2. Search for the origin of iron overload in patients with chronic hepatitis C

2-1. Liver as a key regulatory organ for body iron homeostasis

Hepatocytes are a major site of iron storage and express relatively low amounts of the iron exporter ferroportin on surfaces that face the sinusoids (46). In states of genetic or acquired iron overload, hepatocytes become a major site of iron deposition, presumably because their iron uptake exceeds the capacity for export. Hepatocytes display particularly high uptake rates for non-transferrin-bound iron, a form of iron that is present when iron load exceeds the iron-binding capacity of transferrin (47). Recently, in addition to the function as iron reservoir, the liver is believed to play a central role in the sensing of body iron requirements and regulation of duodenal iron absorption. Although our complete knowledge of these processes is still lacking, recent discoveries of iron regulatory proteins have pushed our understanding forward at a rapid rate. It was previously believed that body iron levels were directly sensed at the level of the small bowel crypt enterocytes by the HFE-transferrin receptor 1 (TfR1) complex (48). These crypt enterocytes would then be programmed to express the appropriate levels of iron regulatory proteins as they matured along the crypt villous-axis. However, this model fails to adequately explain recent experimental findings. These include:

- The lack of significant basolateral HFE and TfR1 protein expression in the crypt enterocytes.

- The rapid change in the rate of iron absorption that can occur within hours of a change in iron status (e.g., blood loss) rather than in the days necessary for the process of enterocyte maturation.

More recently, a new model has emerged in which it is proposed that the sensing of body iron levels occurs in the liver, by regulating the hepatic expression of hepcidin (49).

Hepcidin, exclusively synthesized in the liver, was originally isolated from human serum and urine as having an antimicrobial activity (6, 50). The hepcidin is produced as an 84 amino-acid precursor, and is subsequently processed to give the 60 amino-acid pro-hepcidin plus a smaller active peptide of 20-25 amino-acids. The lack of hepcidin expression in knockout mice leads to iron overload (51), and, conversely, overexpression of hepcidin in transgenic mice causes severe iron deficiency (52). In normal mice, iron overload increases and iron deficiency decreases hepatic mRNA expression of hepcidin; change in hepcidin

expression is associated with inverse changes in intestinal iron absorption (53). Recently, it was demonstrated that hepcidin can bind ferroportin, inducing ferroportin internalization and degradation, and resulting in reduced iron efflux from iron exporting tissues into plasma (9, 54, 55). An effect of hepcidin to another iron transporter, DMT1, was also proposed (56). Moreover, hepcidin mutations are associated with a new type of severe juvenile hemochromatosis not related to HFE mutations (57). More common forms of hereditary hemochromatosis [caused by mutations in HFE, transferrin receptor 2 (TfR2), hemojuvelin (HJV)] also correlate with various degrees of hepcidin deficiency (58-61). Thus, hepcidin is recognized as a major negative regulatory hormone for iron homeostasis.

Recently, a second transferrin receptor isoform, TfR2, has been described predominantly expressed in the liver (62). Its role in iron regulation is exemplified by a study, which identified mutations in human TfR2 associated with a hemochromatosis phenotype independent of HFE status (63). In addition, Fleming and coworkers (64) were able to produce a classical hemochromatosis phenotype in TfR2 knockout mice. TfR2 function, at first is thought to be consistent with its homology to classical transferrin receptor-TfR1 (62, 65); iron uptake by the hepatocytes [TfR2 has limited tissue distribution, with prominent expression in the liver, especially in hepatocytes (62, 66)]. But disabling mutation in TfR2 gene in human (63) and mice (64) leads to significant hepatic iron accumulation despite an absence of TfR2 expression, suggesting that iron uptake in hepatocytes is not the principle function of TfR2, and that TfR2 has important but unknown role for maintenance of iron homeostasis. Recently, Kawabata et al. (67) demonstrated that hepcidin is down-regulated in TfR2 mutant mice, suggesting that TfR2 act as an upstream sensor for hepcidin production in the pathway of iron homeostasis. Its ability to bind transferrin (62) also makes it an ideal candidate as a detector of transferrin saturation to regulate of hepcidin expression. A mechanism for the detection of transferrin saturation by TfR2 was also proposed by Townsend and Drakesmith (68). However, they placed the site of TfR2 action in the crypt cells of the duodenum and the marcrophages of the reticuloendotherial system. We reposition the site of TfR2 activity to the liver as this organ expresses high levels of hepcidin. We propose that the binding of diferric transferrin to TfR2 produces a signal to influence the expression of hepcidin. In this case, as the level of diferric transferrin falls, TfR1 would outcompete TfR2 for diferric treansferrin binding, as TfR1 has a 25-fold greater affinity for diferric transferrin than does TfR2 (69). This would reduce the signal for hepcidin synthesis and, as a result,

stimulate iron absorption at duodenal enterocytes. When iron levels increase, the resultant downregulation in TfR1 expression would shift the competition in favor of TfR2 causing an increase in hepcidin production and a decrease in iron absorption. A recent study has also shown that diferric transferrin upregulates TfR2 expression and causes a redistribution of TfR2 to the cell surface (70), further increasing hepcidin production when iron levels are high. The decrease in serum iron levels that occurs following blood loss is likely to be sensed by TfR2 at the liver as a reduction in serum transferrin saturation leading to decreased hepcidin production and increased enterocyte iron absorption.

The emergence of hepcidin as a strong candidate for the "humoral factor" regulating iron absorption strengthens this proposal and, along with recent data on the biology of TfR2, suggests that the liver plays a key role in body iron homeostasis via the TfR2-hepcidin axis.

2-2. Presumptive molecular mechanisms of iron overload in patients with chronic hepatitis C

What are the molecular mechanisms underlying the iron overload in patients with chronic HCV infection? An increased intestinal iron absorption has been suggested in HCV-infected individuals (71). An intriguing hypothesis is that HCV may affect the expression of proteins important in modifying iron trafficking, such as HFE, ferritin, DMT1, ferroportin, transferrin receptors, or hepcidin. We have investigated the expressions of TfR1, TfR2, feropprtin, and hepcidin mRNAs in the liver of patients with chronic hepatitis B, chronic hepatitis C, and controls. Hepatic TfR2 expression levels were significantly higher in HCV group than in HBV (72), and hepcidin expression levels were relatively low in patients with HCV (73), suggesting that TfR2 and hepcidin may play a role in the pathogenesis of iron overload in patients with chronic hepatitis C. As mentioned above, TfR2 appears to be upstream regulator of hepcidin and is required for hepcidin to respond appropriately to changes in serum transferrin saturation. Thus, up-regulation of TfR2 mRNA in the liver may involve in the down-regulation of hepcidin in patients with chronic hepatitis C. In addition to its response to iron homeostasis, hepcidin is known to be up-regulated by inflammation (74), and it is well recognized as the key mediator of "anemia of inflammation" (8). Therefore, hepatic inflammatory status in chronic hepatitis may influence the hepatic hepcidin expression levels. But, in our previous report, hepatic necroinflammation status seems to be not related to relatively low hepcidin expression in patients with chronic hepatitis C, because serum aminotransferase levels and histological grading of

inflammatory activity were not significantly different between the HCV and HBV groups (73). Further *in vitro* study is required to clarify the distinct interaction between TfR2, hepcidin, and the degree of intracellular iron content in hepatocytes during chronic HCV infection.

3. Clinical impact of iron overload in patients with chronic hepatitis C

3-1. Involvement of iron overload for liver disease progression in patients with chronic hepatitis C

Increased body iron storage in patients with chronic hepatitis C may play a role as a factor for hepatocyte damage during chronic HCV infection. It is reported that excess dietary iron in HCV-infected chimpanzees exacerbates liver injury as shown by elevated aminotransferase levels and histological changes, without influence on viral load (75). HCV-infected females are more likely than males to have consistently normal ALT levels (76, 77), and the reduction of hepatic iron stores as a result of menstruation and child bearing may partially explain the lower ALT levels observed in HCV-infected females (76, 78). Studies in chronic HCV patients with mild to moderate iron excess have shown that phlebotomy reduces serum aminotransferase levels (79). These results strongly suggest that the surplus iron in the body accelerates hepatocytic damage in HCV-infected patients. What are the mechanisms that link hepatic iron accumulation to liver damage in chronic hepatitis C? Iron-induced hepatic injury may occur via oxidant stress, because iron is a potent catalyst of oxidative stress; it reacts with oxygen to generate hydroxyl free radicals (2). The iron-storage capacity of the liver is highly expandable and mainly relies on the induction of ferritin synthesis: Within the ferritin shells, iron is sequestered in a safe status. Nevertheless, this is not an inert state, and redox changes in the cytoplasm, xenobiotics, or other conditions may rapidly mobilize this iron and make it catalytically active (80). This is particularly relevant to for hepatitis C where oxidative stress may have already been produced by HCV-expressing proteins in hepatocytes. *In vitro* and transgenic animal model studies have shown that HCV proteins, such as Core, Nonstructural (NS) 3, or NS5A, directly induce oxidative stress (81-85). There is an increasing evidence that in humans oxidative stress abundantly exists in the body of patients with chronic hepatitis C (86-88). Plasma samples from HCV-infected individuals have increased lipid peroxidation products (89), and peripheral blood mononuclear cells show elevated superoxide dismutase activity (90), that are consistent with an increased cellular reactive oxygen species (ROS) formation in HCV patients. Immunohistochemistry has also documented the presence of oxidative stress in liver

biopsy specimens from HCV patients (87, 89). A subsequent study showed that ROS were associated with the disease activity in chronic hepatitis C (91). Thioredoxin is also an indicator of oxidative stress in various diseases (92). Sumida et al. (93) measured serum thoredoxin levels in various liver diseases and showed a high correlation between thioredoxin and serum ferritin levels in patients with chronic hepatitis C. All these findings suggest that there is the possibility that slight iron overload, although unlikely to be cytotoxic in healthy individuals, may contribute to cause liver tissue injury by increasing the formation of oxidative stress leading to progressive liver inflammation in chronic HCV-infected patients. Our previous study also clearly demonstrated that hepatic 8-hydroxy-2'-deoxyguanosine (8-OHdG) counts, which is considered as a useful marker of oxidatively generated DNA damage (94), were significantly correlated with serum transaminase levels and iron overload condition (serum ferritin levels and hepatic iron score) in patients with chronic hepatitis C, indicating that a role of iron for liver injury through oxidative stress formation during chronic HCV infection (95). Involvement of iron in the pathogenesis of chronic HCV infection through the oxidative stress is also supported by the fact that the iron reduction therapy improved liver injury with the reduction of hepatic oxidative stress in patients with chronic hepatitis C (95).

A close correlation between the amount of iron accumulation and hepatic fibrosis has also been reported in patients with chronic hepatitis C (41). The frequency and the degree of iron overload increases with the stage of fibrosis (36-38, 41, 96-99). Rigamonti et al. (97) found a positive correlation between numbers of hepatic stellate cells (HSCs) and hepatic iron concentration in patients with chronic hepatitis C; HSCs proliferate and differentiate into myofibroblast-like cells and are responsible for the development of liver fibrosis following liver injury. The fibrogenic potential of iron has also been demonstrated in animal models (100). After 6 weeks of subcutaneous iron injection in rodent, HSCs showed a dramatic increase in mRNA for collagen synthesis, and micronodular cirrhosis developed after 4 months with increased expression of transforming growth factor β. A substantial increase in hepatic iron deposition, as occurs in patients with hereditary hemochromatosis, is associated with greater frequency of hepatic fibrosis and cirrhosis (101). However, in patients with chronic hepatitis C from a clinical background, the impact of iron on fibrogenesis remains controversial. Most studies concluded to an association between the amount of liver iron and progression of fibrosis in HCV patients (34, 36, 38, 41), while more recent studies did not find such an association (102, 103). The reasons for these discrepancies have been recently fully

reviewed (48). Presumably, they rely on differences in study population and lack of control for confounding variables. Variables defined in studies on fibrosis progression in HCV-infected patients were male sex, duration of infection, age at contamination > 40 years, daily consumption of alcohol > 50 g/day, and features of the metabolic syndrome (such as high body mass index, diabetes, and liver steatosis) (104). All these variables can also influence iron metabolism: serum ferritin levels increase with age and male sex (105) and iron metabolism disturbances are common in alcoholic liver diseases (106), insulin resistance iron overload syndrome (107) and non-alcoholic steatohepatitis (NASH) (108). Because all the factors responsible for rapid fibrosis progression can also be associated with an increase in ferritin and iron stores, the assertion of a link between iron and fibrogenesis can only rely on an appropriate analysis adjusted for all these confounding factors. Angelucci et al (109) recently reported the highly suggestive and valuable situation ; two hundred eleven patients cured of thalassemia major by bone marrow transplantation, who did not receive any chelation or antiviral therapy, have been followed for a median follow-up of 5 years. In a multivariate Cox progression hazard model, the risk for fibrosis progression correlated with hepatic iron content and HCV infection. Thus, iron overload and HCV infection are independent risk factors for liver fibrosis progression, and their concomitant presence results in a striking increase in risk.

3-2. Iron and HCC in patients with chronic hepatitis C

The risk of HCC in patients with HCV-positive cirrhosis runs between 2% and 7% annually (110) and HCV is the leading cause of HCC in Japan, Italy, and Spain, representing up to 84% (111), but the molecular mechanisms of hepatocarcinogenesis caused by HCV infection is precisely unknown. In contrast to HBV, HCV does not integrate into host DNA. Therefore, extrachromosomal oncogenic pathways seem to be operative. Conclusively, markedly increased hepatic iron itself is the risk for HCC development because the association of severely increased iron accumulation in the liver with hepatocarcinogenesis in hereditary hemochromatosis has been well documented (112). Patients with secondary iron overload conditions, such as with the dietary African variant (113) or with that related to homozygous β thalassemia (114), are also at a higher risk for HCC, but it remains to be elucidated whether mild to moderate increases in hepatic iron accumulation, which is observed in chronic hepatitis C, contribute to HCC development. The effect of even minor increases of hepatic iron, independent of other confounding factors, on the risk to

develop HCC in patients with chronic hepatitis C has been addressed by several studies that yielded controversial findings. Thus, heterozygosity for the HFE mutation which favors mild hepatic iron accumulation (35) correlated with an increased risk of HCC in some studies (115, 116), but no such correlation was found in other studies (117, 118). Recent studies using transgenic mouse model expressing the full HCV polyprotein, have clearly demonstrated a causative link between mild to moderate iron overload by iron supplementation and HCC development under the expression of HCV proteins (119). Hepatic iron accumulation, by feeding a diet containing carbonyl iron, induces mitochondrial alternations, hepatic steatosis, fatty acid oxidation, formation of 8-OHdG, and tumor development. These results strongly support that the mild to moderate iron overload, which unlikely to cause hepatocarcinogensis in healthy conditions, favor DNA damage, genetic instability, and tumorigenesis in HCV-infected liver of patients with chronic hepatitis C, via oxidative stress formation. Several investigators also indicated that the iron overload and HCV proteins cooperatively act to enhance oxidative stress and DNA damage in hepatocytes leading to cancer formation (81, 120). Iron depletion both in the form of dietary iron restriction and phlebotomy also decreased hepatic content of 8-OHdG and prevented HCC development in a 6-year study from Japan that involved 34 patients with chronic hepatitis C (121), definitively indicating the involvement of hepatic iron overload in hepatocarcinogenesis in patients with chronic HCV infection.

3-3. Iron, HCV replication, and immune defense

The effect of hepatic iron overload on HCV replication remains controversial. Iron, which is essential for the growth of all organisms, may facilitate HCV replication (122-124). In fact, in hepatoma cells iron promoted HCV translation by up-regulating expression of the translation initiation factor eIF3 as measured in reporter studies driven by regulatory HCV mRNA stem-loop structures (124). In contrast, iron was also reported to suppress HCV replication by inactivating the RNA polymerase NS5B (125). Clinical data suggest that iron status does not significantly influence HCV replication in vivo, because many studies indicated that the serum HCV viral load did not correlate to the degree of iron accumulation (26, 41, 96), and venesection did not affect hepatitis C viral load (21, 126, 127).

Iron may also directly affect the pathogenesis of HCV infection by impairing the host immune response or by inducing an immune response that is deleterious to the host. Iron impairs many aspects of the immune response, including humoral

immunity, cellular immunity, and the function of iron-loaded antigen-presenting cells (20). The induction of iron overload results in a shift of the ratio between T-helper ($CD4^+$) and T-suppressor/cytotoxic T cells ($CD8^+$), with a relative decrease of the latter (128). In fact, the $CD4^+/CD8^+$ peripheral blood lymphocyte ratio is often high, because of a reduction in the number of circulating $CD8^+$ T lymphocytes, in most hemochromatosis patients with liver damage (129). A similar reduction in the number of $CD8^+$ T lymphocytes was also documented in liver biopsy specimens from hereditary hemochromatosis patients (129). Although this association may hold for the excessive iron accumulation in human hereditary hemochromatosis (112), it remains to be shown if the mild to moderate iron overload which is observed in patients with chronic hepatitis C contributes to the development of HCC. Future studies in this area may lead to better management of HCV infection and improved therapies.

3-4. Influence of iron overload on the IFN-based treatment response in patients with chronic hepatitis C

In patients with chronic hepatitis C, the influence of hepatic iron deposit on treatment response has been discussed for several years. As for IFN monotherapy, two trails clearly showed that the hepatic iron content of non-responders was more prominent than that of responders (23, 24). However, some studies have not observed a decreased response to IFN in association with increased iron load (28, 130). Recently, ribavirin combination with IFN has been shown to be more effective than IFN alone and it has become the current standard for chronic hepatitis C treatment (131). The relevance of hepatic iron metabolism to combination therapy response is also controversial. Patients receiving the combination therapy frequently develop moderate-to-severe hemolytic anemia (132), and the ribavirin itself could be responsible for an increase in liver iron content (133). Thus, the influence of body iron status on treatment response may be different between patients treated with IFN monotherapy and IFN plus ribavirin combination therapy. Several investigators reported no association of iron overload with the treatment response in patients treated IFN plus ribavirin (134, 135), but the others reported poor response in iron-overloaded patients (136, 137). Recently, we have evaluated the influence of hepatic iron accumulation on treatment response in seventy genotype 1b-infected patients treated with a 24-week course of IFN-α and ribavirin (26). Non-sustained responders represented significantly higher hepatic iron concentration, and multivariate logistic analysis identified hepatic iron score as the only independent variable for resistance to treatment. This result implies that hepatic iron reduction may improve

response to treatment in patients resistant to the IFN plus ribavirin combination therapy. Phlebotomy in combination with IFN monotherapy was reported to improve antiviral efficacy compared with IFN alone (126, 138). But as for IFN/ribavirin therapy, combination with phlebotomy seems to be lead to reduced tolerance, because phlebotomy frequently decreases serum hemoglobin levels (139), which required to ribavirin dose reduction or treatment discontinuation. Desferroxamine or dietary iron restriction, which is also effective for serum ALT reduction in patients with chronic hepatitis C (140), may be effective for reduction of hepatic iron content and may improve response to IFN/ribavirin therapy. Additional studies are warranted to determine whether these combination therapies are effective for chronic hepatitis C.

4. Therapy for iron overload in patients with chronic hepatitis C

Ultimate therapeutic goal in patients with chronic HCV infection is the eradication of HCV from the body. Therapies with IFN-α, with or without ribavirin, are the only approved regimens that are capable to eliminate HCV (141). The success rates of treatment, however, are at best 50%, with the most effective regimen being pegylated IFN-α and ribavirin for most pandemic HCV genotype 1b (141). Furthermore, these therapies carry a substantial risk of serious side effects and in quite a considerable proportion of patients require premature discontinuation because of side effects (132). Given this situation, the development of safe and effective therapies in patients with chronic hepatitis C is our high-priority goad. As mentioned above, considering that these is accumulating evidence regarding iron as a risk factor for liver injury at HCV infection, iron reduction therapy is an attractive and potentially promising therapeutic modality for patients with chronic hepatitis C.

In 1994, Hayashi et al. (16) first reported the biochemical effects of phlebotomy on chronic hepatitis C patients. This effect has subsequently been confirmed by many other investigators (19, 127, 142). Although the initial biochemical effect of phlebotomy was significant, the long term effects of iron removal on biochemical parameters and liver histology had been uncertain. In 2002, Yano et al. (139) reported that serum ALT levels were significantly decreased by initial period and maintained at the lower levels during initial and maintenance phlebotomy than those recorded before treatment, and the histological grading scores were also significantly improved in phlebotomy group during the 5 years. This result suggested that maintenance of the iron-deficient state has beneficial effects in preventing disease

progression in patients with chronic hepatitis C. In 2004, a multicenter, prospective, randomized, controlled trial of phlebotomy for HCV patients also confirmed the effectiveness of iron reduction for improvement of serum ALT levels (79). There were no major side effects of iron reduction by phlebotomy requiring special medical attention during the study period (139). Therefore, iron removal treatment is a safe and alternative therapies that might prevent progression to end-stage liver diseases and hepatic failure without any changes in HCV viral titers. The iron-deficient state induced by phlebotomy increases the rate of dietary iron absorption by decreasing hepatic hepcidin expression, and resulting could again accumulate in the liver and be cytotoxic to hepatocytes. Iwasa et al. (140, 143) reported the effectiveness the dietary iron restriction treatment for improvement of hepatic inflammation and serum ALT levels in HCV patients. It is of great interest that the long term iron reduction by combined phlebotomy and low iron diet brought about not only sustained improvement of biochemical parameters but also normalized hepatic levels of 8-OHdG (121). Further studies will clarify whether iron reduction therapy is effective in minimizing the risk of late-onset HCC during chronic HCV infection.

Considering the therapeutic effects of iron removal on both biochemical and histological parameters, the safety-proved economical procedure should be recommended for chronic hepatitis C patients as an option to IFN. Bonkovsky (144), in a review of other treatment options to IFN in chronic hepatitis C, recommended iron reduction therapy as the first line among various regimens including ursodeoxycholic acid and herbal medicines. Conversely, the supplementation of iron in virtually all commercially prepared foods may have adverse effects on the patients with chronic hepatitis C, and iron content in the food should be taken into consideration. Relatively low levels of hepatic hepcidin expression for the degree of iron burden may be involved in the pathophysiologic mechanism of increased iron overload in patients with chronic hepatitis C, and supplementation of hepcidin may be beneficial for these conditions.

Conclusion

In summary, elevated serum iron values and increased hepatic tissue iron deposition are relatively common and associated with severe hepatic inflammation and fibrosis in patients with chronic hepatitis C. We now know that iron depletion is important as a treatment option for this disease. Finally, since HCV-positive patients are at a high risk of HCC, the possible role of increased liver iron storage in liver carcinogenesis and the effect of iron reduction therapy to reduce the incidence

of HCC in patients with chronic hepatitis C should be investigated. The last decade has seen rapid advances in our knowledge of the regulation of iron metabolism in the body. Thus, a better understanding of the molecular mechanisms for iron overload in chronic HCV infection will likely improve our therapeutic armamentarium for patients with chronic hepatitis C.

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Original Contribution

Hepatic oxidative DNA damage correlates with iron overload in chronic hepatitis C patients

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Abstract

Hepatic oxidative stress occurs in chronic hepatitis C (CH-C), but little is known about its producing mechanisms and precise role in the pathogenesis of the disease. To determine the relevance of hepatic oxidatively generated DNA damage in CH-C, 8-hydroxy-2′-deoxyguanosine (8-OHdG) adducts were quantified in liver biopsy specimens by immunohistochemical staining, and its relationship with clinical, biochemical, and histological parameters, and treatment response was assessed in 40 CH-C patients. Hepatic 8-OHdG counts were significantly correlated with serum transaminase levels ($r = 0.560$, $p = 0.0005$) and histological grading activity ($p = 0.0013$). Remarkably, 8-OHdG levels were also significantly related to body and hepatic iron storage markers (vs serum ferritin, $r = 0.565$, $p = 0.0004$; vs hepatic total iron score, $r = 0.403$, $p = 0.0119$; vs hepatic hepcidin messenger RNA, $r = 0.516$, $p = 0.0013$). Baseline hepatic oxidative stress was more prominent in nonsustained virological responder (non-SVR) than in SVR to interferon (IFN)/ribavirin treatment (50.8 vs 32.7 cells/10^5 μm^2, $p = 0.0086$). After phlebotomy, hepatic 8-OHdG levels were significantly reduced from 53.4 to 21.1 cells/10^5 μm^2 ($p = 0.0125$) with concomitant reductions of serum transaminase and iron-related markers in CH-C patients. In conclusion, this study showed that hepatic oxidatively generated DNA damage frequently occurs and is strongly associated with increased iron deposition and hepatic inflammation in CH-C patients, suggesting that iron overload is an important mediator of hepatic oxidative stress and disease progression in chronic HCV infection.

Keywords: Oxidative stress; Free radicals; Fenton reaction; 8-Hydroxy-2′-deoxyguanosine; Iron; Chronic hepatitis C; Immunohistochemistry; Hepcidin; Interferon/ribavirin; Phlebotomy

Abbreviations: CH-C, chronic hepatitis C; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; SVR, sustained virological responder; IFN, interferon; ROS, reactive oxygen species; HCV, hepatitis C virus; MU, million units; PCR, polymerase chain reaction; NSAIDs, nonsteroidal anti-inflammatory drugs; HIS, hepatic iron score; SIS, sinusoidal iron score; PIS, portal iron score; TIS, total iron score; PBS, phosphate-buffered saline; HPLC-ECD, electrochemical detector coupled to a high-pressure liquid chromatography; mRNA, messenger RNA; cDNA, complementary DNA; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; TNF-α, tumor necrosis factor-α; TGF-β, transforming growth factor-β.

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Introduction

Reactive oxygen species (ROS) have been implicated in a number of pathologies, including inflammatory diseases of the gastrointestinal tract [1], alcoholic liver disease [2], and several other types of toxic liver injury [3]. The strongest species among the many ROS is the highly toxic hydroxyl radical, which has been shown to be responsible for a number of base modifications that include thymine glycol, 5-(hydroxylmethyl)uracil, and also 8-hydroxyguanine [4]. Therefore, 8-hydroxy-2′-deoxyguanosine (8-OHdG), an inducer of a point mutation in daughter DNA strands, is considered a useful marker of oxidatively generated DNA damage in several diseases [5].

Hepatitis C virus (HCV) is a worldwide major causative agent of chronic hepatitis and progressive liver fibrosis leading to cirrhosis and hepatocellular carcinoma [6]. Although the mechanisms underlying HCV-associated liver cell injury are not well understood, there is increasing evidence that oxidative stress may play a pathogenic role in chronic hepatitis C (CH-C). Plasma samples from HCV-infected patients have increased lipid peroxidation products [7], and peripheral blood mononuclear cells from CH-C patients show elevated superoxide dismutase activity [8], which are consistent with an increased cellular ROS formation. Immunohistochemistry has also documented the presence of oxidative stress in liver biopsy specimens from CH-C patients [7,9]. A role of oxidative stress in the pathogenesis of CH-C is also supported by the fact that antioxidant therapy improved liver injury in CH-C patients [10]. Despite this evidence, little is understood about the mechanisms that produce oxidative stress in chronic HCV infection. In vitro and transgenic animal model studies have shown that HCV proteins, such as core, NS3, or NS5A, directly induce oxidative stress [11–15], but it is not clear whether HCV proteins directly induce liver oxidative stress in HCV-infected humans.

Another convincing candidate for the source of oxidative stress is excessive accumulated iron in the body of patients with CH-C, because elevated serum iron and mild to moderate iron overload in the liver are common in patients with CH-C [16,17]. It is known that ferrous iron in the presence of hydrogen peroxide generates hydroxyl radicals through the Fenton reaction. Hepatic iron deposits have been associated with the degree of liver inflammation and damage in HCV-infected liver tissues [18]. Moreover, a close correlation between the amount of iron accumulation and hepatic fibrosis has also been reported in CH-C [19]. In the representative iron-related liver injury disorder, genetic hemochromatosis, it is clearly demonstrated that hepatic iron is responsible for liver damage through ROS formation, leading to lipid peroxidation and alteration of the cellular membrane [20]. Therefore, iron may cause liver tissue injury by increasing the formation of toxic hydroxyl radicals leading to progressive liver inflammation, fibrosis, and increased risk for developing liver cancer in CH-C.

The aim of the present study was to evaluate the relation between the degree of hepatic oxidatively damaged DNA assessed by the level of 8-OHdG and the clinical, biochemical, and histological findings in patients with CH-C. In addition, the influence of hepatic oxidative stress on treatment response to interferon (IFN) plus ribavirin and its improvement by phlebotomy were investigated.

Materials and methods

Patients with chronic hepatitis C

The study included 40 patients with chronic hepatitis C (27 males and 13 females, with a median age of 55 (25–70) years) referred to Mie University Hospital and Mie Central Medical Center between October 1999 through December 2003 (Table 1). We selected patients fulfilling the following inclusion criteria: (1) *Liver injury caused by chronic HCV infection.* All patients were seropositive for both anti-HCV antibody (the third-generation enzyme-linked immunosorbent assay; Ortho Diagnostic Systems, Raritan, NJ) and HCV-RNA (Amplicor-HCV assay; Roche Molecular Diag. Co., Tokyo, Japan), and were seronegative for hepatitis B surface antigen/antibody and anti-human immunodeficiency virus antibody. Patients with other liver diseases (drug-induced, autoimmune, metabolic) were excluded by serological tests and anamnesis. None of the patients received any antiviral or immunomodulatory treatment in the preceding 6 months of the study. (2) *Liver biopsy.* Liver tissue was obtained by percutaneous needle biopsy in all patients for diagnostic purposes. Liver biopsy specimens were divided in two parts. One portion was fixed in buffered formalin and embedded in paraffin, and the other part was immediately frozen and stored at −80°C for RNA extraction. (3) *IFN/ribavirin combination therapy.* Patients were treated with a 24-week course of IFN and ribavirin combination immediately after liver biopsy. Patients infected only with HCV genotype 1b were recruited because the Japanese national health insurance covers the IFN/ribavirin combination treatment only in genotype 1b-infected patients. Six million units (MU) of IFN-α (IFN-α2b;

Table 1
Clinical characteristics of chronic hepatitis C

Characteristics	Chronic hepatitis C (n = 40)
Age (year)	55 (25–70)
Gender (M/F)	27/13
Laboratory data	
ALT (IU/l)	77.5 (19–411)
AST (IU/l)	56.5 (23–565)
Platelet count ($\times 10^4$/mm^3)	15.7 (4.9–26.3)
Serum iron (μg/dl)	134.5 (42.2–334)
Transferrin saturation (%)	37.9 (10.7–80.9)
Serum ferritin (ng/ml)	162.0 (17.5–884)
Serum HCV-RNA (KIU/ml)	826.5 (28.1–2100)
HCV genotype (1a/1b/2a/2b)	0/40/0/0
Liver histology	
Inflammatory activity (0/1/2/3)	0/11/23/6
Fibrosis staging (0/1/2/3/4)	1/9/14/12/4
Total iron score	6 (0–22)
Treatment response (SVR/non-SVR)	12/28

ALT, alanine aminotransferase; AST, aspartate aminotransferase; SVR, sustained virological response.
Data are expressed as median (range).

Intron A, Schering-Plough Pharmaceutical Co., Osaka, Japan) was given daily for 2 weeks, and IFN administration frequency was then reduced to 6 MU three times a week for the remaining 22 weeks of combination therapy. Daily ribavirin (Schering-Plough Pharmaceutical Co.) dosage was 600 mg for patients weighing less than 60 kg, 800 mg for patients between 60 and 80 kg, and 1000 mg for patients with more than 80 kg. Only this dose and medication period for standard IFN and ribavirin combination have been authorized, and pegylated IFN plus ribavirin combination treatment have not been authorized in Japan for the treatment of CH-C. Fifteen patients (37.5%) had previously received a 24- to 48-week course of IFN monotherapy (3–9 years preceding IFN/ribavirin combination therapy). Patients with serum HCV-RNA negative by reverse transcription–polymerase chain reaction (PCR) for 6 months after the completion of therapy were defined as "sustained virological responders (SVR)," and the remaining patients categorized as "non-SVR."

Patients with concurrent diseases or those taking medications capable of interfering with free radical production, such as nonsteroidal anti-inflammatory drugs (NSAIDs), vitamins, and iron-containing drugs were excluded from the study. Patients with chronic alcohol consumption of ethanol in excess of 40 g/week for men and 20 g/week for women for at least 5 years were excluded from the study. There was no patient with severe iron deposition by histological examination that may be considered as genetic hemochromatosis, and all patients had no HFE mutations C282Y or H63D. The following data were collected for each patient at entry: serum biochemical, hematological, and iron-related markers [serum iron, serum transferrin saturation (calculated and expressed as a percentage: serum iron / total iron binding capacity × 100%), and serum ferritin] and liver histological findings.

Five patients that did not respond to IFN/ribavirin underwent phlebotomy. Phlebotomy (200 or 400 ml) was performed biweekly or monthly. When the serum ferritin levels reached 10 ng/ml, which is recognized as the level indicating a subclinical iron-deficient state, the initial period of phlebotomy was considered as complete. If the serum ferritin level rebounds, maintenance phlebotomy was performed as needed to maintain the serum ferritin level below 20 ng/ml.

Informed consent was obtained from each patient and the study was approved by the Ethical Committee of Mie University. The study was carried out according to the ethical guidelines of the 1975 Declaration of Helsinki.

Histological evaluation

All of the liver biopsy samples were fixed in 10% buffered formalin and embedded in paraffin for routine histological examination. Slides were stained with hematoxylin-eosin and Masson's trichrome, and were graded for the degree of necroinflammatory activity and staged for the extent of fibrosis using the criteria of Desmet et al. [21]. Liver histological evaluation was done by two independent pathologists without knowledge of the patients' backgrounds and clinical conditions. A grading system was applied to liver samples stained with Perls' Prussian blue to assess iron deposition. The histological quantification of hepatic iron was done according to Deugnier et al. [22] by scoring iron separately within hepatocytes [hepatic iron score (HIS), 0 to 36], sinusoidal cells [sinusoidal iron score (SIS), 0 to 12], and portal tracts or fibrotic tissue [portal iron score (PIS), 0 to 12]. The total iron score (TIS, 0 to 60) was defined by the sum of these scores. This score has been shown to highly correlate with the biochemical hepatic iron index and hepatic iron concentration as measured by the atomic absorption spectrophotometry in patients with chronic liver diseases [18,23,24].

Immunohistochemical study

Hepatic immunohistochemical staining of 8-OHdG was performed as previously reported [25]. Briefly, formalin-fixed and paraffin-embedded sections of liver tissue (5 μm thick) were deparaffinized with xylene and rehydrated through graded ethanol concentrations. After washing three times with phosphate buffered saline (PBS), the tissue sections were treated with 0.5% skim milk in PBS for 30 min to block nonspecific bindings. Subsequently, sections were treated with mouse monoclonal antibody against 8-OHdG (5 μg/ml, Japanese Aging Control Institute, Shizuoka, Japan) for 2 days at room temperature, and then treated with Alexa 488-labeled goat antibody against mouse IgG (1:400 diluted in PBS, Molecular Probes, Eugene, OR) for 5 h. Immunofluorescence was scanned under invert laser scan microscope (LSM 410, Zeiss, Gottingen, Germany), and observation was done using three nonoverlapping microscopic fields containing portal triads under magnification of ×400. Mean total counted hepatocyte number was 139 (106–179) cells/microscopic field in hematoxylin and eosin staining. The degree of immunoreactivity was estimated by counting the number of stained hepatocyte nuclei using Adobe Photoshop version 5.5 and NIH Image free software (Vers. 1.62, National Institute of Health, image program) [25]. The brightness and contrast of each image file were uniformly enhanced using Adobe Photoshop. Image files in Tagged Image File Format were opened in gray scale mode using NIH image software. The hepatocyte nuclei were differentiated from the nuclei of other cells using pixel range of "Measure" command. Cell number was determined using the "Analyze Particles" command. The average of these counts was taken in each specimen.

The specificity of the monoclonal antibody of 8-OHdG was confirmed by (i) comparison with adjacent sections in which the primary antibody was omitted, or (ii) using normal mouse serum instead of the primary antibody, or (iii) absorption with purified 8-OHdG (Sigma, Tokyo, Japan) or guanosine (Sigma), or (iv) RNA digestion. The primary antibody was incubated for 5 h at room temperature in serial dilutions of purified 8-OHdG or guanosine in PBS from 2.5 mg/ml to 2.5 ng/ml and applied to the sections. RNA digestion was performed before the immunostaining procedures in PBS containing DNase-free RNase 5 μg/μl for 1 h at 37°C.

Electrochemical measurements of hepatic 8-OHdG levels

In several liver samples sufficient for DNA extraction, 8-OHdG levels in the liver DNA were also measured by an

electrochemical detector coupled to high-pressure liquid chromatography (HPLC-ECD), as described previously [26]. To prevent oxidation by air exposure, all solutions and instruments that came in contact with the tissue specimen were filled with argon gas. High molecular DNA was purified from each 5 mm of fresh liver biopsy specimen using a DNeasy Tissue Kit (Qiagen GmbH, Hilden, Germany). Each extracted DNA sample was treated with nuclease P1 and alkaline phosphatase and filtrated through an Ultra Free Centrifugal Filter (Millipore Co., Bedford, MA, USA), and 10 μg of DNA was injected into the HPLC apparatus (Simadzu, Kyoto, Japan). The 8-OHdG in the digested DNA was detected by using an electrochemical detector (ECD-300, Eicom Co., Kyoto, Japan). The amount of dG was calculated from the absorbance at 290 nm, and the 8-OHdG levels were represented as the number of 8-OHdG per 10^5 dG in DNA.

Hepcidin messenger RNA (mRNA) quantification in liver biopsy samples

mRNA was extracted from liver biopsy samples using the SV RNA Isolation System (Promega corporation, Madison, WI) according to the manufacturer's instructions. The complementary DNA (cDNA) was generated by reverse transcription of 2 μg-adjusted RNA, with random hexamers and Moloney murine leukemia virus reverse transcriptase (Applied Biosystems, Tokyo, Japan) for 30 min at 48°C. Hepcidin mRNA was determined by TaqMan real-time detection-PCR assay. Primers and probes were designed using the Primer Express Software package (Applied Biosystems), which spanned an intron to avoid coamplification of genomic DNA. The sequences were as follows: forward primer 5′-TTCCCCATCTGCATTTTCTG-3′, reverse primer 5′-TCTACGTCTTGCAGCACATCC-3′, and FAM/TAMURA probe 5′-TGCGGCTGCTGTCATCGATCAA-3′. Five microliters of cDNA was incubated with 20.75 μl of TaqMan Master Mix (Perkin Elmer, Yokohama. Japan), 10 μM forward primer, 10 μM reverse primer, and 5 μM probe. The amplification was performed using the ABIPRISM 7700 Sequence Detection System (Applied Biosystems). Amplification conditions were 2 min at 50°C and 10 min at 95°C, followed by 53 cycles of 15 s at 95°C, and then 1 min at 60°C. Data were analyzed using the Sequence Detector 1.6 software (Applied Biosystems). The results for hepcidin mRNA are expressed as the amount relative to that of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA quantified simultaneously in each liver sample. Experiments were performed in triplicates, and the mean values were calculated.

Statistical analysis

Results were expressed as median with ranges. Comparisons between groups were made using the Mann-Whitney *U* test, paired or unpaired *t* test, or one-way factorial ANOVA and multiple-comparison test for continuous variables, and the χ^2 or Fisher's exact-probability test for categorical data. Correlation coefficients between numerical variables were calculated as Spearman's rho corrected for ties. All tests were two-tailed, and *p* values less than 0.05 were considered as statistically significant. Statistical analysis was performed using the commercially available software StatView (SAS Institute, Inc., NC).

Results

Clinical characteristics of the patients with CH-C

Table 1 shows the baseline clinical characteristics of the study group. The patients consisted of 27 males and 13 females ranging from 25 to 70 years old (median age 55 years). Serum iron overload status was found in many CH-C patients. Among the 40 cases, 14 patients (35.0%) had elevated serum iron values (>170 μg/dl for male and >120 μg/dl for female), 11 (27.5%) elevated transferrin saturation (>45%), and 19 (47.5%) elevated serum ferritin values (>220 ng/ml for male and >100 ng/ml for female). Mild iron deposition in liver tissue (median TIS was 6) was found in the majority of CH-C patients and no hepatic iron deposition (TIS = 0) was seen only in 4 cases.

In situ liver detection of 8-OHdG in CH-C patients

In the liver of CH-C patients before treatment, 8-OHdG immunoreactivity was strongly observed in the nuclei (and weekly in the cytoplasm) of hepatocytes, Kupffer cells, and infiltrated inflammatory cells (Figs. 1A and 1B). 8-OHdG-immunoreactive cells were distributed throughout the whole acinus in liver of CH-C patients. In the liver tissue from CH-C patients, the number of 8-OHdG-positive hepatocytes were counted from 11 to 142 cells/10^5 μm^2, the median being 43.0 cells/10^5 μm^2. In the liver of control (simple fatty liver), immunoreactivity of 8-OHdG was slightly observed in the nuclei of hepatocytes (Fig. 1C).

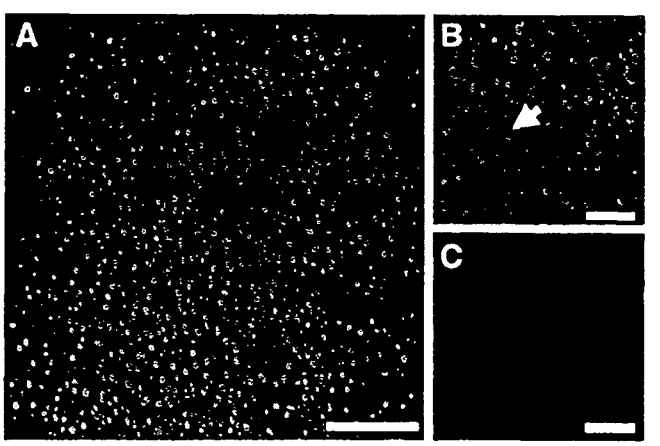

Fig. 1. 8-Hydroxy-2′-deoxyguanosine (8-OHdG) immunohistochemical staining in liver tissue from chronic hepatitis C (CH-C) and control (simple fatty liver) patients. In the liver of CH-C patient [(A) at low-powered and (B) at high-powered magnifications], 8-OHdG immunoreactivity was strongly observed throughout the whole acinus and mainly in the nuclei of hepatocytes and Kupffer cells [arrow in (B)]. In the liver of control [simple fatty liver, (C)], immunoreactivity of 8-OHdG was weak in the nuclei of hepatocytes. (A) Scale bar 200 μm. (B and C) Scale bar 50 μm.