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The liver in itai-itai disease (chronic cadmium poisoning): pathological features and metallothionein expression

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Cadmium (Cd) is a highly hepatotoxic heavy metal, which is widely dispersed in the environment. Acute Cd hepatotoxicity has been well studied in experimental animals; however, effects of prolonged exposure to Cd doses on the liver remain unclear. In the present study, to evaluate chronic Cd hepatotoxicity, we examined specimens from cases of itai-itai disease, the most severe form of chronic Cd poisoning. We compared 89 cases of itai-itai disease with 27 control cases to assess Cd concentration in organs. We also examined 80 cases of itai-itai disease and 70 control cases for histopathological evaluation. In addition, we performed immunohistochemistry for metallothionein, which binds and detoxifies Cd. Hepatic Cd concentration was higher than Cd concentration in all other organs measured in the itai-itai disease group, whereas it was second highest following renal concentration in the control group. In the liver in the itai-itai disease group, fibrosis was observed at a significantly higher rate than that in the control group. Metallothionein expression was significantly higher in the itai-itai disease group than that in the control group. Prolonged exposure to low doses of Cd leads to high hepatic accumulation, which can then cause fibrosis; however, it also causes high expression of metallothionein, which is thought to reduce Cd hepatotoxicity.
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Cadmium (Cd) is an environmental pollutant ranked as one of the most toxic substances,[1] and human industrial activities have markedly increased its distribution in the global environment. Food is the major source of Cd exposure to the general population;[2] therefore, the effects of chronic exposure to this metal are a major concern for humans. Several studies reported that even chronic exposure to low Cd doses can cause serious health effects.[3–5]

Itai-itai disease is the most severe form of chronic Cd poisoning caused by prolonged oral Cd ingestion. It developed in numerous inhabitants of the Jinzu River basin in Toyama Prefecture, Japan, an area most severely polluted by Cd that originated from a zinc mine located upstream. The main target organ of Cd toxicity in itai-itai disease is the kidney, where injury is manifested by tubular and glomerular dysfunction.[6] Renal dysfunction causes an insufficiency of active vitamin D, followed by bone injury consisting of a combination of osteomalacia and osteoporosis. Although femoral pain and lumbago are frequently seen as the initial manifestation, the leakage of low molecular weight proteins is observed in urine test from an earlier stage. Although bone injury is secondary and treatable, renal injury is irreversible and deteriorates culminating in end-stage renal failure in severe cases.[7]

Although chronic Cd intoxication mainly results in renal disease, acute exposure to toxic Cd doses primarily results in liver damage.[8] Acute Cd hepatotoxicity has been well studied in experimental animals, and its mechanism has been elucidated in detail.[8] However, the liver of itai-itai disease patients has not been the focus in research;

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therefore, the effects of prolonged Cd exposure on the liver remain to be clarified. Our faculty, the University of Toyama, located in the region where itai-itai disease has occurred, maintains a large collection of autopsy specimens from itai-itai disease patients. One of the aims of the present study was to assess the histopathological characteristics of the liver of itai-itai disease cases to evaluate the effects of chronic Cd hepatotoxicity.

In previous research on heavy-metal toxicology, antitoxicity protection mechanisms have attracted a great deal of attention. Till date, several antioxidants and metal-chelating agents have been proven to be effective in protecting against Cd-induced hepatotoxicity.[9–11] Among these substances, metallothionein is one of the most important substances because animal and human studies suggest that most Cd in the human body is associated with metallothionein.[12] As it is reported that the induction of metallothionein following Cd exposure mainly occurs in the liver,[13] the other aim of our study was to investigate metallothionein expression in order to clarify the aspect of its protective function against metal toxicity in the liver of itai-itai disease cases.

Materials and methods

Materials

We examined 89 autopsied itai-itai disease cases. All patients were elderly women. The clinical diagnosis was based on the opinion of the Ministry of Health and Welfare, with regard to itai-itai disease in Toyama Prefecture.[14] The diagnosis of itai-itai disease was made when patients fulfill the following three criteria:[15] (1) to live in an area polluted with Cd; (2) have symptoms that appear in adults (especially after menopause) and are not congenital, and (3) exhibit renal tubulopathy. We selected 27 non-Cd-polluted cases as the control group for comparison of Cd concentration in various organs; 70 cases with an organ other than the liver as the site of the main pathological lesion were then selected as the control group for analysis. We chose cases from a different region of Japan without risk of high Cd exposure as non-Cd-polluted cases. The demographic features of these cases are listed in Table 1.

Table 1 Demographic features of itai-itai disease (IID) and control cases

	IID	*Control for Cd concentration*	*Control for pathological analysis*
N	89	27	70
Median age (range)	79 (62–97)	71 (46 – 87)	75 (50 – 97)
Sex (female/male)	89/0	27/0	70/0
Period when autopsies were performed	1979–2009	1955–1990	2000–2010

Cd Concentration

Cd concentration in organs was measured at two institutions to promise precision: (1) the Department of Pharmaceutical Science, International University of Health and Welfare and (2) the Department of Occupational and Environmental Medicine, Graduate School of Medicine and School of Medicine, Chiba University. The degree of discrepancies between the two testing methods is shown in Table 2. We adopted average Cd titers from the two institutions. As described by Kuzuhara *et al*,[16] each tissue (fresh and unfixed, 1–10 g wet weight) was mineralized by heating in the presence of 12 N nitric acid to dryness, and the residue was taken up in 1 N nitric acid. The final preparation was subjected to instrumental analyses for Cd, with a graphite furnace atomic absorption spectrometer at 228.8 nm (Hitachi Z-8200, Hitachinaka, Japan).

Pathological Analysis

Of the 89 itai-itai disease cases, we excluded 9 cases from analysis on account of their samples proving inadequate for further investigation. In addition, we excluded ischemic and/or congestive changes considered to be agonal from the evaluation, along with apparent drug-induced injuries. Fibrosis was assessed on a four-step scale: (1) perisinusoidal or periportal; (2) perisinusoidal and portal/periportal; (3) bridging fibrosis; and (4) cirrhosis. Iron accumulation was graded from 0 to 4, according to the Scheuer's scoring system modified by Searle *et al*[17]: 0 granules absent or barely discernible at $\times 400$; 1 granules barely discernible at $\times 250$ and easily confirmed at $\times 25$; 2 discrete granules resolved at $\times 100$; 3 discrete granules resolved at $\times 25$; 4 masses visible at $\times 10$, or naked eye.

Immunohistochemical Analysis

We performed immunohistochemical analysis on 21 of the 80 itai-itai disease cases and 15 of the 70 control cases to investigate metallothionein expression. These cases were randomly selected from our list. After deparaffinization of specimens, proteinase K antigen retrieval (Histofine Nichirei, Tokyo, Japan) was performed for 5 min, followed by endogenous peroxidase blocking (5% H_2O_2 in methanol) for additional 5 min. After nonspecific binding blocking with 5% bovine serum albumin (Sigma-Aldrich, Tokyo, Japan), incubation with the prediluted primary antibody was carried out overnight at 4 °C, using mouse monoclonal anti-metallothionein antibody (IgG_1, 1:300, clone UC1MT; Abcam, Cambridge, UK). The immunoreaction was visualized with diaminobenzidine–chromogen/EnVison Polymer–horseradish peroxidase (K4001; Dako, Glostrup, Denmark and SK4100; Vector Labs, Burlingame, CA, USA). Mild counterstaining was

Table 2 The degree of discrepancies between cadmium (Cd) measuring tests in two institutions

	Liver	*Kidney cortex*	*Kidney meddula*	*Thyroid grand*	*Pancreas*	*Muscle*	*Aorta*	*Rib*	*Heart*
IID									
IUHW	69.7 (7.4–314)	36.0 (8.0–133)	25.3 (1.0–78.8)	52.8 (7.0–185)	47.9 (9.9–132)	12.4 (3.2–42.1)	3.45 (1.0–17.8)	1.82 (0.5–7.0)	1.1 (0.1–5.0)
CU	69.4 (5.6–287)	35.1 (9.3–123)	25.5 (8.1–82.4)	50.9 (8.1–177)	51.2 (8.9–162)	12.1 (3.6–36.2)	3.27 (1.1–12.3)	1.91 (0.4–4.9)	1.1 (0.3–3.8)
P-value	0.964	0.809	0.914	0.716	0.427	0.746	0.596	0.586	0.795
Control									
IUHW	11.9 (1.7–47.8)	87.3 (5.7–193)	39.1 (3.2–120)	10.1 (2.0–25.9)	10.3 (2.1–25.7)	1.53 (0.3–5.1)	1.06 (0.2–2.9)	0.601 (0.01–2.4)	0.377 (0.01–0.8)
CU	14.0 (3.3–49.6)	102 (5.7–218)	44.9 (3.8–111)	11.0 (2.4–30.9)	13.6 (2.4–32.1)	1.77 (0.4–5.3)	1.22 (0.2–2.6)	0.584 (0.2–1.3)	0.561 (0.1–1.5)
P-value	0.489	0.317	0.440	0.616	0.185	0.464	0.367	0.890	0.0404

Abbreviations: CU, Chiba University; IID, itai-itai disease; IUHW, International University of Health and Welfare.
Values are present as average (min–max).

performed with hematoxylin. Staining intensity was scored on a four-point scale (0, 1+, 2+, 3+): 0, no positive staining; 1+, mild cytoplasmic staining; 2+, moderate-to-severe cytoplasmic staining; and 3+, moderate-to-severe cytoplasmic staining with nuclear staining. Scoring was performed by two independent pathologists (HB and KT), and the average score was adopted.

Statistical Analyses

Statistical analyses were performed using SPSS for Windows version 15.0 software (SPSS, Chicago, IL, USA). Cd concentration was expressed as mean ± s.e.m. and analyzed by Student's *t*-test. Comparison of pathological findings was analyzed by Pearson's χ^2-test. To compare scores obtained from immunohistochemical analysis, Mann–Whitney *U*-test was performed. Differences were considered significant at *P*-values <0.05.

Results

Cd Concentration in Organs

Cd concentration in organs is shown in Figure 1. Cd accumulated at a significantly higher concentration in the liver in the itai-itai disease group than that in the liver in the control group. Figure 1 also shows that in the itai-itai group, the liver had the highest concentration of Cd of organs tested, whereas in the control group, renal cortex had the highest levels, and more Cd than the liver in the disease group.

Pathological Features

The results of pathological analysis are shown in Table 3. In the itai-itai disease group, fibrosis and hemosiderosis were observed at a significantly higher rate compared with the control group ($P=0.0022$ and 0.0006, respectively).

Of 19 cases with fibrosis, periportal fibrosis was observed in 9 (47%) , perisinusoidal and portal/periportal fibrosis in 5 (26%), and bridging fibrosis in 5 (26%) cases. Most fibrosis was observed around the portal region, with slender fibrotic extensions and a stellate pattern. Perivenular and pericellular fibrosis was not observed (Figures 2a and b).

Of 15 cases with hemosiderosis, 6 were graded 2 and 9 were graded 3 according to the modified Scheuer's scoring system. Iron was primarily located in hepatocytes but was also in some Kupffer cells in most cases. It was accumulated in a pelicanalicular pattern in two cases, and in a massive pattern in all other cases (Figures 2c and d). In the liver of the itai-itai disease group, statistically higher Cd concentration was recorded in cases with fibrosis than in those without fibrosis ($t(76)=2.105$, $P<0.05$). In contrast, no significant difference was observed

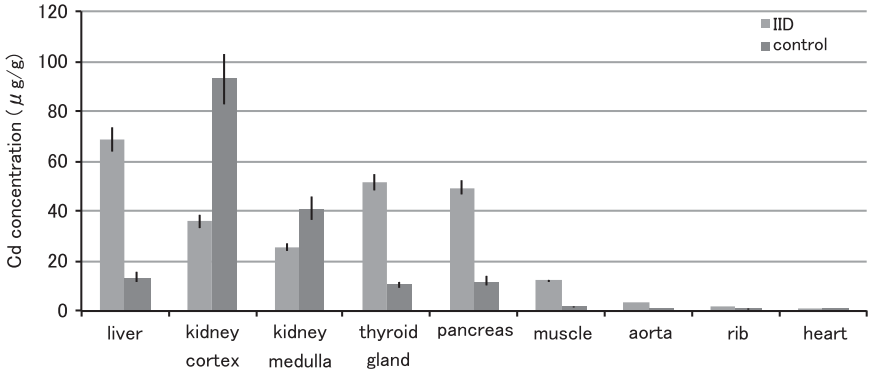

Figure 1 Average cadmium (Cd) concentration in selected organs. Significant differences were observed between the itai-itai disease and control groups for all organs examined ($P<0.01$).

Table 3 Pathological findings observed in liver specimens of 80 itai-itai disease cases and 70 control cases

Pathological finding	*IID*	*Control*	χ^2 P-*value*	*Odds ratio (95% confidence intervals)*
	N *(%)*	N *(%)*		
Fibrosis	19 (24)	4 (6)	**0.0022**	**5.13 (1.66–16.0)**
Hemosiderosis	15 (19)	1 (1)	**0.0006**	**15.9 (2.04–124)**
Inflammation				
Infiltration of lymphocyte	8 (11)	7 (9)	0.585	0.743 (0.255–2.16)
Hepatitis	2 (3)	—	—	—
Steatosis	6 (8)	7 (10)	0.587	0.730 (0.233–2.28)
Carcinoma	0 (0)	—	—	—

Bold *P*-value: <0.05.
Bold odds ratio: >1.

between cases with hemosiderosis and those without hemosiderosis ($t(76)=1.411$, $P>0.05$).

Inflammation observed in 10 cases was mainly located in portal regions. In 4 out of 10 cases, mononuclear aggregates in some portal tracts. In three cases, mononuclear aggregates in all portal tracts (Figure 2e). In three cases, including two cases with hepatitis, large and dense mononuclear aggregates in all portal tracts. The infiltrating cells were mainly lymphocytes, and plasma cells were also confirmed. Eosinophils were observed in several cases.

In six cases with steatosis, macrovesicular and microvesicular fatty changes were mixed (Figure 2f). It was located in a centrilobular pattern in four cases, and diffusely in two cases. Glycogenerated nuclei were observed in two cases with steatosis. Hepatocellular carcinoma and other tumors were not observed in the livers of the itai-itai disease group. Megamitochondria and biliary tract changes were not observed.

Metallothionein Expression

Metallothionein was expressed more strongly in the itai-itai disease group than that in the control group ($P=0.017$) (Figures 2g,h and 3). Whereas metallothionein expression was observed only in the cytoplasm of the liver in the control group, it was observed in the cytoplasm and at times in the nucleus in 6 cases (29%) in the itai-itai disease group. Cases with nuclear metallothionein expression had significantly higher Cd concentration than those with only cytoplasmic metallothionein expression ($P=0.0051$) (Figure 4).

Discussion

Considerable hepatic and renal Cd accumulation is known to occur,[18] and toxicological studies in animals show that hepatic Cd levels are initially the highest, followed by an increase in renal Cd levels after long-term exposure.[19–21] Whereas the results of Cd concentration in our control group are consistent with the above-mentioned data, Cd concentration in the itai-itai disease group showed marked hepatic Cd accumulation, following renal injury because of chronic Cd exposure.

In the liver of the itai-itai disease group, but not the control group, slender, stellate-shaped fibrotic lesions were observed as a characteristic change, and a significant difference was observed in Cd concentration between cases with fibrosis and those without fibrosis. Therefore, fibrosis is thought to reflect Cd-specific changes. Several studies

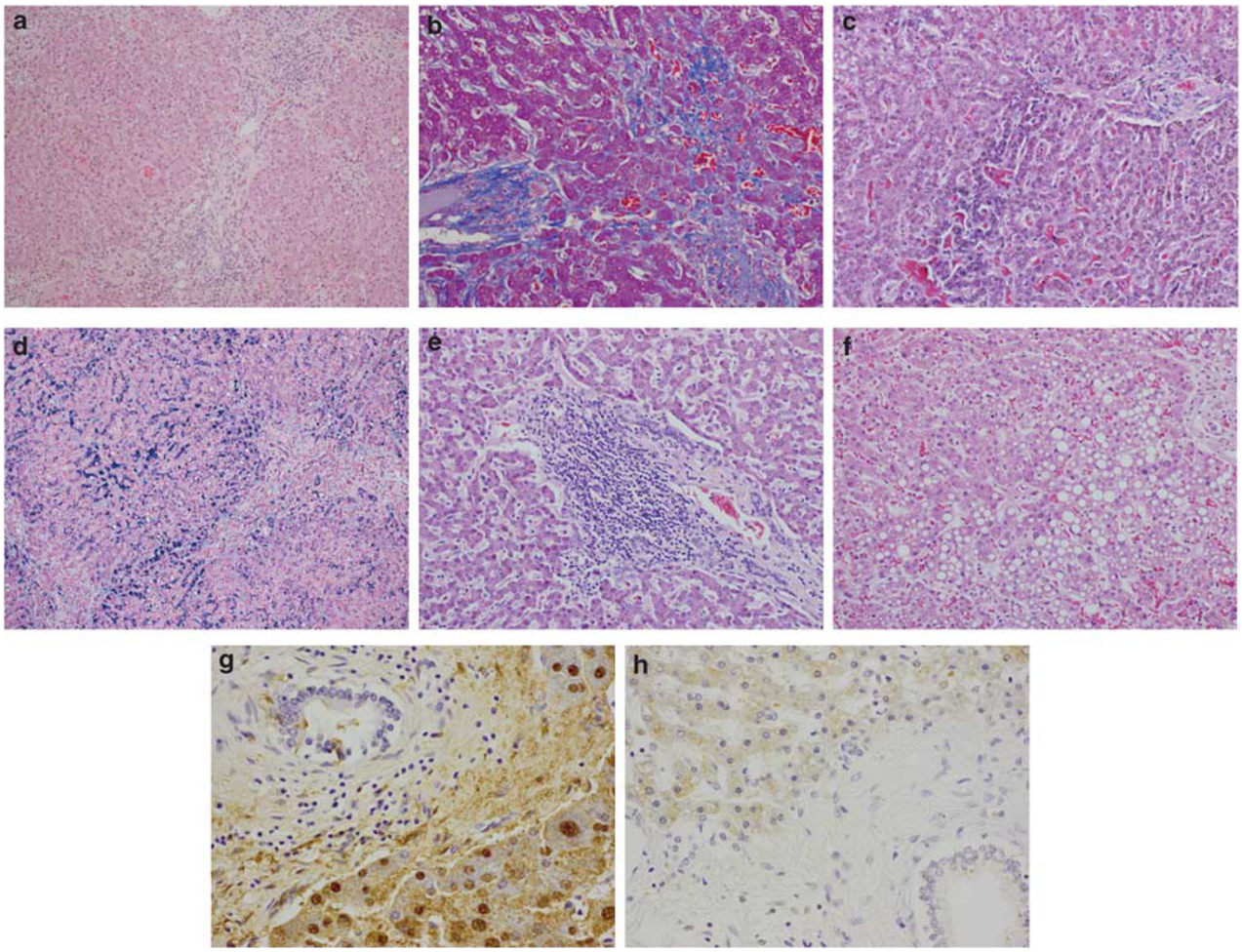

Figure 2 (**a**, **b**) In the liver of itai-itai disease cases, fibrosis was observed in a stellate pattern around the portal region; (**a**) H&E, ×200, (**b**) Azan, ×200. (**c**, **d**) Iron accumulation was mainly in hepatocytes with a massive pattern in itai-itai disease cases with hemosiderosis; (**c**) H&E, ×200, (**d**) Berlin blue, ×200. (**e**) Inflammation observed in itai-itai disease cases was mainly located in portal regions. The infiltrating cells were primarily lymphocytes; H&E, ×200. (**f**) Macrovesicular and microvesicular fatty changes were mixed in itai-itai disease cases with steatosis; H&E, ×200. (**g**) Metallothionein was expressed in the cytoplasm and nuclei of hepatocytes in the liver of itai-itai disease cases; metallothionein immunohistochemistry, ×400. (**h**) Metallothionein was weakly expressed only in the cytoplasm of hepatocytes in the liver of control cases; metallothionein immunohistochemistry, ×400.

have confirmed the fibrogenic effect of Cd.[22–24] Carmen *et al*[25] reported that the role of Cd in liver fibrosis was the production of oxidative stress *in vivo*. The fibrotic liver may be subjected to exposure by continuous oxidative stress induced by Cd. We could not however identify contributing or underlying conditions to the phenotype of itai-itai disease cases with fibrosis from the available information. One of the possible biases is drug history. We are now trying to collect more detailed clinical information from various medical institutions. In further study, we need to clarify the specific backgrounds of fibrosis in itai-itai disease.

Although hemosiderosis was also observed at a significantly higher rate in the itai-itai disease group than that in the control group, various studies have shown that hepatic iron concentration was reduced when Cd was orally ingested through drinking water or through the diet,[26–28] contradicting our findings. Many itai-itai disease patients receive blood transfusion for renal anemia; therefore, we need to consider the impact. Unfortunately, detailed clinical records were unavailable for the patients in our study, and further investigation will be required to determine the significance of hemosiderosis.

Although fibrosis and hemosiderosis can occur in itai-itai disease, the changes observed in this study were less severe compared with the acute hepatotoxicity induced by Cd exposure reported in other studies.[22,29,30] Our results indicate that increased metallothionein expression can inhibit Cd hepatotoxicity. Many studies using metallothionein transgenic animals have demonstrated that metallothionein has an important protective role against Cd hepatotoxicity. Liu *et al*[31] reported that mice overexpressing metallothionein were protected against acute Cd lethality and hepatotoxicity. In contrast, repeated administration of Cd chloride to

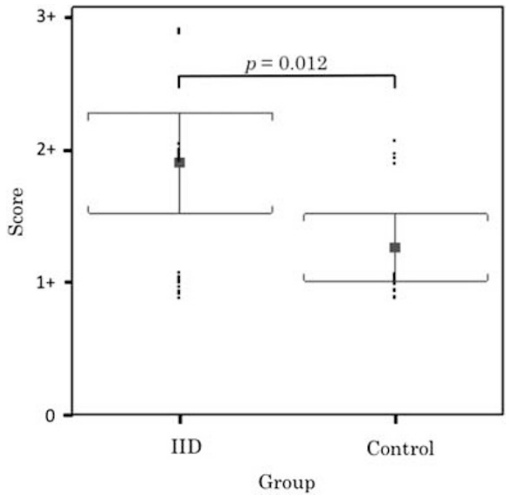

Figure 3 Immunohistochemistry scoring for metallothionein. The error bars indicate 95% confidence interval. A significant difference was observed between the itai-itai disease and control groups ($P = 0.017$).

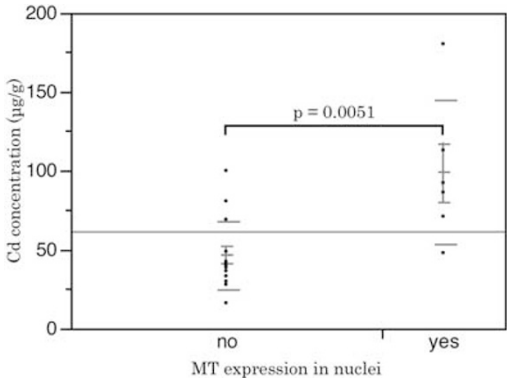

Figure 4 The comparison of cadmium (Cd) concentration between itai-itai disease cases with nuclear metallothionein expression and those without nuclear metallothionein expression. A significant difference was observed between two groups ($P = 0.0051$).

metallothionein-null mice caused nonspecific chronic inflammation in the liver parenchyma and portal tracts, and higher doses produced granulomatous inflammation and preneoplastic proliferative lesions.[32] These studies support the premise that high metallothionein expression in the liver of itai-itai disease patients can reduce hepatic damage.

An interesting point is that metallothionein was expressed in hepatocyte nuclei of some itai-itai disease cases. Numerous studies, both *in vivo* and *in vitro*, showed that the adult animal and human liver has a very low nuclear metallothionein content,[33–35] so metallothionein expression in nuclei is thought to be a finding related to itai-itai disease. Metallothionein does not have nuclear localization signals, so nuclear trafficking of metallothionein requires binding to cytosolic substances.[36,37] Although metallothionein binds to various heavy metals, it is reasonable to consider that metallothionein binds to Cd under Cd-rich condition. Thus, nuclear metallothionein is thought to reflect the distribution of Cd. This is strongly supported by the result that metallothionein expression in nuclei was observed in cases with high Cd concentration. Therefore, the result of immunohistochemistry suggests that Cd accumulates in hepatocyte nuclei in itai-itai disease.

This is the first study to focus on liver pathology in itai-itai disease. Although several studies using animal models have evaluated the chronic effects of Cd exposure, the exposure period in these models was much shorter than that in humans in everyday life; therefore, it is difficult to apply these results directly to the chronic effects of Cd in humans. Therefore, we believe the findings of this study can be beneficial because they represent direct evidence of chronic human hepatotoxicity due to Cd. Considering that Cd exposure is expected to rise globally,[4] further studies focusing on itai-itai disease patients are urgently required.

In conclusion, chronic exposure to low Cd doses leads to not only high hepatic Cd accumulation, which can cause fibrosis, but also high metallothionein expression. Highly expressed metallothionein is thought to reduce the hepatotoxic effects of Cd.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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