Interferon-alpha-induced changes in metallothionein expression in liver biopsies from patients with chronic hepatitis C

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An association between reactive oxygen species and liver damage has been postulated in the course of hepatitis C virus (HCV) infection. Metallothionein (MT), induced by HCV core protein and interferon (IFN), plays a role in scavenging free radicals. MT expression in liver biopsies obtained from 21 patients with chronic HCV infection before and after IFN-alpha therapy was investigated. Changes in Knodell histological activity index (HA1) scores, MT protein levels (immunohistochemistry), MT-I and MT-II messenger (mRNA) expression levels (in situ hybridization) and proliferating cell nuclear antigen (PCNA) labelling index were determined and compared in serial liver specimens. MT staining was clustered around the portal tracts with inflammatory cells and fibrosis. The pattern of MT protein before IFN-alpha therapy was similar in all patients, but was higher in IFN-sustained responders than in nonresponders after IFN-alpha therapy. HA1 scores and PCNA labelling indexes were significantly reduced after IFN-alpha therapy. MT-II mRNA expression correlated positively with PCNA index before therapy and with HA1 scores after therapy (P<0.05). No correlation was found between MT-I mRNA and HA1 scores or PCNA index. The findings indicate that IFN-alpha-induced hepatic MT may participate in the therapeutic effects of IFN-alpha for HCV. In addition, MT-II mRNA expression may be involved in cell proliferation in the livers of patients with chronic HCV infection.

Key Words: Chronic hepatitis C; Interferon-alpha; Knodell histological activity index score; Metallothionein; PCNA labelling index

Oxidative stress has been implicated in the pathogenesis of certain viral infections; in particular, hepatitis C virus (HCV), which can result in chronic hepatitis and subsequently lead to liver cirrhosis and hepatocellular carcinoma (1,2). The precise mechanisms underlying HCV-associated liver cell injury are not well known, but oxidative stress, imposed either directly by the virus or by the host-immune response, has been postulated as a potential mechanism of pathogenesis of HCV infection (3-7). A positive correlation between oxidative stress and histological disease pattern has been demonstrated in the livers of patients infected with HCV (7). Core proteins and nonstructural proteins of HCV have been found to induce oxidative stress in cultured cells and human monocytes (8-11). Recently, the involvement of metallothionein (MT) in the protection against HCV-related oxidative stress was reported. A study in vitro showed that HCV core protein increased both production of reactive oxygen species (ROS) and expression of MT messenger (mRNA), resulting in no changes in cell viability (10,11). The finding that hepatic MT protein inhibited nuclear factor-kappa B activation via scavenging ROS suggests that MT promotes liver cell apoptosis which had been restrained by HCV protein (12,13). On the other hand, low concentrations of hydrogen peroxide suppressed HCV RNA replication in hepatoma cells (14). Thus, the relationship

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between oxidative stress and HCV replication has yet to be determined. MT was originally discovered as a class of small and cysteine-rich proteins that participate in detoxification of heavy metals and in the absorption, storage and homeostasis of essential trace elements (15,16). Accumulated evidence indicates a significant role for MT in the maintenance of redox balance, scavenging free radicals, and in controlling the activity of zinc-containing enzymes (17-20). MT can scavenge most potent hydroxyl and other free radicals very efficiently. A wide range of factors, including metal ions, oxidative stress, hormones and cytokines induce MT synthesis (15,16,21-23). In mammals, four MT genes have been cloned, but MT-I and MT-II are the most widely distributed MT isoforms. Protective roles for MT-I and MT-II isoforms against toxic metals and free radicals have been demonstrated, but the biological roles of MT isoforms and the proportion of MT-I and MT-II depends on a stimulus which remains largely unknown (23-26).

HCV infection is currently treated with interferon (IFN)-based therapy, either alone or in combination with ribavirin. Modern treatment regimens result in viral elimination in 60% of individuals (1,27,28). A variety of viral and host factors, such as HCV RNA titre, HCV genotype and the presence of cirrhosis, have been shown to affect the response to IFN-alpha therapy (1,29,30). MT was among the first genes to be identified as an IFN-alpha/beta-induced gene (31). A putative IFN-stimulated response element is present in the promoter region of MT genes (21,32). Recently, Carrera et al (33) proposed the hepatic MT level as a Biological factor associated both with the severity of HCV infection and with a better response to IFN therapy. In addition, we have previously reported (34) that MT induction by IFN was possibly related to the therapeutic effect of IFN in HCV patients. Thus, MT protein in the liver has attracted interest as a host immune defense mechanism for limiting hepatic inflammation and viral replication in HCV-infected patients. To explore the involvement of IFN-alpha with MT gene and protein expression, we examined the MT gene and protein using serial liver specimens obtained from HCV-infected patients who received IFN-alpha therapy.

PATIENTS AND METHODS

Patients
Twenty-one patients with chronic HCV infection who underwent liver biopsy both before and after IFN-alpha therapy were selected to participate in the present study. The second biopsy was performed approximately one year after the completion of IFN-alpha therapy. The diagnosis of chronic HCV infection was based on abnormal serum alanine aminotransferase levels for more than six months. The patients were positive for HCV antibody (second-generation test, ELISA) and HCV RNA (reverse transcription-polymerase chain reaction). The standard protocol for IFN-alpha therapy was as follows: 6 MU to 10 MU of IFN-alpha daily for the first two weeks then three times a week for 22 weeks. Patients were identified as sustained responders (SR) if their serum alanine aminotransferase levels were normalized and they showed HCV RNA clearance at the end of treatment and if these results were sustained for 12 months; otherwise, they were identified as nonresponders (NR). The clinical characteristics in these two groups of patients were similar, with the exception of HCV RNA titres (Table 1). The patients agreed to undergo liver biopsy and provided informed consent for their participation in the study. The study was approved from the institutional committee for the study of human rights.

Histopathology of chronic hepatitis
Hematoxylin and eosin, Mallory and Periodic Acid-Schiff staining were performed for histological analysis. The Knodell’s histological activity index (HAI) score (35) was assessed before and one year after IFN-alpha therapy. The HAI score was calculated by averaging the scores of three visual fields.

Immunohistochemical studies of MT in the liver
MT protein in the liver was stained immunohistochemically as previously described by Nakajima et al (36). In brief, serial paraffin sections of the liver specimens were incubated in absolute methanol containing 0.3% hydrogen peroxide to block endogenous peroxidase activity, and then treated with normal goat serum for 30 min. They were subsequently incubated with rabbit anti-MT antibody, diluted in phosphate buffered saline at 1:400. After being washed with phosphate buffered saline, the sections were reacted with biotinylated goat antirabbit immunoglobulin G and then processed with a Histofine SAB-PO kit (Nichirei Corporation, Japan), which detects MT. The MT polyclonal rabbit antibody used in the present study was previously prepared by Nakajima et al (36), and recognizes both MT-I and MT-II isoforms of human, rat or rabbit MT.

In situ hybridization of MT-I and MT-II mRNA in the liver
To prepare the probes for mRNA, digoxigenin-labelled human MT-I and MT-II complementary DNA probes were prepared by polymerase chain reaction as previously described by Kubota et al (37), with minor modifications. Briefly, a construct of pUC-MT-I and MT-II as template was amplified by Taq polymerase (TaKaRa taq, Takara Bio Inc, Japan) for 10 to 20 cycles involving denaturation at 95°C for 2 min, annealing at 65°C for 1 min and primer extension at 72°C for 1 min. A matrix (Boehringer Mannheim, Germany) containing digoxigenin was added to the last five cycles to label the probe. Prehybridization events and hybridization procedures were performed according to the methods of Haas et al (38) except for microwave pretreatment. Briefly, liver tissues were fixed in formalin and embedded in paraffin under routine procedures. The tissues were mounted onto slides, deparaffinized in two changes of xylene and rehydrated in a descending series of ethanol. The tissue was digested with 0.2% protease at 37°C, then washed with 0.05 M Tris-hydrochloride (HCl) (pH 7.5). This was followed by fixation in 4% formalin at 4°C for 10 min. The slides were washed again, dehydrated in graded ethanol and air dried.

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<th>TABLE 1 Characteristics of sustained responders and nonresponders of interferon-alpha therapy</th>
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<td>Age (mean years)</td>
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<td>Sex (male:female)</td>
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<td>Mean ALT (U/L)</td>
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<td>Mean AST (U/L)</td>
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<td>Mean HCV RNA (copies/mL)</td>
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*Significant difference (P<0.01) compared with the sustained responders.
After the target DNA was denatured, the hybridization mix (50 ng probe per 100 µL Hybrisol IV) was added. The slides were covered, sealed and stored in a humid chamber at 37°C for 16 h. The slides were washed three times in 0.1 × standard saline citrate at 45°C and then two times for 5 min each in 0.05 M Tris-HCl (pH 7.5). After incubation with a 1:300 dilution of anti-digoxygenin antibody (Roche Diagnostics, Germany) in 0.1% bovine serum albumin/Tris for 1 h at room temperature, the slides were washed again in 0.05 M Tris-HCl (pH 7.5). Detection was performed with nitro-blue tetrazolium chloride/5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (Roche Diagnostics, Germany) according to the manufacturer's instructions. Finally, the tissue sections were counterstained with Mayer's hematoxylin. Both MT-I and MT-II mRNA expression were assessed in 15 of 21 patients (10 SR and 5 NR); it could not be determined in the others because insufficient amounts of liver specimen remained after histology, PCNA and MT protein procedures.

Evaluation of MT gene and protein levels

The degree of MT staining and MT-I and MT-II mRNA expression were quantified using a modified method described by Goulding et al (39) and Endo et al (40). The percentage of MT-positive hepatocytes in the biopsy specimen was calculated by counting at least 100 hepatocytes in three visual fields. One pathologist categorized the staining as rare (less than 5% of the cells stained), mild (5% to less than 30%), moderate (30% to less than 60%) or strong (more than 60% of the cells stained) and scored as follows: rare = 1, mild = 2, moderate = 3 or strong = 4. Standards for each grade of MT staining and MT-mRNA expression are shown in Figures 1 and 2.

PCNA labelling index

Liver specimens were allowed to react with the first antibody, a 1:200 dilution of anti-PCNA monoclonal antibody (DAKO Japan Co Ltd, Japan), at 4°C for 12 h, and then with biotin-labelled anti-rabbit immunoglobulin G antibody and horseradish peroxidase-bound EnVision (DAKO Japan Co Ltd, Japan). The specimens were subjected to 3,3'-diaminobenzidine reaction by the addition of 0.3% hydrogen peroxide solution and nucleus staining with Mayer's hematoxylin, dehydrated and mounted. The proportion of positive cells per 400 liver cells was calculated as the PCNA labelling index.

Statistical analysis

Data are expressed as mean ± SD. Statistical analyses were performed with the StatView-J4.5 computer program (Abacus Concepts Inc, USA). Differences in HAI score, MT mRNA score, and PCNA labelling index before and after IFN therapy were analysed with Wilcoxon’s signed rank test. Nonparametric data were compared using the Mann-Whitney U test. All P-values were two-tailed, and P<0.05 was regarded as statistically significant.

RESULTS

MT staining in the liver

Immunohistochemical expression of MT was demonstrated in the cytoplasm and nuclei of hepatocytes. The MT staining was clustered around the portal tracts that were enlarged because of inflammatory cells and fibrosis, and focally in the interlobular areas (Figures 1 and 3). Serial changes of hepatic MT staining...
in typical SR and NR patients are shown in Figure 3. The MT in the SR patient was stained markedly in the hepatocytes pre- and post-therapy; however, the MT-positive hepatocytes in the NR patients were diminished post-therapy. The MT staining scores before therapy were similar in the SR and NR patients. The MT scores after IFN-alpha therapy were higher in SR patients than in NR patients, although the difference was not significant (Figure 4). In total patients, the MT staining scores after therapy were decreased compared with those before therapy. Reduction of MT staining after therapy was significant in NR patients and slight in SR patients (Figure 4).

MT mRNA expression in the liver
MT-I and -II mRNA were detected in the cytoplasm of hepatocytes (Figure 2). There is a positive correlation between MT-I and MT-II mRNA expression. Before IFN-alpha therapy, the MT-II mRNA scores were higher than the MT-I mRNA scores. MT-I mRNA scores were reduced after IFN-alpha therapy in both NR and SR patients. MT-II mRNA scores after therapy were reduced in SR patients; however, the scores were unchanged in NR patients. In total patients, the reduction of MT mRNA expression after therapy was significant in the MT-I isoform, but not in the MT-II isoform (Figure 5).

Changes in HAI score and PCNA labelling index by IFN-alpha therapy
As shown in Table 2, HAI scores before therapy were similar in SR and NR patients. HAI scores after therapy decreased significantly in SR patients but remained unchanged in NR patients.

PCNA was positive for the nuclei of hepatocytes. Before therapy, the PCNA labelling index in the liver was similar in SR and NR patients. The PCNA index in total patients was decreased significantly after IFN-alpha therapy, but the reduction of PCNA index was larger in SR than in NR patients (Table 2).

Relationship among MT and HAI score and PCNA labelling index
Before IFN-alpha therapy, the grade of MT-II mRNA staining showed a positive correlation with PCNA labelling index ($r=0.58$, $P<0.05$) (Figure 6), but not with HAI scores. In total patients, the grade of MT-II mRNA after IFN-alpha therapy positively correlated with HAI score ($r=0.56$, $P<0.05$) (Figure 7). Neither MT protein nor MT-I mRNA correlate with HAI scores or PCNA labelling index.

DISCUSSION
In the present study, MT protein was mainly found around the portal tracts with inflammatory cells and fibrosis, and this finding complements a previous finding by Tanimoto et al (41). Localization of oxidative stress markers in the livers of patients infected with HCV has been demonstrated around the portal tract with piecemeal necrosis (3). These findings suggest that MT is expressed in hepatocytes for protecting against the oxidative stress in chronic HCV infection.

The grade of MT staining in the liver was similar in SR and NR patients before IFN-alpha therapy, whereas it was higher in SR than in NR patients after therapy. These data are in agreement with our previous study (34), suggesting that IFN-alpha-induced hepatic MT may participate in the therapeutic effects of IFN-alpha for HCV patients. In comparison with pretreatment levels of MT, the grade of hepatic MT protein was decreased one year after IFN-alpha therapy. Changes in hepatic MT protein expression after therapy differed according to IFN response; MT was reduced significantly in NR but only slightly in SR. The mechanism by which MT was reduced one year after stopping IFN-alpha therapy in chronic HCV infection remains to be determined, but two possibilities have been
proposed. Induction of MT is achievable by a variety of cytokines (22,32). Chronic HCV infection is associated with a T helper (Th)2-like (42) or a combined Th1-Th2-like cytokine (43) pattern and resolving infection with a Th1-like cytokine profile (44). With regard to post-IFN therapy, Marinho et al (45) reported a reduced production of Th1-Th2-like cytokines 15 months after stopping IFN therapy; however, they did not find any characterizing Th1/Th2 response between the SR and NR patients. Therefore, a decrease in MT one year after the therapy is, at least in part, possible for association with the reduction of these cytokines, and other factors may cause different MT levels between SR and NR patients. Zinc is considered to exert a protective action on liver cell activity and to prevent damage caused by oxidative stress through induction of MT (15,16). MT gene expression and protein synthesis are induced by IFN during normal or adequate zinc status, but not during periods of zinc deficiency (46). As was observed in our previous study (34), serum zinc levels were decreased in NR patients after completion of IFN therapy, consequently diminishing MT protein in the liver. In addition, the induction of the hepatic MT gene by IFN is so rapid and transient that the occasionally performed liver biopsy probably influences MT levels (32). In the present study, the liver samples taken one year following completion of IFN-alpha therapy were evaluated, which might have caused difficulty in defining the precise induction of MT by IFN-alpha. Additional studies using liver samples taken during IFN-alpha therapy are needed to fully assess MT induction by IFN-alpha and its potential for providing a beneficial role for HCV patients.

MT-I and MT-II genes are actively expressed in many cell types in different organs and tissue, as well as in most cultured cells. The MT-II isofrom is the predominant form in human tissue (25), but the relative proportion of the isoproteins depends on the particular stimulus. IFN-alpha and -beta are well known for inducing MT-II genes in cultured cells (32), but little is known on expression patterns of MT isoforms in the liver of HCV-infected patients who have received IFN-alpha therapy. To explore changes in hepatic MT isofrom expression resulting from the influence of IFN-alpha, we stained for MT-I and MT-II mRNA in HCV-infected patients both pre- and post-therapy. Because MT-II mRNA increased in correspondence with an increase in PCNA labelling index before therapy, MT-II may be involved in cell proliferation in liver that has been damaged by continuous HCV infection. In addition, MT-II mRNA after therapy correlated positively with HAI scores. The expression of MT-II mRNA before therapy tended to be higher than that of MT-I mRNA. On the other hand, MT-I mRNA correlated with neither HAI score nor PCNA index. These data suggest that it is predominantly the MT-II isofrom, rather than the MT-I isofrom, which is involved in the pathogenesis of HCV infection.

Carrera et al (33) demonstrated that hepatic MT levels are a biological factor associated with the severity of HCV infection; however, our data using immunohistochemical staining for MT could not confirm their hypothesis. Further study using quantitative assays for MT protein is necessary to explore the association of MT with the pathogenesis of HCV infection.

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REFERENCES
Nagamine et al.
