Evaluation of Distribution Patterns for Copper and Zinc in Metallothionein and Superoxide Dismutase in Chronic Liver Diseases and Hepatocellular Carcinoma Using High-Performance Liquid Chromatography (HPLC)

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It has been reported that the copper (Cu) content of hepatocytes increases in chronic liver diseases and small hepatocellular carcinoma (HCC). In cells, Cu exists mainly as Cu-metallothionein (MT) or Cu, zinc (Zn)-superoxide dismutase (SOD). In this study, we investigated the biochemical state of Cu in the hepatocytes of patients with HCC using high-performance liquid chromatography (HPLC). The subjects of present study were 23 patients with HCC who underwent liver resection. The cancerous tissue and non-cancerous hepatic parenchyma with chronic disease were analyzed. In addition, as a normal control, hepatic tissue was collected at autopsy from 13 patients with no liver disease. Each sample was diluted with buffer, chilled, homogenized, and centrifuged. The supernatant was fractionated using HPLC. The metal contents of each fraction were measured using a desktop-type inductively coupled plasma (ICP) emission spectrochemical analyzer. HPLC analysis showed that MT existed mainly as Zn-MT in the normal hepatic tissue. The case of Cu,Zn-MT was significantly greater than Zn-MT in the non-cancerous, but diseased hepatic parenchyma than in the normal hepatic tissue (p<0.01). In comparison with non-cancerous hepatic parenchyma, the Cu-MT in the cancerous section was significantly greater than the Cu,Zn-MT (p<0.01). The Cu content for MT was significantly higher in small HCC (<=40 mm) (p<0.01), and the absence of Cu or Zn in the MT fraction was significantly more frequent in the large HCC (>=40 mm) (p<0.01). The Cu and Zn content for SOD in the samples showed no significant difference. Increase in the Cu content in the cancerous hepatic tissue were, thought to be reflecting changes in the distribution of Cu in the MT fraction of hepatic tissues.

Key words metallothionein; superoxide dismutase; high-performance liquid chromatography; hepatocellular carcinoma; particle-induced X-ray emission

Metallothionein (MT) was initially discovered from a horse kidney in 1957 as a protein that contained cadmium. Metallothionein is a heat-stable, metal-binding protein with a molecular weight of 6500 to 7000. A number of physiological and biological roles have been suggested for MT, including detoxification of heavy metals, metabolic regulation of Zinc (Zn) and Copper (Cu) (two trace elements that are essential to the body), and a radical-scavenging action that is closely related to the biological defense mechanism. It has also been reported that in chronic liver diseases, the Cu concentration in the liver parenchyma increases with the progression of disease. Tashiro-Itoh et al., in their study on human tissue bearing hepatocellular carcinoma (HCC), reported that the levels of both Cu and MT were increased in well-differentiated cancer but were lower in poorly differentiated cancer, which is in contrast with results from pericancerous hepatic tissue.

Ebara et al. evaluated the presence and localization of Cu-MT in HCC and non-cancerous hepatic parenchyma in surgically resected specimens from HCC patients using the gel-filtration method, and concluded that accumulation of Cu in small HCC, in which Cu was present as Cu-MT or Cu,Zn-MT, was greater than that in the surrounding liver parenchyma. Liaw et al. studied SOD, another form in which Cu can exist in cells, and concluded that reductions in the contents of trace elements (such as Cu and Zn) would cause a reduction in the intracellular SOD concentration in HCC. However, they did not investigate Cu-MT, the other major form in which Cu can be found in cells. In this study, we focused on both MT and SOD, the two major forms in which Cu and Zn exist in cells. Using sam-
samples taken from human HCC and noncancerous hepatic parenchyma with chronic liver disease obtained by surgery or autopsy, the ratios of Cu and Zn bound to MT and SOD were measured in these tissues; and the results were compared with those of the normal hepatic tissue.

MATERIALS AND METHODS

Subjects After obtaining informed consent from each patient, cancers and surrounding non-cancerous tissues were removed from 23 patients with HCC (18 men and 5 women; mean age, 59.23 ± 8.98 years) who underwent hepatic resection at Chiba University Hospital and affiliated hospitals between 1 January 1995 and 31 December 1998. Hepatitis virus was associated with HCC for all patients; hepatitis C virus was detected in 20 patients, and hepatitis B virus was associated with HCC for all patients; hepatitis C virus was detected in 20 patients, and hepatitis B virus was detected in 20 patients. Alcohol abuse were evident in 8 of the 23 patients. Between March 1996 and November 1998, autopsies were conducted on 13 patients who were free of hepatic disease. Their liver tissues were collected and used as normal controls.

Methods Part of the tissue that had been collected was fixed in 10% formalin, embedded in paraffin and stained with hematoxylin–eosin. The remaining tissue was frozen rapidly and preserved at −80 °C. According to the method by Suzuki et al., each sample (weighing 314 to 643 mg) was placed in 3 volumes of buffer (100 mM Tri–HCl [pH 7.4] 0.25 M glucose), chilled in argon gas, homogenized for one minute using a Polytron Homogenizer (Kinematica, Lucerne, Switzerland), and centrifuged for 60 min at 17000 g at 2 °C. During HPLC (Nihon Bunko Tosoh, Tokyo, Japan; column, TSK gel G3000 SW 7.5 mm × 60 mm; buffer, 19 ms Tris–HCl [pH 8.0] 0.1% NaN₃) the supernatant was separated for fractions taken every 30 s. The metal content of each fraction was quantified using a desktop-type inductively coupled plasma (ICP) emission spectrochemical analyzer (Seiko SPS7000A, Tokyo, Japan).

SOD was detected in 14 to 15 min, and MT in 17 to 19 min. The fraction in which Zn was mainly bound to MT was designated as Zn-MT (Zn/Cu > 20); the one in which both Cu and Zn were bound to MT as Cu,Zn-MT (0.05 ≤ Zn/Cu ≤ 20); and the one in which Cu was mainly bound to MT as Cu-MT (Zn/Cu > 0.05). The levels of MT in tissue were determined by radioimmunoassay according to the method by Sakurai et al. The MT and SOD fractions within the HPLC fraction were determined using authentic samples.

Statistical Methods The Wilcoxon signed-ranks test was used for the determination of metal contents in the tissue, SOD and MT. For the distribution pattern of Cu and Zn in MT examined by the χ² test. The significance level was set at p < 0.05.

RESULTS

(1) The contents of Cu and Zn for SOD were determined for each sample by ICP emission spectrochemical analyzer and the results were compared: normal liver, Cu, 0.65 ± 0.37 μg/g wet weight (w.w.); Zn, 0.98 ± 0.45 μg/g w.w.; noncancerous hepatic parenchyma, Cu, 0.92 ± 0.93 μg/g w.w.; Zn, 1.78 ± 1.28 μg/g w.w.; tumors with a diameter less than 40 mm, Cu, 0.97 ± 1.24 μg/g w.w.; Zn, 1.45 ± 1.49 μg/g w.w.; and tumors with a diameter measuring 40 mm or more, Cu, 0.54 ± 0.28 μg/g w.w., Zn, 0.82 ± 0.51 μg/g w.w. No significant differences were noted between the samples (Table 1).

(2) The contents of Cu and Zn for MT were determined for each sample by ICP emission spectrochemical analyzer and the results were compared: normal liver, Cu, 0.48 ± 0.43 μg/g w.w.; Zn, 26.31 ± 19.22 μg/g w.w.; noncancerous hepatic parenchyma, Cu, 2.61 ± 2.77 μg/g w.w.; Zn, 17.24 ± 22.83 μg/g w.w.; tumors with a diameter less than 40 mm, Cu, 5.73 ± 12.86 μg/g w.w.; Zn, 4.70 ± 9.64 μg/g w.w.; and tumors with a diameter measuring 40 mm or more, Cu, 1.84 ± 2.53 μg/g w.w., Zn, 0.65 ± 0.45 μg/g w.w. No significant differences were noted between the samples (Table 1).

(3) An example of the HPLC analysis of a normal liver is shown in Fig. 1. Metallothionein was found to exist as Zn-MT. Specimens from all 13 normal livers showed a similar pattern of distribution (Table 3).

Examples of HPLC analysis of non-cancerous hepatic parenchyma are shown in Figs. 2a and 3a. Although Zn was predominant in MT, binding with Cu was also noted. Thus MT existed as Cu,Zn-MT. The noncancerous hepatic parenchyma are chronic hepatitis in 8 and hepatic cirrhosis in 15. Between 1 January 1995 and 31 December 1998. Hepatitis virus was detected in 20 patients, and hepatitis B virus was detected in 3 patients. But none were infected by both B and C. Alcohol abuse were evident in 8 of the 23 patients.

Between March 1996 and November 1998, autopsies were conducted on 13 patients who were free of hepatic disease. Their liver tissues were collected and used as normal controls.

Table 1. The Contents of Cu and Zn for SOD

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Cu</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal hepatic tissue</td>
<td>13</td>
<td>0.65±0.37</td>
<td>0.98±0.45</td>
</tr>
<tr>
<td>Non cancerous hepatic tissue</td>
<td>23</td>
<td>0.92±0.93</td>
<td>1.78±1.28</td>
</tr>
<tr>
<td>Hepatocellular carcinoma (diameter&lt;40 mm)</td>
<td>10</td>
<td>0.97±1.24</td>
<td>1.45±1.49</td>
</tr>
<tr>
<td>Hepatocellular carcinoma (diameter≥40 mm)</td>
<td>13</td>
<td>0.54±0.28</td>
<td>0.82±0.51</td>
</tr>
</tbody>
</table>

μg/g wet weight.

Table 2. The Contents of Cu and Zn for MT

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Cu</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal hepatic tissue</td>
<td>13</td>
<td>0.48±0.43</td>
<td>26.31±19.22</td>
</tr>
<tr>
<td>Non cancerous hepatic tissue</td>
<td>23</td>
<td>2.61±2.77</td>
<td>17.24±22.83</td>
</tr>
<tr>
<td>Hepatocellular carcinoma (diameter&lt;40 mm)</td>
<td>10</td>
<td>5.73±12.86</td>
<td>4.70±9.64</td>
</tr>
<tr>
<td>Hepatocellular carcinoma (diameter≥40 mm)</td>
<td>13</td>
<td>1.84±2.53</td>
<td>0.46±0.32</td>
</tr>
</tbody>
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<sup>a</sup><sup>—</sup><sup>c</sup>p < 0.01. μg/g wet weight.
in Figs. 2b and 3b. When the tumor diameter was 28 mm, a Cu,Zn-MT pattern was seen in Fig. 2b, with Cu being dominant. Figure 3b shows the results of a sample from a tumor 80 mm in diameter: only minute quantities of Cu and Zn were detected bound to MT. In HCC lesions that measured less than 40 mm, only 2 of the 10 exhibited a Cu,Zn-MT pattern. Six showed the Cu-MT pattern and only trace amounts of Cu and Zn were detected in the remaining 2. In the lesions of HCC where the tumor diameter measured 40 mm or more, 3 of the 13 produced a Cu,Zn-MT pattern; 3 exhibited the Cu-MT pattern; and in the remaining 7, only minute quantities of Cu and Zn were detected. The number of Cu,Zn-MT pattern in the non-cancerous liver parenchyma was significantly greater than that in normal liver tissue \( (p < 0.01) \). The number of Cu-MT pattern in cancerous tissue \( (\text{diameter} \geq 40 \text{ mm}) \) was significantly greater than that in the non-cancerous liver parenchyma \( (p < 0.01) \).

(4) The levels of MT in tissue were determined by radioimmunoassay. The levels of MT in noncancerous hepatic parenchyma; \( 14.88 \pm 9.86 \mu \text{g/g w.w.} \), tumors with a diameter less than 40 mm, \( 20.87 \pm 17.92 \mu \text{g/g w.w.} \), and tumors with a diameter measuring 40 mm or more, \( 11.25 \pm 12.89 \mu \text{g/g w.w.} \) (Table 4).

**DISCUSSION**

Reduction in the intracellular Zn and increase in Cu have been reported in association with chronic liver diseases by Hatano et al.\(^{11}\); an increase in the Cu content in a small HCC by Ebara et al.\(^{17}\) In hepatocytes, it is believed that Cu exists mainly as Cu,Zn-SOD, Cu,Zn-MT and Cu-MT.\(^{9,10}\) It is likely that Cu, which increases in advanced chronic hepatitis and small HCC, exists in one of these forms. Liaw et al. found a reduction in the SOD level in HCC and concluded that the lower Cu and Zn content in the hepatic tissue and sera accounts for this change.\(^{15}\) In this study, there was no significant difference in the contents of Cu and Zn for SOD between normal liver tissue, non-cancerous hepatic parenchyma and cancerous tissue obtained from patients with HCC (Table 1). It should be noted that Liaw et al.\(^{15}\) did not include Cu-MT, a major form of intracellular Cu.

Sakurai et al., in their gel-filtration analysis of the Cu content that increases in the hepatocytes of LEC rats, reported that most of the Cu existed as Cu-MT.\(^{16}\) Tashiro-Itoh et al. employed an immunostaining technique and reported an increase in the Cu content and enhanced staining for MT in human HCC, especially in well-differentiated HCC.\(^{13}\) Ebara et al. reported that accumulation of Cu was greater in small HCC than in noncancerous hepatic parenchyma and that Cu was present as Cu-MT analyzed by the gel filtration analysis.\(^{14}\) However, they did not analyze Cu,Zn-SOD, and normal hepatic tissue. The HPLC method proposed by Suzuki et al. is considered to be well suited for the analysis of not only Cu-MT and Zn-MT, but also Cu,Zn-SOD.\(^{9,10}\) In the present study, we used the method of Suzuki et al. for the analysis of Cu and Zn in MT and SOD.\(^{9,10}\)

SOD exists in cells mainly in two forms Cu,Zn-SOD and Mn-SOD. In this study, no significant difference was noted between the contents of Cu and Zn for SOD of the normal hepatic tissue and tissue from patients with chronic liver diseases. A definite change was noted in the binding ratio of Cu and Zn to MT. Normal hepatic tissue from all 13 subjects showed the pattern depicted in Fig. 1, with the absence of Cu-MT, while the noncancerous hepatic parenchyma obtained from 16 of the 23 patients with HCC showed the Cu,Zn-MT pattern. The cancerous section of these patients, on the other hand, showed diverse patterns. In small HCCs, in particular, Cu frequently existed in the form of Cu-MT, suggesting a high Cu-MT level at the early stage of oncogenesis. Among the samples of large HCC, both the Cu and Zn contents that were bound to MT were frequently low, probably reflecting a reduction in the MT content in the samples.
Fig. 2a. An Example of Analysis of a Non-cancerous Hepatic Parenchyma Obtained from Patient with HCC (Diameter 28 mm)
MT existed as Cu,Zn-MT and Zn was dominant in MT. HCC, hepatocellular carcinoma; SOD, superoxide dismutase; MT, metallothionein; Zn, zinc; Cu, copper.

Fig. 2b. An Example of Analysis of the HCC Lesion (Diameter 28 mm)
MT existed as Cu,Zn-MT and Cu was dominant in MT. HCC, hepatocellular carcinoma; SOD, superoxide dismutase; MT, metallothionein; Zn, zinc; Cu, copper.

Fig. 3a. An Example of Analysis of a Non-cancerous Hepatic Parenchyma Obtained from Patient with HCC (Diameter 80 mm)
MT existed as Cu,Zn-MT and Zn was dominant in MT. HCC, hepatocellular carcinoma; SOD, superoxide dismutase; MT, metallothionein; Zn, zinc; Cu, copper.
These findings correspond to those reported by Tashiro-Itoh et al., in which they found that Cu and MT increased in well-differentiated cancers and reduced in poorly differentiated cancers. Furthermore, these findings suggest that increases (or reductions) in the Cu content in cases of human liver diseases are mainly associated with an increase (or decrease) of Cu-MT but not SOD.

In addition to tumor size, this study included an evaluation of the degree of liver tumor differentiation. Unfortunately, there was an uneven distribution of the various levels of differentiation, with small numbers of cases (3 well-differentiated, 19 moderately differentiated, and 1 poorly differentiated cancer), and no statistical analysis was possible. The type of infection, age, and gender were also evaluated but no significant differences were found with any of these parameters.

A number of studies have been conducted using LEC rats to investigate the relationship between HCC and Cu-MT. Because of their congenital anomaly of Cu metabolism, abnormal copper accumulation in the hepatic tissue, and spontaneous hepato-oncogenesis, LEC rats are used as animal models for Wilson’s disease and HCC. Sakurai et al. reported that the Cu-MT level increases markedly in the hepatocytes of LEC rats and that this Cu-MT produces hydroxyl radicals by Fenton-like reaction. They suggest the increased free radical produced by Cu-MT in hepatocyte may cause hepatic injury.

In this study, it was found that the quantity of Cu, which is higher in human HCC, exists in the form of Cu-MT, as it does in LEC rats. We believe that it is important to investigate the relationship between the development of HCC and free radical generation by Cu-MT.

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**REFERENCES**