Distribution of Copper in Biopsied Canine Liver

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Abstract: Biopsied canine liver tissues were investigated histochemically by electron microscopy to demonstrate copper localization. The samples were fixed and stained with the modified sulfide-silver method with a combination of iron and zinc removal. Using this method, the hepatic copper, even at a low concentration of 30 µg/g dry weight, was detectable. Copper was found in the hepatocyte lysosomes or in the cytoplasm as electron-dense granules.

Key words: canine, copper, liver

The liver is known to develop copper-associated hepatitis due to an excessive accumulation of this material in the hepatocytes. These include hepatic disease in the Bedlington terrier and Wilson's disease in man, as well as other hepatobiliary diseases that disturb the homeostasis of copper metabolism [1–5]. For the diagnosis and management of copper associated hepatopathy, detection of a small amount of copper in hepatocytes at an early stage is useful because dietary restriction of copper absorption can delay the onset of the disease [6]. The histochemical localization of copper may also provide information on the mechanisms of copper accumulation in the hepatocytes.

Currently available staining methods for histologic demonstration of copper such as rhodanine and rubeanic acid staining are applicable only when the hepatic copper concentration exceeds 400 µg/g dry weight (dw) [7]. The orcein staining detects copper binding proteins but not copper itself in the liver tissue. Timm's method, regarded as the most sensitive for the detec-

tion of copper, also detects other metal elements such as iron and zinc [8].

The object of this study is to establish a new histochemical method for demonstrating copper localization in biopsied hepatic tissue by electron microscopy. We have combined a method for iron and zinc removal reported previously by Kozma *et al.* [9], with a modified sulfide-silver method reported elsewhere by Kodama *et al.* [10]. For comparison, the specimens were stained with hematoxylin and eosin (HE), rhodanine and orcein and examined under a light microscope. Specimens with routine fixation were also examined as a control under a transmission electron microscope (JEM2000-FX, JEOL, Tokyo, Japan). The hepatic copper concentrations in the same specimen were also measured with a Hitachi Z-8100 atomic absorption spectrophotometer (Hitachi, Tokyo, Japan).

Two beagles with no clinical signs of diseases, free of abnormality on complete blood count (CBC) or serum biochemistry screening, were used for hepatic

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biopsy (No. 1, 2-month-old male, 2.3 kg BW; No. 2, 7-month-old female, 6.5 kg BW). The dogs were fasted for 12 hours before hepatic biopsy and anesthetized by induction with intravenous sodium thiopental (15 mg/kg BW), then with inhalation of isoflurane. Through median ventrotomy, two pieces of liver specimen were taken with an 18G biopsy needle (Microvasive, Boston Scientific., Massachusetts, U.S.A.) in the caudal lobe for light and electron microscopic observations, and then about 1 g of hepatic tissue was resected from a hepatic lobe by ligation with Teflon and for measurement of copper content.

In the modified sulfide-silver method, the biopsy samples were soaked in 0.9% sodium sulfide for 1 min at 38°C, and then transferred into 0.9% sodium sulfide at 4°C before dissection under a stereoscopic microscope (SDZ-TR-PL, Kyowa Optical, Tokyo, Japan). Then the samples were fixed in a mixture of 0.1% glutaraldehyde, 2% paraformaldehyde with 0.1 M phosphate buffer (PB; pH 7.4) for 2 hr, and washed with 0.1 M PB three times every 10 min. After removal of iron and zinc sulfides by placing the samples in 15% trichloroacetic acid (TCA) for 15 min, the samples were washed with 0.1 M PB as stated above, then stained with the modified Timm's reagent in darkness for 80 min. This reagent was made of a mixture of 60 ml of 30% gum arabic (Wako, Osaka, Japan), 39 ml of hydroquinone solution and 1 ml of 10% silver nitrate. The hydroquinone solution consisted of 2 g hydroquinone, 6 ml of 0.5 M citrate buffer at pH 3.8 and 33 ml distilled water. After washing with 0.1 M PB, the samples were postfixed in 2% glutaraldehyde buffered with 0.1 M PB, and finally postfixed in 1% OsO₄. Following dehydration through a graded series of ethyl alcohol, the samples were embedded in Quetol 812.

For comparison, the other biopsied samples were immersed in a routine fixative consisting of 2% paraformaldehyde, 2.5% glutaraldehyde, and 0.1 M PB for 2 hr for comparison with our method. The samples were washed with 0.1 M PB three times and fixed in 1% OsO₄. Apart from fixation, the tissues were processed according to the modified sulfide-silver method. Ultrathin sections were made and stained with uranyl acetate and lead citrate, then examined with the electron microscope.

With HE staining in light microscopy, the hepatocytes of the two dogs showed no significant lesion.

Neither rhodanine, rubanic acid nor orcein staining could detect copper granules or copper binding proteins in the hepatocytes. In electron microscopic examination, the samples with regular fixation and staining showed no electron-dense deposit suggestive of copper.

The hepatic copper concentrations in dogs No. 1 and 2 were 95 µg/g dw and 30 µg/g dw, respectively. By using the modified sulfide-silver method and electron microscopy, lysosomes containing electron-dense particles were occasionally found in the hepatocytes of dog No. 1 (Fig. 1). Some lysosomes were free of those particles. In dog No. 2, a diffuse localization of fine electron-dense granules was recognized in the cytoplasm of hepatocytes.

Biopsy specimens of the liver have been examined with various staining methods in light microscopy, but a small amount of copper was not detected by these methods. Copper is generally found only in the late stage of copper-loading diseases, in both man and animals. In liver specimens without specific staining, copper at a high concentration is observed by electron microscopy as electron-dense granules, but it is not possible to distinguish copper from other metal elements such as iron and zinc. Although it has been attempted experimentally to examine the localization of copper in the liver and other organs of mice with systemic fixative perfusion for a modified sulfide-silver method [10], systemic perfusion is not possible for obtaining biopsy samples from clinical cases. Furthermore, biochemical determination of the copper concentration can not provide information on localization in hepatocytes to study the pathogenesis of copper-loading hepatopathy in the early stage.

In the present study, we have succeeded in detecting small amounts of copper in hepatocytes under an electron microscope. Our procedure could show the subcellular localization of this material. In this study, the detection of copper was based on the reduction of silver ions by heavy metal sulfides [8]. It has been documented that TCA could effectively eliminate iron and zinc sulfides by solubilizing them [9, 10], and a combination of TCA treatment and Timm's method is suitable for the detection of copper, although the staining was only possible by perfusion treatment.

In many hepatic disorders, such as fetal and neonatal hepatitis, Wilson's disease, alcoholic hepatitis in man,

Fig. 1. Electron micrograph of the biopsied liver from dog No. 1 with a hepatic copper concentration of 95 $\mu\text{g/g}$ dry weight. Numerous copper particles are present in the lysosomes and there are a small number in the cytoplasm. Some lysosomes are free of particles. Modified sulfide-silver method. $\times 37,000$.

Fig. 2. Electron micrograph of the biopsied liver from dog No. 2 with a hepatic copper concentration of 30 $\mu\text{g/g}$ dry weight. Copper particles are found in the hepatocyte cytoplasm but not in the lysosomes. Modified sulfide-silver method. $\times 19,000$.

and copper associated hepatitis in the Bedlington terrier, the distribution of copper in the hepatocytes can be vary according to the stage of the illness, the hepatic lobe biopsied, and individual differences. Thus, in biopsied liver tissue samples, in spite of the small size (1.2 mm in diameter and 10 mm in length), copper localization in each hepatocyte was determined without severe stress applied to the subjects, by applying the modified sulfide-silver method. By this method, early detection of copper in the biopsied liver tissue with an electron microscope was made possible. This will provide invaluable information for the diagnoses and pathogenesis studies of diseases with copper accumulation in the liver.

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