei.

As summarized in Table 2, the copper content of the ultrathin sections (80-nm thick), expressed as the Cu Kα X-ray counts per 1000 counts of P Kα X-ray, ranged between 0.132 and

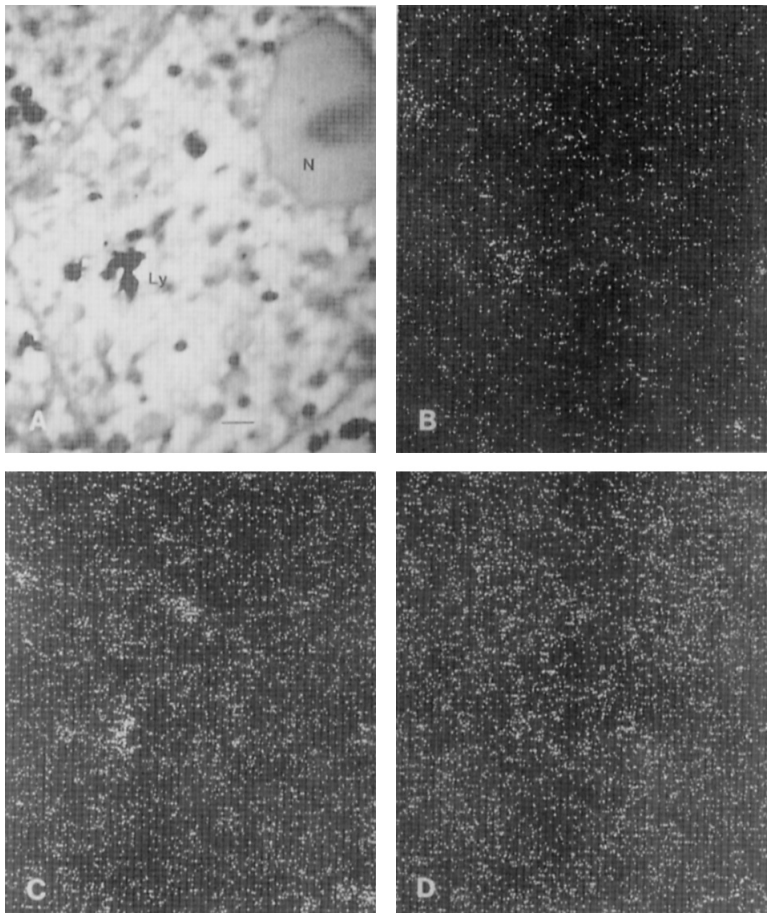


Fig. 1. Mapping of hepatocytes by different specific X-rays. A) Scanning transmission electron microscopic image of a glutaraldehyde-fixed, unstained, ultrathin section. The nucleus (N) of this hepatocyte is localized in the upper right corner of the field. The scattered electron dense bodies (Ly) are considered to be lysosomes because of their iron and copper content. For the selected area analysis, small areas of the nuclei, lysosomes and lysosome-free cytoplasm were chosen at random. B) Mapping of hepatocytes by Kα X-ray of copper. C) Mapping of hepatocytes by Kα X-ray of sulphur. D) Mapping of hepatocytes by Kα X-ray of phosphorus. Highly dense foci of Cu and S are seen in the lysosomes. The distribution of P is even throughout the cytoplasm, but it is a little higher in the nucleus. When the thickness of sections was adjusted by direct determination, the nucleus/cytoplasm ratio of X-ray intensity of P was found to be constant, at 1.9. Thus the copper content was compared between the nuclei and lysosome-free cytoplasm. The length of the bar is equivalent to 1 μm.

Table 2. Intracellular Copper Accumulation.

| Case No. | Nucleus | | Cytoplasm | | N/C | |
|---|---|---|---|---|---|---|
| | X-ray intensity | Atomic percentage | X-ray intensity | Atomic percentage | X-ray intensity | Atomic percentage |
| 1 | 0.469 | 0.423 | 0.276 | 0.249 | 1.699 | 1.699 |
| 2 | 0.351 | 0.350 | 0.271 | 0.251 | 1.394 | 1.295 |
| 3 | 0.271 | 0.260 | 0.136 | 0.149 | 1.745 | 1.993 |
| 4 | 0.132 | 0.131 | 0.177 | 0.141 | 0.929 | 0.746 |
| 5 | 0.263 | 0.199 | 0.267 | 0.178 | 1.118 | 0.985 |

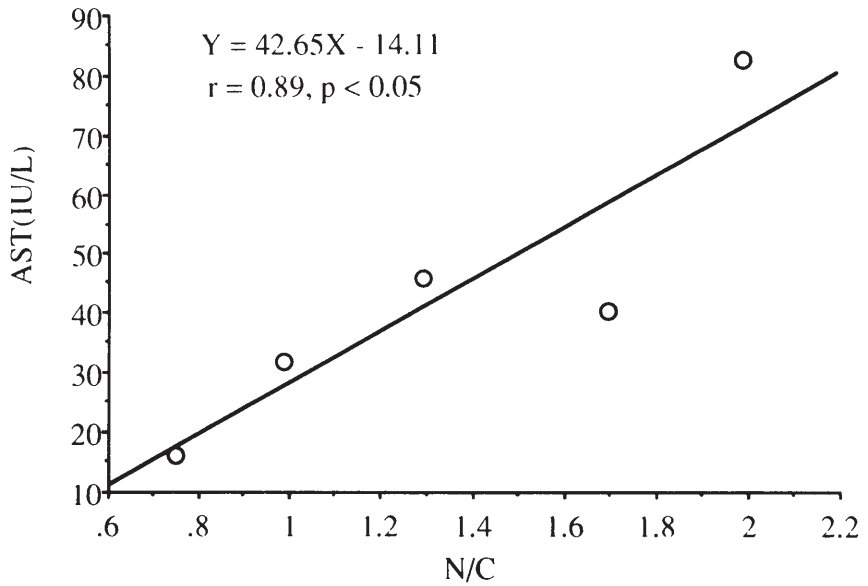


Fig. 2. Correlation between the nucleus/cytoplasm ratio of Cu content (N/C) and the values of aspartate aminotransferase (AST) in patients with Wilson's disease. When the Cu content was expressed by X-ray counts adjusted by phosphorus, the internal standard element, a correlation between N/C and AST was obtained. The close relationship between the copper gradient and serum levels of aminotransferases suggests that nuclear damage by copper is associated with hepatic necrosis in Wilson's disease.

0.469 in the nuclei, and between 0.136 and 0.276 in the lysosome-free cytoplasm. The nucleus/lysosome-free cytoplasm ratio of copper (N/C) correlated with the serum levels of AST ($P < 0.05$, Fig.2) but did not correlate with those of ALT ($P=0.06$). When the atomic percentage of copper was adjusted by that of phosphorus, the nucleus/lysosome-free cytoplasm ratio of copper (N/C) correlated with the serum levels of ALT ($P < 0.05$). No correlation was found between the N/C ratio and serum levels of AST ($P=0.09$).

## DISCUSSION

There is no question that copper plays a role in the pathogenesis of Wilson's disease, but the information obtained from light microscopic procedures is too limited to understand copper cytotoxicity. In Wilson's disease, excess copper can not be excreted into the bile for unknown reason, but is stored in hepatocyte lysosomes. However, there is no evidence that lysosomal copper causes hepatocellular necrosis. Considering that copper is incorporated into cuproproteins and stored in lysosomes, lysosomal copper is less cytotoxic than copper elements in the nucleus and cytoplasm. Goldfischer & Sternlieb reported some changes in the distribution of hepatic copper in relation to the progression of Wilson's disease[1]: in the early stages of the disease, fatty liver and active hepatitis stages, the hepatic copper concentration was high and copper distributed diffusely in the cytoplasm, while in the late stages hepatic copper content decreased and lysosomal copper deposits increased. As far as we know, light microscopic procedures, either histochemical analysis for the copper element or orcein for thiol residues, have not demonstrated any relation between lysosomal copper deposits and biochemical indices of hepatocellular damage.

In an experimental study using copper-loaded rats, Fuentealba et al. reported that copper was identified within the nuclei of the hepatocytes and was associated with irreversible nuclear damage[9]. Though the acute copper intoxication in rats is different from the chronic copper toxicosis seen in patients with Wilson's disease, nuclear damage caused by copper might precede hepatocellular necrosis.

Our observations, demonstrating the correlations between the nucleus/lysosome-free cytoplasm ratio of copper content and serum levels of aminotransferases, suggest copper accumulation in the nuclei is associated with cell necrosis. And these results were first obtained by means of EDX with introduction of the internal standard element, phosphorus, to estimate the gradient of the copper content between the nuclei and lysosome-free cytoplasm. Theoretically, the Cu Kα X-ray energy yielded by electrons correlates with the amount of atomic copper in the exposed tissue. Cu Kα counts therefore vary in relation with the thickness of the ultrathin sections when the analysis conditions, including the scanned area, are mechanically pre-set. However, it is technically difficult to attain ultrathin sections of specified thickness. This problem has been solved by using chlorine as the internal standard element[4]. Hanaichi et al. used phosphorus as the internal standard element because of its homogenous distribution in the cytoplasm[5]. However, the distribution of phosphorus in the nuclei is different from that in the cytoplasm because of abundant nucleic acids in the nuclei. To revise the differences in phosphorus content, we measured the thickness of ultrathin sections and found that the P Kα X-ray count of the nuclei was 1.9 times as much as that of the cytoplasm. Considering the fact that phosphorus is not a constituent of cuproproteins, phosphorus content, when adjusted as described above, can be an internal standard for the specified thickness.

It might be emphasized that the gradient is more important for hepatocyte necrosis than the absolute content of copper in the nuclei and/or in the lysosome-free cytoplasm. However, it remains unresolved why or how variations in the gradient of copper content between the nuclei and the cytoplasm can lead to hepatocyte necrosis.

To our knowledge this is the first study in which it has been demonstrated that the gradient of copper content between the nuclei and lysosome-free cytoplasm is associated with hepatocyte necrosis through irreversible nuclear damage.

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