to form the SHAP-HA complex, which is associated with many inflammatory diseases.[47] Some researchers suggest that the SHAP-HA complex level is a better indicator for the progression of the stages of liver fibrosis in patients.[48] On the other hand, the SHAP-HA complex can also inhibit the development of inflammation.[49] Thus, our results of this study may provide an incentive for evaluating the effect of HC deficiency in UTI-KO mice on inflammation and liver fibrosis in future studies.

In conclusion, our results provide evidence for the potential that UTI endogenously released from the liver inhibits TGF-β production as well as proteases involved in TGF-β activation, thereby modulating the process of liver fibrosis.

Author contributions: TK, YK and SK conceived and designed the study; YK, NC, TA and YE performed the animal experiments; TK, YK and TA performed the molecular biology experiments; TK and YE performed the histological experiments; and TK and MY analyzed the data and wrote the manuscript. TK and YK contributed equally to the study.

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The authors report no conflicts of interest.

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Polaprezinc prevents ongoing thioacetamide-induced liver fibrosis in rats

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ABSTRACT

Aims: Cirrhotic patients commonly have a liver zinc deficiency, which may aggravate liver fibrosis due to the lack of antioxidative effects of zinc. This study examined the ability of polaprezinc, N-(3-aminopropionyl)-L-histidinato zinc, to prevent fibrosis in a rat model of thioacetamide (TAA)-induced hepatic fibrosis.
Main methods: Liver cirrhosis was induced by orally administering TAA for 20 weeks. The rats were cotreated with one of the following for the last 10 weeks of TAA treatment: (1) polaprezinc (50 or 200 mg/kg/day); (2) L-carnosine (155 mg/kg/day), which contained equal amounts of L-carnosine as 200 mg/kg/day polaprezinc; (3) zinc sulfate (112 mg/kg/day) or (4) zinc-L-aspartic complex (317.8 mg/kg/day). Both zinc supplementations contained equal amounts of zinc as high-dose polaprezinc.
Key findings: Hepatic zinc levels fell significantly in rats treated with TAA for 20 weeks. Cotreating with high-dose polaprezinc and zinc-L-aspartic complex for 10 weeks prevented hepatic zinc loss. Hepatic hydroxyproline and tissue inhibitor of metalloproteinases-1 (TIMP-1) were significantly higher in rats treated with TAA for 20 weeks than 10 weeks, whereas polaprezinc prevented changes in these fibrosis markers and reduced hepatic transforming growth factor-β1 protein concentration, macroscopic and histologic changes. TAA caused oxidative stress-related changes in the liver that were prevented by high-dose polaprezinc and partially by zinc-L-aspartic complex. Treatment with L-carnosine, low-dose polaprezinc or zinc sulfate for 10 weeks did not affect liver fibrosis progression or oxidative stress-related changes.
Significance: Polaprezinc may prevent ongoing fibrosis by preventing zinc depletion, oxidative stress and fibrosis markers in cirrhotic livers.

Introduction

During hepatopathy that is induced by chronic inflammation (i.e., chronic hepatitis), the damaged tissue is repaired by the accumulation of connective tissue, and the liver ultimately becomes cirrhotic. Because fibrosis is the most important factor in the onset of both carcinogenesis and hepatic failure, one important prophylactic and therapeutic strategy is to control the progression of hepatic fibrosis (Bataller and Brenner, 2005; Kisseleva and Brenner, 2008). In addition, the continuous production of free radicals in chronic hepatitis accelerates liver fibrosis (Friedman, 2003) and exacerbates hepatocellular damage (Britton and Bacon, 1994; Loguercio and Federico, 2003). Therefore, eliminating continuous free radical production may inhibit the progression of liver fibrosis.

Zinc is an essential trace element that exerts important antioxidant, anti-inflammatory, and antiapoptotic effects (Powell, 2000; Stamoulis et al., 2007). Cirrhosis is commonly associated with zinc deficiency, and for many years, zinc has been thought to have protective effects against liver fibrosis (Stamoulis et al., 2007). Zinc deficiency causes a decline in the activity of collagenase that leads to liver fibrosis (Seltzer et al., 1977). Moreover, zinc exerts membrane-stabilizing activity on liver lysosomes and has cytoprotective activities that protect hepatocytes from oxidative stress (Stamoulis et al., 2007; Zhou et al., 2005).

Polaprezinc, N-(3-aminopropionyl)-L-histidinato zinc, contains the compound L-carnosine, which forms a chelate complex with bivalent metal zinc ions, and has been used to treat gastritis and gastric ulcers in Japan (Matsukura and Tanaka, 2000; Yamaguchi et al., 1996). Several in vitro and in vivo studies have also shown that polaprezinc has potent antioxidant effects (Odashima et al., 2006; Ohkawara et al., 2006; Ohkawara et al., 2005; Yoshikawa et al., 1991a). Among the active components of polaprezinc, L-carnosine has been shown to have antioxidant activity (Hipkiss and Brownson, 2000). Incidentally, it has been reported that polaprezinc has more potent antioxidant effects than zinc or L-carnosine alone (Odashima et al., 2002; Ohkawara et al., 2005).

These findings raise several important questions. In particular, it is unknown whether the antioxidative activities and antifibrotic effects of polaprezinc are due to the prepotency on the complex structure or

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whether the efficacy of polaprezinc is due to zinc supplementation alone.

In this study, we used a rat model of long-term thioacetamide (TAA)-induced hepatic fibrosis to compare the antioxidative and antifibrotic effects of a continuous supply of different zinc amino acid complexes and zinc sulfate, all contained the same amount of zinc, except for the low-dose polaprezinc, as well as the effects of L-carnosine, a component of polaprezinc.

Materials and methods

Animal treatment and induction of chronic liver damage

Male Wistar rats (aged 5 weeks at the start of the protocols) were used in all experiments. The rats were kept in the animal breeding house at Asahikawa Medical University with free access to food (Purina rodent powder diet CE-2, Hokudo, Sapporo, Japan) and water. All animals received humane care during the study. The experimental protocols were approved by the Animal Care Committee of Asahikawa Medical University and were in accordance with the National Institute of Health's "Guide for the Care and Use of Laboratory Animals".

Liver cirrhosis was induced by orally administering TAA (300 mg/L) in the drinking water for 20 weeks as described previously (Muller et al., 1988). The age-matched control animals received normal tap water.

The experimental protocol is illustrated in Fig. 1. Nine groups, each containing 15 animals, were examined. In group 1, TAA was continuously administered for 20 weeks. Starting 10 weeks after the initiation of TAA administration, the animals in groups 2, 3, 4, 5 and 6 were also administered the following in their powdered food for 10 weeks: polaprezinc (50 mg/kg/day, zinc 11.3 mg or 200 mg/kg/day, zinc 45.2 mg; Zeria Shinyaku Pharmaceutical Co., Ltd., Tokyo, Japan); zinc sulfate (112 mg/kg/day, zinc 45.2 mg; Wako Pure Chemical Industries, Osaka, Japan); zinc-L-aspartic chelate complex (317.8 mg/kg/day, zinc 45.2 mg; Zinc100; KAL, Knoxville, TN, USA); or L-carnosine (155 mg/kg/day, NOW, Bloomingdale, IL, USA), which contained equal amounts of L-carnosine as polaprezinc 200 mg/kg/day. Zinc sulfate and zinc-L-aspartic complex contained equal amounts of zinc ions as polaprezinc (200 mg/kg/day). The rats in group 7 were sacrificed after 10 weeks of TAA administration. The rats in group 8 received no drug treatment during the 20-week experimental period. The rats in group 9 received polaprezinc (500 mg/kg/day) for the last 10 weeks of the experimental period. Body weight and food intake were monitored weekly and daily throughout the experimental period, respectively. At the end of the experimental period, the rats were anesthetized with sodium pentobarbital (50 mg/kg). Blood was drawn using a heparinized syringe from the abdominal aorta, and serum was obtained by centrifugation.

Analysis of the zinc content in the liver

At the end of the study period, liver tissue was obtained from the rats in each group, frozen in liquid nitrogen, and stored at −80 °C. The zinc concentration in each sample was determined using an absorption spectrophotometer (Z-6110, Hitachi, Tokyo, Japan).

Assessment of liver fibrosis

At the end of the study period, the resected livers were either fixed overnight in 4% paraformaldehyde in phosphate-buffered saline at 4 °C or frozen immediately in liquid nitrogen. Serial 5-μm sections were prepared after the samples had been dehydrated in graded ethanol solutions, cleared in chloroform, and embedded in Paraplast (Fisher Scientific Japan, Tokyo, Japan). Collagenous and noncollagenous proteins were differentially stained with 0.1% Sirius Red using 0.1% Fast Green as a counterstain in saturated picric acid in order to conduct a semiquantitative morphometric analysis of liver fibrosis using an image analysis system (Nikon Digital Sight DS-L1, Tokyo, Japan). The percentage of area that was stained with Sirius Red at a 40× magnification and the mean percentage area for each sample were calculated.

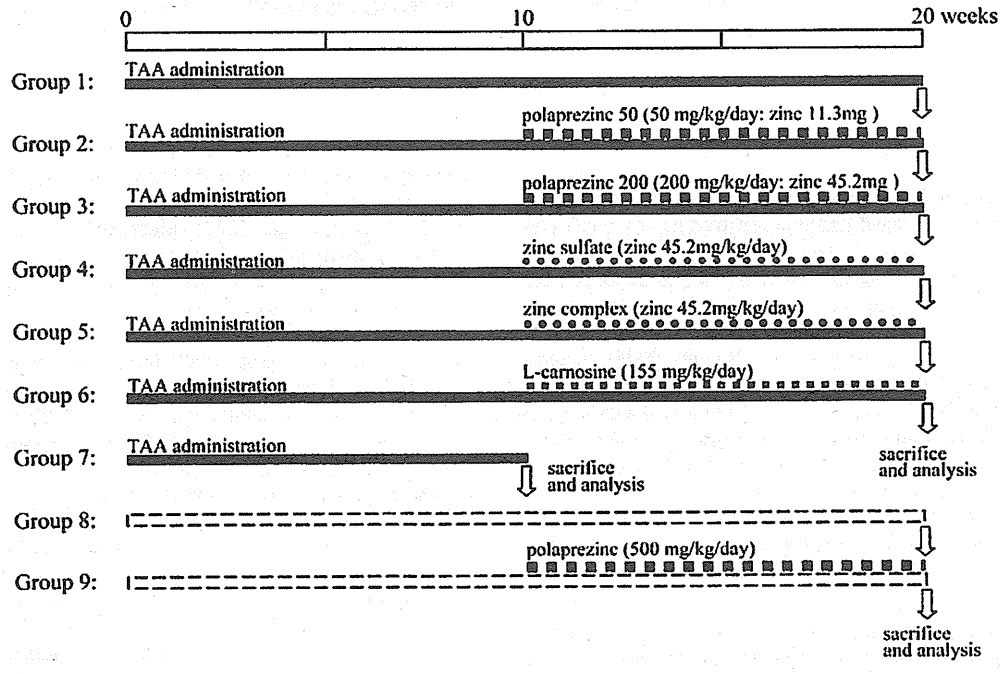

Fig. 1. Experimental protocol. Rats were divided into 9 groups of 15 animals each. In groups 1–6, thioacetamide (TAA) was administered continuously for 20 weeks. The rats in group 7 were administered TAA for 10 weeks. In addition to TAA, the animals in groups 2, 3, 4, 5, and 6 received polaprezinc (50 mg/kg/day or 200 mg/kg/day), zinc sulfate (112 mg/kg/day), zinc-L-aspartic complex (317.8 mg/kg/day) and L-carnosine (155 mg/kg/day) in their powdered food for the final 10 weeks of the experiment, respectively. The rats in group 8 received no drug treatment. The rats in group 9 received polaprezinc at 500 mg/kg/day only for the last 10 weeks of the experimental period.

The levels of α-smooth muscle actin (SMA), a specific marker of hepatic stellate cell (HSC) activation, in the liver were detected by immunohistochemistry using an anti-SMA antibody (Sigma, St. Louis, MO, USA). The Vectastain avidin–biotin kit (Vector Laboratories, Burlingame, CA, USA) was used according to the manufacturer's instructions.

For a semiquantitative morphometric analysis, we assessed the mean value of the area of SMA-positive cells in three visual fields per specimen as the percent area at a 40× magnification using an image analysis system (NIH image 1.62, Bethesda, MD, USA). The SMA-positive cells were expressed as a percentage of the total specimen area.

Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were assessed using routine laboratory methods.

The collagen concentration was determined by digesting fresh liver samples with acid and then measuring the hepatic hydroxyproline (HP) content (Sakaida et al., 1996). The liver specimens were weighed, and 20 mg of frozen sample was autoclaved in 6 M HCl for 24 h. After centrifugation, the supernatant was mixed with 1% phenolphthalein and 8N KOH to obtain a solution with pH 7 to 8. This solution was stirred with chloramine T solution for 60 min at 0 °C. The resulting solution was stirred with KCl and borate buffer (pH 8.2) for 15 min at room temperature and for another 15 min at 0 °C. After adding 3.6 M sodium thiosulfate, the solution was incubated for 30 min at 120 °C and stirred with toluene for 20 min. The reaction was centrifuged at 2000 rpm at 4 °C and Ehrlich's solution was added to the resulting supernatant. The final product was incubated for 30 min at room temperature. The absorbance at 560 nm was measured, and the HP content was expressed in μg/g of wet liver.

Hepatic TGF-β1 protein concentration was measured by the Predicta assay kit (Genzyme Diagnostics, Cambridge, MA, USA) after determining total hepatic protein content with Isogen (Nippon Gene Co., Toyama, Japan) (Sakaida et al., 1998). All TGF-β_1 protein levels were assessed as the active form by the addition of HCl. The total liver protein content was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). TGF-β_1 protein concentration was expressed as ng/g of total liver protein.

Assessment of overall extent of fibrinolysis in liver

For the study of MMP activity, frozen liver samples were mechanically homogenized and centrifuged and clarified supernatant were used for protein quantifcation by Bradford method. Aliquots (30 μg protein) from liver extracts were subjected to electrophoresis in SDS-PAGE gels under non-reducing conditions. The gelatin substrate was present at 0.1% final concentration in the gel. The gels were electrophoresed at 100 V for 2 h at 4 °C in a Bio-Rad MiniProtean II system (Bio-Rad Laboratories, Inc., Richmond, CA, USA). Following electrophoresis, gels were washed by gentle shaking at room temperature with 2.5% Triton X-100 (two changes) for 1 h. The gels were incubated for 24 h in 50 mM Tris–HCl (pH 8.4) containing 5 mM CaCl2 and 1 μM ZnCl2 at 37 °C. After incubation, gels were stained by Coomassie Brilliant Blue R-250. Areas of proteolysis appeared as clear zones against a blue background. Molecular mass determinations were made with reference to prestained protein standards (Bio-Rad Laboratories) co-electrophoresed into the gels. The integrated optical density (IOD) spectrophotometry was determinated in an image analysis system (NIH image 1.62, Bethesda, MD, USA). The values of IOD were analyzed statistically and plotted in histograms.

For the detection of tissue inhibitor of MMP (TIMP) in liver, enzyme-linked immunosorbent assay (ELISA) was used. Frozen liver samples were mechanically homogenized and centrifuged, and protein extracted on supernatant was quantified by Bradford method. The supernatants were quantified by the Quantikine immunoassay kits from R&D systems raised against rat TIMP-1 (Wako, Osaka, Japan) by following the instructions provided by the manufacturers. The absorbances at 450 nm were measured in a microtest plate spectrophotometer (Immuno Mini NJ-2300, Biotec, Tokyo, Japan), and antigen levels were determined by appropriate calibration curves.

Assessment of oxidative stress-related parameters in the liver

Oxidative stress was evaluated based on the levels of lipid peroxide (LPO), superoxide dismutase (SOD) activity and reduced glutathione (GSH) in the liver. LPO and SOD were measured using the LPO-586 and SOD-525 commercial assay kits, respectively (Bioxytec, Portland, OR, USA). LPO and SOD were measured in liver homogenates following the manufacturer's guidelines, as previously described (Sun et al., 1988; Zhou et al., 2001). The GSH-400 commercial kit (Bioxytec) was used to measure GSH in liver extracts.

Statistical analysis

Data from each experimental group are expressed as the means ± SD. Multiple group comparisons were performed by Kruskal–Wallis one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test. *P*-values <0.05 were considered statistically significant.

Results

The body weight and daily food intake are shown in Table 1. The starting weight did not differ among the groups. TAA caused a marked decrease in total body weight in all the groups except for the normal control group. However, cotreatment with polaprezinc (200 mg/kg/day) and zinc complex attenuated this change in body weight. By contrast, cotreatment with zinc sulfate exacerbated this change in body weight and also resulted in reduced daily food intake compared to other groups.

Among rats that were treated with TAA for 20 weeks, the hepatic zinc levels started to fall at 10 weeks and were significantly lower at 20 weeks than those of the controls (from 39.5 ± 3.5 μg/g to 24.2 ± 4.6 μg/g, $P<0.01$). However, the hepatic zinc content was significantly higher in animals cotreated with oral polaprezinc (200 mg/kg/day) and zinc-L-aspartic complex (34.5 ± 5.2 μg/g and 32.8 ± 7.2, $P<0.01$,

Table 1
Body weight (g) and daily food intake (g).

Treatment	Start	5 weeks	10 weeks	15 weeks	20 weeks
Control	119 ± 4.2	253 ± 5.7	318 ± 9.6	357 ± 14.4	388 ± 15.3
	20	20	24	24	24
TAA 10 weeks	120 ± 3.0	187 ± 7.6*	218 ± 11.5*		
	20	12	15		
TAA 20 weeks	119 ± 3.8	181 ± 11.4*	222 ± 18.3*	233 ± 26.7*	239 ± 28.0*
	20	12	15	15	15
TAA 20 weeks + polaprezinc 50	120 ± 4.0	182 ± 9.8	219 ± 13.4	235 ± 26.1	242 ± 27.0
	20	12	15	15	15
TAA 20 weeks + polaprezinc 200	118 ± 3.7	181 ± 10.8	217 ± 16.3	245 ± 22.9**	259 ± 20.1**
	20	12	15	18	19
TAA 20 weeks + zinc sulfate	119 ± 3.8	182 ± 10.5	220 ± 15.4	227 ± 25.6	224 ± 28.4**
	20	12	15	14	13
TAA 20 weeks + zinc complex	118 ± 3.9	183 ± 12.2	219 ± 17.9	241 ± 20.0	252 ± 20.8**
	20	12	15	17	18
TAA 20 weeks + L-carnosine	119 ± 3.0	184 ± 11.6	217 ± 15.3	230 ± 26.0	238 ± 31.8
	20	12	15	15	15
Polaprezinc 500	120 ± 4.1	256 ± 6.4	319 ± 10.4	356 ± 12.7	386 ± 13.8
	20	20	24	24	24

Note: Results are expressed as the means ± SD (n = 15). The controls received no drug treatment. The remaining groups received 300 mg/L thioacetamide (TAA) in their drinking water for 10 or 20 weeks. Six groups of rats were also cotreated with polaprezinc (50 or 200 mg/kg/day), zinc sulfate (112 mg/kg/day), zinc-L-aspartic complex (317.8 mg/kg/day) or L-carnosine (155 mg/kg/day) after 10 weeks of TAA monotherapy. The polaprezinc 500 group only received polaprezinc (500 mg/kg/day) after 10 weeks. Abbreviations: TAA, thioacetamide.

* $p<0.01$ versus control.

** $p<0.05$ versus TAA 20 weeks.

respectively) than in rats treated with TAA alone for 20 weeks, and these levels were close to the 10-week TAA value (37.1 ± 5.4 μg/g). There were no significant differences between the groups that received polaprezinc (200 mg/kg/day) and zinc-L-aspartic complex (Fig. 2).

Fig. 3 shows representative photographs of the gross appearance of the liver after 10 and 20 weeks of TAA treatment alone and after polaprezinc was added to the treatment regimen for the last 10 weeks of TAA administration. Among rats treated with TAA alone, the hepatic surface was slightly rough at 10 weeks, indicating that hepatic fibrosis had already developed. At 20 weeks, large nodules were observed on the surface of the liver, indicating that cirrhosis had developed by this stage. Rats cotreated with polaprezinc (200 mg/kg/day) had decreased hepatic fibrosis progression after 10 weeks of TAA treatment. However, L-carnosine cotreatment did not prevent the progression of liver fibrosis.

The degree of hepatic fibrosis was confirmed by histologically examining Picrosirius Red-stained liver sections (Fig. 3). In the age-matched control rats, there was no spontaneous hepatic fibrosis. In the group treated with TAA for 10 weeks, mild but noticeable fibrosis developed in the liver, which was consistent with the macroscopic appearance of the liver. In the group treated with TAA for 20 weeks, there was a marked increase in the extracellular matrix collagen content and bridging fibrosis was prominent. There were bundles of collagen surrounding the lobules that resulted in large fibrous septa and distorted tissue architecture. In sharp contrast, only mild fibrotic changes were detected at 20 weeks in the polaprezinc group. These changes were similar to those seen after 10 weeks of TAA monotherapy. Semiquantification of collagen deposition in the liver showed that rats treated with TAA for 20 weeks (19.3 ± 5.2%) had significantly higher collagen deposition than that of the control rats (0.8 ± 0.4%) and rats treated with TAA for 10 weeks (6.5 ± 1.5%). High doses of polaprezinc (200 mg/kg/day) significantly prevented the progression of TAA-induced fibrosis (6.8 ± 1.5%), but this was not observed in the groups cotreated with low-dose polaprezinc (17.3 ± 2.9%), zinc sulfate (17.4 ± 4.8%), zinc complex (14.7 ± 3.3%) or L-carnosine (19.2 ± 4.6%) (Fig. 4A).

Fig. 3 also shows the hepatic expression of SMA. Positive SMA staining was clearly detected along the sinusoidal endothelium in the hepatic lobules in the TAA-treated groups. Very few SMA-positive cells were found in the sinusoidal walls in the control animals, although the portal area contained SMA-positive vessels, which were thought to be hepatic arteries. Semiquantification of SMA-positive cells showed that the number of SMA-positive cells significantly increased during the latter 10-week treatment period (control, 0.3 ± 0.1%; 10-week TAA, 1.2 ± 0.5%; 20-week TAA, 4.3 ± 1.5%). However, this increase in SMA expression was significantly inhibited in rats that were cotreated with high-dose polaprezinc (200 mg/kg/day) (1.3 ± 0.5%), but not low-dose polaprezinc (3.9 ± 1.4%), zinc sulfate (4.0 ± 2.1%), zinc complex (3.0 ± 1.6%) or L-carnosine (4.4 ± 1.7%) (Fig. 4B).

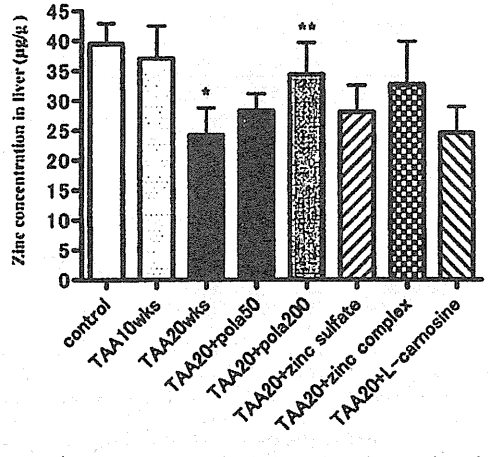

Fig. 2. Mean zinc concentrations (± SD, μg/g) in the rat liver. The controls received no drug treatment. The remaining groups received 300 mg/L thioacetamide (TAA) in their drinking water for 10 or 20 weeks. Two groups of rats were also cotreated with polaprezinc (50 or 200 mg/kg/day) after 10 weeks of TAA monotherapy. Five groups of rats were also cotreated with polaprezinc (50 or 200 mg/kg/day), zinc sulfate (112 mg/kg/day), zinc-L-aspartic complex (317.8 mg/kg/day) or L-carnosine (155 mg/kg/day) after 10 weeks of TAA monotherapy. Each bar represents the mean ± SD. All assays were performed in duplicate with samples that were obtained from 15 different animals. *$p<0.01$, a significant difference from the control. **$p<0.01$, a significant difference from the group treated with TAA for 20 weeks.

To evaluate hepatic collagen production after chronic TAA administration, hepatic HP was analyzed (Fig. 5). HP markedly increased in rats that were treated with TAA for 20 weeks (707 ± 223.1 μg/g), and this increase was significantly greater than that in the control group (92 ± 36.2 μg/g) and animals treated with TAA for 10 weeks (199 ± 62.7 μg/g). The increase in HP during the latter 10 weeks of TAA administration was significantly prevented in rats cotreated with polaprezinc during this period (200 mg/kg/day) (156 ± 65.5 μg/g). Cotreatment with low-dose polaprezinc (410 ± 207.1 μg/g), zinc sulfate (607 ± 280.4 μg/g), zinc-L-aspartic complex (307 ± 53.8 μg/g) or L-carnosine (518 ± 236.1 μg/g) did not prevent this increase in HP.

Fig. 6 shows hepatic TGF-β1 protein concentration. Hepatic TGF-β1 protein concentration remarkably increased in the 10-week TAA treatment group (69.9 ± 17.6 ng/mg) from the control level (7.4 ± 1.4 ng/mg), and declined but maintained a significant increase in the 20-week TAA treatment group (34.5 ± 11.9 ng/mg) compared to the control level. This increase of hepatic TGF-β1 protein concentration was significantly prevented in rats cotreated with polaprezinc during this period (200 mg/kg/day) (17.3 ± 4.7 μg/g). However, cotreatment with low-dose polaprezinc (28.2 ± 8.4 μg/mg), zinc sulfate (31.3 ± 11.8 μg/mg), zinc-L-aspartic complex (22.7 ± 7.1 μg/mg) or L-carnosine (32.3 ± 8.5 μg/mg) did not reduce the hepatic TGF-β1 protein concentration.

Data regarding MMP-2 and -9 activities are shown in Fig. 7. A significant increase in the activity of active MMP-2 (59 kDa) occurred in rats treated with TAA for 20 weeks (3.9 ± 2.0 arbitrary units), and this increase was significantly greater than that in animals treated with TAA for 10 weeks (1.7 ± 0.4 arbitrary units). The increase in MMP-2 activity during the latter 10 weeks of TAA administration was not observed in rats cotreated with polaprezinc during this period (50 and 200 mg/kg/day) (1.2 ± 0.4 and 1.1 ± 0.7 arbitrary units), respectively. Cotreatment with zinc sulfate (4.5 ± 2.3 arbitrary units), zinc-L-aspartic complex (3.3 ± 2.6 arbitrary units) or L-carnosine (6.7 ± 3.2 arbitrary units) resulted in an increase in MMP-2 activity. Pro-MMP-2 (72 kDa) activity also showed a similar trend of pro-MMP-2 activity in liver. Pro-MMP-9 (92 kDa) was significantly higher in all groups than in normal rats. However, there were no significant differences among the groups.

As shown in Fig. 8, TAA treatment caused a marked and time-dependent increase in the amount of TIMP-1 protein levels (from 1427 ± 321 pg/mL to 5467 ± 321 pg/mL after TAA 10 weeks, and to 12,733 ± 306 pg/mL after TAA 20 weeks). Polaprezinc cotreatment caused a gradual and dose-dependent decrease in the amount of TIMP-1 protein from the starting point of polaprezinc cotreatment (from 5467 ± 321 pg/mL to 3033 ± 115 pg/m after low-dose polaprezinc, and to 1867 ± 208 pg/mL after high-dose polaprezinc). High-dose polaprezinc decreased the level of TIMP-1 protein to the control level. Zinc-L-aspartic complex prevented the increase of TIMP-1 protein (4900 ± 100 pg/mL). However, cotreatment with L-carnosine and zinc sulfate, the increased the TIMP-1 protein levels to 13,367 ± 839 and 13,833 ± 737 pg/mL, respectively, which were comparable to the levels seen in rats treated with TAA for 20 weeks.

As shown in Table 2, TAA administration for 10 and 20 weeks significantly increased hepatic LPO, which was significantly inhibited by

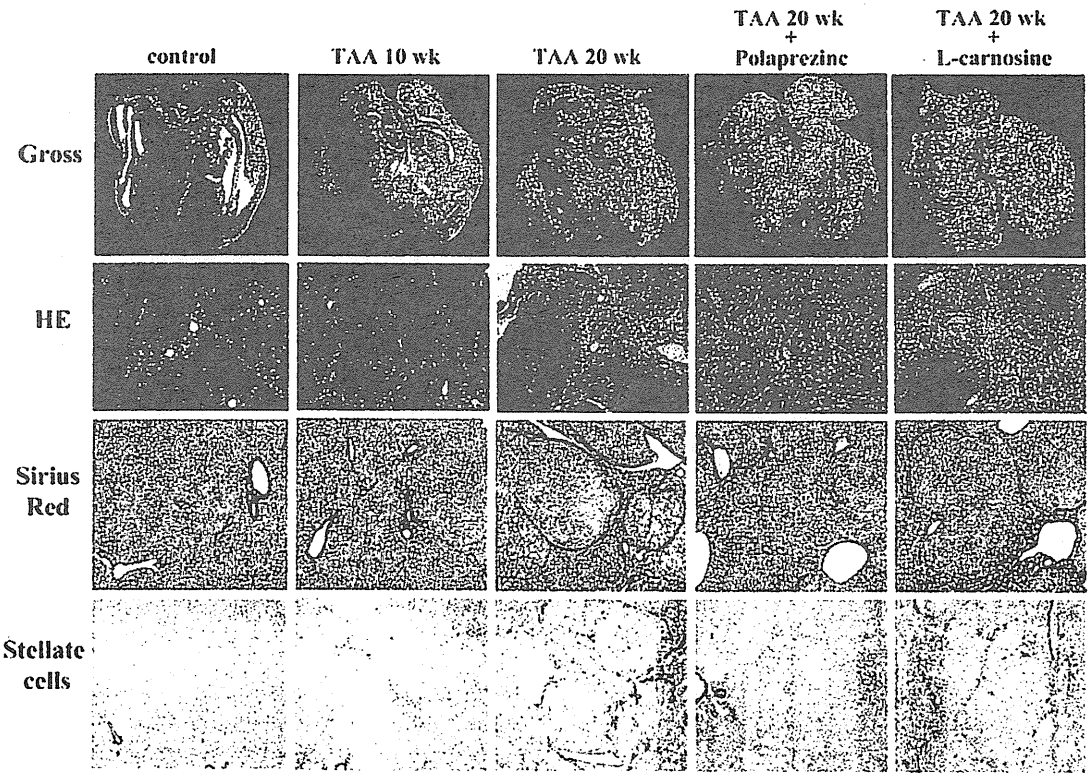

Fig. 3. Representative photographs of the macroscopic appearance of liver specimens and histologic analyses of liver sections. The controls received no drug treatment. The remaining groups received 300 mg/L thioacetamide (TAA) in their drinking water for 10 or 20 weeks. Two groups of rats were cotreated with polaprezinc (200 mg/kg/day) or L-carnosine (155 mg/kg/day) after 10 weeks of TAA monotherapy. The sections were stained with hematoxylin–eosin (HE) and Sirius Red to detect the extracellular matrix and collagen, respectively. Activated hepatic stellate cells were detected by immunohistochemistry with a monoclonal antibody for α-smooth muscle actin. Original magnification 40×.

cotreatment with high-dose polaprezinc. In contrast, LPO was not affected by cotreatment with low-dose polaprezinc, zinc sulfate, zinc-L-aspartic complex or L-carnosine. GSH and SOD in the liver were significantly reduced in both TAA-treated groups. Cotreatment with high-dose polaprezinc significantly inhibited these changes in the TAA-treated animals, and zinc-L-aspartic complex cotreatment significantly inhibited the TAA-induced reduction in GSH, but not SOD (Table 2). Cotreatment with low-dose polaprezinc, zinc sulfate or L-carnosine did not affect the TAA-induced reduction in GSH.

The serum levels of ALT and AST did not change significantly during the development of liver cirrhosis in any of the groups except for the group treated with TAA for 10 weeks (Table 3). The serum ALP levels steadily increased in TAA-treated rats at both 10 and 20 weeks. Cotreatment with high-dose polaprezinc (200 mg/kg/day) significantly inhibited this increase in ALP. Furthermore, very high-dose polaprezinc (500 mg/kg/day) monotreatment did not alter the serum ALT, AST and ALP levels.

Discussion

It is critical to prevent fibrosis in chronic liver disease. Once cirrhosis is established, patients are at increased risk of developing liver injury, portal hypertension and carcinomas (Bataller and Brenner, 2005; Friedman, 2003). Cytokines and oxidative stress are thought to induce fibrosis that results from the activation of stellate cells (Cruz et al., 2005; Salguero Palacios et al., 2008). Indeed, free radical generation, mitochondrial dysfunction and antioxidant depletion contribute to the progression of fibrosis and cirrhosis (Natarajan et al., 2006). TAA is widely used to induce liver cirrhosis in rats and the resulting disease is similar to human cirrhosis (Muller et al., 1988; Natarajan et al., 2006; Strnad et al., 2008). TAA is metabolically activated to thioacetamide sulfoxide and further to thioacetamide-S,S-dioxide, and the toxic effects of TAA are attributed to these reactive metabolites (Chilakapati et al., 2007; Chilakapati et al., 2005). TAA-induced liver cirrhosis is associated with lipid peroxidation and the depletion of antioxidants (Abul et al., 2002; Low et al., 2004; Sanz et al., 2002; Sun et al., 2000). Accordingly, reducing oxidative stress appears to facilitate the regression of fibrosis and cirrhosis. Thus, several previous studies have suggested that radical scavengers and antioxidants can be used to prevent TAA-induced liver fibrosis (Balkan et al., 2001; Bruck et al., 2001; Cruz et al., 2005; Hsieh et al., 2008).

Polaprezinc, a zinc-L-carnosine complex, has been clinically used as an antifibrotic agent to treat chronic hepatitis in Japan (Abul et al., 2002; Himoto et al., 2007; Low et al., 2004; Matsuoka et al., 2009; Murakami et al., 2007; Sanz et al., 2002; Sun et al., 2000; Takahashi et al., 2007), although the precise antifibrotic mechanisms of polaprezinc are not fully understood. Polaprezinc treatment has been shown to attenuate fibrosis due to reduced lipid peroxidation, suppressed hepatic stellate cell activation and inhibited mRNA expression of pro-inflammatory cytokines (Sugino et al., 2008). Polaprezinc is comprised of approximately 22.4% (w/w) zinc and 77.6% (w/w) L-carnosine (Yamaguchi et al., 1996). Although both zinc and carnosine have radical scavenging and antioxidant activities (Bray and Bettger, 1990; Hipkiss and Brownson, 2000; Prasad, 2009), many researchers believe that zinc is the main active agent in polaprezinc that impacts the development of liver fibrosis. In this study, carnosine did not attenuate fibrosis, based on its lack of effect on liver HP levels and histopathological findings. A recent study further supported these observations using more than a 12-fold higher dose of L-carnosine than was used in our studies (Aydin et al., 2010).

It is important to compare the antioxidative effects and antifibrotic effects across the different zinc amino acid complexes and zinc sulfate, all of which contain the same amount of zinc, to determine whether the effects of the complex are important or whether it is a

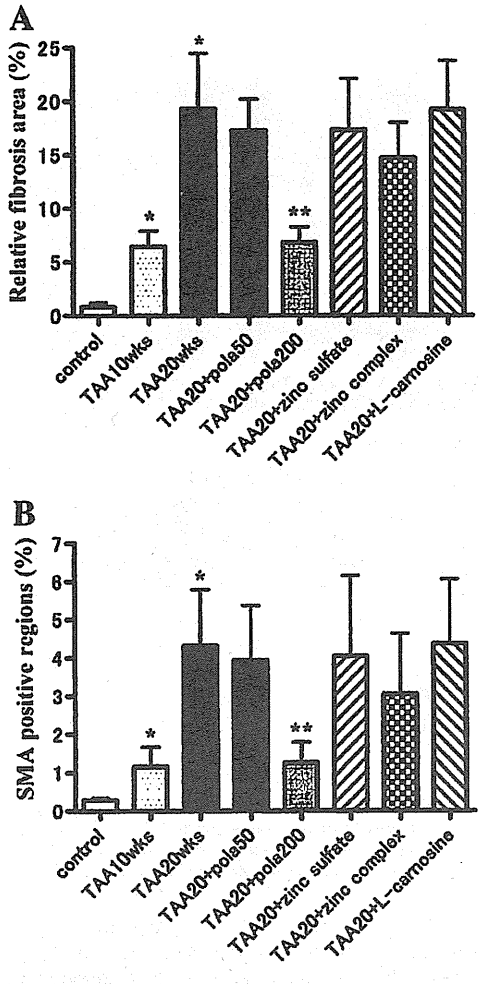

Fig. 4. Semi-quantitative morphometric analysis. (A) Relative fibrosis area (expressed as the % of the total liver area) was assessed by analyzing Sirius red-stained liver sections for each animal. Each field was acquired at 40× magnification and then analyzed with a computerized morphometry system. (B) The percent area of the α-smooth muscle actin (SMA)-positive region. The final net fibrosis area was determined by subtracting the vascular luminal area from the total field area.The controls received no drug treatment. The remaining groups received 300 mg/L of thioacetamide (TAA) in their drinking water for 10 or 20 weeks. Five groups of rats were cotreated with polaprezinc (50 or 200 mg/kg/day), zinc sulfate (112 mg/kg/day), zinc-L-aspartic complex (317.8 mg/kg/day) or L-carnosine (155 mg/kg/day) after 10 weeks of TAA monotherapy. Each bar represents the mean ± SD. Each group consisted of 15 rats. *p<0.001, a significant difference from the control. **p<0.01, a significant difference from the group treated with TAA for 20 weeks.

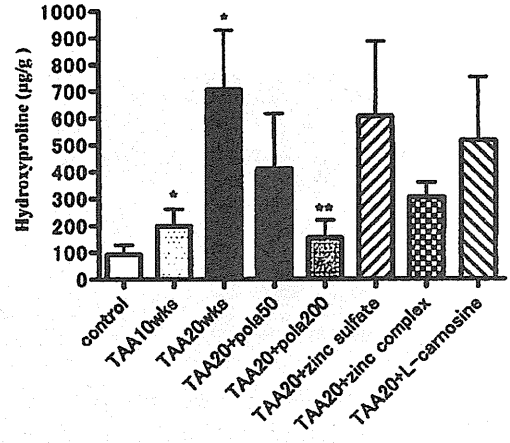

Fig. 5. Hydroxyproline analysis of liver samples. The controls received no drug treatment. The remaining groups received 300 mg/L thioacetamide (TAA) in their drinking water for 10 or 20 weeks. Five groups of rats were also cotreated with polaprezinc (50 or 200 mg/kg/day), zinc sulfate (112 mg/kg/day), zinc-L-aspartic complex (317.8 mg/kg/day) or L-carnosine (155 mg/kg/day) after 10 weeks of TAA monotherapy. Each bar represents the mean ± SD of the hydroxyproline content in micrograms per gram of liver tissue. Each group consisted of 15 rats. *p<0.01, a significant difference from the control. **p<0.01, a significant difference from the group treated with TAA for 20 weeks.

zinc-specific effect. The dose of zinc sulfate used in this study was approximately half of that used in previous liver fibrosis animal model studies (Dashti et al., 1997; Gimenez et al., 1994; Sidhu et al., 2005; Song and Chen, 2003). Therefore, it is possible that the dose used in this study was not sufficient to mediate these antioxidative and antifibrotic effects. However, we found that this dose suppressed diet-associated weight gain in the zinc sulfate-treated group. This might be due to the adverse effects of zinc sulfate on the gastrointestinal system (Samman and Roberts, 1987), and this side effect should be considered when zinc sulfate is clinically administered.

Furthermore, our comparisons of the different zinc amino acid complexes and zinc sulfate indicated that zinc-L-carnosine complex had the greatest antioxidative and antifibrotic effects. A recent study clearly showed that zinc ions in zinc complexes had superior antioxidant effects than that of zinc salts, including the zinc sulfate (Pavlica and Gebhardt, 2010). Moreover, polaprezinc and Zn-SOD have been shown to have similar structures (Yoshikawa et al., 1991a). Thus, polaprezinc may act as an antioxidant in cirrhotic livers, although the precise mechanisms of action are unclear.

To date, the relationship between the effects of zinc ions on membrane stabilization and antioxidation remain unclear. Polaprezinc may provide significant protection compared to the lack of protection that is achieved with zinc-L-aspartic complex and zinc sulfate because polaprezinc binds more tightly to membrane lipids (Pavlica and Gebhardt, 2010). This is in good agreement with the reported observation that zinc ions obtained from a zinc complex stabilized peroxidized membranes and minimized their peroxidative damage, resulting in a retained membrane structure rather than a decrease in the levels of oxidant formation (Pavlica and Gebhardt, 2010). Both zinc complexes significantly inhibited TAA-induced LPO production in

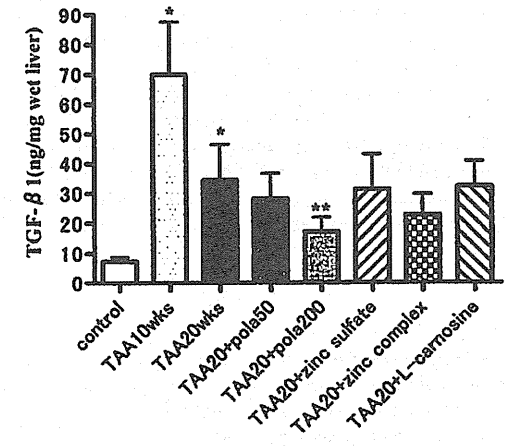

Fig. 6. Transforming growth factor (TGF)-β1 protein concentration analysis of liver samples. The controls received no drug treatment. The remaining groups received 300 mg/L thioacetamide (TAA) in their drinking water for 10 or 20 weeks. Five groups of rats were also cotreated with polaprezinc (50 or 200 mg/kg/day), zinc sulfate (112 mg/kg/day), zinc-L-aspartic complex (317.8 mg/kg/day) or L-carnosine (155 mg/kg/day) after 10 weeks of TAA monotherapy. Each bar represents the mean ± SD of the TGF-β1 content in micrograms per milligram of liver tissue. Each group consisted of 15 rats. *p<0.001, a significant difference from the control. **p<0.05, a significant difference from the group treated with TAA for 20 weeks.

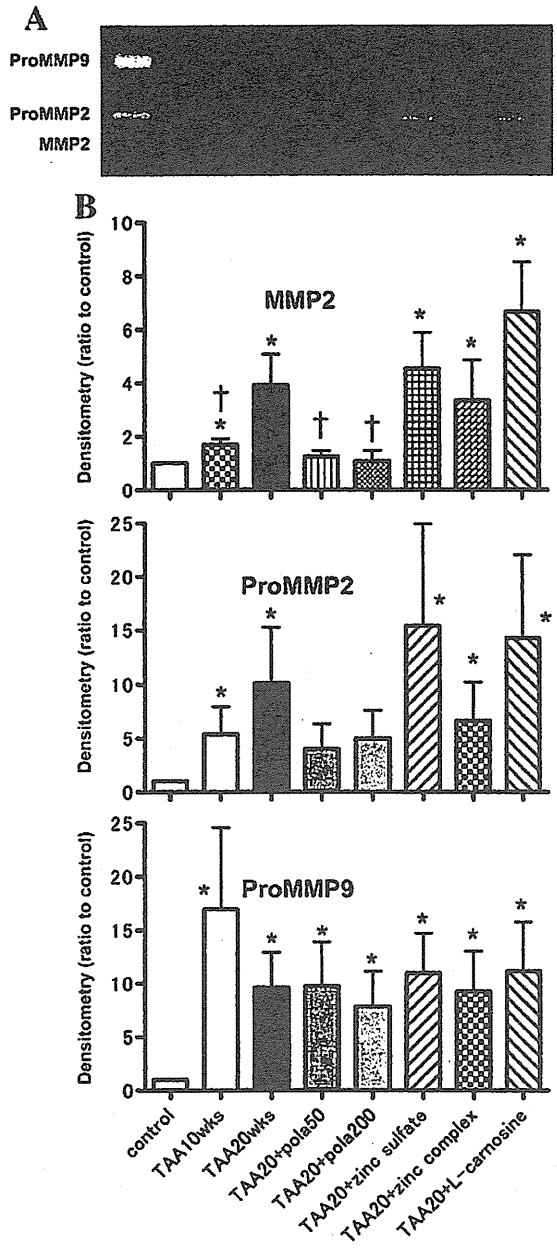

Fig. 7. A. Representative gelatin zymography from liver samples. The controls received no drug treatment. The remaining groups received 300 mg/L thioacetamide (TAA) in their drinking water for 10 or 20 weeks. Five groups of rats were also cotreated with polaprezinc (50 or 200 mg/kg/day), zinc sulfate (112 mg/kg/day), zinc-L-aspartic complex (317.8 mg/kg/day) or L-carnosine (155 mg/kg/day) after 10 weeks of TAA monotherapy. The clear bands of 92, 72 and 59 kDa corresponds to the pro-MMP-9, pro-MMP-2 and active-MMP-2, respectively. B. Densitometric analysis of gelatolytic bands for MMP-2 and MMP-9. The values are expressed as average of relative integrated optical density, normalized to control group values. Each bar represents the mean ± SD. *p<0.05, a significant difference from the control. †p<0.05, a significant difference from the group treated with TAA for 20 weeks.

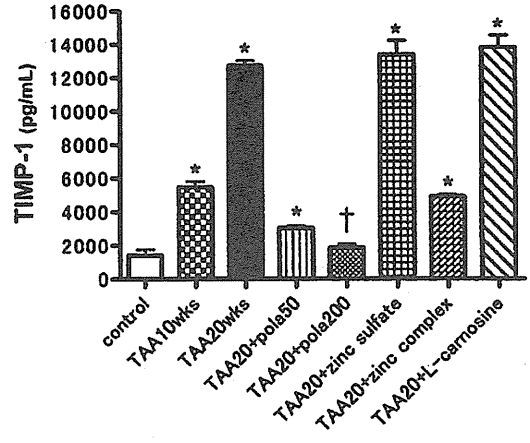

Fig. 8. Tissue inhibitor of metalloproteinases-1 (TIMP-1 by ELISA) in rat liver samples. The controls received no drug treatment. The remaining groups received 300 mg/L thioacetamide (TAA) in their drinking water for 10 or 20 weeks. Five groups of rats were also cotreated with polaprezinc (50 or 200 mg/kg/day), zinc sulfate (112 mg/kg/day), zinc-L-aspartic complex (317.8 mg/kg/day) or L-carnosine (155 mg/kg/day) after 10 weeks of TAA monotherapy. Each bar represents the mean ± SD. *p<0.01, a significant difference from the control. †p<0.01, a significant difference from the group treated with TAA for 10 weeks.

the liver. However, the precise mechanism by which polaprezinc inhibits hepatic LPO could not be ascertained from the present study.

The cirrhotic rat liver has been shown to have decreased zinc levels (Marchesini et al., 1996). In addition, the levels of zinc in liver tissue are significantly lower in cirrhotic patients than in healthy controls (Capocaccia et al., 1991). Intestinal zinc absorption has been found to be significantly reduced in cirrhotic patients and to correlate with the degree of liver dysfunction (Solis-Herruzo et al., 1989). The rate of zinc absorption from polaprezinc is approximately 11% in rats, which is comparable to that of zinc-L-aspartic complex (Sano et al., 1991). In this study, zinc deficiency was restored by administering polaprezinc or zinc-L-aspartic complex and these treatments did not result in zinc overdosing. In addition, there were no differences in the hepatic zinc content between the groups that received polaprezinc and zinc-L-aspartic complex, but polaprezinc treatment resulted in significantly greater preventative effects than zinc-L-aspartic complex based on serum markers, hepatic fibrotic markers, and liver histology. These findings might indicate that the effects of the complex, especially for L-carnosine complex, are more important than zinc supplementation alone.

SMA expression is reportedly a biomarker of HSC activation and activated HSCs are the main source of TGF-β1 (Gressner et al., 2002; Tsukada et al., 2006). TGF-β1 is one of the most powerful profibrogenic mediators in the liver and increased levels of TGF-β1 have been found both in patients with liver fibrosis and in experimental models (Chen et al., 2002; Salguero Palacios et al., 2008). Interestingly, the maximal expression of transiently elevated TGF-β1 was observed which is in accordance with previous results using TAA-treated animals (Salguero Palacios et al., 2008). The blockade of

Table 2
Effects of polaprezinc on oxidative stress-related parameters in the liver.

Treatment	LPO (μmol/g)	GSH (μmol/g)	SOD (U/g)
Control	5.5 ± 2.0	6.4 ± 1.3	11.6 ± 3.5
TAA 10 weeks	9.1 ± 2.1*	3.3 ± 0.8*	9.0 ± 1.9*
TAA 20 weeks	11.4 ± 2.9*	1.7 ± 0.5*	4.3 ± 2.2*
TAA 20 weeks + polaprezinc 50	9.3 ± 2.7	2.0 ± 0.6	5.6 ± 3.0
TAA 20 weeks + polaprezinc 200	6.3 ± 2.7**	6.1 ± 1.2**	10.4 ± 4.0**
TAA 20 weeks + zinc sulfate	10.6 ± 3.6	1.9 ± 0.7	6.3 ± 4.1
TAA 20 weeks + zinc complex	8.4 ± 3.7	3.8 ± 1.4***	8.6 ± 4.1
TAA 20 weeks + L-carnosine	11.1 ± 3.8	1.8 ± 0.9	4.7 ± 3.7

Note: The controls received no drug treatment. The remaining groups received 300 mg/L of thioacetamide (TAA) in their drinking water for 10 or 20 weeks. Six groups of rats were also cotreated with polaprezinc (50 or 200 mg/kg/day), zinc sulfate (112 mg/kg/day), zinc-L-aspartic complex (317.8 mg/kg/day) or L-carnosine (155 mg/kg/day) after 10 weeks of TAA monotherapy. Data are expressed as the means ± SD. Each group contained 15 rats. Abbreviations: TAA, thioacetamide; LPO, lipo peroxide; GSH, reduced glutathione; SOD, superoxide dismutase; U, activity units.
* p<0.01 versus the control.
** p<0.01.
*** p<0.05 versus TAA 20 weeks.

Table 3
Effects of polaprezinc on serum markers.

Treatment	ALT (U/L)	AST (U/L)	ALP (U/L)
Control	50 ± 3.1	83 ± 4.8	519 ± 81
TAA 10 weeks	74 ± 10.8*	123 ± 19.6*	960 ± 96*
TAA 20 weeks	47 ± 3.7	95 ± 7.3	1439 ± 384 *
TAA 20 weeks + polaprezinc 50	48 ± 2.8	93 ± 5.2	1193 ± 251
TAA 20 weeks + polaprezinc 200	49 ± 2.8	92 ± 6.4	878 ± 172**
TAA 20 weeks + zinc sulfate	51 ± 3.2	95 ± 5.9	951 ± 212
TAA 20 weeks + zinc complex	50 ± 4.1	94 ± 6.5	1006 ± 145
TAA 20 weeks + L-carnosine	51 ± 3.2	93 ± 2.9	1251 ± 365
Polaprezinc 500	51 ± 5.2	85 ± 7.4	524 ± 60

Note: Results are expressed as the means ± SD (n = 15). The controls received no drug treatment. The remaining groups received 300 mg/L thioacetamide (TAA) in their drinking water for 10 or 20 weeks. Six groups of rats were also cotreated with polaprezinc (50 or 200 mg/kg/day), zinc sulfate (112 mg/kg/day), zinc-L-aspartic complex (317.8 mg/kg/day) or L-carnosine (155 mg/kg/day) after 10 weeks of TAA monotherapy. The polaprezinc 500 group only received polaprezinc (500 mg/kg/day) after 10 weeks. Abbreviations: TAA, thioacetamide; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase.
* $p<0.01$ versus the control.
** $p<0.01$ versus TAA 20 weeks.

TGF-β1 synthesis is one of the primary targets for the development of antifibrotic approaches (Gressner et al., 2002). Polaprezinc treatment prevented both the activation of HSCs, which are stained with an anti-SMA antibody only upon activation, and the increase of TGF-β1 content in the TAA treatment liver. It has been reported that zinc deficiency results in a depletion of intracellular glutathione in HSCs followed by the subsequent activation of HSCs that triggers collagen synthesis (Kojima-Yuasa et al., 2003). Recent report suggested that polaprezinc could be used as an immunosuppressive agent through its inhibitory effect on calcineurin activity which plays a crucial role in cytokine expression (Zhang et al., 2011), although the precise mechanism of TGF-β1 down-regulation remains undetermined. Our results showed that preventing hepatic zinc reduction was associated with decreased SMA expression in the liver of rats that were cotreated with polaprezinc. This finding may also be due to the role of oxidative stress in the activation of HSC and collagen production. It has been suggested that polaprezinc reduces mitochondrial-dependent free radical generation in animals subjected to ischemic-reperfusion gastric mucosal injury (Yoshikawa et al., 1991b). In light of previous findings, our data suggest that polaprezinc prevents HSC activation with concomitant TGF-β1 down-regulation and inhibits SMA accumulation during ongoing fibrogenesis in TAA-treated livers as a result of antioxidative stress.

The present study showed the dynamic changes in MMP-2 and -9 activities during the progression of TAA-induced liver fibrosis in rats. In this chronic fibrosis model, MMP-2 is mainly produced by the activated HSCs, while MMP-9 is produced by Kupffer cells and leukocytes, particularly by the neutrophils in response to extracellular matrix changes (Consolo et al., 2009; Salguero Palacios et al., 2008). We demonstrated that continuous administration of TAA led to significant increases in both MMP-2 and -9 activities. By contrast, MMP-2 activity did not increase whereas MMP-9 activity did in rats cotreated with polaprezinc. Therefore, the observed differences in the MMPs may be associated with a reduction in the type of cells. Although MMPs are zinc-dependent endopeptidases, we speculated that the antifibrotic effect of polaprezinc was not caused by the increased MMPs.

MMPs, which remove extracellular matrix components, are decreased in hepatic fibrosis, mainly because of the increased expression of TIMP-1, an MMP-specific inhibitor. In hepatic fibrosis, an imbalance develops between excess collagen synthesis and/or decreased removal of extracellular matrix material, resulting in liver scarring. Very few previous reports on in vivo effects of polaprezinc on TIMP-1 protein level in liver tissue are available. In clinical practice, polaprezinc suppressed serum levels of TIMP-1 and type IV collagen in patients with advanced chronic liver disease (Takahashi et al., 2007). In an animal model of non-alcoholic steatohepatitis, polaprezinc supplementation for 10 weeks resulted in a significant reduction in the mRNA expression of TIMP-1 in the liver tissue (Sugino et al., 2008). These results are consistent with our findings. A major source of TIMP-1 is activated HSCs (Arthur and Fibrogenesis, 2000; Iredale et al., 1996). Our results showed that polaprezinc suppressed the activation of HSCs. Therefore, the observed reductions in the level of TIMP protein may be associated with a reduction in the numbers of activated HSCs. Because the MMP-2 activity, which is produced in activated HSCs, was reduced by polaprezinc, the reduction in the level of TIMP-1 protein level is likely due to the loss of activated HSCs. Although the precise mechanism responsible for this reduction remains uncertain, an antifibrotic effect can be achieved through the down-regulation of TIMP-1.

In a toxicity study with polaprezinc-treated rats, the toxic effects of polaprezinc became apparent at doses of 600 mg/kg/day (zinc, 134.4 mg/kg/day) or more (Yamaguchi et al., 1996). Therefore, we selected a very high-dose of 500 mg/kg/day (zinc, 102 mg/kg/day) in order to determine the adverse effects that result from treatment with polaprezinc over a long period. Fortunately, this dose of polaprezinc did not result in any adverse effects, and high-dose polaprezinc did not affect body weight. Thus, our findings indicate that polaprezinc may be administered over a long period to patients with chronic liver disease.

Conclusions

In conclusion, our results strongly suggest that polaprezinc can be safely administered during ongoing liver fibrosis to inhibit the progression of liver fibrosis. Polaprezinc prevents oxidative stress and HSC activation in the liver, leading to a reduction in the liver HP, TIMP-1 and TGF-β1 contents that are proportional to the reduction in collagen production.

Conflict of interest statement

No conflict of interest.

Acknowledgments

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Original Article

Anti-hepatitis B surface immunoglobulin reduction in early postoperative period after liver transplantation in hepatitis B virus-positive patients

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Aim: We investigated a protocol that lowered the necessary dose of anti-hepatitis B surface immunoglobulin (HBIg) with frequent monitoring of hepatitis B surface antigen (HBsAg) and antibody (HBsAb) levels in the early post-transplant period.

Methods: Fifteen hepatitis B virus (HBV)-positive patients were studied. We administered a nucleoside analog from the preoperative period, high dose HBIg was used intraoperatively (200 IU/kg in the patients who weighed less than 50 kg, and 10 000 IU in those who weighed more than or equal to 50 kg) and was continued every day (5000–10 000 IU/day). Thereafter, HBIg was administered to keep the target trough titers. We evaluated the effectiveness and safety of this protocol for preventing HBV reactivation.

Results: The average use of HBIg during the first three postoperative months (POM) was 27.9 ± 9.6 Kilo International Units. The average cost was $US11 800 in the first three postoperative months, compared with other previously reported protocols (about $20 000–40 000). HBV reactivation was detected in only one patient (6.7%) during the median follow up of 64 months (range: 12–86 months).

Conclusions: The present protocol for HBIg administration, which used frequent monitoring of HBsAg and HBsAb levels to determine the minimum required dose, was both safe and effective, and contributed to overall cost saving after liver transplantation.

Key words: cost, early preoperative period, anti-hepatitis B surface immunoglobulin, hepatitis B virus, liver transplantation

INTRODUCTION

LIVER TRANSPLANTATION FOR hepatitis B virus (HBV) positive patients was considered to be a relative contraindication until the beginning of 1990s, because of the high recurrence of viremia. Furthermore some cases showed rapid disease progression; termed fibrotic cholestatic hepatitis, which features aggressive cholestasis and fibrosis in the graft and subsequent hepatic failure even within several months postoperatively.[1,2] However, following the introduction of nucleos(t)ide analogs and anti-hepatitis B surface immunoglobulin (HBIg), the circumstances drastically changed for affected individuals, as use of that combination of prophylaxis resulted in a recurrence rate of 0–10% in HBV-positive patients who underwent liver transplantation.[3–7] That protocol has been shown to very effective and safe, and recently became the gold standard of prophylaxis for HBV reactivation after liver transplantation.

On the other hand, an important issue associated with that combination therapy is the extremely high cost of HBIg administration. Although the standard protocols for HBIg differ among institutions, but in general, high-dose HBIg is generally administered for the first 5–7 days after transplantation in a blind manner, after which lifelong maintenance administrations of HBIg are necessary to maintain a high titer of hepatitis B surface antigen (HBsAg). Previous reports have showed that the cost of such a regimen; more than $US100 000 in the first year and $5000 per year afterwards were needed

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according to the previous published reports.[8–10] So the high burden of cost of this drug cannot be ignored not only for personal expense but also for the budget of the national healthcare in each country. So a plan to save the cost for HBV prophylaxis is advocated and becomes a worldwide matter to resolve.

There could be some strategies for saving the cost of HBIg. Lamivudine monotherapy for prevention of HBV reactivation after liver transplantation was tried in some studies, but the recurrence rate was quite high.[10–12] So until now, combination of HBIg with nucleos(t)ide analog is considered to be the safe and standard strategy. HBIg reduction was tried in some reports, to set extremely low HBs Ab threshold[13] or even to withdraw HBIg[14–16] in the relatively distant postoperative period. But until recently, there had been no reports that tried to reduce HBIg dose starting from the first perioperative period when its cost is the highest over the entire postoperative period.

In the present study, we show the trial to reduce the dose of HBIg with frequent monitoring of HBsAg and hepatitis B surface antibody (HBsAb) especially in the early postoperative period starting from anhepatic phase, when the highest cost should be spent during these protocols for preventing HBV reactivation after liver transplantation, and evaluate the effect, safety, and cost of this protocol compared with the previously reported protocols.

METHODS

Patients

SEVENTEEN HBV-POSITIVE patients with liver cirrhosis who received living donor liver transplantation from April 2003 to October 2009 in our institute. Two patients were excluded because one patient died within one year and one patient was followed up for less than one year, so 15 patients were analyzed in this study.

Cost for HBIg and measurement of HBsAg, HBsAb

Dried polyethylene-glycol (PEG) treated anti-HBs human immunoglobulin (Hebsbulin; Mitsubishi-Welpharma, Tokyo, Japan) was used. It costs approximately $400 per vial (one vial includes 1000 IU of HBsAb). HBsAg was measured by enzyme immuno assay (EIA) and cut off level was 0.1 signal to cut off ratio (S/CO), and in some recent cases, measured by chemiluminesence immunoassay (CLIA) and cut off levels were 0.05 IU/mL. HBsAb was measured by CLIA and cut off level was 10.0 IU/mL. Measurement of HBsAg costs $2.90 and that of HBsAb costs $9.50 so this combination costs $12.40 for every combination measurement.

Protocol for HBIg administration

Nucleos(t)ide analogs (Patient 1–10; Lamivudine, Patient 11,12,14 and 15; Entecavir, Patient 13; combination of Lamivudine and Adefovir) were commenced before transplantation to reduce HBV-DNA as much as possible with the aim of HBV-DNA negativity in serum until the operation. During the anhepatic phase, high dose HBIg (200 IU/kg in the patients weighing <50 kg, and 10 000 IU in those weighing ≥50 kg) was administered intravenously. Nucleos(t)ide analogs were restarted from the next day of transplantation, and HBIg was administered according to the following principles: (i) administer intraoperative dose of HBIg when HBsAg titer is above 1.0 IU/mL, or half dose when HBsAg titer is positive but below 1.0 IU/mL in CLIA, every day until the HBsAg disappears from serum; (ii) after attaining negativity of HBsAg, additional administration of 1000–2000 IU of HBIg to maintain >500 IU/mL of HBsAb for one week; (iii) >300 IU/mL of HBsAb in the first month; (iv) >200 IU/mL in the second and third months; and (v) >100 IU/mL afterwards. All HBIg was administered intravenously over the entire period.

Schedule for measurement of HBsAg, HBsAb, and HBV-DNA after liver transplantation

Hepatitis B surface antigen and HBsAb were measured preoperatively, and principally on 1, 2, 3, 4, 5, 7, 9, 11, 14, 16, 18, 21, 23, 25, 28, 32, 35, 39, 42, 49, 56 postoperative days, and every 2–4 weeks thereafter whenever patients visited as outpatients, and measured additionally if HBsAb levels were decreased beyond expectation for patients' safety. HBV-DNA was also measured preoperatively and every month after transplantation to monitor HBV recurrence along with HBsAg.

Safety

We evaluated the safety of this protocol in two aspects regarding: (i) HBV reactivation during this protocol; and (ii) adverse effect of HBIg administration itself. HBV recurrence was confirmed when either HBV-DNA positive or HBsAg reappearance was detected and continued

for more than 3 months. We compared recurrence with the protocols reported by others and tried to convince no inferiority from others.

RESULTS

Patients' background

TABLE 1 SHOWS the preoperative backgrounds of the patients analyzed in this study. Recipients include 12 males and three females and ages were 40–61 (median 52) years old. Donors were seven males and eight females and ages were 21–60 (median 41) years.

Durations of preoperative nucleoside analog administration were 1–4 months in the older 10 patients with lamivudine (Patients 1–10), and the following newer patients with entecavir, durations were 11–22 months (Patients 11, 12, 14, 15). In patient 13, lamivudine was used in the first 24 months and viral and hepatitis breakthrough occurred, then adefovir was added and double therapy was continued for the following 20 months. In most patients. HBV-DNA was lower than detection limits except for five patients (Patient 7, 9, 10, 11, 15) all of which were below 4 log copy/mL. HBc Abs were positive in 8/15 (53.3%) and HBs Abs were positive in 11/15 (73.3%) of these donors.

Cost saving effect of this protocol

As described in Methods, all patients were administered large amounts of HBIg up to 10 000 IU intraoperatively, and then, until HBsAg became negative (average: 2.67 ± 2.02 days, Table 2), 5000–10 000 IU of HBIg was administered every day, and afterwards, smaller amounts (1000–2000 IU) of HBIg were added to keep the target titers according to the protocol.

Table 2 shows the summary of the necessary dose of HBIg until up to 12 postoperative months in this protocol. HBIg was administered 25.3 ± 8.8V (Range: 14–48) in the first postoperative month, 2.5 ± 2.7V (Range: 0–8) in the following 2 months, and 8.2 ± 5.2V (Range: 0–18) in the last 9 months. Interestingly, some patients (Patient 3, 8, 12, 15) showed spontaneous elevation of serum HBsAb without additional HBIg administration on the way of follow up for long times (Fig. 1).

Average cost of HBIg was $10 120 in the first 1 month, $11 160 in the first 3 months, and $14 400 within 12 months postoperatively. If the costs of HBV markers were included, they were $10 320 in the first month, $11 440 in the first 3 months, and $14 700 within 12 months that were much cheaper than the protocols in the previous published reports (Table 3).

Table 1 Preoperative patients' background

ID	Age	Gender	Donor age	Donor gender	Preoperative			
					HBV-DNA (Logcopy/mL)	NA	Duration of NA (Months)	Donor HBV status (HBsAg/HBcAb/HBsAb)
1	52	M	50	F	<2.6†	Lam	4	0.3/87.7/>1000
2	51	M	20	M	<2.6†	Lam	4	0.4/13.9/19.9
3	57	M	60	F	<2.6†	Lam	1	0.4/93.4/>1000
4	61	M	27	M	<2.6†	Lam	1	0.3/10.6/0.0
5	51	M	50	F	<2.6†	Lam	2	0.4/94.1/274.0
6	61	M	34	M	<2.6†	Lam	1	0.4/15.5/0.0
7	49	F	54	M	3.1†	Lam	2	0.4/40.3¶¶/23.8
8	40	M	41	F	<2.6†	Lam	1	0.4/74.8¶¶/>1000
9	48	M	43	F	3.2†	Lam	1	0.4/93.4¶¶/>1000
10	50	F	22	M	3.6†	Lam	1	0.3/8.9¶¶¶/289.0
11	41	M	30	F	3.4‡	Env	17	0.00¶/0.1¶¶¶/247.3
12	59	M	57	F	<1.8‡	Env	11	0.00¶/1.9¶¶¶/>1000
13	53	M	49	F	<1.8‡	Lam + Adv	44,20§	0.00¶/0.1¶¶¶/0.6
14	53	F	24	F	<1.8‡	Env	22	0.01¶/10.4¶¶¶/867.3
15	42	M	31	F	3.5‡	Env	4	0.01¶/7.3¶¶¶/>1000

†Amplicor, ‡TaqMan, NA: Nucleos(t)ide Analog, Lam: Lamivudine, Lam+Adv: Lamivudine+Adefovir, Env: Entecavir, §Duration of Lam(44),Lam + Adv(20).
Hepatitis B surface antigen (HBsAg): enzyme immunoassay, signal to cutoff ratio (EIA, S/CO) (cut off <1.0), ¶CLIA, IU/mL (cut off <0.05) HBcAb: EIA, % (cut off <50), ¶¶×200 Dilution, ¶¶¶CLIA, S/CO (cut off <1.0), HBsAb: CLIA (cut off <10.0). F, Female; M, Male.

Table 2 Summary of anti-hepatitis B surface immunoglobulin (HBIg) doses and blood tests in each patient

ID	Age	Gender	POD of HbsAg negativity (Days)	0–12 POM HBIg (Blood tests) (V/1000 IU)	0–1 POM HBIg (Blood tests) (V/1000 IU)	1–3 POM HBIg (Blood tests) (V/1000 IU)	3–12 POM HBIg (Blood tests) (V/1000 IU)
1	52	M	2	56 (41)	38 (17)	8 (9)	10 (15)
2	51	M	2	36 (37)	24 (12)	8 (4)	8 (17)
3	57	M	8	50 (52)	48 (20)	0 (11)	2 (21)
4	61	M	1	35 (45)	23 (15)	2 (9)	10 (21)
5	51	M	1	28 (28)	17 (11)	2 (7)	9 (10)
6	61	M	2	41 (41)	21 (14)	2 (10)	18 (17)
7	49	F	2	34 (34)	19 (13)	5 (11)	10 (10)
8	40	M	2	22 (36)	20 (14)	0 (8)	2 (14)
9	48	M	2	24 (45)	14 (13)	2 (11)	8 (21)
10	50	F	7†	45 (45)	29 (16)	4 (13)	12 (16)
11	41	M	2	37 (35)	26 (15)	1 (7)	10 (13)
12	59	M	2	28 (37)	28 (18)	0 (9)	0 (10)
13	53	M	2	37 (41)	29 (16)	8 (13)	10 (12)
14	53	F	2	31 (34)	17 (14)	0 (8)	14 (12)
15	42	M	3	29 (29)	27 (15)	0 (5)	2 (9)

†HBsAg became negative once at day 2 but low grade positive at day 5 (1.2 signal to cutoff ratio, enzyme immunoassay (S/CO, EIA), cut off <1.0) and confirmed persistent negative after day 7.
F, Female; M, Male; POD, postoperative days; POM, postoperative months.

Safety and effectiveness of this protocol

In these 15 patients, there were no obvious adverse effects of HBIg administration. During median follow up of 64 months (Range: 12–86 months), one patient (Patient 7) was judged as having HBV reactivation. In this patient, low levels of HBsAg (1.0 S/CO in EIA) appeared transiently in 10 postoperative months, and became negative again with a high rescue dose of HBIg administration. But in 14 postoperative months, HBsAg became positive again and continued more than 3 months afterwards though a high dose of HBIg was administered for rescue, so we gave up HBIg administration and switched to lamivudine monotherapy. In this patient, HCC recurrence was seen at 12 postoperative months. HBV-DNA level was continuously negative and the biochemical markers also remained stable for 58 months of follow up. Figure 2 shows the detailed dynamics of serum HBsAb levels in the first postoperative year in this patient.

The overall recurrence rate was 6.7% (1/15), which might be reasonable compared with the previous protocols (Table 3).

DISCUSSION

COMBINATION OF NUCLEOS(T)IDE analog and HBIg is now a standard regimen for the prophylaxis of HBV reactivation after liver transplantation, which shows satisfied results: the recurrence rate was reported to be 0–10%.[3–7] But the high cost of HBIg is a worldwide problem of this combination treatment that should be a heavy economical burden not only for each patient but for the national health budget in each country.

In this study, we have shown our protocol for the prevention of HBV reactivation after liver transplantation by deciding the minimum necessary dose of HBIg after measurement of HBsAg and HBsAb. We have also succeeded in saving the dose of HBIg, which is the most costly agent, especially in the earliest postoperative period. In brief, our protocol requires only approximately half of the cost of HBIg within the first 3 postoperative months compared with the protocols reported previously.

To reduce the long term use of HBIg, various methods have been reported until now. One strategy is the combination of HBV vaccine to induce self-producing HBsAb by active immunization and thereby to wean HBIg. HBV vaccine was used in many centers and there are several reports about the effect of HBV vaccine. First, two controversial results were reported (82%[17] vs. 29%[18]). Thereafter, several centers reassess vaccine effect, but in these recent published reports, responses were relatively poor at most.[19–22] One strategy to improve the effect of HBV vaccine is the adjuvant co-administration. Bienzle *et al.* showed that 80% of the response was achieved with adjuvant vaccine containing

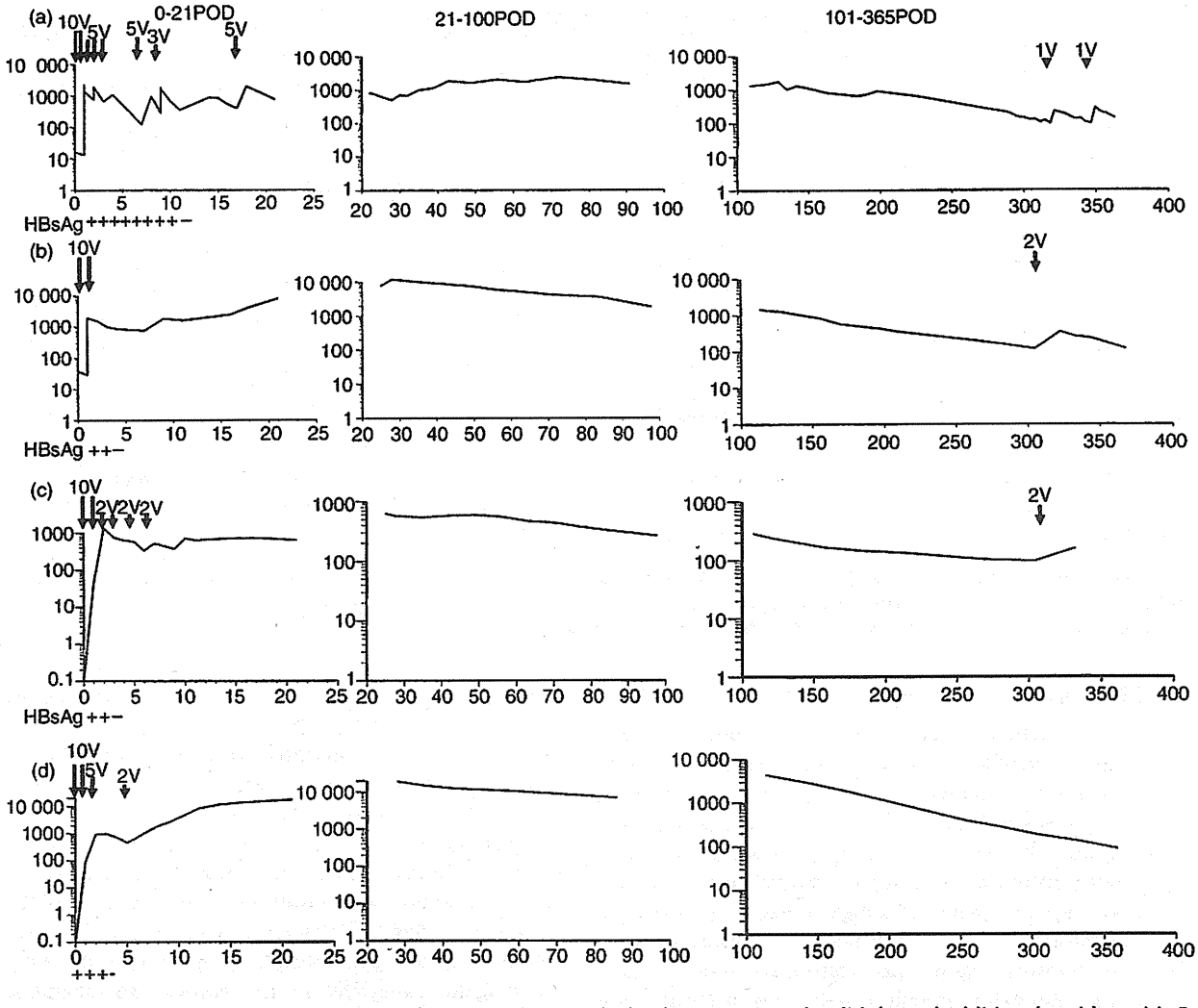

Figure 1 Dynamics of serum hepatitis B surface antibody (HBsAb) levels in patients who didn't need additional anti-hepatitis B surface immunoglobulin (HBIg) for a long time. (a) Patient 3, (b) Patient 8, (c) Patient 12, (d) Patient 15. Arrows represent the timing of HBIg administration.

3-deacetylated monophosphoryl lipid A (MPL) and natural saponin.[23] In a recent study, Tahara *et al.* showed that minimization of immunosuppression using monitoring with MLR (mixed lymphocyte culture) by 5,6-CaboxylFluoresecin diacetate Succinimidyl Ester (CFSE)-labeled recipients' lymphocytes with donors' or third parties' lymphocytes, and showed good response (64.7% response rate) for vaccine and HBIg could be eventually withdrawn in HBV carriers.[24] In another study by Weber *et al.*, 12 patients underwent HBIg withdrawal substituted by HBV vaccination with continuation of nucleos(t)ide analogs, and no viral or hepatitis recurrence was seen, though the effect of vaccine was relatively poor.[25] This strategy is still open to debate and needs investigation to attain further improvement in the effect of vaccination and saving the dose of HBIg in lengthy postoperative periods.

Another strategy is to try to reduce HBIg itself. In most centers, high doses of HBIg were used rather conventionally in a blind manner. Consequently, approximately $20 000–40 000 is spent for only HBIg even in the first 3 months.[3,5–7,26–32]

To reduce such a heavy economic burden, some HBIg saving protocols were tried and showed good results.

Table 3 Comparison of amount and cost of anti-hepatitis B surface immunoglobulin (HBIg) in the combination prophylaxis with nucleos(t)ide analog in previous published reports[26]

References	Patients (*n*)	Protocol (HBIg)	Follow up months (median or average)	HBV Recurrence (*n* [%])	Amount of HBIg (V/1000 IU/ 3months)	HBIg cost (3 months) ($US)
Markowitz, *et al.*[6]	14	80 KIU i.v. 1st month 10 KIU i.v./month thereafter	12	0	100	40 000
Yao, *et al.*[27]	10	80 KIU i.v.+3.3KIUi.m. 1st month 1.48 KIU i.m./month thereafter	15.6	1 (10%)	86.26	34 500
Yoshida, *et al.*[28]	7	43.4 KIU i.m. 1st month 4.3–6.8 KIU i.m./month thereafter	18	0	52–67	20 800–26 800
Mazano, *et al.*[7]	33	46.5 KIU i.v. 1st week 5 KIU/month thereafter	30	1 (3%)	56.5	23 000
Rosneau, *et al.*[5]	21	45 KIU i.v. 1st week HBsAb >500 until 14POD HBsAb >200 thereafter	21	2 (9.5%)	>45	>18 000
Roche, *et al.*[31]	15	80 KIU i.v. 1st month 10 KIU i.v. thereafter	>120	1 (6.6%)	100	40 000
Han, *et al.*[3]	59	80 KIU i.v. 1st month 10 KIU i.v. thereafter	35	0	100	40 000
Seehofer, *et al.*[32]	17	80 KIU i.v. 1st month 1.5–2 KIU/month to maintain HBsAb >100	25	3 (18%)	83–84	25 200–25 600
Wong, *et al.*[14]	21	70 KIU i.v. 1st month 10 KIU/month thereafter	60	2 (9.5%)	90	36 000
Takaki *et al.*[11]	18	200 IU/kg i.v. for 1 week HBsAb >100 following 5 months HBsAb >10 thereafter	18	0	47	18 800
Buti *et al.*[15]	14	10 KIU i.v. anhepatic phase 5 KIU i.v. following 6 days 4 KIU i.m. following 3 weeks	18	1 (7.1%)	52	20 800
Current	15	10 KIU every day until HBsAg negative HBsAb >300–500 1st month HBsAb >200 2nd-3rd month HBsAb >100 thereafter	53	1 (6.7%)	27.9	11 400

US is calculated as 100 yen. Cost of HBIg was estimated as $400.
Including cost of HBV markers.
HBsAb, hepatitis B surface antibody; HBsAg, hepatitis B surface antigen; KIU, kilo international unit.

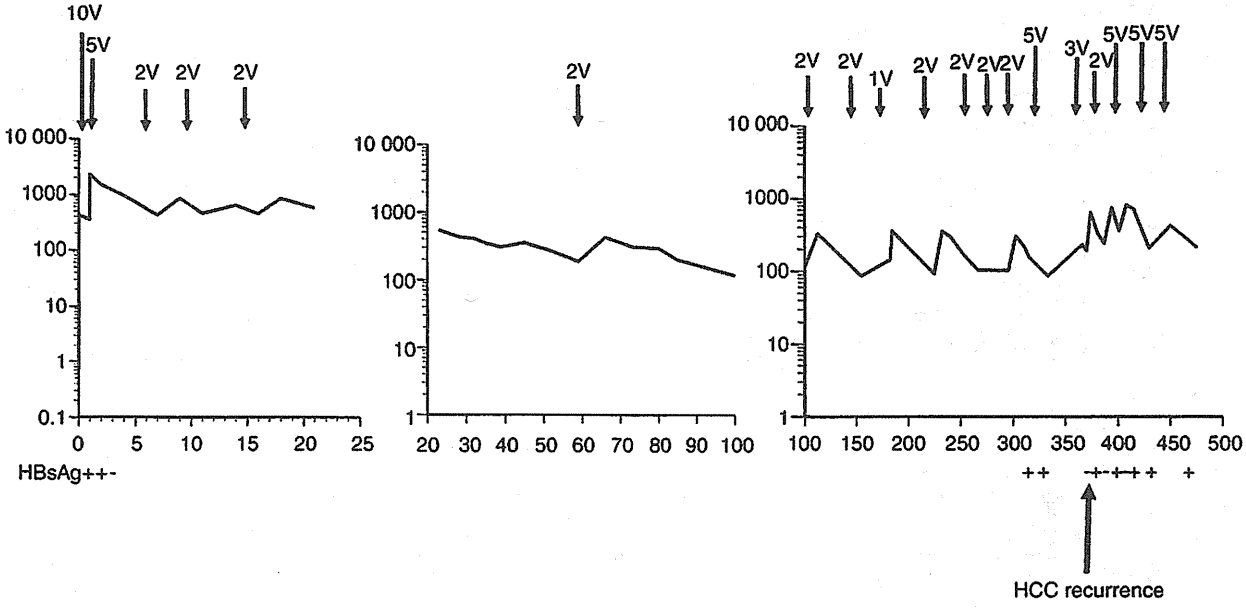

Figure 2 Dynamics of serum hepatitis B surface antibody (HBsAb) levels in Patient 7. Arrows represent the timing of anti-hepatitis B surface immunoglobulin (HBIg) administration. Arrows below the graph represent the timing of hepatocellular carcinoma (HCC) recurrence of this patient.

Takaki *et al.* reported that threshold levels can be reduced down to 10 IU/mL of HBsAb from later than 6 months after liver transplantation and the cost can be reduced down to about $400 per month from later than 2 months after transplantation. This was less than half that compared with the standard protocols.[13] Wong *et al.* reported that in 21 selected patients out of 40 HBV-related liver disease patients, HBIg was withdrawn after a median duration of HBIg for 26 months and followed up for a median of the following 40.2 months, and one patient (4.8%) developed HBV recurrence.[14] Buti *et al.* reported that 15 patients whose HBV-DNA was negative spontaneously or induced by lamivudine preoperatively was switched to lamivudine monotherapy after combination with HBIg for one month after transplantation, and HBV-DNA became positive only in one case (6.7%).[15,16]

As shown in Table 3, we spent $11 400 in the first three postoperative months including the cost of frequent measurement of HBsAg and HBsAb, which is about half of the previously reported protocols. Trials to reduce maintenance HBsAb titers[13] or in some studies, withdrawal of HBIg[14–16] were tried and their effectiveness and safety concerns discussed (Table 3). But these studies focused on the dose of HBIg in the relatively distant postoperative period compared with this study. Needless to say, these strategies were very important because an effective substitute of HBIg, like HBV vaccine, was not completely established until now, so we ought to use HBIg on a lifelong basis for the safety of patients. But we thought that a saving of the earliest phase HBIg is also important because the cost of HBIg was greatest during the first postoperative year. Every combination of HBsAg and HBsAb costs $12.40, so if these measurements were done every day for 3 months for example, only about $1100 was added to the cost, which is less than only 3000 IU of HBIg.

Interestingly, four patients (Patient 3, 8, 12, 15, Fig. 1) showed spontaneous elevation of serum HBsAb levels without additional HBIg administration for long periods. Lo *et al.* showed 42% of patients receiving lamivudine monotherapy after liver transplantation could produce spontaneous HBsAb and remained positive for around 200 days in some patients.[33] So we suggest that some patients do not need a high dose of HBIg because of the spontaneous elevation of HBsAb, and from this observation we should clarify the character of such patients. Because of the limitation of the patient number, we cannot elucidate this point, and need a future observation in this point.

In the safety concerns, serum HBsAg reappeared in one patient (Patient 7) in our protocol. The first thing

we should discuss is whether this specific recurrence was due to early preoperative HBIg reduction or not. HBV recurrence was undoubtedly lowered after induction of nucleos(t)ide analog and HBIg combination prophylaxis, but in fact, it still exists in less than 10% of patients.[9] According to the published reports, 1–3% of patients had recurrence of HBV even within 12 months in the protocol of the relatively low dose of HBIg and nucleos(t)ide analog,[34–36] in contrast to the high dose HBIg regimen, where the recurrence rate was 1.4%, which is comparable with the low dose HBIg regimen.[37] In the distant postoperative period (5 years), recurrence was increased to 4–8% in these reports.[34–37] From these studies, the dose of HBIg might be irrespective of the recurrence rate.

In regard to this patient, one interesting issue is the relationship between HCC recurrence and HBV recurrence after liver transplantation. In recent reports, HBV recurrence was significantly correlated with HCC recurrence in HBV positive post-transplant patients.[38–40] In these reports, some HBV recurrences were seen even within 1 year after liver transplantation though the standard preventive regimens with HBIg and nucleos(t)ide analog were continued.[38–40] Faria *et al.* showed the possibility that HBV replication in tumor cells may contribute to HBV recurrence by showing high rates of detection of HBVcccDNA in explanted liver.[38] In the current study, Patient 7 was the only patient not only with HBV recurrence but also with HCC recurrence and it might be the mechanism for early recurrence of HBV after liver transplantation in this specific patient. In another recent prospective study by the National Institute of Health Hepatitis B Virus Orthotropic Liver Transplantation (NIH HBV OLT) Study Group, HBV recurrence was not found as an independent predictor of recurrence of HCC in HBV positive patients.[41] But these authors pointed out a small number of HBV recurrence might be a cause of inability to demonstrate this association,[41] so this issue is still interesting and is open to debate.

Collectively, in the recent study, this only, but one recurrence seemed noticeable because of the small number in this study. This aspect remains open for discussion with increasing numbers in future observations. Although further careful discussion is needed, we think this recent prophylaxis protocol seems to be relatively safe, or at least not as dangerous (median 64 months, 6.7% of recurrence, Table 3) compared with other previous protocols.[34–41]

Another point that should be considered is more effective cost saving prophylaxis protocols in the overall postoperative periods in these patients. As described above, some excellent results were reported about the saving of HBIg in the distant preoperative period including the withdrawal of HBIg.[13–16] In two recent studies, intramuscular HBIg administration from the early preoperative period could save the cost of prophylaxis of HBV after liver transplantation.[34,35] To date, intramuscular HBIg is not available for clinical use in Japan, but we should consider using it or a combination of our protocols and prophylaxes for the long follow up periods described above in the hope of achieving more powerful cost saving effects for HBIg administration and releasing the heavy burden not only for patients but also for worldwide national health budgets.

In conclusion, we show that the alternative strategy to save HBIg doses during the early postoperative period with frequent monitoring of HBsAg and HBsAb titer is cost-effective and relatively safe. This strategy required about half of the cost compared with the previously reported strategy, and it could be an alternative way to combine cost-saving in the long postoperative periods that have been previously reported.

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