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Original Research Communication

Thioredoxin Levels in the Sera of Untreated Viral Hepatitis Patients and Those Treated with Glycyrrhizin or Ursodeoxycholic Acid

TOSHIAKI NAKASHIMA,[1] YOSHIO SUMIDA,[1] TAKAHARU YOH,[1] YUKO KAKISAKA,[1] YOSHIKI NAKAJIMA,[1] HIROKI ISHIKAWA,[1] HIRONORI MITSUYOSHI,[1] KEI KASHIMA,[1] HAJIME NAKAMURA,[2] and JUNJI YODOI[2]

ABSTRACT

Thioredoxin (TRX), a thiol-containing protein, is induced by various oxidative stresses. Serum TRX levels were measured with a sandwich enzyme-linked immunosorbent assay kit in 210 hepatitis C virus (HCV)-infected patients, 39 hepatitis B virus (HBV)-infected patients, and 17 healthy volunteers. The effects of hepatoprotective drugs on TRX levels were also examined. The median TRX levels were significantly higher in HCV-infected patients than in controls (34.2 vs. 23.5 ng/ml, respectively; $p < 0.005$), but were not elevated in HBV-infected patients (26.7 ng/ml). The TRX levels were significantly correlated with serum lipid peroxide levels and indocyanine green exclusion test values, and were markedly decreased following treatment with Stronger Neo-Minophagen C or ursodeoxycholic acid. In conclusion serum TRX levels, a marker of oxidative stress, were higher in patients with HCV infection than those with HBV infection and healthy controls. The therapeutic efficacy of hepatoprotective drugs may be connected with the decrease in oxidative stress in hepatitis patients. Antiox. Redox Signal. 2, 687–694.

INTRODUCTION

THIOREDOXIN (TRX) contains a dithiol-active site (-Cys-Gly-Pro-Cys-) (Tagaya *et al.*, 1989) and has a variety of biological activities (Nakamura *et al.*, 1997), including scavenging of active oxygen radicals (Nakamura *et al.*, 1995), and redox regulation of molecules (Makino *et al.*, 1996). TRX is induced by many forms of oxidative stress (Sachi *et al.*, 1995; Hoshi *et al.*, 1997), and therefore a good marker of oxidative stress.

Stronger Neo-Minophagen C (SNMC) and ursodeoxycholic acid (UDCA) are popular hepatoprotectives used in Japan to treat chronic viral hepatitis (Takano *et al.*, 1994; Mitsuyoshi *et al.*, 1996). SNMC is an injection containing glycyrrhizin, a major component of the herb licorice, and is known to decrease serum transaminase levels in chronic hepatitis more effectively than other hepatotherapeutic drugs (Suzuki *et al.*, 1983). Glycyrrhizin has been found to protect the hepatocyte membrane via various mechanisms including enhancement of glucocorticoid effects and inhibition of phospholipase A_2 activity (Shiki *et al.*, 1986; Arase *et al.*, 1997). UDCA is a bile acid and the suggested mechanisms of UDCA action include induction of hypercholeresis, immunomodulatory effects, and protection of hepatocytes

[1]Third Department of Internal Medicine, Kyoto Prefectural University of Medicine, Kyoto 602-8566, Japan.
[2]Department of Biological Responses, Institute for Virus Research, Kyoto University, Kyoto 606-8507, Japan.

against the toxicity of hydrophobic bile acids (Attili *et al.*, 1986; Makino and Tanaka, 1998).

In the present study, serum TRX levels were measured in patients with hepatitis C virus (HCV)- or hepatitis B virus (HBV)-related chronic liver diseases, and the effects of SNMC and UDCA on these levels were investigated.

PATIENTS AND METHODS

Patients

The subjects of this study were 210 serum HCV RNA-positive patients (122 males and 88 females, aged 57.4 ± 11.9 years, mean ± SD), including 180 chronic hepatitis (CH) patients (104 males and 76 females, aged 56.1 ± 12.2 years), and 30 liver cirrhosis (LC) patients (18 males and 12 females, aged 64.7 ± 7.1 years). There were 39 serum HBV DNA-positive patients (30 males and 9 females, aged 43.6 ± 15.8 years), including 35 CH patients (27 males and 8 females, aged 42.4 ± 16.2 years) and 4 LC patients (3 males and 1 female, aged 53.3 ± 8.7 years). Seventeen healthy volunteers (9 males and 8 females, aged 46.4 ± 16.3 years) were also included. CH and LC were diagnosed clinically on the basis of the ultrasonography findings, laboratory data, and histological findings in the liver. Patients with decompensated LC exhibiting ascites or jaundice and patients with hepatocellular carcinoma (HCC) were excluded from the present study. All patients were human T-lymphotropic virus type-I (HTLV-I) and human immunodeficiency virus (HIV) negative, and nondrinkers, and were not suffering from a fatty liver.

Nine HCV-infected patients were receiving SNMC, consisting of 0.2% glycyrrhizin, 0.1% cysteine, and 2.0% glycine in physiological saline solution (Minophagen Pharmaceutical Co., Tokyo, Japan, 40 ml/day) alone intravenously three times/week for 1–2 months. Twelve HCV-infected patients were receiving UDCA (Urso, Mitsubishi-Tokyo Pharmaceutical Co., Tokyo, Japan, 600 mg/day) alone orally for 6 months. High serum transaminase levels persisted for more than 6 months before SNMC or UDCA treatment in all patients. The 4 out of 9 SNMC-treated patients and 5 out of 12 UDCA-treated patients failed to improve on interferon (IFN) therapy at least 1 year before SNMC or UDCA treatment.

The study design was approved by the Ethics Committee of Kyoto Prefectural University of Medicine. All patients and healthy volunteers in this study gave informed consent before entering the study.

Serum TRX, lipid peroxides, and ICG measurement

Serum was obtained from venous blood drawn in the fasting state, and was stored frozen at −30°C until use. Serum hemoglobin concentrations were measured with a diagnostic kit for serum hemoglobin (Sigma Chemicals Co., St. Louis, MO), and serum samples containing more than 10 mg/dl hemoglobin were excluded from TRX measurements (Holmgren and Luthman, 1978). Serum TRX levels were measured with a recently established sandwich enzyme-linked immunosorbent assay kit (ELISA) (Fujirebid Inc., Tokyo, Japan) (Kogaki *et al.*, 1996). Briefly, adult T-cell leukemia-derived factor (ADF) 21 antibody-precoated 96-microwell plates were incubated for 2 hr at room temperature with 20 μl of serum or standard solution in the presence of 200 μl of 50 m*M* sodium phosphate buffer, pH 6.0, containing 150 m*M* NaCl, 1.0 m*M* $MgCl_2$, 1.0% bovine serum albumin (BSA), and 0.1% NaN_3. The plates were washed five times with 10 m*M* sodium phosphate buffer, pH 7.5, containing 0.05% Tween 20 and 150 m*M* NaCl (Wash Solution), and then 200 μl of the horseradish peroxidase-labeled anti-ADF antibody was added to the plates for incubation at room temperature for 2 hr. After the plates were washed five more times with Wash Solution, 100 μl of 100 m*M* triethanolamine-succinate buffer, pH 4.4, containing 1.5 m*M* H_2O_2 and 0.13% ABTS was added, and the plates were incubated for 1 hr at room temperature. The reaction was then stopped by the addition of 100 μl of 1% oxalic acid solution, and the optical density of the plates was measured at 415 nm with a microplate reader (Benchmark, Bio-Rad Laboratories, CA). All measurements were made in duplicate, and the average value was adopted.

Lipid peroxide levels in serum was quanti-

tated according to the methods of Ohkawa *et al.* (1979). Lipid peroxide content was expressed as nmoles of malonic dialdehyde, namely, thiobarbituric acid-reactive substance (TBARS) formed per 1 ml of serum.

The indocyanine green (ICG R_{15}, Daiichi Pure Chemicals Co., Tokyo, Japan) concentration in serum was measured spectrophotometrically (UV-2100S spectrophotometer; Shimadzu, Kyoto, Japan) from the absorbance at 805 nm in serum 15 min after intravenous administration of 0.5 mg/kg body weight of ICG (Paumgartner *et al.*, 1970).

Statistical analysis

Statistical differences between groups for quantitative data were determined by the Mann-Whitney U test and the Kruskal-Wallis test when applicable (Fig. 1). Correlation coefficients were calculated using Spearman rank correlation analysis (Figs. 2 and 3). The statistical analysis in Fig. 4 was performed by using the Wilcoxon signed-rank test. Differences

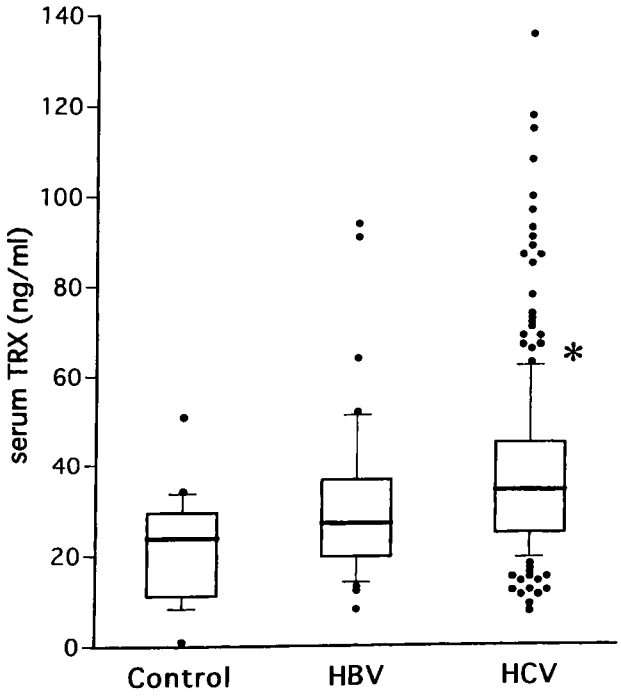

FIG. 1. Serum TRX levels in HCV-(n = 210) or HBV-(n = 39) infected patients and healthy controls (Control, n = 17). The box encloses the values between the 25th and 75th percentiles and the box horizontal line is the median; the error bars stretch from the 10th and to the 90th percentile, and individual outliers are presented by circles. Overall significance of differences among three groups according to nonparametric Kruskal-Wallis analysis of variance was $p < 0.0001$. Therefore, the significance of differences among groups was determined by the Scheffe's method: $^*p < 0.005$, compared to the values in the Control.

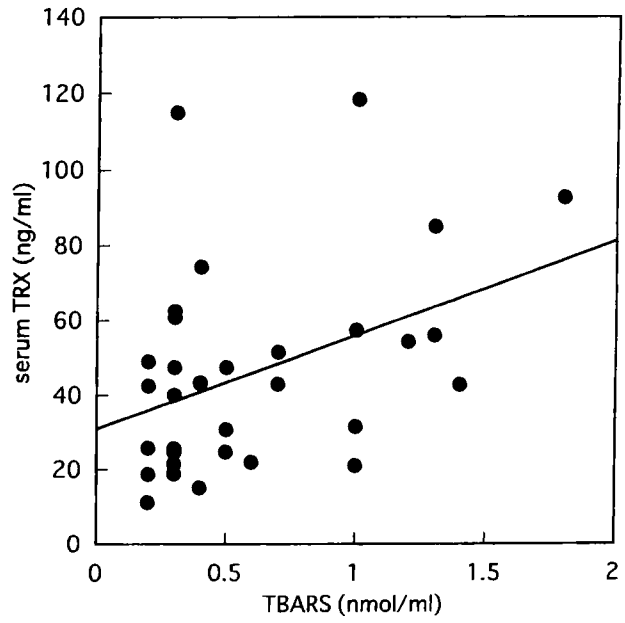

FIG. 2. Comparison between the serum TRX levels and lipid peroxidation in 33 HCV-infected patients with chronic hepatitis or liver cirrhosis. The normal value of serum TBARS is 0.4–1.3 nmol/ml.

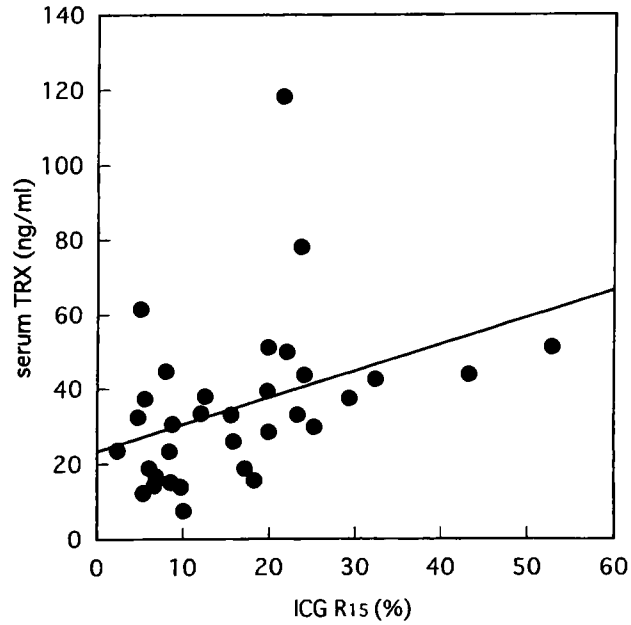

FIG. 3. Comparison between the serum TRX levels and indocyanine green (ICG R_{15}) values in 33 HCV-infected patients with chronic hepatitis or liver cirrhosis. Normal value of ICG R_{15} is 0–10%.

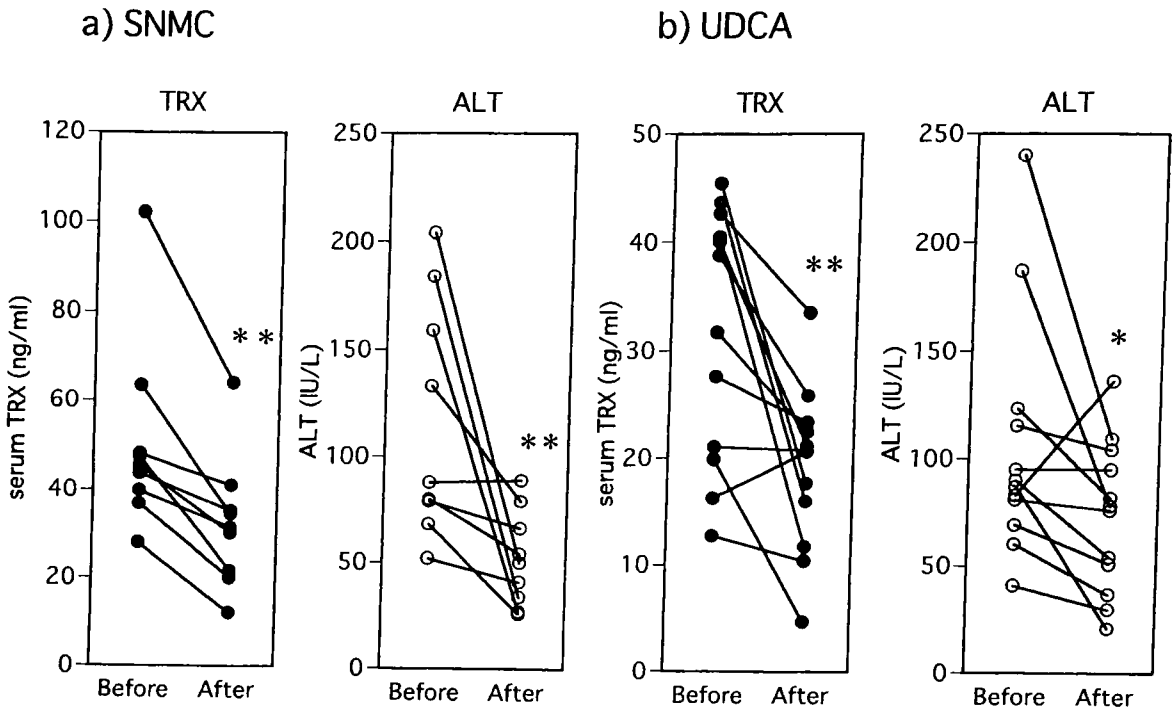

FIG. 4. Effects of SNMC (a) and UDCA (b) on the serum TRX and ALT levels in HCV-infected patients (SNMC, $n = 9$; UDCA, $n = 12$). SNMC was administered at 40 ml/day, 3 times/week for 1–2 months, and UDCA, at 600 mg/day for 6 months. Normal value of serum ALT is 0–35 IU/ml. $^{*}p < 0.05$, $^{**}p < 0.01$, compared to the value before the beginning of SNMC or UDCA treatment.

were considered statistically significant when the p value was less than 0.05.

RESULTS

Clinical laboratory parameters of hepatitis patients

As shown in Table 1a, age and serum ferritin level were significantly higher in the HCV-infected patients than in the HBV-infected patients. The clinical parameters of SNMC- or UDCA-treated patients are shown in Table 1b.

Serum TRX levels in the hepatitis patients

Serum TRX levels (median and [range], ng/ml) were significantly higher in patients with chronic HCV infection ($n = 210$, 34.2

TABLE 1a. CLINICAL LABORATORY PARAMETERS OF HBV- OR HCV-INFECTED PATIENTS

	HBV-infected n = *39*	*HCV-infected* n = *210*	p *value*
Male/female	30/9	122/88	0.027
Age (years)	44 [18–85]	56 [19–90]	<0.0001
Cirrhosis/chronic hepatitis	4/35 (10%)	30/180 (14%)	n.s.
ALT (IU/L)	45 [17–451]	58 [12–459]	n.s.
Hyaluronic acid (ng/ml)	68 [16–193]	77 [10–835]	n.s.
IV collagen (ng/ml)	4.4 [3.2–9.4]	5.7 [0.9–18]	n.s.
Ferritin (ng/ml)	86 [9–266]	137 [2–1564]	0.034
TBARS (nmol/ml)	0.5 [0.2–1.3]	0.5 [0.2–1.8]	n.s.

Results are presented as numbers for qualitative data or as medians and [ranges] for quantitative data. p values for sex and cirrhosis/chronic hepatitis were calculated by χ^2 analysis, and the p values for the other parameters were calculated by Mann-Whitney U-analysis with correction for ties.

Normal values and unit: ALT (alanine aminotransferase), 0–35 IU/L; hyaluronic acid, <50 ng/ml; IV collagen (type IV collagen-7S domain), <5.0 ng/ml; ferritin, 21–247 ng/ml; TBARS, 0.4–1.3 nmol/ml.

n.s. (not significant).

TABLE 1b. CLINICAL LABORATORY PARAMETERS OF SNMC- OR UDCA-TREATED HCV-INFECTED PATIENTS

	SNMC-treated n = 9	*UDCA-treated* n = 12
Male/female	6/3	7/5
Age (years)	66 [45–73]	62 [46–71]
Cirrhosis/chronic hepatitis	1/8 (11%)	2/12 (17%)
ALT (IU/L)	108 [63–204]	105 [41–515]
Hyaluronic acid (ng/ml)	193 [108–456]	163 [42–271]
IV collagen (ng/ml)	5.3 [4.5–8.4]	5.3 [3.6–8.2]
Ferritin (ng/ml)	124 [51–429]	94 [2–479]
TBARS (nmol/ml)	0.2	0.5
HCV-RNA (kcopy/ml)	200	151 [57–300]

The parameters were determined in the patients within 1 month before SNMC or UDCA administration. Results are presented as numbers for qualitative data or as medians and [ranges] for quantitative data.

Normal values and unit: ALT (alanine aminotransferase), 0–35 IU/L; hyaluronic acid, <50 ng/ml; IV collagen (type IV collagen-7S domain), <5.0 ng/ml; ferritin, 21–247 ng/ml; TBARS, 0.4–1.3 nmol/ml.

n.s. (not significant).

[7.4–135.6]) than in healthy controls (n = 17, 23.5 [1.3–50.7]) ($p < 0.005$; Fig. 1). Among the chronic HCV-infected patients, the TRX levels were significantly higher in patients with LC (n = 30, 41.9 [21.4–114.9] than in those with CH (n = 180, 33.8 [7.4–135.6]) ($p <$ 0.05).

No significant differences in the serum TRX levels were found between patients with chronic HBV infection (n = 39, 26.7 [7.5–93.7]) and healthy controls. Among the chronic HBV-infected patients, the TRX levels were 25.8 [14.4–26.5] in patients with LC (n = 4) and 27.8 [7.5–93.9] in those with CH (n = 35); there were no significant differences among them.

Furthermore, the TRX levels in patients with CH, type C tended to be higher than in patients with CH, type B, but the difference was not significant.

Correlation between serum TRX levels and lipid peroxides or ICG

The correlation between the serum TRX levels and the TBARS or ICG R_{15} values was examined in 33 patients with HCV infection. There was a significant correlation between the levels of serum TRX and TBARS ($p < 0.05$) (Fig. 2), and between the TRX levels and the ICG R_{15} values ($p < 0.01$) (Fig. 3).

Serum TRX levels in patients treated with hepatoprotective drugs

Both the serum TRX (median and [range], ng/ml) and ALT (IU/ml) levels were significantly improved from 45.5 [27.9–102.1] and 88.0 [52–204] before SNMC treatment, respectively, to 31.3 [12.1–64.1] and 50.0 [26–89] after treatment, respectively ($p < 0.01$) (Fig. 4a).

Serum TRX and ALT levels were also significantly improved from 35.3 [12.7–45.4] and 88.5 [41–240] before, respectively, to 20.7 [4.7–33.5] and 77.0 [21–136], respectively, after UDCA treatment ($p < 0.01$, $p < 0.05$, respectively) (Fig. 4b).

DISCUSSION

HCC patients were excluded from this analysis, because previous studies have shown that HCC cells specifically induce TRX. Immunohistochemistry showed that TRX expression is increased in HCC tissues (Nakamura *et al.*, 1992), and serum TRX levels decrease after surgical removal of HCC (Miyazaki *et al.*, 1998).

Viral infection may be associated with increased oxidative stress. In a previous study, serum TRX levels were found to be significantly higher in HIV-infected individuals than in controls (Nakamura *et al.*, 1996). HCV is

more common than HIV in Japan, and recent reports have suggested that oxidative stress may contribute to HCV-related liver diseases (Farinati *et al.*, 1995; Larrea *et al.*, 1998). Consistent with this, the present study revealed that serum TRX levels were elevated in patients with HCV infection, but not in patients with HBV infection. We have revealed that the serum TRX levels of HCV-infected patients is not influenced by the amount of HCV or the HCV serotype, and that the TRX levels are significantly correlated with the serum ferritin levels that are assumed to reflect iron storage of the liver (Sumida *et al.*, 2000). Therefore, it is suggested that the iron-induced oxidative stress may contribute to the cytopathic effects of HCV, but not HBV, on the liver (Bonkovsky *et al.*, 1997).

Lipid peroxides, measured as TBARS formation in sera, have been used as a clinical marker of oxidative stress. Previous studies have revealed that serum lipid peroxide levels are significantly increased in various alcoholic liver diseases and viral chronic liver diseases (Suematsu *et al.*, 1977; Sato *et al.*, 1978). The present study found a significant correlation between the serum levels of TRX and TBARS, but the TBARS levels were almost within the normal range. Therefore, TRX is a more sensitive marker than TBARS for monitoring oxidative stress in hepatitis patients.

The present data also showed a significant correlation between the TRX levels and the ICG R_{15} values. The levels of ICG generally increase in proportion to the development of hepatic fibrosis. The precise pathophysiological mechanisms involved in hepatic fibrogenesis are poorly understood, but the present findings suggest that oxidative stress may accelerate hepatic fibrogenesis in patients with HCV infection. Hepatic stellate cells are the principal collagen-producing cells in the liver (Friedman, 1993). Therefore, oxidative stress may enhance the fibrogenesis activity of hepatic stellate cells (Houglum *et al.*, 1997). The source of the serum TRX in HCV-infected individuals remains uncertain.

IFN is currently the best agent for the treatment of viral hepatitis, but its therapeutic efficacy is not always satisfactory (Hoffnagle and Di Bisceglie, 1997). Many hepatoprotective drugs have been used in patients resistant to IFN. The antioxidative effects of the hepatoprotective drug glycyrrhizin have not been proven to date. As SNMC includes cysteine, which is a thiol (-SH)-containing amino acid, SNMC may influence the redox status of the body. The present data clearly indicate that SNMC treatment decreases serum TRX levels with improvement of serum alanine aminotransferase (ALT) levels. We found no correlation between serum TRX and ALT levels in patients with chronic HCV infection in the present study (data not shown), suggesting that the decrease in TRX by SNMC treatment is not directly related to improvement of damaged hepatocytes.

Glutathione (GSH) contains thiol (-SH) and protects against oxidative stress. Recently, we found that UDCA increases levels of GSH and thiol-containing proteins in hepatocytes, thereby protecting primary cultured rat hepatocytes against oxidative injury (Mitsuyoshi *et al.*, 1999). The present data indicate that UDCA decreases serum TRX levels with improvement in ALT. As serum TRX levels increase in connection with an decrease in GSH (Nakamura *et al.*, 1996), the decrease in TRX in these patients is suggested to be due to the increase in GSH by UDCA therapy.

In conclusion, oxidative stress may contribute to HCV-related liver diseases. The serum TRX level increased with the progression of liver fibrosis. Because TRX regulates the intracellular redox state and influences various signal transduction pathways in the cell (Hayashi *et al.*, 1993; Hirota *et al.*, 1997), the decrease in oxidative stress, namely, the decrease in TRX may be one mechanism that accounts for the therapeutic effects of SNMC or UDCA.

ABBREVIATIONS

ADF, Adult T-cell leukemia-derived factor; ALT, alanine aminotransferase; CH, chronic hepatitis; GSH, glutathione; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HIV, human immunodeficiency virus; ICG, indocyanine green; IFN, interferon; LC, liver cirrhosis; SNMC, Stronger Neo-Minophagen C; TBARS, thiobarbituric

acid-reactive substance; TRX, thioredoxin; UDCA, ursodeoxycholic acid.

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Address reprint requests to:
Dr. Toshiaki Nakashima
Third Department of Internal Medicine
Kyoto Prefectural University of Medicine
Kawaramachi-Hirokoji, Kamigyo-ku
Kyoto 602-8566, Japan

E-mail: nakashim@koto.kpu-m.ac.jp

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