Abstract. Hepatitis C is the commonest form of chronic viral hepatitis in most western countries. A significant proportion of patients develop cirrhosis, hepatic failure and hepatocellular carcinoma. The results of controlled trials have shown that interferon α is an effective treatment for hepatitis C. Treatment results in normalization of elevated transaminase levels in up to 50% of patients, although only 15–25% of patients have a sustained response. Recent studies have shown that iron influences the response of chronic hepatitis C to treatment and the natural history of hepatitis C. The mechanisms responsible for the effects of iron are not clear but emerging data suggest that the cellular location of iron within the liver lobule and the subsequent effects on immune function are likely to be critical determinants for these effects. It is likely that therapies for chronic hepatitis C which either remove iron or interfere with the action of iron at the cellular level may not only prove useful clinically but may also elucidate further the mechanisms of cellular injury in this disease. (Keio J Med 48 (3): 124–131, September 1999)

Key words: hepatitis C virus, interferon, iron metabolism, ferritin

Introduction

Hepatitis C has emerged as the commonest form of chronic viral hepatitis in most developed countries and predisposes patients to the development of cirrhosis, hepatic failure and hepatocellular carcinoma. Clinical trials have shown that interferon α is an effective treatment for hepatitis C. Treatment results in normalization of elevated transaminase levels in up to 50% of patients, although only 15–25% of patients have a sustained response. Furthermore, interferon α is costly with well-documented side effects. Thus, there is a need to identify alternative or complementary therapies which increase the proportion of patients who have a sustained response. Well characterized pretreatment characteristics of response include age, sex, duration of infection, mode of acquisition, liver histology, hepatitis C virus (HCV) RNA levels and genotype. More recently, considerable interest has been generated regarding the role of iron in the pathogenesis of chronic hepatitis C and the role of de-ironing therapies as an adjunctive treatment for chronic liver disease which results from HCV infection. Whilst iron is an essential element for the survival of cells, excess amounts can result in tissue injury. It is now apparent that iron can also modulate disease states and cellular function at levels much below those observed in classical iron overload. The aim of this article is to review the current state of knowledge pertaining to the role of iron in chronic hepatitis C.

Serum and Hepatic Iron Studies in Hepatitis C

The study of serum and hepatic iron parameters in chronic liver disease is readily achieved through the use of several standard methods. Serum transferrin saturation and ferritin levels, whilst useful in the assessment of iron overload in conditions such as hereditary hemochromatosis, are not as useful in the determination of iron status in chronic inflammatory liver diseases due to the effect of inflammation and pro-inflammatory mediators on serum iron levels and hepatic ferritin synthesis. The gold standard for defining hepatic iron content is biochemical measurement of the non-heme hepatic iron concentration (HIC). The HIC can be determined from fresh or paraffin embedded
tissue using colorimetric or atomic absorption spectrophotometry based methods. A semi-quantitative grading of iron deposition and cellular distribution can be accomplished using histological assessment of sections stained for iron using Perls' Prussian blue method.

It has long been known that serum and non-heme hepatic iron parameters can be increased in chronic liver diseases of diverse aetiologies excluding hereditary haemochromatosis and other secondary iron overload disorders. Blumberg and colleagues described abnormal iron studies in patients with hepatitis B. Interest in the role of iron in chronic hepatitis C commenced in 1992 when Di Bisceglie et al. noted that up to 36% of patients with chronic hepatitis had elevated serum iron parameters, but only 5% had elevated HICs in the order generally seen in haemochromatosis. Similar observations have also subsequently been reported by other groups. Following this Van Thiel et al. reported that in a group of patients with chronic viral hepatitis of varying aetiologies, the HIC of responders was less than that of non-responders and that HIC predicted response to interferon. Olynyk et al. studied the effect of HIC on response to interferon therapy of chronic hepatitis C. This study demonstrated that the HIC was higher in non-responders to IFN therapy compared with responders. More specifically, an HIC > 1100 µg/g predicted non-response in nearly 90% of patients (Fig. 1). In contrast, HICs ranging up to 700 µg/g were also frequently seen in responders. In keeping with the biochemical measurements of HIC, more patients demonstrated low-grade stainable hepatic iron in the non-responder group. Additionally, the HIC was similar in cirrhotic and non-cirrhotic patients, suggesting that hepatic iron was not related to histological severity of disease. Serum ferritin concentrations were significantly higher in the 'high' iron non-responder group than in the 'low' iron non-responder and the responder groups. However, the marked overlap of ferritin concentrations between these groups precluded using an increased serum ferritin concentration to predict response (Fig. 2). The overlap in ferritin concentrations may be due to the acute-phase reactant properties of ferritin in the setting of chronic inflammatory liver disease. The responders and non-responders had similar HCV RNA levels (Fig. 3). There were no significant relationships between HCV RNA levels and the HIC, the presence of an elevated serum ferritin levels, or the alanine aminotransferase (ALT) level. In the last 4 years many additional studies have been published regarding the role of iron in chronic hepatitis C. Most have confirmed that increased serum and/or hepatic iron parameters are associated with a lower likelihood of response to interferon therapy.

Several studies have indicated that the distribution of iron within the liver lobule and the cell type effected by the iron may be important in determining the effect which iron has on chronic hepatitis C. Iron deposition within zone 1, portal tracts and sinusoidal lining cells is associated with a higher likelihood of non-response to interferon therapy. In our study, we noted that 18 of 24 responders had no stainable iron in hepatocytes or Kupffer cells. The remaining 6 responders had grade I stainable iron distributed equally between hepatocytes and Kupffer cells. However, 19 of 34 non-responders showed grade I stainable iron distributed equally between hepatocytes and Kupffer cells. Banner et al. conducted a study of the frequency with which stainable iron occurred in sections of liver biopsies from patients with chronic hepatitis C. These investigators noted that non-responders to treatment had greater accumulation of iron in the sinusoids and portal tracts. Ikura et al. found that the presence and degree of portal iron deposition correlated inversely with the response to interferon treatment. The presence of stainable iron has been shown to correlate with inflamma-

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### Fig. 1 Hepatic iron concentration in responders and non-responders to interferon therapy in chronic hepatitis C. The shaded region represents HICs of <1100 µg/g. *P < 0.05, non-responders vs responders. (Reproduced from 24).
tion and fibrosis in chronic hepatitis C, suggesting that the iron came from damaged hepatocytes. In contrast, the absence of stainable iron is associated with a higher likelihood of response. Other groups have suggested that iron may be a more significant factor in certain genotypes, in particular genotype 1b. In a study by D’Alba et al., patients with chronic hepatitis C and genotype 1b had higher hepatic iron concentrations compared with other genotypes. Genotype and hepatic iron concentration remained predictive factors of nonresponsiveness on multivariate analysis.

**HFE Mutations and Hepatitis C**

The recent discovery of the *HFE* gene containing two missense mutations (C282Y and H63D) which are strongly associated with disordered iron metabolism raises the possibility that abnormal *HFE* genotypes could contribute to iron-related cell injury in chronic hepatitis C. Recent studies have analysed the relationship of *HFE* mutations and iron overload in chronic hepatitis C. Patients with hepatitis C have frequencies of *HFE* mutations that are no different than the general population. However, heterozygosity for the C282Y mutation is often associated with increased iron stores and with more advanced liver fibrosis. There is a much stronger association between *HFE* gene mutations, abnormal iron status and HCV infection in patients with porphyria cutanea tarda. Thus in this group of patients it is possible that iron could play a more significant role in the pathogenesis of hepatitis C-related liver injury, but this remains to be confirmed in prospective studies.

**Pathophysiology of Iron Toxicity in Hepatitis C**

The mechanisms by which iron may cause liver disease have been recently reviewed. The concept that iron can act in a synergistic fashion with other hepato-
toxins has been previously described. Iron has been shown to be a synergistic factor in the pathogenesis of alcohol and carbon tetrachloride induced liver diseases. It is generally accepted that iron increases the formation of reactive oxygen intermediates which can result in lipid peroxidation which can result in oxidative damage to proteins and nucleic acids. This can result in organelle dysfunction, fibrosis and eventually hepatocellular carcinoma. Whilst these findings were initially based on studies in iron overload, lipid peroxidation products have been shown in the plasma and liver biopsies of patients with chronic hepatitis C. Farinati et al. studied whether HCV may have a direct cytopathic effect on hepatocytes through the occurrence of iron-dependent lipid peroxidation. Patients with chronic hepatitis C had significantly greater lobular inflammation, steatosis, serum ferritin levels and liver iron accumulation in chronic hepatitis C. This may be related to a specific effect of the virus on parenchymal or non-parenchymal cell function. In liver tissue, lipid peroxidation products are mainly observed in portal tract macrophages. Lipid peroxidation products have been shown to stimulate collagen production in activated hepatic stellate cells and cultured human fibroblasts. Alternatively, lipid peroxidation products may increase production of transforming growth factor (TGF)-β or other profibrogenic substances by Kupffer cells which might then stimulate hepatic stellate cell activation.

It is well known that the risk for development of hepatocellular carcinoma is substantially increased in both hereditary hemochromatosis and chronic hepatitis C. The mechanisms responsible for the development of hepatocellular carcinoma in chronic liver disease are not clear but several potential mechanisms exist. Chronic infection with HCV may be directly oncogenic. Alternatively, HCV-induced chronic liver injury may culminate in cirrhosis and an increased risk for hepatocellular carcinoma. As cirrhosis develops, hepatocyte necrosis is followed by an attempted secondary proliferative response of mature hepatocytes. However, this proliferative response is often impaired in chronic liver disease. An alternative mechanism for hepatocyte regeneration in chronic liver disease involves stem cell proliferation and differentiation into hepatocytes. In humans, oval cells have been reported in hepatitis B-associated hepatocellular carcinoma and chronic liver disease associated with ductular proliferation. We have recently shown that oval cells are present in patients with hereditary hemochromatosis and chronic hepatitis C. Furthermore, oval cell numbers increase significantly with progression of disease severity in each of the groups studied, suggesting that oval cell proliferation is not disease specific but occurs in response to progressive liver injury and fibrosis. The association between severity of liver disease and increasing number of oval cells is consistent with the hypothesis that oval cell proliferation is associated with the increased risk for development of hepatocellular carcinoma with advancing liver disease, particularly when cirrhosis is present. Finally, iron could contribute to the increased risk of hepatocellular carcinoma in chronic hepatitis C through DNA damage from iron-induced adduct formation and chromosomal damage.

The pathophysiological mechanisms whereby iron exerts its effects in chronic hepatitis C are unknown. Much evidence has accumulated supporting an immunopathological mechanism which underlies liver injury in chronic hepatitis C. Virus specific T cells are present in the liver tissue and peripheral blood of patients with HCV infection and are able to contribute to hepatocellular injury, but are not able to eliminate viral infection. Previous studies have shown that persistent hepatitis B virus infection is associated with iron overload. It is also known that patients with iron overload are more susceptible to bacterial infections. Iron has been shown to impair antigen-specific immune responses and generation of cytotoxic T-cells, decrease functional T-helper precursor cells, and enhance T-suppressor activity. Ferritin molecules, particularly those rich in heavy (H) subunits, bind to activated T-cells and H-ferritin receptors are expressed by T-cell lines. These data suggest that iron could impair host lymphocyte-dependent clearance of HCV virus. Interferon α possesses multiple actions including direct antiviral effects and enzyme modulation. The actions of IFN are not known to be dependent on intracellular iron although it is possible that iron might also interfere in some way with these actions resulting in a reduced antiviral activity. It has been suggested that transferrin and non-transferrin-bound iron-uptake pathways may be affected in necro-inflammatory conditions. As a result, non-responders might have increased iron uptake and hepatic iron deposition when compared with responders. Increased hepatic iron deposition in hepatitis C may then result in increased oxidative stress in the liver, decreased glutathione levels and lipid peroxidation and formation of malondialdehyde adducts. The type of storage molecule from which iron is released could modulate these effects. It is well known that ferritin and hemosiderin release iron to different degrees, a property which may...
influence the ability of iron to participate in biological reactions.\(^{94}\)

It is possible that iron deposition in sinusoidal cells, especially Kupffer cells, could alter the immune responsiveness of macrophages. This hypothesis is supported by observations that iron deposition within zone 1, portal tracts and sinusoidal lining cells is associated with a higher likelihood of non-response to interferon therapy.\(^{27-34}\) There are reports of impaired phagocytic function by monocytes in hereditary hemochromatosis.\(^{95,96}\) Intracellular killing of microorganisms may also be impaired by iron overload.\(^{96,97}\) Interleukin 2 production by cytotoxic T-cells is reduced in the presence of iron overload.\(^{83}\) We have studied the effect of chronic iron overload on Kupffer cell cytokine production.\(^{98}\) Kupffer cells from iron loaded animal exhibit reduced proinflammatory cytokine production compared with Kupffer cells from control animals. Thus iron loading may impair immune clearance mechanisms via impaired macrophage function or interfere with the actions of interferon \(\alpha\) on macrophage function.

### Iron Status and Likelihood of Response to Interferon Therapy

Following the reports of the relationship between iron status and likelihood of response, investigators began evaluating the possibility that patients might benefit by being depleted of iron by repeated therapeutic phlebotomy before treatment with interferon or to improved response rates in previous non-responders. Hayashi et al.\(^ {34}\) reported that iron reduction alone led to the normalization of serum ALT levels in 5 of 10 patients with chronic hepatitis C. Four to 13 phlebotomies, with removal of 1–3 g of iron, over 2–9 months were required to achieve iron removal as judged by serum ferritin levels less than 10 ng/ml. Seven patients underwent repeat biopsy within 2 months of iron depletion, with no apparent change in the severity of portal fibrosis or inflammation. In another study of 8 patients with chronic hepatitis C who had previously failed to respond to treatment with interferon \(\alpha\), serum ALT levels fell in 7 of 8 following iron reduction.\(^ {38}\) Van Thiel et al.\(^ {99}\) randomized 30 non-responders to iron depletion followed by interferon \(\alpha\) or interferon \(\alpha\) alone. Twelve of 15 (80%) of patients treated with iron depletion and interferon had a virological response at 6 months compared with 6/15 (40%) in the interferon-alone group. Significantly higher sustained virological response rates were seen in the iron depleted group (60%) compared with interferon-alone group (13%). Iron chelation with desferoxamine has also been shown to improve response to interferon therapy.\(^ {100}\) However, there have been no clear effects of iron reduction on levels of HCV RNA in serum.\(^ {100-102}\)

Fong et al.\(^ {43}\) recently conducted a randomized study which evaluated the effect of iron depletion on aminotransferase activity, HCV RNA levels and response to interferon \(\alpha\) therapy in patients with chronic hepatitis C. Serum ALT levels decreased in 15 of 17 patients after phlebotomy. Changes in iron indices and ALT levels were not accompanied by changes in HCV RNA levels. At the end of 24 weeks of interferon therapy, similar numbers of phlebotomized patients (7 of 17) had a response compared to control patients (6 of 21). However after 6 months of follow up, 5 of 17 phlebotomized patients remained HCV RNA negative compared with 1 of 21 controls \((p = 0.07)\). Tsai et al.\(^ {103}\) have also shown that phlebotomy therapy may result in a sustained response in up to 15% of patients who have previously not responded to treatment with interferon but who are retreated following phlebotomy therapy.

Boucher et al.\(^ {27}\) provided additional information on the possible relationships between hepatic iron metabolism and chronic hepatitis C. In their study, 55 patients were treated with interferon for six months and the HIC and distribution of iron were evaluated before and after therapy. They found no difference in HIC between non-responders and responders. However, they did identify a relation between HIC and inflammatory activity such that the iron load was higher in those patients with the greatest degree of histological inflammatory activity. Interestingly, HIC decreased following treatment with interferon. This was related to iron depleted from sinusoidal cells and was apparent regardless of whether patients responded to interferon therapy or not. These findings suggest that increased iron stores may be present in patients with chronic hepatitis C predominantly as a result of the degree of inflammatory activity, presumably correlating with cell injury or necrosis. with subsequent phagocytosis by Kupffer cells resulting in progressive increases in Kupffer cell iron loading.

In summary, iron influences the response of chronic hepatitis C to treatment and perhaps the natural history of hepatitis C. The mechanisms responsible for the effects of iron are not clear but emerging data suggest that the cellular location of iron within the liver lobule and the subsequent effects on immune function are likely to be critical determinants for these effects. Continued evaluation of therapies for chronic hepatitis C which either remove iron or interfere with the action of iron at the cellular level may not only prove useful clinically but may also elucidate further the mechanisms of cellular injury in this disease.

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