Electron Microscopic Study and X-ray Probe Microanalysis of the Liver of LEC Rat, an Animal Model of Wilson Disease

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Microanalysis using JEOL JEM-2010 and JEM-2800 could provide important information on trace elements in LEC rat livers. Copper appeared with sulfur, while iron coexisted with phosphorus and oxygen in acid phosphatase-positive dense bodies. It is likely that cuprothionein and iron protein are stored in the lysosomal system of hepatocytes and Kupffer cells. Therefore, LEC rats may be a good model for studying the pathogenesis of progressive liver disease in Wilson disease.

Introduction

Morphological identification of lysosomes was established by acid phosphatase reaction [1]. Pericanalicular lysosomes exhibited lead deposits after incubating liver slices with a lead and phosphate solution under acidic condition. Transitional elements, copper and iron, are stored in lysosomes as detoxified forms of cuprothioneins and hemosiderins, respectively. Genetic background of adult-onset storage diseases in the liver remained undetermined until 1993 when ATP7B was cloned in patients with Wilson disease (WD) [2]. In 1996, HFE was first cloned in patients with hemochromatosis, followed by TFR2 of non-HFE hemochromatosis. These storage diseases of the liver were named secondary lysosomal diseases due to genetic defects of extra-lysosomal molecules [3].

Light microscopy is a standard laboratory examination of the modern pathology, but has a drawback in detecting heavy metal toxicoses. Special staining methods such as rhodanine and rubeanic acid for copper, and Berlin blue for iron are needed. It must be kept in mind that negative results do not rule out overloading, namely toxic effect of transition metals. Aggregation of metal-rich lysosomes may be needed in positive histochemical staining, or minimal overloading of metal in lysosomes may be toxic [4].

Electron Microscopy is widely used in clinical diagnosis and pathological investigation. Structural changes of intracellular organelles provided by electron microscopy are important information for understanding disease mechanism of patients and animal models. In addition, X-ray microanalysis, especially energy dispersive X-ray microanalysis (EDX), realizes detection of not only trace element deposits but also compositional pattern of heavy metal complexes [5].

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The liver is a central organ for homeostasis of trace elements of iron and copper. Iron absorption in the gut is regulated by hepcidin molecules secreted from the liver, while excess copper is secreted from the liver into bile. The hepatic copper transporter ATP7B is defective in patients with WD whose biliary excretion of copper was completely blocked [6, 7]. Excess copper is first stored in the liver, and then in extrahepatic organs including the central nervous system. In the liver, copper is stored in hepatocellular lysosomes as cuprothioneins. Recent studies indicate that lysosomes are also loaded with iron, and may be replaced by iron during long-term copper cheletion because of more severe hypoceruloplasminemia [8]. Both iron and copper are transition elements and primary sources in radial generation. As a result, multi-organ damage may be inevitable when excess amounts of iron and copper are accumulated in the liver and other organs.

Long-Evans cinnamon (LEC) rats are an animal model of WD with an ATP7B mutation and hypoceruloplasminemia [9]. There is evidence to suggest that transition metals of both iron and copper may be involved in variety of fulminant hepatitis and chronic hepatitis of LEC rats [10, 11]. Because these transition elements are finally stored in hepatocellular lysosomes as detoxified forms, ultrastructural element analysis on LEC rats may provide information of cytoprotective response to hepatotoxic elements during chronic hepatitis leading to hepatic fibrosis and malignancy in ATP7B-linked copper toxicosis.

Materials and Methods

Two male LEC rats aged 16 weeks were purchased from Chubu Kagaku Shizai Inc. (Nagoya). Hepatectomy was performed under anesthesia. As a standard study using transmission electron microscopy, pieces of liver specimens (1 mm^3) were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer for 3 days, and post-fixed with 0.1% osmic acid solution buffered with 0.1 M cacodylate buffer, pH 7.2, for 45 min [12]. To identify lysosomes under

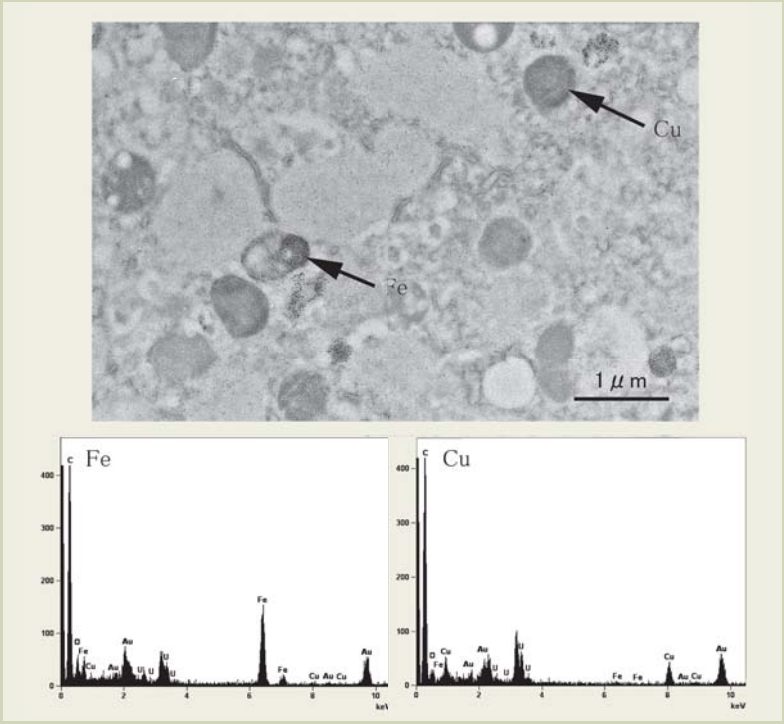

Fig. 1 Microanalysis of LEC rat hepatocytes. Transmission electron micrograph shows a variety of intracellular dense bodies (upper). There are 2 subtypes, Cu-rich dense bodies and Fe-rich ones identified by X-ray microanalysis (lower). Note that Fe-rich dense bodies are heterogeneous in matrices with fine grains. Fixed with 0.1% osmic acid and stained with uranyl acetate. (taken with a JEM-2010)

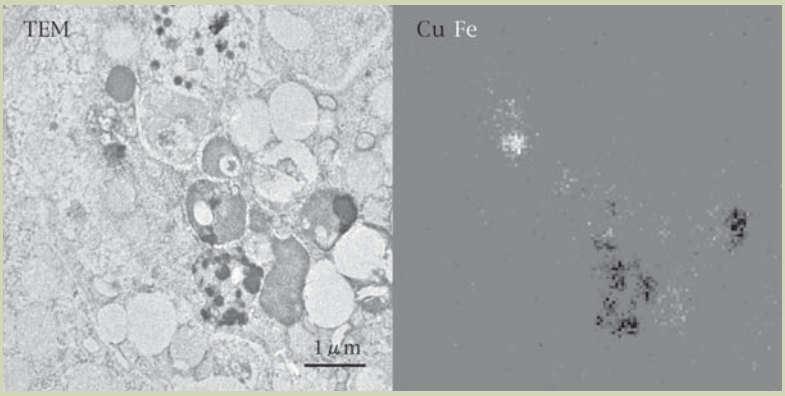

Fig. 2 Microanalysis of LEC rat Kupffer cells using a JEM-2010. Transmission micrograph shows a variety of intracellular dense bodies (left). Two dimensional imaging shows different distribution of Cu and Fe in dense bodies and their matrices (right). Fixed with 0.1% osmic acid and unstained.

electron microscopy, portions of liver tissues were fixed in 4% paraformaldehyde at 4°C, and 0.1 M cacodylate buffer, pH 7.2, for 60 minutes. After rinsing in 0.1 M cacodylate buffer at 4°C, pH 7.2, with 0.25 M sucrose, specimens were sliced in agar gel. Sections were incubated in 0.05 M acetate buffer, pH 5.0, with lead nitrate, sucrose, and 3% β-glycerophosphate, pH 5.0-5.2, at 37°C for 10 minutes, followed by a short rinsing period in distilled water [13]. Liver pieces were finally embedded in epoxy resin, TAAB812. Ultra-thin sections were mounted on gold grids. Sections unstained and stained with a 2% uranyl solution were examined under a transmission electron microscope (TEM; JEOL) and other JEOL microscopes (JEM-2010, JEM-2800) equipped with an EDX.

Electron microscopic study was first performed to visualize intracellular distribution of dense bodies in hepatocytes, Kupffer cells, and bile duct epithelial cells. Using an EDX of a JEM-2010, dense bodies in the hepatocytes, Kupffer cells, and bile duct epithelial cells were randomly exposed to electron beams for a short period. Lead was always identified in the dense bodies with acid phosphatase reaction. When copper or iron was identified in a particular dense body, the total number of X-rays yielded from the dense body was counted for 200 sec to complete an X-ray spectrum. Identification of the specific Kα X-rays of copper and iron was performed using an autoanalysis system equipped with the EDX. Co-existence of copper and iron was expressed as isolated Cu, compound Cu and Fe, and isolated Fe. Finally, the 2 dimensional distribution of iron or copper with other elements were mapped using scans of the dense body-rich cytoplasm with the EDX of a JEM-2800 for 480 sec. Multi-element X-ray spectrums at any pin point area were available after 2 dimensional scanning.

Results

Under electron microscopy, hepatocellular organelles including the nucleus, mitochondria, and dense bodies were visualized in ultrathin sections (**Fig. 1**). Combined with EDX, some characters of Cu- and Fe-positive dense bodies of hepatocytes were visualized. Cu-rich particles were diffusely dense, while Fe-rich particles were granularly dense. Kupffer cells contained a variety of dense bodies. Distribution of Cu and Fe was apparently different in these dense bodies (**Fig. 2**). After the acid phosphatase reaction, hepatocellular dense bodies became positive for lead precipitation. As a result, co-deposits of intrinsic elements of copper and iron, and extrinsic lead were identified in some hepatocyte dense bodies (**Fig. 3**). There was no significant

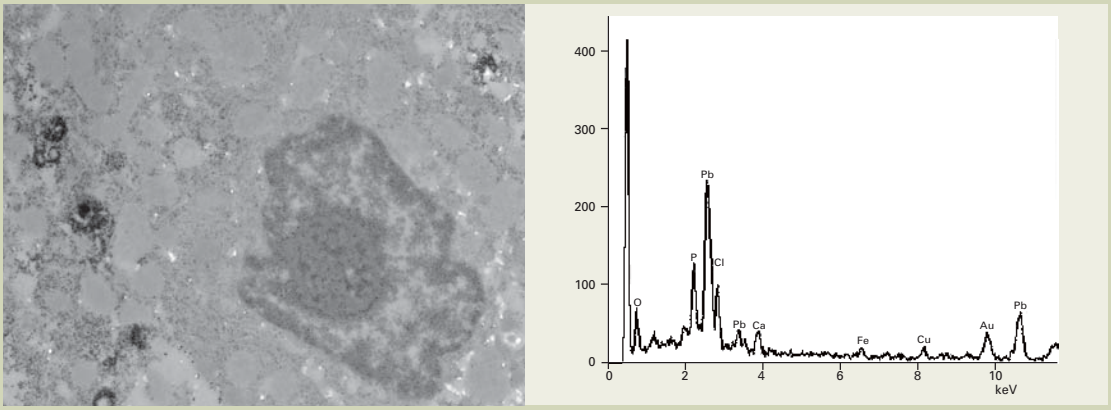

Fig. 3 Microanalysis of LEC rat hepatocytes with acid phosphatase reaction. Transmission electron micrograph shows intracellular dense bodies scattered in the cytoplasm (left). Extrinsic elements of Pb and Au, and intrinsic elements of O, P, Ca, Fe, and Cu appear in the spectrum obtained from a dense body. Fixed with a 4 % paraformaldehyde solution, and incubated with a Gomori-medium for acid phosphatase reaction, and observed without staining. (taken with a JEM-2010)

lead precipitation outside of the dense bodies.

Two dimensional distribution of copper and iron was clearly visualized within lysosomes (**Fig. 4**). Iron was co-existed with phosphorus and oxygen, while copper appeared with sulfur. Intralysosomal localization of copper and iron seemed different.

Discussions

Wilson disease is primary copper toxicosis due to mutant ATP7B which can not transport Cu molecules inside the Golgi net work. As a result, copper accumulates in the cytoplasm secondary to impaired secretion of copper into bile. However, recent studies indicate the co-existence of copper and iron in patients with WD, especially in male patients after copper chelation therapy [8]. Similar observations with WD patients were obtained in the current study using the LEC rats, animal model of WD. Iron complex consists of phosphorus and oxygen, while copper makes complex with sulfur. Dense bodies either in hepatocytes or Kupffer cells were positive for acid phosphatase, supporting that both copper and iron are accumulated in lysosomes of parenchymal cells and reticuloendothelial cells, as cuprothionein and hemosiderin, respectively. The etiology of iron overload in the animal model of WD may be complex as human disease [14], but the main reason may be due to hypoceruloplasminemia. In the animal model, dietary iron may be fortified for higher fertility. LEC rats are representative of primary copper toxicosis and secondary iron overloading due to ferroxidase deficiency and dietary fortification.

In spite of primary copper toxicosis, liver specimens of young LEC rats around 16 weeks of age are not positive for histochemical copper staining such as rhodanine and rubeanic acid. Aggregation of copper-positive lysosomes around bile canaliculi is prerequisite for histochemical staining so that biochemical determination of hepatic copper is widely applied in most reports of LEC rat studies. In fact, not histochemical study but chemical determination of copper is recommended in international scoring system for diagnosis of WD [15]. Microanalysis used in this study is not routine examination in practice, but all X-rays appear in a spectrum, clarifying multi-metal accumulation within lysosomes.

Energy dispersive X-ray microanalysis opened a way to study in vivo interaction of multi-elements. Using the JEM-2800, both information on intralysosomal distribution and coexistence with other elements was simultaneously obtained from LEC rat liver. Different pattern of copper deposits was obtained in hepatocytes and Kupffer cells, as well. Although the sensitivity of element detection is not different, but the time period consumed for one analysis is quite different between the JEM-2010 and the JEM-2800 (8 hrs vs. 8 min/one imaging). Information on X-rays accumulated in a 2 dimensional analysis is also different. X-rays yielded from any spot area within the imaged field are reserved in a computer, and are reproducible after analysis.

Twenty years ago, using an EDX, we met a question of coexisting copper and iron in a male patient with WD. Now, we understand that iron also accumulates in the liver of WD due to concomitant low levels of serum ceruloplasmin, namely ferroxidase deficiency [8]. This hypothesis was supported by double overloading of copper and iron in animal model of LEC rats [9] and discovery of aceruloplasminemia by Miyajima [16]. The genetic disease was not a copper disease but one of iron overload syndromes.

Lysosomal deposits of iron complexes and cuprothioneins do not mean pathological conditions, but detoxification process by cellular defense mechanism. It is more important to detect toxic metal ions around the organelles essential to survive and resume the ability of reproduction. In the case of copper with a physiologically secretory route of bile ducts, copper retention is inevitable in WD and idiopathic copper toxicosis, but unusual in secondary pathological conditions. These include primary biliary cirrhosis and non-alcoholic steatohepatitis. Copper toxicosis in these conditions may be overcome with UDCA treatment [17].

In the case of iron which has no physiological route for excretion, iron overload may occur in a variety of pathological conditions. A small amount of lysosomal iron in CHC indicates iron-induced oxidative stress to hepatocytes infected with HCV. Complete removal of lysosomal iron is an effective treatment of refractory liver disease [18]. However, a large amount in lysosomal iron in hepatocytes does not affect hepatocytes of aceruloplasminima [16]. Liver structures are intact even in aged patients. Low levels of serum iron and transferrin saturation indicates hepatocytes usually face with iron deficient state, probably except for a short postprandial period when iron is actively absorbed by inappropriately low setting of hepcidin regulation system. Iron, once trapped in lysosomes, can not utilize outside of lysosomes

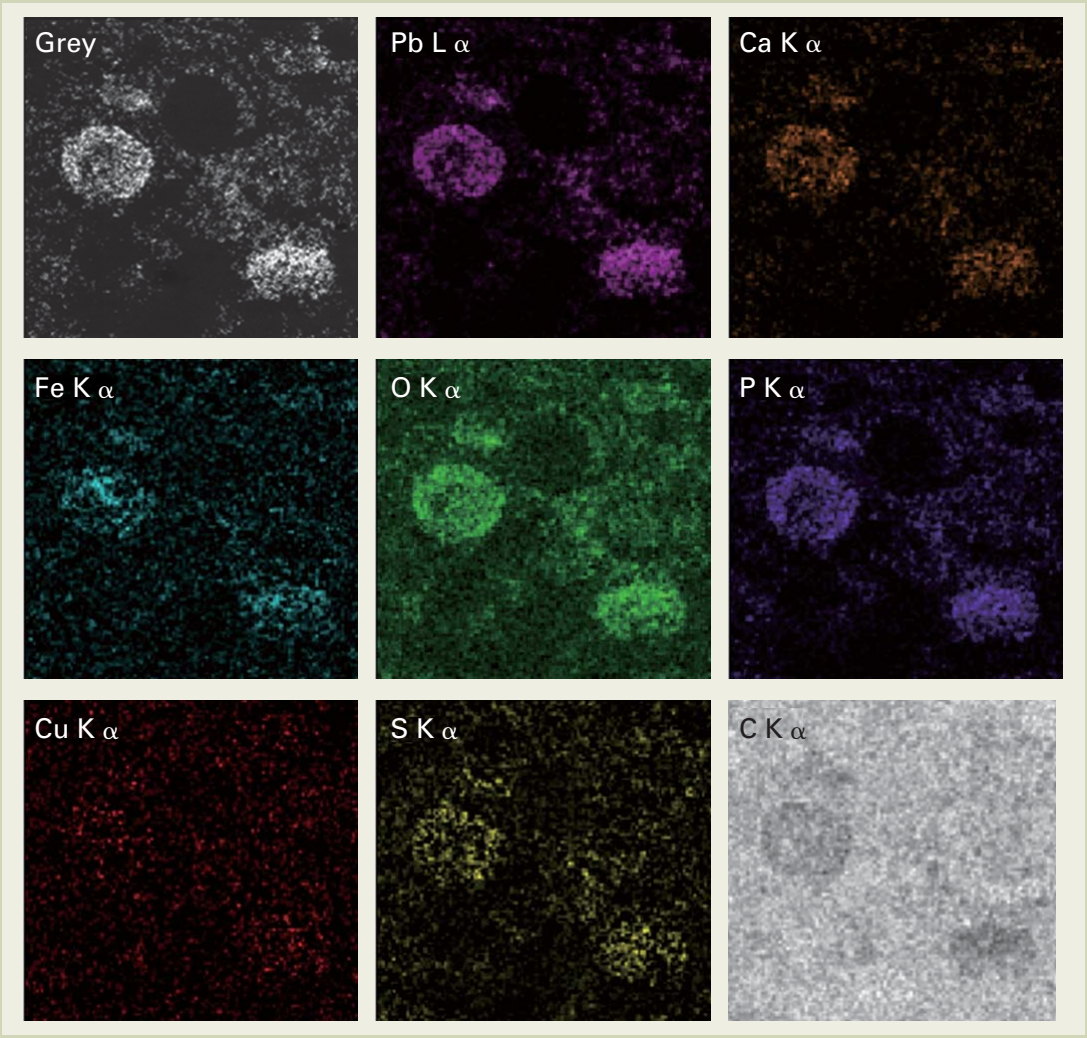

Fig. 4 Microanalysis of LEC rat hepatocytes using a JEM-2800. A series of 2 dimensional imaging using specific X-rays visualize intrinsic elements of Ca, Fe, O, P, Cu, S, and C. Pb is a reaction product demonstrating these particles lysosomal in origin. Note diffuse distribution of Cu in the cytoplasm.

probably because transport across membrane is disrupted in aceruloplasminima. This may be a reason why patients with aceruloplasminima are not affected by iron-induced liver damage in spite of heavy iron deposits in the liver.

Conclusions

Compound overloading of copper and iron was confirmed in lysosomes of LEC rat livers. Copper appeared with sulfur, while iron coexisted with phosphorus and oxygen. Microanalysis using JEOL JEM-2010 and JEM-2800 could provide important information on the state of trace elements in LEC rats, which may be a good model for studying the pathogenesis of progressive liver disease in WD.

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