— **Minireview** —

# Iron Cytotoxicity in Chronic Hepatitis C

**Hisao Hayashi**[*,a] **and Motoyoshi Yano**[b]

[a]*Department of Medicine, Faculty of Pharmaceutical Sciences, Hokuriku University, Ho-3 Kanagawa-machi, Kanazawa 920–1181, Japan and* [b]*Third Department of Internal Medicine, Nagoya University School of Medicine, Tsuruma-cho, Nagoya 466–8550, Japan*

(Received January 7, 2002)

Iron-induced cytotoxicity in chronic hepatitis C was described in detail based on effectual iron reduction therapy. Without treatment, chronic hepatitis C may progress to cirrhosis and develop hepatocellular carcinoma. The first choice treatment for the disease is interferon. However, the cost-benefit of interferon treatments is so poor that options other than interferon are needed for poor responders. A minimum requirement for managing these patients is good control of disease activity shown by maintaining a low serum level of alanine aminotransferase activity. An index accepted widely is 80 IU/l or less of the enzyme activity. When patients were treated with repeated phlebotomy to remove body iron stores, the mean serum levels of enzyme activities changed from 128 to 63 IU/l. Maintaining patients at an iron deficient state for 5 years or more did not change the staging of liver histology, in contrast with the advanced liver histology in the control patients. Iron generates hydroxyl radicals that mediate peroxidation of membrane lipids. After the proposal of our empirical treatment based on the ultrastructural study of hepatic iron stores, evidence has been accumulated that oxidative stress *via* lipid peroxides plays an important role in the pathogenesis of chronic hepatitis C. The treatment is not yet authorized in Japan, but is approved in the United States as the first of the options to interferon. Considering the therapeutic effects of iron removal on both biochemical and histological parameters, the safety-proved economical procedure might be recommended for patients as an option to interferon.

**Key words** —— iron toxicity, phlebotomy, hepatitis C

## INTRODUCTION

Iron plays an important role in biology. Body growth may be impaired and mental retardation may occur if the iron supply is inadequate before the late teens. Iron demand after adolescence differs from a minimum for males to a maximum for pregnant females. In males, iron balance should be zero where iron absorbed in the intestine merely compensates for the iron loss from the gut mucosa and skin sequestration. Though iron absorption is regulated in the intestine, most males have a positive balance so that iron begins to accumulate in the liver as storage iron.

The transition property of iron from ferrous ion to ferric ion and vice versa is essential for energy generation in mitochondria. Because the free radicals generated by transition element iron have high potential, all cell organelles are protected by a variety of anti-oxidative systems. Under pathological conditions, however, potential iron, even though small in amount, generates free radicals, resulting in peroxidation of membranous organelles known as oxidative stress.[1] Iron-induced oxidative stress may be negligible in healthy subjects, but can be a trigger of organ damage in some sensitive hosts.

Hepatitis C virus (HCV) infection causes chronic hepatitis C (CHC) in the majority of individuals who are infected, and may result in hepatic cirrhosis and hepatocellular carcinoma. The optimal results of CHC treatments are the elimination of HCV-RNA and cessation of the disease state. However, the sustained elimination rate of HCV-RNA by interferon (IFN) therapy is far from perfect in patients with CHC.[2] New antiviral treatments for CHC include a combination of IFN with ribavirin. Recently, it was reported that pegylated interferon alpha-2a improves the rate of complete response in CHC.[3,4] However, the elimination rates of HCV-RNA by these treatments are less than 50%. More than half of all pa-

*To whom correspondence should be addressed: Department of Medicine, Faculty of Pharmaceutical Sciences, Hokuriku University, Ho-3 Kanagawa-machi, Kanazawa 920–1181, Japan. Tel. & Fax: +81-76-229-6203; E-mail: h-hayashi@hokuriku-u.ac.jp.

tients fail to respond to these treatments and require additional treatment.[5)]

The therapeutic effects of iron reduction therapy on CHC,[6,7)] which we first empirically introduced based on lysosomal iron stores detected under an X-ray microanalyzer,[8)] suggested that most patients are exposed to iron-induced oxidative stress. This is particularly important for male patients with a positive balance of iron. When patients have an active form of CHC, iron reduction therapy might be recommended as an option to the antiviral agent.[9)]

**Iron Load in the Liver of CHC Patients**

A histochemical study on liver specimens is a standard technique for detecting hepatic iron overload. Some patients with chronic hepatitis, regardless of etiology, were associated with iron overload.[10)] After the identification of HCV, we found that most patients with CHC were complicated with hepatic iron deposits. Microanalysis of the biopsy specimens demonstrated that almost all patients with CHC had at least a lysosomal iron load in the liver, even though histochemical study was negative.[8)] For example, lysosomal iron deposits were found in the pre-treatment liver of a patient with a serum ferritin level as low as 13 ng/ml. Immediately after complete removal of the body iron store, as judged by the serum ferritin levels of 10 ng/ml or less, most hepatocytes still had iron deposits in their lysosomes. The complete disappearance of such iron required an iron deficient period of a few months. Thus, there is little difference between two markers of the iron store, a biochemical standard of serum ferritin concentration and ultrastructural index of lysosomal iron deposits, and it is most likely that a patient with a serum ferritin level higher than 10 ng/ml has potential iron in the liver to generate free radicals.[11)] In a study of male blood donors with anti-HCV antibody, serum ferritin levels were correlated with serum alanine aminotransferase (ALT) activity, suggesting that iron not only accumulates in the liver but also induces liver damage.[12)] It is unclear where the hepatotoxic iron comes from in CHC patients. The body iron store estimated during iron reduction therapy was not increased in these patients and iron absorption from their gut was similar to HCV-free iron-deficient subjects.[13)] Thus, it appears likely that hepatic iron is derived from other organs inside the body, due to active inflammation in the liver.

**Short-term Effect of Iron Reduction Therapy**

A minimum requirement for options to IFN is good control of disease activity, because CHC progresses to liver cirrhosis and is highly complicated by hepatocellular carcinoma. The lower the serum ALT levels obtained by a treatment, therefore, the better is treatment. An index accepted widely for controlling the serum ALT levels is 80 IU/l or less.[14)]

In the first trial,[6)] 10 patients with CHC and excess hepatic iron were treated by initial phlebotomy to remove body iron to a deficient state. Phlebotomy was performed bi-weekly with 200 or 400 ml of blood removed depending on the patient's response. When the serum ferritin levels reached 10 ng/ml or less, the initial period of phlebotomy was considered complete. If the serum level rebounded, maintenance phlebotomy was performed repeatedly as long as the hemoglobin levels remained at 10.0 g/dl or greater. A total blood volume of 800–3600 ml was removed by 4–13 phlebotomies performed over a period of 2–9 months. The serum ALT levels decreased during treatment in all patients. The treatment reduced the mean serum levels of ALT from $152 \pm 49$ to $55 \pm 32$ IU/l ($p < 0.01$; *t*-test); this level became normal in 5 of 10 patients. Serum ferritin levels fell from $242 \pm 129$ to $9 \pm 2$ ng/ml ($p < 0.01$). The concentrations of hemoglobin decreased from $14.3 \pm 1.2$ to $11.3 \pm 1.6$ g/dl ($p < 0.01$), but there were no adverse effects attributable to the mild anemia.

In the second trial,[7)] 7 of 40 patients studied were negative for histochemical iron in the liver. All patients, however, had some iron-overload in the liver. Pre-treated liver biopsy specimens, when available, were analyzed under an electron microscope equipped with X-ray analyzer. Iron deposits were found even in the hepatocyte lysosomes of a patient with a serum ferritin level as low as 13 ng/ml. A mean blood volume of $2400 \pm 1100$ ml was removed by $8 \pm 4$ phlebotomies performed over a period of $5 \pm 3$ months. The results obtained were similar to the first trial. Serum levels of ALT activity decreased during treatment in all but one patient. The treatment reduced the mean serum levels of ALT from $128 \pm 74$ to $63 \pm 28$ IU/l ($p < 0.01$; *t*-test), with a mean reduction of $65 \pm 56$ IU/l. A high correlation was noted between the baseline levels of serum ALT activity and their reductions after treatment. These results suggest that the disease activity was higher; therefore, the treatment effect was greater. This short-term effect was independent of that of the

hepatoprotective agents used in practice such as ursodeoxycholic acid and herbal medicines. Therefore, the combination of phlebotomy with ursodeoxycholic acid had an additive effect on the biochemical parameters.[15]

**Long-term Effect of Iron Reduction Therapy[16]**

Thirteen patients with biopsy-proven CHC were treated by initial phlebotomy to reduce the serum ferritin levels to 10 ng/ml or less. Then, they were followed for more than 5 years and, if needed, given maintenance phlebotomy to maintain the iron-depleted state. A second liver biopsy was performed to evaluate histological changes after the 5-year study period. The liver histology was evaluated according to the classification of Desmet *et al*.[17] Nineteen patients with CHC who were virological non-responders to IFN and had undergone two liver biopsies with an interval of 4–5 years between the pre- and post-IFN periods were selected as the control. Patient backgrounds, pre-treatment laboratory findings and liver histology (grading and staging) were evaluated in the two groups, and no significant differences were found in any of the three areas. Serum ALT levels were decreased significantly by the initial phlebotomy and were maintained at the same levels throughout the study period ($p < 0.05$; *t*-test). The grading scores were decreased significantly in the study group ($2.3 \pm 0.5$ *vs*. $1.7 \pm 0.5$, $p < 0.05$) and were unchanged in the control group ($1.7 \pm 0.6$ *vs*. $2.0 \pm 0.4$). The staging scores remained unchanged in the study group ($1.8 \pm 0.9$ *vs*. $2.0 \pm 1.1$) but progressed in the control group ($1.9 \pm 1.0$ *vs*. $2.8 \pm 1.0$, $p < 0.01$); there was a significant difference between the two groups in progression of the staging score ($p < 0.05$; chi-square test). There were no major side effects of iron reduction therapy requiring special medical attention during the study period. These findings suggest that the long-term treatment improves the disease activity index and suppresses progressive liver fibrosis. Considering that CHC is highly predisposed to hepatic cirrhosis and a late complication of hepatocellular carcimona, the second stage of maintaining the iron deficient state is more important as an option to IFN.

**Oxidative Stress in CHC**

Trace metals of iron and copper facilitate the task for organisms to utilize oxygen made possible by the oxidation-reduction reactions. When anti-oxidative systems do not work properly, the potential ions induce free radicals, resulting in a variety of membrane damage and nuclear injury. Since our proposal of iron reduction therapy,[6] iron-induced oxidative stress *via* radical generation has been reported to be one of the factors involved in the pathogenesis of CHC. Patients with CHC showed increased hepatic levels of malondialdehyde (MDA) and reduced levels of glutathione.[18] A subsequent study showed that reactive oxygen species were associated with the disease activity of CHC.[19] In another study,[20] two major aldehyde metabolites of lipid peroxidation, MDA and 4-hydroxy-2-nonenal (HNE), were measured in CHC liver specimens. The histochemical detection rates of their adducts were 77 and 30%, respectively. The MDA index was correlated with both fibrosis score and the grade of activity. Kageyama *et al*.[21] determined HNE adducts in paired liver specimens of pre- and post-IFN treatment. HNE adducts disappeared from the livers of responders similar to histochemical iron, while both histochemical parameters remained positive in the livers of poor responders. They concluded that hepatic lipid peroxidation and iron may potentially play contributory roles in the pathogenesis of CHC. Thioredoxin is also an indicator of oxidative stress in various diseases. Sumida *et al*.[22] measured serum thioredoxin levels in various chronic liver diseases and showed a high correlation between thioredoxin and ferritin in CHC. All these findings suggest that iron-induced oxidative stress contributes to CHC.

## DISCUSSION

A body iron store is needed in an acute blood loss which includes gastrointestinal bleeding. People living in urban areas, however, can receive iron supplements whose bioavailability is not different from endogenous storage iron. In an emergency, transfusion is needed regardless of iron store, because post-hemorrhage erythropoiesis takes more than one week even though an iron store is adequate. Therefore, evaluation should be made of whether storage iron is really needed for a healthy life in modern society.

The present observations suggest that slight iron overload, although unlikely to be cytotoxic in healthy individuals, may contribute to hepatic injury in patients with CHC. Measurement of the body iron store during iron reduction therapy was almost within normal ranges in patients,[13] supporting the hypothesis that small amounts of iron are sufficient for such iron-induced hepatotoxicity. Therefore, it is impor-

tant to keep CHC patients iron-free during iron reduction treatment. In addition to the iron stored in the liver, absorbed iron in the intestine may generate hepatotoxic free radicals when transported to the liver. In fact, iron restriction therapy using a low iron diet[23,24] was recommended for CHC patients.

Storage iron is one of the predictive parameters of IFN effects, but iron removal neither changes circulating HCV RNA levels nor enhances IFN efficacy.[25] Iron reduction therapy is non-specific elimination of oxidative stress and does not affect viral replication in the patients infected with HCV. Unfortunately, the iron reduction for iron overload diseases is not yet authorized in Japan. This is in contrast to Caucasians because of the different incidence of an iron overload disorder genetic hemochromatosis.[26,27] In Europe and the United States, individuals with C282Y mutation are so common that iron-induced oxidative stress and its prophylaxis are major issues even in patients with CHC. Bonkovsky, in a review of other options to IFN in CHC,[5] recommended iron reduction therapy as the first line among various regimens including ursodeoxhycholic acid and herbal medicines.

Male patients are more often exposed to progressive hepatic lesions than are female patients. Little is known about this gender difference, but it is clear that males are more sensitive to oxidative stress than females who have a long-term physiological blood loss before menopause. As mentioned above, the therapeutic effects of phlebotomy were not related to the serum iron indices but to the disease activity expressed by serum ALT levels.[7] Therefore, the treatment should be recommended to the patient group with a higher disease activity that might be exposed to a more rapid progression to cirrhosis. Suppression of progressive liver fibrosis rather than biochemical control of active disease is a more important issue for managing patients with CHC. In these respects, the long-term treatment might be an option for a large number of patients who need alternatives to IFN. It is of 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-hydroxy-2′-deoxyguanosine which is closely related to carcinogenesis.[28] More studies are needed to clarify whether iron reduction therapy is effective in minimizing the risk of late-onset hepatocellular carcinoma in CHC.

The iron reduction therapy described here provides a safety-proven, economical option for patients who have failed to respond or have contraindications to the antiviral agent IFN. After the proposal of our empiric treatment based on an ultra-structural study of the liver, histochemical evidence has increased that iron-induced oxidative stress is definitely involved in CHC.

## REFERENCES


1) Britton, R. (1996) Metal-induced hepatotoxicity. *Semin. Liver Dis.*, **16**, 2–12.
2) National Institutes of Health (1997) National Institutes of Health consensus development conference panel statement: Management of hepatitis C. *Hepatology*, **26** (Suppl. 1), 2S–10S.
3) Zeuzem, S., Feinman, S. V., Rasenack, J., Heathcote, E. J., Lai, M. Y., Gane, E., O'Grady, J., Reichen, L., Diago, M., Lin, A., Hoffman, J. and Brunda, M. J. (2000) Peginterferon alfa-2a in patients with chronic hepatitis C. *N. Engl. J. Med.*, **343**, 1666–1672.
4) Heathcote, E. J., Shiffman, M. L., Cooksley, W. G., Dusheiko, G. M., Lee, S. S. and Balart, L. (2000) Peginterferon alfa-2a in patients with chronic hepatitis C and cirrhosis. *N. Engl. J. Med.*, **343**, 1673–1680.
5) Bonkovsky, H. L. (1997) Therapy of hepatitis C: Other options. *Hepatology*, **26** (Suppl. 1), 143S–151S.
6) Hayashi, H., Takikawa, T., Nishimura, N., Yano, M., Isomura, T. and Sakamoto, N. (1994) Improvement of serum aminotransferase levels after phlebotomy in patients with chronic active hepatitis C and excess hepatic iron. *Am. J. Gastroenterol.*, **89**, 986–988.
7) Hayashi, H., Takikawa, T., Nishimura, N. and Yano, M. (1995) Serum aminotransferase levels as an indicator of the effectiveness of venesection for chronic hepatitis C. *J. Hepatol.*, **22**, 268–271.
8) Isomura, T., Yano, M., Hayashi, H. and Sakamoto, N. (1992) Excess iron in the liver of patients with chronic hepatitis C. *J. Clin. Electron Microsc.*, **25**, 231–237.
9) Bonkovsky, H. L., Banner, B. F. and Rothman, A. L. (1997) Iron and chronic viral hepatitis. *Hepatology*, **25**, 759–768.
10) Di Bisceglie, A. M., Axiotis, C. A., Hoofnagle, J. H. and Bacon, B. R. (1992) Measurements of iron status in patients with chronic hepatitis. *Gastroenterology*, **102**, 2108–2113.
11) Yano, M., Hayashi, H., Wakusawa, S., Takikawa, T. and Ikeda, R. (1997) Hepatic iron storage in patients with chronic hepatitis C but no hepatic iron detectable histochemically. *Med. Electron Microsc.*, **30**, 70–75.

12) Takikawa, T., Nishimura, N., Hayashi, H., Yano, M., Isomura,T. and Sakamoto, N. (1994) Correlation between serum levels of alanine aminotransferase and ferritin in male blood donors with antibody to hepatitis C virus. *J. Gastroenterol.*, **29**, 593–597.
13) Shiono, Y., Hayashi, H., Wakusawa, S., Sanae, F., Takikawa, T., Yano, M., Yoshioka, K. and Saito, H. (2001) Body iron stores and iron restoration rate in Japanese patients with chronic hepatitis C as measured during therapeutic iron removal revealed neither increased body iron stores nor effects of C282Y and H63D mutations on iron indices. *Nagoya J. Med. Sci.*, **64**, 51–57.
14) Tarao, K., Rino, Y., Ohkawa, S., Shimizu, A., Tamai, S., Miyakawa, K., Aoki, H., Imada, T., Shindo, K., Okamoto, N. and Totsuka, S. (1999) Association between high serum alanine aminotransferase levels and more rapid development and higher rate of incidence of hepatocellular carcinoma in patients with hepatitis C virus-associated cirrhosis. *Cancer*, **86**, 589–595.
15) Wakusawa, S., Ikeda, R., Takikawa, T., Hayashi, H., Yano, M. and Yoshioka, K. (2000) Combined phlebotomy and ursodeoxycholic acid treatment in the patients with chronic hepatitis C. *Hepatol. Res.*, **18**, 54–62.
16) Yano, M., Hayashi, H., Wakusawa, S., Sanae, F., Takikawa, T., Shiono, Y., Arao, M., Ukai, K., Ito, H., Watanabe, K. and Yoshioka, K. (2002) Long-term effects of phlebotomy on biochemical and histological parameters of chronic hepatitis C. *Am. J. Gastroenterol.*, **97**, 133–137.
17) Desmet, V. J., Gerber, M., Hoofnagle, J. H., Manns, M. and Scheuer, P. J. (1994) Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology*, **19**, 1513–1520.
18) Farinati, F., Cardin, R., De Maria, N., Della Libera, G., Marafin, C., Lecis, E., Burra, P., Floreani, A., Cecchetto, A. and Naccarato, R. (1995) Iron storage, lipid peroxidation and glutathione turnover in chronic anti-HCV positive hepatitis. *J. Hepatol.*, **22**, 449–456.
19) De Maria, N., Colantoni, A., Fagiuoli, S., Liu, G. J., Rogers, B. K., Farinati, F., Van Thiel, D. H. and Floyd, R. A. (1996) Association between reactive oxygen species and disease activity in chronic hepatitis C. *Free Radic. Biol. Med.*, **21**, 291–295.
20) Paradis, V., Mathurin, P., Kollinger, M., Imbert-Bismut, F., Charlotte, F., Piton, A., Opolon, P., Holstege, A., Poynard, T. and Bedossa, P. (1997) In situ detection of lipid peroxidation in chronic hepatitis C: correlation with pathological features. *J. Clin. Pathol.*, **50**, 401–406.
21) Kageyama, F., Kobayashi, Y., Kawasaki, T., Toyokuni, S., Uchida, K. and Nakamura, H. (2000) Successful interferon therapy reverses enhanced hepatic iron accumulation and lipid peroxidation in chronic hepatitis C. *Am. J. Gastroentero.*, **95**, 1041–1050.
22) Sumida, Y., Nakashima, T., Yoh, T., Kakisaka, Y., Nakajima, Y., Ishikawa, H., Mitsuyoshi, H., Okanoue, T., Nakamura, H. and Yodoi, J. (2001) Serum thioredoxin elucidates the significance of serum ferritin as a marker of oxidative stress in chronic liver diseases. *Liver*, **21**, 295–299.
23) Matsumoto, M., Imamura, S., Sato, H., Takamatsu, M., Okanoue, T. and Kashima, K. (1999) Dietary iron reduction on patients with chronic hepatitis C [in Japanese with English abstract]. *Acta. Hepatol. Jpn.*, **40**, 436–444.
24) Iwasa, M., Kaito, M., Ikoma, J., Kobayashi, Y., Tanaka, Y., Higuchi, K., Takeuchi, K., Iwata, K., Watanabe, S. and Adachi, Y. (2002) Dietary iron restriction improves aminotransferase levels in chronic hepatitis C patients. *Hepatogastroenterology*, **49**, 529–531.
25) Piperno, A., Sampietro, M., D'Alba, R., Roffi, L., Fargion, S., Parma, S., Nicoli, C., Corbetta, N., Pozzi, M., Arosio, V., Boari, G. and Fiorelli, G. (1996) Iron stores, response to alpha-interferon therapy, and effects of iron depletion in chronic hepatitis C. *Liver*, **16**, 248–254.
26) Merryweather-Clarke, A. T., Pointon, J. J., Shearman, J. D. and Robson, K. J. (1997) Global prevalence of putative haemochromatosis mutations. *J. Med. Genet.*, **34**, 275–278.
27) Shiono, Y., Ikeda, R., Hayashi, H., Wakusawa, S., Sanae, F., Takikawa, T., Imaizumi, Y., Yano, M., Yoshioka, K., Kawanaka, M. and Yamada, G. (2001) C282Y and H63D mutations in the HFE gene have no effect on iron overload disorders in Japan. *Intern. Med.*, **40**, 852–856.
28) Kato, J., Kobune, M., Nakamura, T., Kuroiwa, G., Takada, K., Takimoto, R., Sato, Y., Fujikawa, K., Takahashi, M., Takayama, T., Ikeda, T. and Niitsu, Y. (2001) Normalization of elevated hepatic 8-hydroxy-2′-deoxyguanosine levels in chronic hepatitis C patients by phlebotomy and low iron diet. *Cancer Res.*, **61**, 8697–8702.