Hepatocyte-protective and anti-oxidant effects of rifampicin on human chronic hepatitis C and murine acute hepatocyte disorder

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Abstract. Rifampicin (RFP) is a semisynthetic antibiotic derived from the rifamycins and is one of the most commonly used pharmaceutical compounds worldwide in the treatment of tuberculosis. We previously reported that low-dose and long-term oral administration of RFP to 6 hepatitis C virus-related liver cirrhosis patients who were at high risk for presenting with hepatocellular carcinoma (HCC) resulted in a marked suppression of the occurrence of HCC without showing an adverse effect. The underlying mechanism was found to be due to the anticancer effect based on the potent anti-angiogenic properties of RFP. The present study revealed that RFP has an additional hepatocyte-protective effect by lowering the release of hepatic enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in chronic hepatitis C patients. Experimentally, we were able to show that RFP had hepatocyte-protective effects in acute hepatocyte disorder models of mice and rats induced by concanavalin A and by D-galactosamine, respectively: RFP significantly prevented an increase in the levels of ALT, AST and lactate dehydrogenase in these animal models. In addition, we found that RFP had a strong anti-oxidant action which was approximately three times stronger than the action of silibinin, an anti-inflammatory agent of human hepatic stellate cells, implicating that the hepatocyte-protective effects of RFP are mediated by its anti-oxidant activity. These results reveal that oral administration of RFP exerts not only a prophylactic effect on the occurrence or recurrence of HCC for an extensive period of time, but also exerts hepatocyte-protective effects on both human chronic hepatitis C and acute hepatocyte disorder in rodent models, and the anti-oxidant activity of RFP is implicated to participate in the latter effects.

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Introduction

Rifampicin (RFP) is a semisynthetic antibiotic derived from the rifamycins, the common structure of which is a naphthohydroquinone or naphthoquinone chromophore spanned by an aliphatic chain. Previously, we reported the inhibitory effects of oral RFP on human cancer progression and its anti-angiogenic properties, which were comparable to those of the angiogenesis inhibitor endostatin (1). Clinically, low-dose and long-term oral administration of RFP to 6 hepatitis C virus (HCV)-related liver cirrhosis patients who were at high risk of presenting with hepatocellular carcinoma (HCC), resulted in only a single occurrence of HCC during the extensive follow-up period of 97.3±29.1 (mean ± SD) months, suggesting that RFP markedly suppressed the progression of HCC of the high-risk patients by inhibiting angiogenesis and also its direct anticancer activity. During that study, we noted that RFP also had a hepatocyte-protective effect by lowering the release of hepatic enzymes, alanine transaminase (ALT) and aspartate transaminase (AST), which was not explicitly described in the previous report. Here, we report the hepatocyte-protective effect of RFP by revealing clinical data obtained from a group of advanced cirrhosis patients, who had a high risk of presenting with HCC. We also revealed that oral RFP had a hepatocyte-protective effect in acute hepatocyte disorder models of rats and mice. Based on these results and a finding that RFP has a strong free-radical oxygen scavenging activity, we speculated that RFP has a hepatocyte-protective effect in hepatitis patients by lowering oxidative stress, which is greatly increased in HCV-infected liver cells (2).

Materials and methods

Clinical follow-up. The 6 patients (1) were followed-up monthly at Tokyo Medical and Dental University Hospital, after liver cirrhosis was diagnosed by liver biopsy or a combination of clinical features or both. During monthly visits, these patients underwent physical examination of serum ALT and AST related to hepatic function. The protocol was approved by the Ethics Committee of the Tokyo Medical and Dental University, and informed consent was received from the patients.

Animal experiments. Animal procedures were approved by the Committee for the Institutional Care and Use of Animals at GeneCare Research Institute in accordance with the guidelines for animal experimentation prepared by the Japanese Association for Laboratory Animal Science.

Mouse concanavalin A (ConA) model. The efficacy of RFP was evaluated in a mouse hepatocyte-disorder model induced by ConA injection. Doses of 50, 100 and 200 mg/kg RFP were orally administered to 8 mice (C57BL/6NCrlCrlj) once a day for 4 days; a single dose of 200 mg/kg RFP was administered to one group of 8 mice. One hour after the last drug administration, 0.2 mg ConA in phosphate-buffered saline was injected into the tail vein. Under ether anesthesia, blood was collected by vena cava puncture 24 h after the ConA injection. ALT, AST and lactose dehydrogenase (LDH) levels in the plasma were measured.

Rat D-galactosamine (D-Gal) model. The efficacy of RFP was evaluated in a rat hepatocyte-disorder model induced by D-Gal injection. Doses of 50, 100 and 200 mg/kg RFP were orally administered to 8 rats [Crlj:CD(SD)] once a day for 4 days; 200 mg/kg of RFP was administered to one group of 8 rats. One hour after the last drug administration, 350 mg/kg of D-Gal in saline was injected into the abdominal cavity. Under ether anesthesia, blood was collected by vena cava puncture 24 h after the D-Gal injection. ALT, AST and LDH levels in the plasma were measured.

Assay of anti-oxidant activity. Proton-donative anti-oxidant activity of RFP was assessed by using stable free radical α,α-diphenyl-β-picrylhydrazyl (DPPH) as a substrate according to the method by Nomura *et al* (3). Briefly, DPPH and test samples were mixed, and the amount of DPPH was determined spectrophotometrically at the absorbance (A) at OD_{550}. The anti-oxidant activity was calculated by using: anti-oxidant activity = [A_{550} (sample) - A_{550} (blank)]/[A_{550} (trolox) - A_{550} (blank)] x trolox concentration/sample concentration. Trolox is a standard anti-oxidant reagent.

Statistical analyses. First, homoscedasticity was tested and statistical analyses between control and test groups were carried out using Dunnett type multiple comparison for homoscedasticity and by Dunnett type multiple comparison (joint type) for non-homoscedasticity. For histopathological studies, statistical analyses were carried out using the Wilcoxon test. For comparison, vitamins C and E, silibinin and trolox were used as anti-oxidant reagents.

Results

Clinical study. The liver function of the 6 HCC high-risk patients with HCV-related cirrhosis was studied by monitoring the serum values of ALT and AST. Detailed clinical information of the 6 patients was described in our previous study (1). All 6 patients had hepatitis virus type C of 1b genotype, suggesting resistance to interferon-α therapy; either they were resistant to interferon-α or their interferon-α therapy was discontinued because of adverse reactions. Most patients received administration of ursodeoxycholic acid (UDCA) before or during administration of RFP (Fig. 1Aa-Fa, dotted lines). They received low-dose RFP therapy of 150 or 300 mg daily during most of the follow-up period from 121 to 209 months. Notably, serum levels of α-fetoprotein, a marker of the growth of hepatocytes and HCC cells, decreased after RFP administration in 3 patients. Notably, 5 patients showed no sign of presenting with HCC during the long REP treatment, despite a high statistical frequency of the occurence of HCC in patients with advanced cirrhosis. Only 1 patient had HCC during the long follow-up period of 97.3±29.1 months. These clinical results strongly suggest that RFP is effective in suppressing HCC, as previously reported (1).

In this context, we investigated whether low-dose and long-term RFP therapy also improves liver function of these high-risk patients with HCV-related cirrhosis.

Patient 1 (Fig. 1A). During ~90 months of RFP therapy, the values of ALT and AST tended to improve from 0 to ~40 months of RFP administration: AST from 100 to ~50 IU/l; ALT from 80 to ~50 IU/l. However, the strict evaluation of the efficacy of long-term administration of RFP became difficult after the first 40 months when the patient developed bile duct stenosis and acute pancreatitis and received treatment for these two conditions.

Patient 2 (Fig. 1B). ALT and AST values improved markedly during 0-80 months of RFP administration: AST values decreased from 200 to <100 IU/l; ALT from 150 to <50 IU/l. This patient received treatment of UDCA before and sometimes during RFP therapy, but the treatment of UDCA alone apparently failed to lower the ALT and AST values. RFP lowered the ALT and AST values without UDCA

Patient 3 (Fig. 1C). ALT and AST values markedly improved throughout ~80 months of RFP therapy: AST from 100 to approximately 50 IU/l; ALT from 175 to ~50 IU/l. This patient received treatment of UDCA before and during RFP treatment, but the treatment of UDCA alone failed to lower the ALT and AST values.

Patient 4 (Fig. 1D). High values of ALT (>150 IU/l) and AST (>100 IU/l), which were often observed before RFP administration, were not observed throughout ~60 months of RFP therapy (150 mg/day), and most values remained <100 IU/l. This patient received treatment of UDCA before and during the period of RFP treatment, but the treatment of UDCA alone did not seem to lower the ALT and AST values.

Patient 5 (Fig. 1E). ALT and AST values markedly improved throughout ~145 months of RFP therapy: AST values decreased from >250 to <100 IU/l; ALT values decreased from >150 to ~50 IU/l by 150 mg/day administration of RFP. This patient received treatment of UDCA before and during RFP treatment, but the results showed that UDCA alone was ineffective in lowering ALT and AST values.

Patient 6 (Fig. 1F). High levels of ALT (150-200 IU/l) and AST (>150 IU/l), which were often observed before RFP administration, were not observed throughout ~76 months of RFP therapy, and most values remained <100 IU/l. This patient

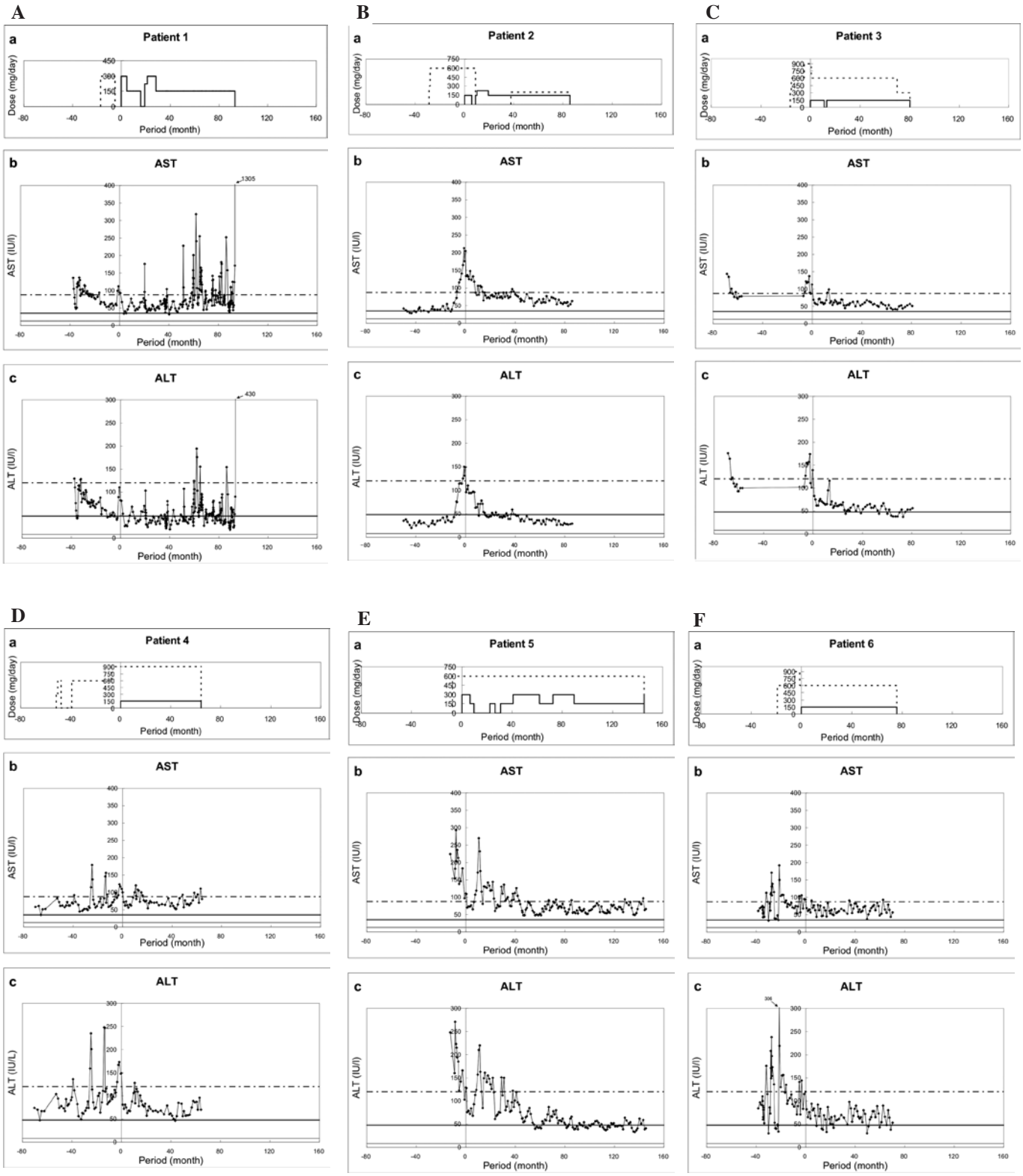

Figure 1. Changes in the plasma levels of AST and ALT of patients 1-6 (A-F) before and after RFP treatment. (a) Time schedule of low-dose RFP therapy of 150, 225 or 300 mg daily (shown by solid line), and UDCA therapy of 200, 300, 600 or 900 mg (dotted line). Plasma levels of (b) AST and (c) ALT, respectively: the solid line and dotted line indicate the upper level of normal individuals and the grade 1 cirrhosis patients, respectively.

received treatment of UDCA before and during the RFP treatment. The treatment of UDCA alone tended to lower ALT and AST values, while the effects were not as consistent as those obtained with combined UDCA and RFP therapy.

These results indicated that low-dose (150 mg/day) and long-term oral administration of RFP markedly decreased ALT and AST values. All patients received oral administration of UDCA before and throughout REP therapy, but UDCA alone

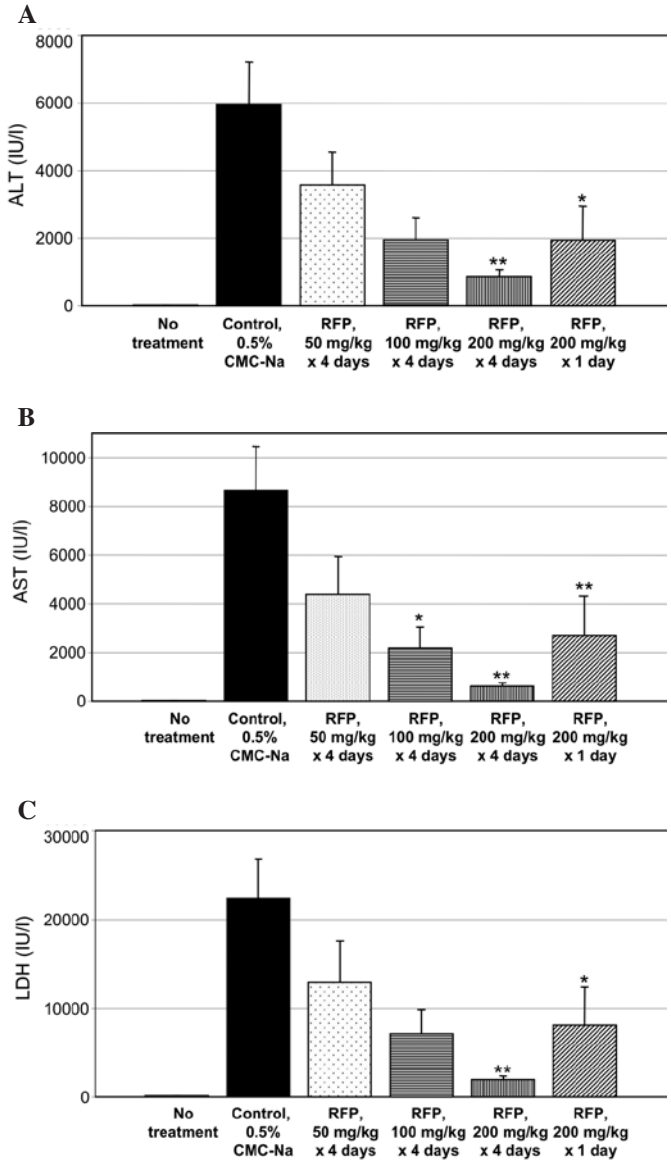

Figure 2. Effect of RFP on ConA-induced hepatic disorder in mice. The mouse model of hepatitis was designed by injection of ConA into a group of mice following the method of Tiegs and Gantner (4). Mice (n=8) were treated by pre-oral administration of varying concentrations (50-200 mg/kg) of RFP for 4 days, or were treated once with 200 mg/kg RFP. The liver-protective effect of RFP is shown, representing a reduction in the plasma levels of (A) ALT, (B) AST and (C) LDH, respectively. Statistical differences of *p<0.05 and **p<0.01, respectively, between the group treated with ConA alone and the groups treated with ConA together with RFP.

was ineffective in lowering the values of ALT and AST of most patients (Fig. 1B-F). By contrast, the results of patients 1 and 2 revealed that administration of RFP alone lowered the values of ALT and AST. All these results together indicated that RFP therapy improved liver function of the HCV-related cirrhosis patients with a high-risk of presenting with HCC. The decrease in ALT and AST values was not considered to be due to a decrease in hepatic enzymes associated with hepatic cirrhosis, as no marked decrease in platelets was observed during RFP therapy (data not shown). The stabilization of platelet values during long-term RFP administration supports the idea that progression of hepatic cirrhosis is prevented by RFP therapy.

Effect of RFP studied in the experimental animal hepatocyte-disorder models. We studied the effect of oral administration of RFP in animal models of acute hepatic disorder. A mouse hepatocyte-disorder model induced by ConA and a rat hepatocyte-disorder model induced by D-Gal were used in the following studies.

Effect of RFP on Con A-induced hepatic disorder in mice

Biochemical study. Fig. 2 shows the effects of RFP on the plasma levels of biochemical markers of liver function in ConA-induced hepatic disorder mice. The mouse hepatic disorder is induced by ConA that activates T cells and therefore has a common background with human hepatitis C, as both disorders are induced by immune reactions (4). Pre-treatment with oral RFP at 50 mg/mouse x 4 days and 100 mg/mouse x 4 days prevented the increase in the plasma levels of ALT (Fig. 2A), AST (B) and LDH (C) in the mouse model of ConA-induced experimental liver disorder. RFP at 200 mg/kg x 4 days or single administration of 200 mg/kg x 1 day also prevented ConA-induced increase in serum ALT, AST and LDH levels.

Histopathological study. Nest coagulation necrosis of mouse hepatocytes was induced by ConA treatment. The effect of RFP to protect this hepatocyte disorder was evaluated by histopathological studies. The results were: i) ConA alone resulted in 5 severe cases and 3 moderate cases; ii) ConA and then oral administrations of RFP at 50 mg/mouse x 4 days RFP resulted in 1 severe case, 6 moderate and 1 weak case; iii) ConA and then RFP at 100 mg/mouse x 4 days resulted in 2 moderate cases, 4 weak, 1 slight and 1 case without nest coagulation; iv) ConA and then RFP at 200 mg/mouse x 4 days resulted in 6 weak cases and 2 slight cases; v) ConA and then RFP at 200 mg/mouse x 1 day resulted in 2 moderate cases, 2 weak, 2 slight and 2 cases without nest coagulation.

Statistical analysis using the Steel test of nest coagulation necrosis showed a significant difference ($p<0.01$) between the group treated with ConA alone and the groups treated with ConA together with RFP at 100 mg/mouse x 4 days, 200 mg/mouse x 4 days and 200 mg/mouse x 1 day. These histopathological results indicate that RFP markedly improves hepatic disorder induced by ConA in mice.

Effect of RFP on D-Gal-induced hepatic disorder in rats

Biochemical study. Fig. 3A-C shows the effects of RFP on liver function in D-Gal-induced hepatic disorder model rats. The plasma levels of ALT, AST and LDH were measured to monitor the liver-protective effect of oral RFP. D-gal induces various cytokines, including tumor necrosis factor responsible for hepatic injury (5). Administrations of RFP at 50 mg/rat x 4 days and 100 mg/rat x 4 days prevented an increase in the plasma levels of ALT (Fig. 3A), AST (B) and LDH (C) in D-Gal-induced experimental hepatic disorder in rats. RFP pre-treatment with 200 mg/kg x 4 days or a single pre-treatment with 200 mg/kg strongly prevented an increase in serum ALT, AST and LDH levels in D-Gal-induced experimental hepatic disorder in rats.

Histopathological study. D-Gal-treated rats had hepatocytes that showed pale staining of cytoplasm and nuclei. The rats also had hepatocytes that showed diffuse necrosis and

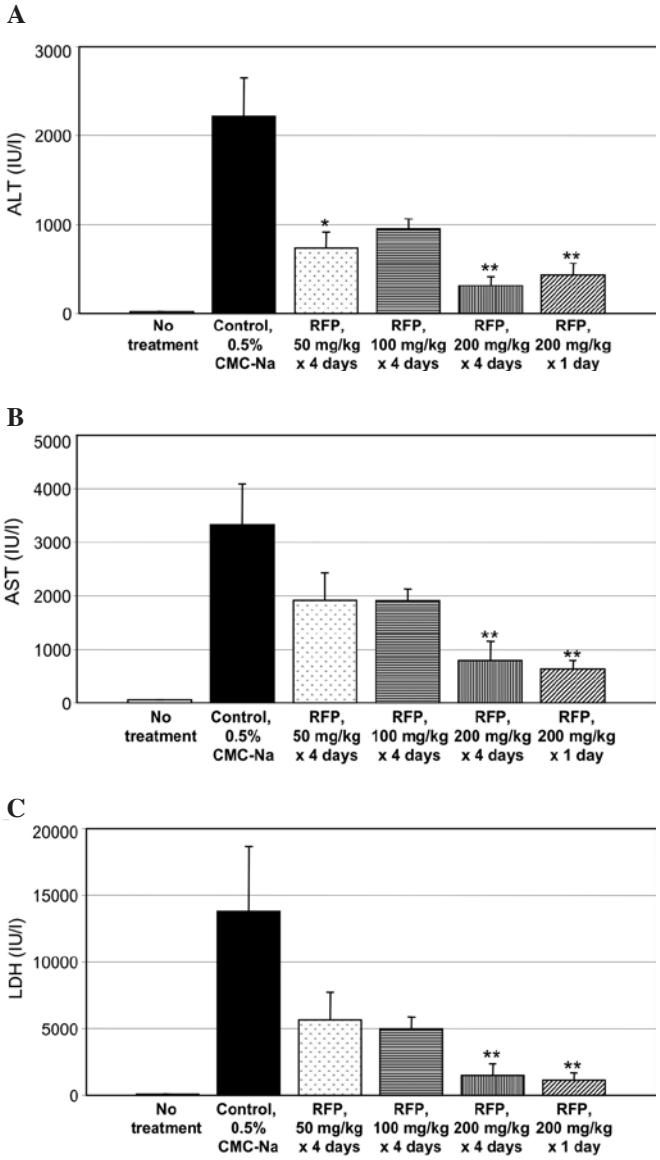

Figure 3. Effect of RFP on plasma (A) ALT, (B) AST and (C) LDH levels in D-Gal-induced hepatocyte disorder in rats. The rat model of hepatitis was designed by injection of D-Gal to a group of rats following the method of Czaja *et al* (5). Rats (n=8) were treated by pre-oral administration of varying concentrations (50-200 mg/kg) of RFP for 4 days, or were treated once with 200 mg/kg RFP

inflammatory intralobular cellular infiltration. Administration of RFP resulted in the following therapeutic effects: i) the group of rats treated with D-Gal and then with RFP at 200 mg/kg x 4 day showed significantly ($p<0.05$) reduced pale staining of cytoplasm and nuclei of hepatocytes, and inflammatory intralobular cellular infiltration, compared to the group treated with D-Gal alone; ii) the group of rats treated with D-Gal and then with RFP of 200 mg/kg x 1 day showed markedly ($p<0.01$) reduced pale staining of cytoplasm and nuclei of hepatocytes, inflammatory intralobular cellular infiltration and significantly reduced ($p<0.05$) diffuse necrosis of hepatocytes compared with the group treated with D-Gal alone.

These results indicate that oral administration of RFP protects rat liver from D-Gal-induced hepatic injury.

Table I. Anti-oxidant activity of rifampicin.

	Specific activity (mol/mol)[a]
Rifampicin	0.95±0.04
Vitamin E	1.11±0.10
Vitamin C	1.11±0.14
Silibinin	0.33±0.08

[a]Specific activity is expressed as described in Materials and methods.

Anti-oxidant activity of RFP. To understand the chemical nature of RFP that may explain the mechanism behind several therapeutic effects of RFP observed in clinical studies with liver cirrhosis patients and in experiments with murine models of acute hepatic injury, we measured the anti-oxidant activity of RFP and compared the values to those of several compounds that have anti-oxidative properties. The anti-oxidant activities of RFP, vitamins E and C and silibinin as expressed by the specific activity of mol/mol trolox (a water-soluble vitamin E derivative) were 0.95±0.04, 1.11±0.10, 1.11±0.04 and 0.33±0.08 (n=3), respectively (Table I). These results indicated that RFP has anti-oxidant activity comparable to vitamins C and E, and is approximately three times stronger than the activity of silibinin, which was found to exhibit anti-inflammatory and anti-fibrogenic effects on human hepatic stellate cells (6).

Discussion

This study showed that low-dose and long-term oral administration of RFP markedly improved plasma hepatitis markers in 6 HCV-related liver cirrhosis patients with a high risk of presenting with HCC. Also, RFP markedly improved acute hepatocyte disorder in mouse and rat models induced by ConA and D-Gal, respectively. Huang *et al* (7) reported that RFP cures liver injury in mice caused by carbon tetrachloride. These results suggest that RFP has an anti-inflammatory effect on hepatocyte disorder by preventing the release of hepatic enzymes, including ALT and AST.

RFP has a naphthohydroquinone chromophore spanned by an aliphatic chain and is implicated to have anti-oxidant and free-radical scavenging activities (8). This study showed that RFP indeed has a strong anti-oxidant activity, comparable to the activities of vitamins C and E, which is approximately three times stronger than silibinin (also known as sylimarin or silybin), an anti-inflammatory agent for human hepatic stellate cells (6).

Peroxynitrite, formed by the reaction of superoxide and nitric oxide, is an important tissue-damaging species generated at sites of inflammation. Whiteman and Halliwell (9) examined *in vitro* the ability of RFP to protect against peroxynitrite-dependent inactivation of α1-antiproteinase and to inhibit tyrosine nitration by peroxynitrite. They showed that RFP was highly protective in both assay systems, suggesting that RFP prevents tissue injury at sites of inflammation by scavenging peroxynitrite. Kalpana *et al* (10) showed that ascorbic acid

(vitamin C) and RFP have free-radical scavenging activity by using DPPH *in vitro* and a deproteinated blood method. Data have accumulated that implicate the participation of oxidants and free radicals in the pathogenesis of hepatitis (11). Morisco *et al* (12) studied the effect of interferon-α and ribavirin treatment on the oxidative state in 52 chronic hepatitis C patients who received a combination of the two drugs for 6 or 12 months. The results revealed that patients with a successive long-term response had a significantly lower basal serum hydroperoxide concentration than non-responders, and that the mean hydroperoxide concentration decreased significantly during the treatment. Berkson (13) reported a conservative triple anti-oxidant approach to the treatment of hepatitis C; 3 patients who received this therapy recovered quickly and their laboratory values improved markedly. RFP has the excellent property of accumulating in the liver (14), which supports the notion that the effect of RFP is markedly increased and thus is preferentially efficient in the liver.

These results collectively support strongly the idea that hepatocyte-protective effects of RFP on human chronic hepatitis C and on acute hepatocyte disorder in the murine experimental models of this study are due to its anti-oxidant and free-radical scavenging activities. Although the pathological reasons behind these human and animal hepatic disorders differ in many details, the observed hepatocyte-protective effect of RFP seems to derive from the fundamental reaction acting on a common and basic cellular event. Destruction of cells occurs in various inflammatory events caused by free radicals produced endogenously by lymphokines and cytokines. This is exemplified by the destruction of cells in inflammation, which includes attack on cells by other exogenous chemical substances, such as carbon tetrachloride.

RFP protects against acute liver injury induced in mice by carbon tetrachloride (7,15). In the models of this study, RFP reduced plasma levels of ALT and AST, and hepatocyte necrosis. Takeda *et al* (15) speculated that RFP suppresses the expression of cytochrome P2E1 and protects CCl_4-mediated DNA damage in hepatocytes by inhibiting cytochrome P2E1-mediated formation of free radicals from CCl_4.

Cytokines resulting from ConA or D-Gal treatment participate in ConA-induced hepatocyte disorder of mice and D-Gal-induced hepatocyte disorder of rats (4,5). Activated T lymphocytes appear to be responsible for liver damage in chronic hepatitis, and the mouse and rat systems are sometimes considered to be hepatitis models in which activated T lymphocytes participate (16). For instance, ConA is a T-cell mitogen and induces release of systemic tumor necrosis factor, interferon-γ, interleukin 12 and various other cytokines. Passive immunization against interleukin 12 (17) or pre-treatment with immunosuppressive drugs to suppress tumor necrosis factor protects mice from liver injury (18), supporting the idea that cytokines participate in liver injury of mice induced by ConA. Suppressive effects of RFP on the immune system have been reported. For instance, secretion of tumor necrosis factor-α is suppressed markedly by RFP in lipopolysaccharide-stimulated monocytes (19,20). These results suggest that RFP indirectly suppresses liver injury by inhibiting secretion of cytokines. Therefore, in the present study RFP possibly suppressed hepatocyte disorder in the murine models also by inhibiting cytokine release, in addition to its anti-inflammatory effects by its anti-oxidant and free-radical scavenging activities.

In our previous clinical study (1), low-dose and long-term oral administration of RFP to 6 HCC high-risk patients with HCV-related liver cirrhosis, who were also the targets of this study, resulted in reduced occurrence of HCC, in which the angiogenesis-inhibitory effect of RFP was implicated (1,21). Since angiogenesis is well known to be stimulated in inflammatory lesions, the angiogenesis-inhibitory effect of RFP is considered to be partly due to its anti-inflammatory effect. Conversely, long-term suppression of inflammation in chronic hepatitis patients might also have contributed to the suppression of HCC in these patients. Recently, Pal *et al* (2) analyzed the evidence of oxidative stress in association with HCV-induced chronic inflammation. They reported that human hepatoma cells infected with HCV showed 30- to 60-fold increases in the level of reactive oxygen species (ROS) and 6-fold increases in oxidatively modified guanosine levels compared to uninfected cells. RFP is apparently multifunctional, but the mechanisms of RFP to cure chronic hepatitis C and to prevent HCC generation may be partly related to RFP scavenging ROS in HCV-infected hepatic cells.

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