

Fig. 5. Molecular hepatocarcinogenesis in HCV infection. Oxidative stress together with hepatic steatosis induced by the HCV core protein would play a pivotal role in the development of HCC. Alterations in cellular gene expressions, such as TNF- $\alpha$  or SOCS-1, and those in the intracellular signaling pathways including JNK would be co-accelerators to hepatocarcinogenesis in HCV infection. The latter pathway has not been found in NASH while the former may be common in the pathogenesis of hepatitis C and NASH. HCC, hepatocellular carcinoma; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; SOCS-1, suppressor of cytokine signaling-1; NASH, non-alcoholic steatohepatitis.

established between non-alcoholic steatohepatitis (NASH) and HCC, it needs to be pursued energetically in view of histological resemblance of NASH to chronic hepatitis C.

When hepatitis C and NASH are compared, there are a number of similarities between these two medical conditions (Table 2). Steatosis, which is one of the definitions in NASH, is a characteristic trait of chronic hepatitis C [4–6,13]. Disturbances in the lipid metabolism are present in both conditions, although the phenotypes may show a distinction: hypo- $\beta$ -lipoproteinemia in hepatitis C but hyperlipidemia in NASH. As described above, insulin resistance often arises in chronic hepatitis C, and is also a feature frequently observed in NASH [18]. Some cytokines, such as TNF- $\alpha$ , are considered to be critical in the pathogenesis of these conditions. TNF- $\alpha$  levels are increased in patients with hepatitis C and are implicated in insulin resistance. Single nucleotide polymorphism in TNF- $\alpha$  gene is significantly found in NASH patients [18]. Overproduction of oxidative stress or reactive oxygen species (ROS) plays a pivotal role in the progression of hepatitis and development of HCC in both the conditions: ROS is overproduced

in the liver of the core gene transgenic mice in the absence of inflammation, contributing, at least in part, to the development of HCC [13,19,20]. Functional abnormalities in the mitochondria are implicated, in both hepatitis C and NASH, in the pathogenesis of liver diseases including HCC. In HCV core gene transgenic mice, the malfunction of the electron transfer system of mitochondria has been suggested and is assumed to be an origin of ROS overproduction (Table 2).

Finally, HCC develops both in chronic hepatitis C and NASH. However, the association between NASH and HCC is not strong yet while there is a definite connection in the case of hepatitis C. Nevertheless, HCC develops in patients with NASH, regardless of the frequency. Hence the underlying mechanism of HCC development in NASH awaits further investigation. The analogy between chronic hepatitis C and NASH, as described above, may be a clue to solve a puzzle in the pathogenesis of NASH including hepatocarcinogenesis. In the pathogenesis of HCC in HCV infection, one of intracellular signaling MAPK systems, JNK, is activated in the liver. In the downstream of JNK, transcription factor AP1 and cell cycle machineries, CDK4 and cyclin D1, are subsequently activated, conferring advantage to cell proliferation [16,20]. However, such activations in cellular genes or signaling systems have not been identified yet for NASH. Overproduction of oxidative stress together with the presence of steatosis may be a common pathway to liver hepatocarcinogenesis in both hepatitis C and NASH (Fig. 5, upper half).

However, the alterations in cellular gene expressions and/or intracellular signaling systems are solely with hepatitis C in the presence of the viral protein(s), putting chronic hepatitis C onto the fast track for the development of HCC (Fig. 5, upper half). This aspect of NASH should be investigated. The

Table 2  
Comparison of hepatitis C and NASH

Hepatitis C	NASH
Steatosis	Steatosis
Hypo- $\beta$ -lipoproteinemia	Hyperlipidemia
Insulin resistance	Insulin resistance
Cytokines (TNF- $\alpha$ , etc.)	Cytokines (TNF- $\alpha$ , etc.)
Oxidative stress	Oxidative stress
Mitochondrial abnormality	Mitochondrial abnormality
Obesity?	Obesity
HCC	HCC?

analogy between hepatitis C and NASH would give a solution to problems in the pathogenesis of NASH.

## 7. Conclusion

Although HCV targets at the liver, it has become increasingly evident that HCV can induce diseases of many organs. Recently, much attention is drawn to metabolic disorders in HCV infection. First, hepatic steatosis and derangement in lipid metabolism have been found characteristic of HCV infection, and later on correlation was noted between HCV infection and diabetes as well as insulin resistance. We have demonstrated that HCV by itself can induce insulin resistance by means of disturbing the insulin signaling pathway by an HCV protein. The fact that HCV infection induces insulin resistance by the virus itself may influence the progression of chronic liver disease and open up novel therapeutic approaches. HCV infection would need to be viewed not only as liver disease but also a metabolic disease, which would be a clue to open up a novel way to the molecular understanding of pathogenesis of NASH.

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## Molecular Basis of Hepatitis C Virus-Associated Hepatocarcinogenesis: Lessons From Animal Model Studies

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Despite numerous lines of epidemiologic evidence connecting HCV infection and the development of hepatocellular carcinoma (HCC), it remains controversial whether HCV itself plays a direct role or an indirect role in the pathogenesis of HCC. Through the use of transgenic mice, it has become evident that the core protein of HCV has oncogenic potential. HCV is directly involved in hepatocarcinogenesis, albeit other factors such as inflammation and environmental factors might also play a role. The direct involvement of HCV in hepatocarcinogenesis would be achieved via 2 pathways. In one pathway, the core protein acts on the function of mitochondria, leading to the overproduction of oxidative stress, which yields genetic aberrations in cell growth-related genes. The other pathway involves the modulation of cellular gene expressions and intracellular signal transductions, such as mitogen-activated protein kinase pathway, which results in the activation of transcription factors and cell cycle machineries. The combination of these alterations would be hypothesized to provoke the development of HCC in HCV infection. This would be a mechanism for HCC development in HCV infection that is distinct from those for other cancers. The presence of the HCV core protein, to which an oncogenic potential is ascribed, might allow some of the multiple steps to be bypassed in hepatocarcinogenesis. Therefore, unlike in other cancers, HCV infection can elicit HCC in the absence of a complete set of genetic aberrations. Such a scenario, "non-Vogelstein type" carcinogenesis, may explain the unusually high incidence and multicentric nature of HCC development in HCV infection.

Worldwide HCV chronically infects hundreds of millions of people and induces a spectrum of chronic liver diseases.<sup>1</sup> Hence, it impacts the society in a number of domains including medical, sociologic, and economic. Hepatocellular carcinoma (HCC) has become the most frequent cause of death in individuals persistently infected with HCV. In particular, HCV has received increasing attention because of its wide and deep penetration in the community, coupled with a very high incidence of HCC. Once cirrhosis is established in Japanese patients infected with HCV, HCC develops at a yearly rate of 5%–7%.<sup>2</sup> Knowledge about the mecha-

nism of HCC development in chronic HCV infection, therefore, is required for the prevention of HCC.

### Pathogenesis of Hepatocellular Carcinoma in Persistent Hepatitis C Virus Infection

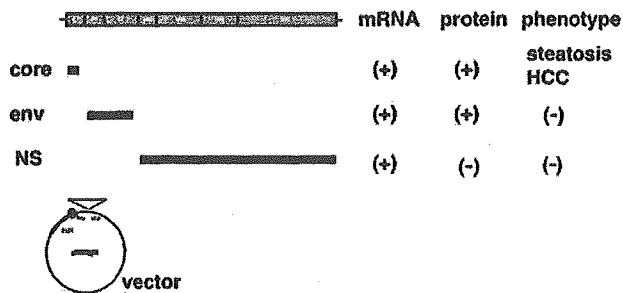
How HCV induces HCC is not yet clear, despite the fact that more than 70% of patients with HCC in Japan are infected with HCV.<sup>1,3,4</sup> HCV infection is also common in patients with HCC in other countries, albeit to a lesser extent. These epidemiologic facts are a stimulus to determine the role of HCV in hepatocarcinogenesis. Inflammation induced by HCV, manifesting itself in various forms of hepatitis, should be considered in a study of the carcinogenic capacity of hepatitis viruses. It has been proposed repeatedly that necrosis of hepatocytes as a result of chronic inflammation and ensuing regeneration enhances mutagenesis in host cells, which can culminate in HCC. This theory presupposes an indirect involvement of hepatitis viruses in HCC via hepatic inflammation. However, this leaves specialists in hepatology with a serious question: can inflammation per se result in the development of HCC in such a high incidence or multicentric pattern in HCV infection? The secondary role of HCV would have to be weighed against the extremely rare occurrence of HCC in patients with autoimmune hepatitis in whom severe inflammation in the liver persists indefinitely.

This background and line of reasoning lead to the hypothesis that viral proteins might play a role in inducing HCC. This possibility has been evaluated by introducing genes of HCV into hepatocytes in culture with little success. One of the difficulties in using cultured cells is that the carcinogenic capacity of HCV, if any, appears to be weak and would thus take a long time to manifest itself. Actually, it takes 30–40 years for

*Abbreviations used in this paper:* HCC, hepatocellular carcinoma; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase.

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**Figure 1.** HCV gene transgenic mouse lines. Among the 3 different transgenic mouse lines established, only the transgenic mice carrying the HCV core gene develop HCC after an early phase with hepatic steatosis in 2 independent lineages. The mice transgenic for the envelope (*env*) genes or nonstructural (*NS*) genes do not develop HCC.

HCC to develop in individuals infected with HCV. Another constraint common to studies of carcinogenesis is the development of HCC by transformed cells that might have resulted from uncontrolled growth and escaped surveillance of the host. If this is the case, the analysis of transformed cells would not be sufficient for solving the mystery of carcinogenesis. On the basis of these viewpoints, we initiated a study of carcinogenesis in chronic viral hepatitis by transgenic mouse technology.

### Core Protein of Hepatitis C Virus With an Oncogenic Activity in Vivo

As illustrated in Figure 1, transgenic mouse lines with parts of the HCV genome were engineered by introducing genes excised from the cDNA of the HCV genome of genotype 1b.<sup>5,6</sup> The background of the mouse lines is a C57BL/6 strain, which is known for a rare spontaneous occurrence of HCC.<sup>7</sup> Three different transgenic mouse lines are established, which carry the core gene, envelope genes, or nonstructural genes under the same transcriptional control element. Among these mouse lines, only the transgenic mice carrying the core gene develop HCC in 2 independent lineages (Figure 1).<sup>6</sup> The envelope gene transgenic mice do not develop HCC, despite high expression levels of both E1 and E2 proteins.<sup>8,9</sup> The transgenic mice carrying the entire nonstructural genes have not developed HCC.

The transgenic mice carry the core gene and express the core protein of an expected size, approximately 21 kd, the level of which in the liver is similar to that in the liver of chronic hepatitis C patients. Early in life, these mice develop hepatic steatosis, which is one of the histologic characteristics of chronic hepatitis C, along with lymphoid follicle formation and bile duct damage.<sup>10</sup> Thus, the core gene transgenic mouse model reproduces

well this feature of chronic hepatitis C. Of note, evidence of significant inflammation is observed in the liver of this animal model. Late in life, these transgenic mice develop HCC. Most hepatic nodules exhibit a pathology characterized by "nodule in nodule," and HCC with a low degree of differentiation develops within an adenoma as well as within HCC with a higher degree of differentiation.<sup>6</sup> Although numerous lipid droplets are found in cells forming an adenoma, as in nontumorous cells, they are rarely observed in HCC cells. These histologic features closely resemble those observed in HCC developing in chronic hepatitis C patients, in which prominent lipid droplets are found in small, differentiated HCC and its precursors; poorly differentiated HCC without lipid droplets develops from within differentiated HCC.<sup>6</sup> Notably, the development of steatosis and HCC has been reproduced by other HCV transgenic mouse lines, which harbor the entire HCV genome or structural genes including the core gene.<sup>11</sup> These outcomes indicate that the core protein of HCV has an oncogenic potential when expressed *in vivo*.

### Mechanism of Hepatocarcinogenesis in Animal Model for Hepatitis C Virus-Associated Hepatocellular Carcinoma

It is difficult to determine the mechanism of carcinogenesis, even for our simple model in which only the core protein is expressed in otherwise normal liver tissues. There is a notable feature in the localization of the core protein in hepatocytes; whereas the core protein predominantly exists in the cytoplasm associated with lipid droplets, it is also present in the mitochondria and nuclei.<sup>6,12</sup> On the basis of this finding, the pathways related to these 2 organelles, the mitochondria and nuclei, were meticulously analyzed.

One activity of the core protein is an increased production of oxidative stress in the liver. We would like to draw particular attention to the fact that the production of oxidative stress is increased in our transgenic mouse model in the absence of inflammation in the liver, *ie*, hepatitis. This reflects a state of an overproduction of reactive oxygen species in the liver or predisposition to it, which is staged by the HCV core protein without any intervening inflammation.<sup>13,14</sup> The overproduction of oxidative stress results in the generation of deletions in the mitochondrial DNA, an indicator of genetic damage. Thus, the core protein induces excessive oxidative stress in the absence of inflammation and might, at least in part, contribute to hepatocarcinogenesis in HCV infec-

**Table 1.** Biomolecular Alterations With the Core Protein Expression Observed in the Transgenic Mouse Model

1. Induction of cytokines including tumor necrosis factor- $\alpha$  and interleukin-1 $\beta$ <sup>19</sup>
2. Activation of MAPK pathway and enhancement of AP-1 activation<sup>19,21</sup>
3. Overproduction of oxidative stress or reactive oxygen species in the absence of inflammation<sup>13</sup>
4. Synergy of HCV core and alcohol in inducing oxidative stress and activating MAPK<sup>13,21</sup>
5. Interaction of HCV core and retinoid X receptor- $\alpha$  and peroxisome proliferator activated receptor- $\alpha$ <sup>20</sup>
6. Induction of insulin resistance<sup>17</sup>
7. Development of steatosis by inhibiting microsomal triglyceride transfer protein activity<sup>5,14,24</sup>
8. Interaction of HCV core and proteasome activator PA28 $\gamma$ <sup>25</sup>
9. Inhibition of suppressor of cytokine signaling-1<sup>26</sup>

tion. If inflammation is induced in the liver with the HCV core protein, the production of oxidative stress is escalated to an extent that cannot be further scavenged by a physiologic antagonistic system. This indicates that the inflammation in chronic HCV infection would have a characteristic different in its quality from those of other types of hepatitis, such as autoimmune hepatitis. The basis for the overproduction of oxidative stress might be ascribed to the mitochondrial dysfunction.<sup>13,15</sup> The function of the electron transfer system of the mitochondrion is suggested in association with the presence of the HCV core protein.<sup>16</sup> Hepatic steatosis in hepatitis C might work as fuel for oxidative stress overproduction.<sup>14,17,18</sup>

Other possible pathways would be alteration in the expression of cellular genes, interacting with cellular proteins, and modulation of intracellular signaling pathways (Table 1). For example, tumor necrosis factor- $\alpha$  and interleukin-1 $\beta$  have been found to be transcriptionally activated.<sup>19</sup> The core protein has also been found to interact with some cellular proteins, such as retinoid X receptor- $\alpha$ , that play pivotal roles in cell proliferation and metabolism.<sup>20</sup> The mitogen-activated protein kinase (MAPK) cascade is also activated in the liver of the core gene transgenic mouse model. The MAPK pathway, which consists of 3 routes, c-Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase, is involved in numerous cellular events including cell proliferation. In the liver of the core gene transgenic mouse model before HCC development, only the JNK route is activated. In the downstream of the JNK activation, transcription factor AP-1 activation is markedly enhanced.<sup>19,21</sup> Far downstream, both the mRNA and protein levels of cyclin D1 and CDK4 are increased. Thus, the HCV core protein modulates the intracellular signal-

ing pathways and gives an advantage for cell proliferation to hepatocytes.

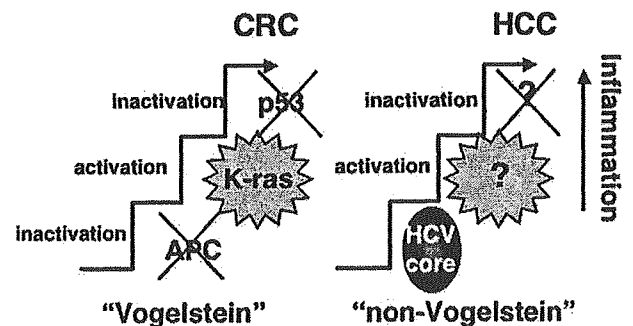
Such an effect of the core protein on the MAPK pathway, combined with that on oxidative stress, might explain the extremely high incidence of HCC development in chronic hepatitis C.

### Hepatocarcinogenesis Induced by Hepatitis C Virus: A Mechanism Distinct From Those in Other Cancers

The results of our studies on transgenic mice have indicated a carcinogenic potential of the HCV core protein *in vivo*; thus, HCV might be directly involved in hepatocarcinogenesis.

In research studies of carcinogenesis, the theory of Kinzler and Vogelstein<sup>22</sup> has gained wide popularity. They have proposed that the development of colorectal cancer is induced by the accumulation of a complete set of cellular gene mutations. They have deduced that mutations in the *APC* gene for inactivation, those in *K-ras* for activation, and those in the *p53* gene for inactivation accumulate, which together lead toward the development of colorectal cancer. Their theory has been extended to the carcinogenesis of other cancers as well, so-called Vogelstein-type carcinogenesis (Figure 2).

On the basis of the results we obtained for the induction of HCC by the HCV core protein, we would like to introduce a mechanism different from that of Kinzler and Vogelstein<sup>22</sup> for the hepatocarcinogenesis in HCV infection. We do allow multistages in the induction of all



**Figure 2.** Mechanism of HCV-associated hepatocarcinogenesis. Multiple steps are required in the induction of all cancers; it would be mandatory for hepatocarcinogenesis that genetic mutations accumulate in hepatocytes. However, in HCV infection, some of these steps might be skipped in the development of HCC in the presence of the core protein. The overall effects achieved by the expression of the core protein would be the induction of HCC, even in the absence of a complete set of genetic aberrations, required for carcinogenesis. By considering such a non-Vogelstein-type process for the induction of HCC, a plausible explanation might be given for many unusual events happening in HCV carriers. CRC, colorectal cancer.

cancers; it would be mandatory for hepatocarcinogenesis that many mutations accumulate in hepatocytes. Some of these steps, however, might be bypassed in the development of HCC in HCV infection to which the core protein would contribute (Figure 2). The overall effects achieved by the expression of the viral protein would be the induction of HCC, even in the absence of a complete set of genetic aberrations, required for carcinogenesis.

By considering such a non-Vogelstein-type process for the induction of HCC, a plausible explanation might be given for many unusual events happening in HCV carriers.<sup>23</sup> Our theory might explain why HCC develops in persistent HCV infection at such a high incidence. Our theory might also account for the nonmetastatic and multicentric de novo occurrence characteristics of HCC, which would be the result of persistent HCV infection.

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# Hepatitis C Virus Infection Can Present with Metabolic Disease by Inducing Insulin Resistance

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## Key Words

Diabetes · Hepatitis C virus · Insulin resistance · Insulin receptor substrate · Transgenic mouse

## Abstract

Although hepatitis C virus (HCV) targets the liver, it has become increasingly evident that HCV can induce diseases of many organs. Recently, much attention is drawn to metabolic disorders in HCV infection. First, hepatic steatosis and derangement in lipid metabolism have been found characteristic of HCV infection, and later on, a correlation was noted between HCV infection and diabetes as well as insulin resistance. We have demonstrated that HCV by itself can induce insulin resistance through disturbing the insulin signaling pathway by HCV proteins. The fact that HCV infection induces insulin resistance by the virus itself may influence the progression of chronic liver disease and open up novel therapeutic approaches. In conclusion, towards the future, HCV infection needs to be viewed not only as a liver disease but also as a metabolic disease.

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## Introduction

Hepatitis C virus (HCV) infects approximately 1.8 million people in Japan alone and as many as 200 million over the world and induces liver disease ranging from

chronic hepatitis through cirrhosis to hepatocellular carcinoma (HCC) [1, 2]. It has been noticed soon after the discovery that the infection with HCV does not exclusively involve the liver. In fact, type II cryoglobulinemia [3] and membranoproliferative glomerulonephritis [4] frequently occur in patients infected with HCV. Furthermore, strong associations of HCV infection with Sjogren's syndrome [5] and lichen planus [6] have been noted, which is verified in the animal model [7]. In addition, the relation between HCV infection and B cell lymphoma has attracted attention especially in Europe [8].

Recently, there have been increasing lines of evidence to indicate metabolic disturbances in HCV infection which, in turn, would influence the pathogenesis of chronic hepatitis C. The discovery of HCV in 1989 [9] enabled a comparison between chronic hepatitis C and other chronic liver diseases. As shown in the results, it has been repeatedly reported that steatosis is significantly associated with chronic hepatitis C [10, 11]. Steatosis in HCV infection is reproduced in animal models [12–14] to reinforce a pathologic role of HCV. Furthermore, patients infected with HCV have abnormalities in serum lipids, such as hypocholesterolemia and abnormal levels of apolipoproteins in serum [15, 16]; they are rectified in sustained virological responders to interferon (IFN) [16]. Thus, the association between HCV infection and a derangement in lipid metabolism has become increasingly strong, both in patients and experimental systems in animals. Finally, patients with chronic hepatitis C accompanied by severe steatosis develop hepatic fibrosis with an

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increased velocity [17]. All in all, we could say that abnormal lipid metabolism in HCV infection is deeply involved in the pathogenesis of hepatitis C.

### HCV Infection and Diabetes

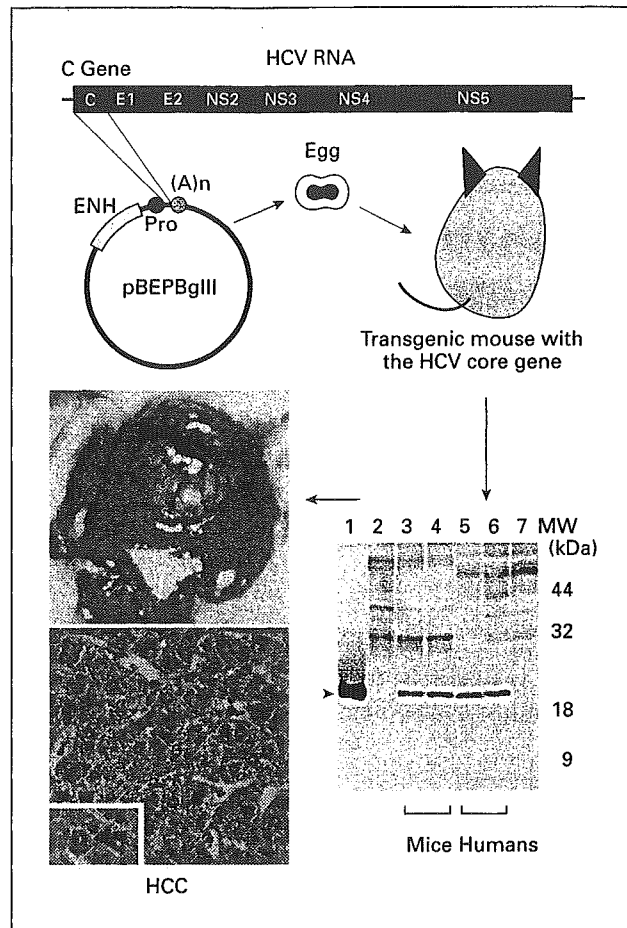
Diabetes is suggested as another metabolic disease in association with HCV infection. In 1994, Allison et al. [18] reported an epidemiological link between diabetes and HCV infection. However, doubts were cast on the association in view of a decreased glucose tolerance in advanced chronic hepatitis as well as an increased opportunity for HCV infection in diabetics who frequently receive determination of blood sugar. Several reports from the same group and others followed along this line. The trend to accept the solid association between diabetes and HCV infection seems to have been triggered in the United States by the population study by Metha et al. [19].

However, the association between diabetes and HCV infection is blemished by factors responsible for decreased glucose tolerance, such as advanced cirrhosis, obesity and ageing common in patients with hepatitis C; they make it difficult to prove this association. Hence, there is a need to evaluate the association by basic studies in experimental systems.

### HCV Infection Induces Insulin Resistance

We set out to demonstrate the association between HCV infection and diabetes using the animal model. Mice transgenic for the HCV core gene were employed to this end [12, 13]. These mice are engineered to have the HCV core gene of genotype 1b in the absence of other viral genes. They express HCV core protein of the expected size in the liver, in levels comparable with those of patients with chronic hepatitis C (fig. 1). Half of them develop HCC later during their lives [13]. These transgenic mice were fed with their normal littermates, and the glucose metabolism was compared between them [20].

Although mice transgenic for the core gene did not develop overt diabetes, they had markedly elevated serum levels of insulin. Plasma glucose levels were somewhat higher in transgenic mice than in their normal littermates, both in the fast and after ample feeding, with no significant differences between them. In remarkable contrast, serum insulin levels were significantly higher in transgenic than in normal mice in both conditions (fig. 2). Since such a combination of normal glucose levels and

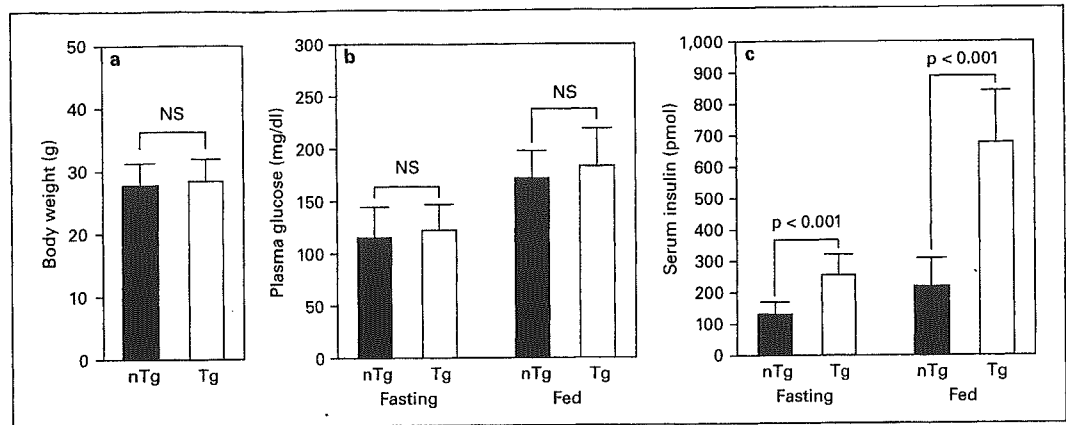


**Fig. 1.** Expression of HCV core gene in transgenic mouse. It carries the core gene of HCV genotype 1b alone and expresses the core protein of expected size in the liver, at levels similar to those in human patients. Mice eventually develop HCC later in their lives.

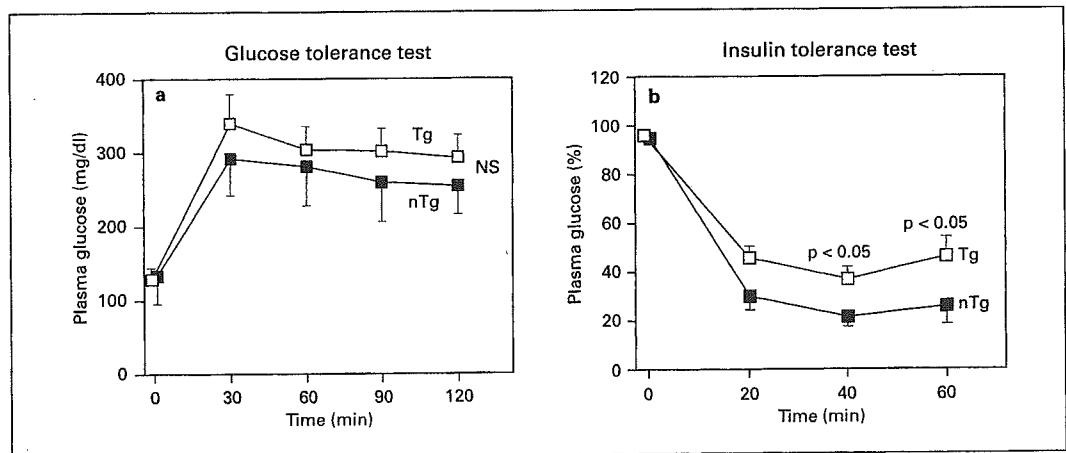
hyperinsulinemia points to insulin resistance, glucose and insulin tolerance tests were conducted.

Mice transgenic for the HCV core gene exhibited glucose levels a little higher than those in normal littermates, without any significant differences between them. In insulin tolerance tests, glucose levels were significantly higher in transgenic than in normal mice, both 40 and 60 min after they were injected with insulin intraperitoneally (fig. 3). These results indicate suppression of the activity of insulin to decrease blood glucose levels for inducing insulin resistance in core-transgenic mice. Since only the HCV core gene had been incorporated into these transgenic mice, HCV core protein was able to induce insulin resistance *in vivo*.





**Fig. 2.** Altered homeostasis of glucose in mice transgenic for the HCV core gene. Body weight of 2-month-old mice (a), plasma glucose levels in fasting or fed mice (b) and serum insulin levels in fasting or fed mice (c) are shown. Values represent means  $\pm$  SE. NS = Not significant statistically; nTg = nontransgenic mice; Tg = transgenic mice.



**Fig. 3.** Insulin resistance in transgenic mice. Glucose tolerance in mice after overnight fasting (a). *D*-Glucose (1 g/kg body weight) was given intraperitoneally to conscious mice, and plasma glucose levels were determined at time points indicated. b Insulin tolerance in mice fasted overnight. Human insulin (1 U/kg body weight) was injected intraperitoneally, and glucose concentrations were determined sequentially. Values were normalized to the baseline glucose concentration at the time of insulin administration. NS = Not significant statistically; nTg = nontransgenic mice; Tg = transgenic mice.

By what mechanism does insulin resistance arise in this animal model? The insulin resistance is considered to involve two factors, namely central and peripheral insulin resistances (table 1) [21]. The hyperinsulinemic-euglycemic clamp method was employed to differentiate between them. In this method, hepatic glucose production (HGP) is calculated on the basis of amounts of glu-

cose required to keep plasma glucose levels within a certain range at serum insulin levels higher than physiological ones. In normal control mice, HGP was suppressed by 60% by the administration of insulin, in contrast to core-transgenic mice in which there was no appreciable suppression of HGP by insulin (fig. 4). These results indicate a hepatic (central) origin of the insulin resistance

**Table 1.** Two types of insulin resistance

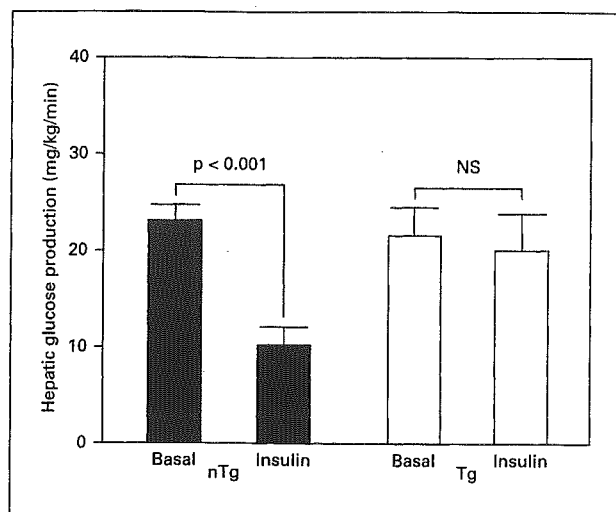
Type	Mechanism
Peripheral	A shortage of insulin action in the muscle due to deficit in the insulin-induced uptake of glucose into muscles
Central	A shortage of insulin action in the liver due to deficit in the insulin-induced suppression of glucose production in hepatocytes

in transgenic mice. For further confirmation, an uptake of glucose into muscle was determined. There were no differences in the uptake in response to administration of insulin between normal and transgenic mice. Therefore, the insulin resistance in mice transgenic for the HCV core gene is central and hepatic.

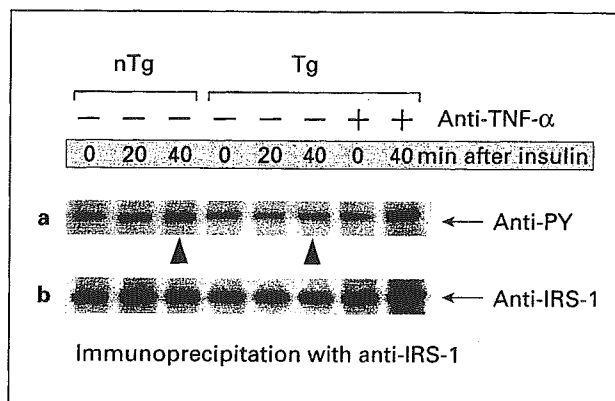
#### HCV Core Protein Suppresses the Transduction of Insulin Signaling in Hepatocytes

Next, we evaluated how insulin resistance elicits in mice transgenic for the HCV core gene. For this purpose, liver homogenate was immunoblotted with antiphosphotyrosine and antiphosphoserine antibodies after insulin receptor substrate (IRS)-1 and IRS-2 had been immunoprecipitated. Tyrosines in IRS-1 were weakly phosphorylated both in normal and transgenic mice before they received insulin, with no differences between them. However, after the administration of insulin, the phosphorylation of tyrosines in IRS-1 increased in normal but not in transgenic mice (fig. 5). Obtained results suggested disturbance in tyrosine phosphorylation as one of the factors responsible for insulin resistance in the liver. There were no differences in phosphorylation of serines in IRS-1 or tyrosines in IRS-2 between normal and transgenic mice. Combined, they provided experimental evidence for the development of insulin resistance by the presence of HCV in the liver that would occur by disturbing the transduction of insulin signaling in hepatocytes (fig. 6).

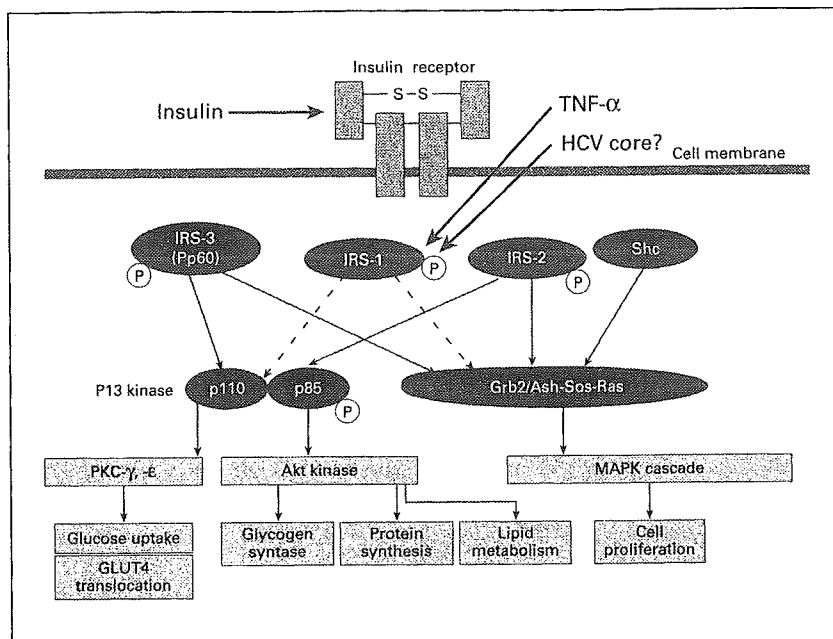
There remains a possibility for the HCV core protein to directly prohibit phosphorylation of tyrosines, or else, it might inhibit tyrosine phosphorylation via certain cytokines. In our extensive searches for the expression of cytokines in the liver of transgenic mice, only TNF- $\alpha$  and IL-1 $\beta$  have been found with an increased expression [22]. Therefore, for the purpose of evaluating the role of



**Fig. 4.** Characterization of glucose metabolism in transgenic mice. Glucose production in the liver was calculated using the hyperinsulinemic-euglycemic clamp method. NS = Not significant statistically; nTg = nontransgenic mice; Tg = transgenic mice.



**Fig. 5.** Phosphorylation of tyrosine in IRS-1 in response to insulin stimulation. Liver tissues from control mice, transgenic mice with or without anti-TNF- $\alpha$  antibody treatment, were analyzed before and 20 as well as 40 min after administration of insulin. Samples were subjected to immunoprecipitation with anti-IRS-1 antibody and then immunoblotted with indicated antibodies. Experiments were performed in triplicate, and a representative picture is exhibited. Immunoblotting with antiphosphotyrosine (PY) antibody (lane a) did not augment phosphorylation of tyrosine in IRS-1 after stimulation with insulin in the core gene transgenic mice (Tg), in contrast to tyrosine phosphorylation markedly enhanced in control mice (nTg). Insulin-stimulated tyrosine phosphorylation was restored 40 min after treatment with anti-TNF- $\alpha$  antibody. Note differences in the intensity of bands 40 min after the administration of insulin (arrowheads). Immunoblotting with anti-IRS-1 antibody (lane b) served as control for the IRS-1 load.



**Fig. 6.** A proposed mechanism for insulin resistance in HCV infection. HCV itself or elevated levels of cytokines such as TNF- $\alpha$  may inhibit tyrosine phosphorylation of IRS-1 in the liver, suppress intracellular transduction of insulin signal and lead to insulin resistance. PKC = Protein kinase C; MAPK = mitogen-activated protein kinase.

TNF- $\alpha$  in insulin resistance in transgenic mice, serum insulin was determined and an insulin tolerance test performed after they had received anti-TNF- $\alpha$  intraperitoneally. Pretreatment with anti-TNF- $\alpha$  partially improved insulin resistance in mice transgenic for the HCV core gene. Albeit a direct anti-insulin activity of core protein and direct or indirect factors for insulin resistance are not to be excluded, high levels of TNF- $\alpha$  in the liver would be one of the factors for expression of insulin resistance in this mouse model.

### Insulin Resistance in Patients with Chronic Hepatitis C

Concurrently with our report in experimental systems, Aytug et al. [23] investigated insulin signaling in biopsied liver specimens from patients with chronic hepatitis C. Specifically, they evaluated changes in IRS-1, IRS-2 and phosphatidyl inositol (PI)3 kinase levels in the liver of patients. With insulin stimulation of biopsied liver samples, insulin receptor proteins and IRS-1 increased, while phosphorylation of tyrosines in IRS-1 decreased to one half of the baseline value, along with a diminished activity for PI3 kinase associated with IRS-1, in patients with chronic hepatitis C. The authors went on to propose a possibility for disturbed transduction of the insulin sig-

naling pathway in the liver to induce insulin resistance in patients with chronic hepatitis C [23]. Their report is quite intriguing in that it opens up the way for evaluating an association between HCV infection and insulin resistance in clinical samples at the molecular level.

The results of Aytug et al. [23] inadvertently coincide with ours in analyzing the mechanism of insulin resistance with the experimental system in mice (vide supra). They unanimously incriminate impaired tyrosine phosphorylation in IRS-1 in the induction of insulin resistance by HCV infection. It struck us as a surprise that the mechanism of insulin resistance induced by HCV infection has been in agreement between clinical samples and experimental animals, in spite of hepatic IRS-2 that was preferred to IRS-1 for its role in development of insulin resistance in former studies [24]. HCV infection is peculiar in that IRS-1 weighs heavier than IRS-2 in the induction of hepatic insulin resistance.

Although our data strongly indicate a hepatic character of insulin resistance in HCV infection, they by no means exclude roles of other factors in the induction of this resistance. There is little expression of the HCV core gene in muscles of our animal model; it is not known if HCV infects muscular cells in patients with chronic hepatitis C. Factors not intrinsic to the liver would have to be evaluated to sort this out, including dysfunction of mitochondria for induction of insulin resistance [25].

## Insulin Resistance for Advanced Hepatic Fibrosis

Insulin resistance in HCV infection may have an additional significant clinical implication. In 260 patients with chronic hepatitis C, Hui et al. [26] have tried to establish the relationship between liver histology and indicators of glucose metabolism, as well as insulin resistance represented by the homeostasis model assessment of insulin resistance. They have found that insulin resistance already exists in hepatitis C patients with stage 0 or 1 fibrosis in the liver. This indicates that insulin resistance in HCV infection is not attributable to advanced liver disease. In their study, independent predictors of insulin resistance in HCV infection were body mass index, non-response to antiviral treatment, intensity of portal inflammation and infection with HCV genotype 3 [26]. Furthermore, the homeostasis model assessment of insulin resistance was a significant and independent predictor of the stage and velocity of hepatic fibrosis. The results of the study are of much importance, because they implicate a role of insulin resistance and hyperinsulinemia by inference, in promoting the progression of hepatic fibrosis. Insulin has been proven as an aggravating factor not only in atherosclerosis, but also in systemic inflammation and fibrosis. The liver is no exception to this.

## Conclusions: Hepatitis C Viewed as a Metabolic Disease and Outlook for Therapeutic Strategies in the Future

We have demonstrated that HCV per se induces insulin resistance in the animal model. Superimposed high-fat diet and obesity may lead to overt diabetes. Since insulin resistance accelerates the progression of chronic hepatitis C, it would naturally influence the development of HCC. Although the association has not been established between nonalcoholic steatohepatitis and HCC, it needs to be energetically pursued in view of the histological homology of nonalcoholic steatohepatitis to chronic hepatitis C. Drugs for improving glucose metabolism and insulin resistance need to be kept in store in the treatment of hepatitis C patients who have failed to respond to antivirals, because they may well prevent progression of fibrosis and development of HCC in such patients. Traditional 'high-protein and high-calorie' diet, especially advocated in Japan after World War II, is obviously detrimental, except in some patients with advanced cirrhosis. Consultation on the dietary habit with hepatitis C patients should include iron restriction [27] as well as weight control, because high-calorie intakes are likely to accelerate hepatic fibrosis by aggravating insulin resistance.

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Review

# Oxidative stress and hepatitis C viral infection

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## Abstract

The involvement of oxidative stress in the pathogenesis of hepatitis and hepatocellular carcinoma has been strongly suggested. Oxidative stress is produced by inflammatory processes that occur in hepatitis via immunological mechanisms. In addition, in hepatitis C virus (HCV) infectious disease, some role has been assigned to viral proteins in the induction of oxidative stress. In the presence of hepatic steatosis, insulin resistance and increased levels of some cytokines, all of which are also induced by viral protein expression, oxidative stress is enhanced in HCV infection. In this sense, the role of oxidative stress in the progression of chronic hepatitis and hepatocarcinogenesis is greater in hepatitis C than in other types of hepatitis such as hepatitis B or autoimmune hepatitis. The additive effects of oxidative stress caused by the inflammatory process and that induced by HCV proteins may, furthermore, exert synergistic effects with alterations in intracellular signaling systems such as mitogen-activated protein kinases (MAPK), which are also induced by HCV proteins. These synergistic effects may be responsible for rare characteristics, that is, the high incidence and multicentric nature of hepatocarcinogenesis in HCV infection.

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**Keywords:** Oxidative stress; Hepatitis C virus; Hepatocarcinogenesis; Lipid peroxidation; Steatosis; Insulin resistance

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## 1. Introduction

There are approximately 200 million people infected with hepatitis C virus (HCV) worldwide, of which about 1.8 million are in Japan. It is one of the most serious causes of liver disease. It was reported that approximately 70% of those with HCV infection suffer from persistent infection, causing active or inactive chronic hepatitis and that about 30% of patients with chronic hepatitis are assumed to develop cirrhosis within their lifetime. Once HCV infection develops into cirrhosis, hepatocellular carcinoma (HCC) develops at an annual rate of 5–7% [1]. The strong association of oxidative stress with HCV infection has been demonstrated recently and it has become possible to explain at least part of the clinical progression of the disease. The pathogenesis of chronic hepatitis C is not merely ascribed to inflammation caused by viral infection, but the role of viral proteins in the pathogenesis was also reported [2–4]. Of proteins constituting HCV, the core protein, in particular, has various functions with respect to host cells [5] and is closely related to oxidative stress. In this overview, the relationship between HCV infection and oxidative stress is reviewed focusing on the pathological effect of the core protein of HCV, and the significance of oxidative stress in the pathogenesis of liver disease will be discussed.

## 2. Oxidative stress, reactive oxygen, and the liver

### 2.1. Oxidative stress and reactive oxygen

The main source of reactive oxygen species (ROS) in hepatocytes is the mitochondria. Outside of hepatocytes, ROS also originate from nicotinamide adenine dinucleotide phosphate

(NADPH) oxidase and xanthine oxidase in Kupffer cells and inflammatory cells. Several percent of consumed oxygen is constantly converted into ROS in the mitochondria accompanied by oxygen consumption in the electron transport system (ETS, Fig. 1). Hepatocytes contain many mitochondria and therefore have a high ROS production. Generated ROS are very unstable and highly reactive, and attack biomolecules such as DNA, lipids, and proteins. The liver not only produces much ROS but is also the center of the anti-oxidative effect in the form of protein synthesis. Oxidative stress refers to the oxidation-reaction-dominant state of the living body induced by an imbalance between the oxidation reaction caused by ROS and the anti-oxidation reaction. Main ROS include superoxide ( $^{\circ}\text{O}_2^-$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and the hydroxyl radical ( $\text{HO}^{\circ}$ ). ROS are mainly produced from  $^{\circ}\text{O}_2^-$  and converted into stable  $\text{H}_2\text{O}_2$  through dismutation reaction.  $\text{H}_2\text{O}_2$  is converted into highly reactive  $\text{HO}^{\circ}$  in the presence of a transition metal.

### 2.2. Antioxidation system and oxidative stress markers

Antioxidants include glutathione (GSH), thioredoxin (TRX), vitamin E, vitamin C, and  $\beta$ -carotene. Reactive oxygen elimination enzymes include superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase. SOD is induced by oxidative stress and dismutates  $^{\circ}\text{O}_2^-$  to  $\text{H}_2\text{O}_2$  and oxygen. GSH is a compound belonging to the SH group and is highly abundant in the living body, and the SH group provides electrons to free radicals to stabilize the radicals. GSH exists in a reduced form in cells. Because it is converted into dimeric oxidized glutathione (GSSG) and becomes stable after donating electrons, GSSG prevents free radicals from continuously scrambling for electrons. GPx decomposes  $\text{H}_2\text{O}_2$  into water

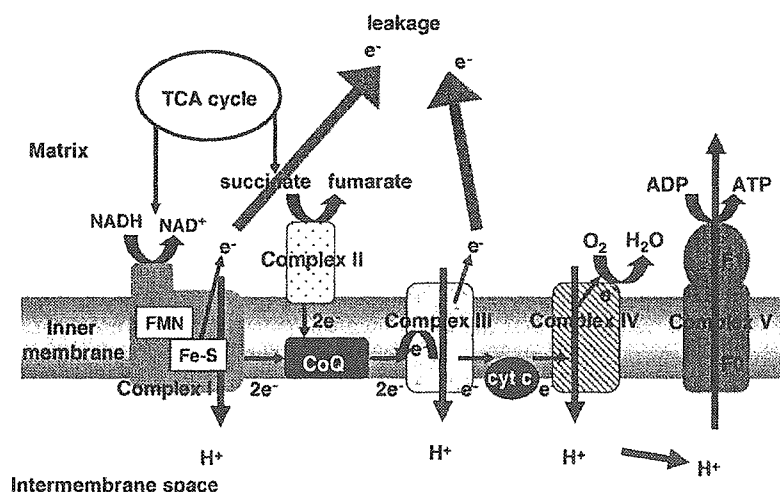


Fig. 1. The electron transfer system (ETS) of the mitochondrion. Most of the oxygen, consumed by mammalian cells, is converted to water via the mitochondrial ETS. However, up to 5% of the electrons entering the mitochondrial ETS can become uncoupled and singly leak out onto oxygen to form superoxide. Therefore, if there is impairment in the mitochondrial ETS function, it can be a cause of the overproduction of reactive oxygen species (ROS). TCA, tricarboxylic acid; NADH, nicotinamide adenine dinucleotide phosphate; FMN, flavine mononucleotide; CoQ, coenzyme Q; cyt c, cytochrome c.

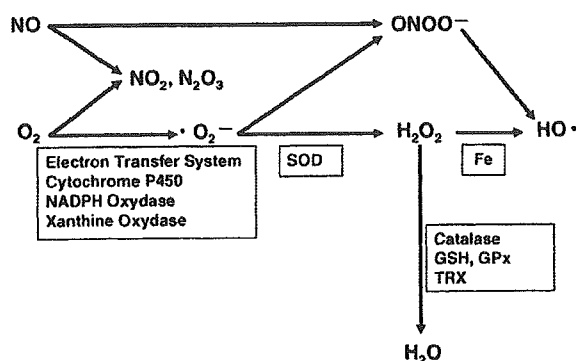


Fig. 2. Generation and scavenging of oxidative stress. SOD, super-oxide dismutase; GSH, reduced glutathione; GPx, glutathione peroxidase; TRX, thioredoxin.

and oxygen with GSH as an electron donor and reduces lipid peroxide to become neutralized. GSSG is converted back to GSH when glutathione reductase transfers an electron from NADPH to GSSG. Catalase in peroxisomes also decomposes  $H_2O_2$  to water and oxygen. TRX is also a protein induced by oxidative stress, and is reduced via the S–S binding of the substrate protein by two SH groups in TRX and acts on the  $H_2O_2$  elimination system via peroxiredoxins (Fig. 2).

ROS cause various forms of cellular damage. 4-Hydroxy-2-nonenal (HNE) and malondialdehyde (MDA) are the peroxidation reaction products of lipids, and 8-hydroxydeoxyguanosine (8-OHdG) is the product of DNA base modification (Fig. 3). These products serve as oxidative stress markers.

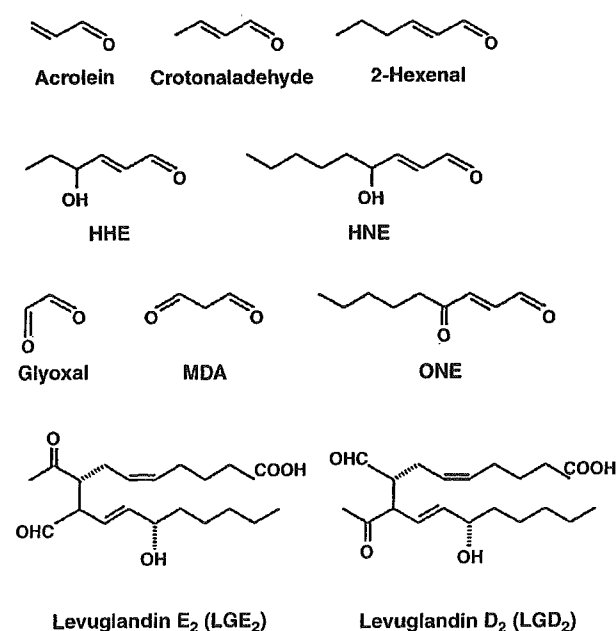


Fig. 3. Representative aldehyde species generated via lipid peroxidation reaction.

### 3. Viral infection and oxidative stress

#### 3.1. ROS production associated with viral infection

Upon viral infection, ROS are produced by NADPH oxidase and xanthine oxidase in neutrophils and macrophages. In particular, NS3, one of the non-structural proteins of HCV, was reported to induce ROS production by NADPH oxidase in neutrophils [6]. Furthermore, in viral hepatitis, ROS are also produced in hepatocytes through the release of inflammatory cytokines such as  $TNF-\alpha$  and  $IL-1\beta$  from inflammatory cells. Increased hepatic or serum 8-OHdG, HNE and MDA levels are observed in chronic hepatitis C, indicating an increase in ROS production [7–13]. Findings that indicate an increase in the activity of the ROS elimination system including decreased hepatic and blood GSH levels, an increased GSSG/GSH + GSSG ratio, and an increased serum TRX level have been reported [13–16]. The findings of markedly decreased HNE level following viral eradication with interferon [12] and decreased serum ALT and TRX levels following the administration of vitamin E, an antioxidant [17], also demonstrated that oxidative stress plays an important role in chronic hepatitis C.

#### 3.2. Nitric oxide production associated with viral infection

In the presence of an inflammation, inducible nitric oxide synthase (iNOS) is induced in macrophages and hepatocytes by  $TNF-\alpha$  and  $IFN-\gamma$  [18–20]. Other investigators reported that protein kinase (PKR) activated by double-stranded RNA formed during virus reproduction in turn activates the transcripts of  $NF-\kappa B$  and IRF-1 to induce iNOS [21]. In the case of HCV, it was reported that its constituent proteins (E2 and non-structural (NS) protein 5A) inhibit PKR activity [22,23], but iNOS induction by viral RNA via PKR is also suspected. Indeed, iNOS synthesis correlates with intrahepatic viral load in chronic hepatitis C [24].

NO is generally synthesized as a non-specific defense reaction to infectious diseases; however, in viral infection, antiviral activity may be present or absent in various viral types [20]. NO is reported to exhibit no antiviral activity against a tick-borne encephalitis virus (TBE-V), flavivirus [25], and NO may also have no antiviral activity against HCV. On the contrary, NO causes cellular damage upon its reaction to  $O_2$  or simultaneously produced  $O_2^{\cdot -}$  (reactive nitrogen species, RNS). Upon reaction to  $O_2^{\cdot -}$ , in particular, NO acts as a strong oxidant with the generation of peroxynitrous acid ( $ONOO^-$ ), and  $ONOO^-$  also produces nitrotyrosine through the nitration of aromatic amino acid residues in the presence of a transition metal. Nitrotyrosine accumulation was observed in correlation to inflammation severity in chronic hepatitis C tissue [26]; suggesting that the production of both NO and ROS increased. ROS and RNS are produced as defense factors for biological viral clearance, but these factors also have cytotoxic effects that



are assumed to contribute to the exacerbation of the disease state.

#### 4. Oxidative stress caused by viral protein

The HCV genome comprises the genes of four structural proteins and six non-structural proteins (Fig. 4), and it has been reported that at least two viral proteins cause oxidative stress in cells. The core protein, a structural protein, was found to have various actions, including the induction of oxidative stress and accumulation of lipids, in experimental studies using cultured cells and transgenic mice [2,27]. Experiments using mice transgenic for the core gene showed an increased ROS production, an increased intrahepatic catalase activity, a decreased intrahepatic GSH level and a decreased GSH/GSH – GSSG ratio indicating an anti-oxidation effect inhibition, although there was no increase in serum ALT level nor a histological finding of hepatitis. Increased levels of intrahepatic peroxide lipids in the core gene transgenic mice with aging as compared with those in the control mice also indicate increased oxidative stress. As a mechanism underlying oxidative stress induction by the core protein, mitochondrial damage is considered. Morphological abnormalities of the mitochondria were observed in the core gene transgenic mouse liver [2], and an increased ROS production caused by damage of the mitochondrial electron transport system was noted in core-protein-expressing cells [27]. Mitochondrial DNA, which has no protective proteins such as histone, is susceptible to damage by ROS [28,29]. Mitochondrial DNA in the core gene transgenic mice showed damage as early as 3-months old. This mitochondrial damage disrupts the synthesis of proteins constituting the electron transport system complex and could also increase oxidative stress caused by damage of the electron transport system.

A study using a cell culture system demonstrated that non-structural protein 5A (NS5A) also causes oxidative stress. NS5A induces endoplasmic reticulum calcium release via

endoplasmic reticulum stress, and this leads to an increased ROS production in the mitochondria [4]. Although the effect of NS5A has not been confirmed yet by other study groups, HCV has the direct action of increasing intracellular ROS production via its proteins, separate from oxidative stress induction by inflammation caused by viral infection. A report that oxidative stress was also observed in HCV carriers with a normal ALT level [13] indicates that it is caused by a direct oxidative stress induction without being mediating inflammatory reactions.

#### 5. Relationship of HCV infection with insulin resistance

The relationship of HCV infection with insulin resistance and type 2 diabetes has been suggested epidemiologically [30–32]. Insulin resistance was also observed in core gene transgenic mice before the onset of hepatic steatosis [33]. A disrupted tyrosine phosphorylation of the insulin receptor substrate (IRS-1) was observed in the liver of these transgenic mice. The analysis of hepatic tissues in patients with chronic hepatitis C not complicated by diabetes showed that insulin receptor and IRS-1 expression levels are elevated in patients with HCV infection, whereas the tyrosine phosphorylation of IRS-1 induced by insulin is inhibited. An excessive oxidative stress may be another potential cause of this insulin resistance. Oxidative stress indirectly blocks the phosphorylation of tyrosine residues of insulin receptors and IRS-1 and inhibits insulin signaling [34].

These reported results thus indicate an insulin signaling disorder in the liver infected with HCV [35]. There has been no report to date directly proving that hepatic insulin signaling disorder in patients with HCV infection is attributable to oxidative stress. However, because diabetes, which is the state of having abnormally high blood sugar levels that cannot be self-regulated by individual organisms, also induces oxidative stress [34], the close relationship between insulin resistance or diabetes and oxidative stress as the cause and the

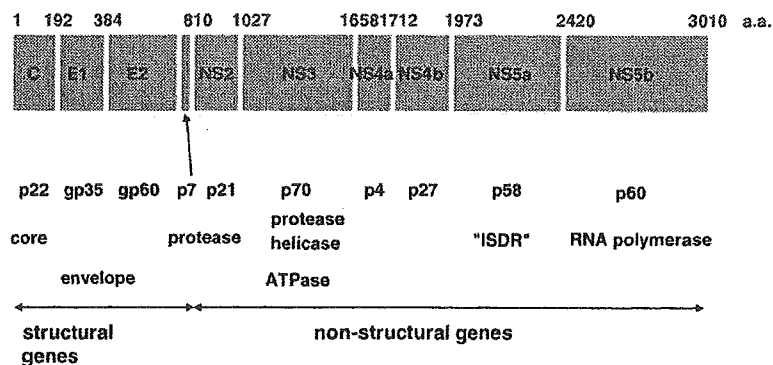


Fig. 4. Structure of hepatitis C virus genome. The genome of HCV consists of two parts, structural and non-structural regions. The former comprises the core and envelope regions, and the latter consists of NS2 to NS5a, which regions chiefly code enzymes necessary for viral replication. NS, non-structural; ISDR, interferon sensitivity-determining region.

result, respectively, is a very interesting issue to investigate in the future.

## 6. Relationship of HCV infection with hepatic steatosis

Hepatic steatosis is frequently observed in patients with HCV infection. The relationships of HCV infection with intrahepatic viral loads and core protein levels, different prevalence of hepatic steatosis by viral genotype [higher incidence for genotype 3a), and improved steatosis following viral eradication were reported [36–38]. It is presumed from these reports that HCV itself causes hepatic steatosis. A similar hepatic steatosis caused even by the core protein alone was observed in a study using an expression system in cultured cells and transgenic mice, and it was thus suggested that the core protein plays a significant role in hepatic steatosis as the direct action of HCV [39,40]. Hyperinsulinemia induced by insulin resistance mentioned above causes the overloading of the liver with fatty acids from fat cells, and mitochondrial damage inhibits the  $\beta$ -oxidation of fatty acids [41]. Furthermore, the core protein was reported to inhibit microsomal triglyceride transfer protein (MTP) activity that is required when neutral fat is released as very low-density lipoproteins (VLDLs) [42]. All these actions could cause hepatic steatosis. In the liver of non-alcoholic steatohepatitis (NASH) patients, it was reported that  $\beta$ -oxidation in the mitochondria and peroxisomes or the metabolism of fatty acids by cytochrome P450 2E1 (CYP2E1) in microsomes is promoted under an excessive load of fatty acids, resulting in ROS production [43,44]. In HCV infection as well, intrahepatic fat accumulation possibly increases ROS production as in NASH. Because hepatic steatosis in chronic hepatitis C was reported to be a factor for disease progression [45–47], increased oxidative stress associated with hepatic steatosis is presumably involved in disease progression.

## 7. Iron and reactive oxygen

The iron content in the liver and spleen is high, and transition metals facilitate electron transfer and play an important role in the production of free radicals. Iron in combination with transferrin and ferritin is stable, but an unstable iron ion is freed when ferritin is decomposed by lysosomes [48]. ROS additionally promote iron release from ferritin [49]. A free iron ion catalyzes changes from relatively poor reactive  $O_2^-$  and  $H_2O_2$  to a highly reactive  $HO^\bullet$  (Fenton reaction) [50,51].  $HO^\bullet$  oxidizes membrane phospholipids, which compose cells and intracellular organelles, and iron forms radicals from produced peroxide lipids, thereby enhancing lipid peroxidation. Iron site-specifically combines with DNA and promotes DNA damage caused by ROS. Iron also increases ROS production by CYP2E1 [52]. A report that an enhanced peroxidation of intrahepatic lipids is attenuated by exsan-

guination in hemochromatosis also supports the involvement of iron in oxidative stress [53].

An excessively high iron content in the liver was observed in chronic hepatitis C [8,54]. Other investigators reported that iron removal therapy by exsanguination of chronic hepatitis C patients significantly improves serum ALT level without affecting viral load [55–57]. Another study showed that hepatic impairment is exacerbated following the administration of iron to chimpanzees with chronic hepatitis C [58]. Furthermore, oxidative stress is decreased by the iron removal therapy for chronic hepatitis C using intrahepatic 8-OHdG level as an index [57]. The above-mentioned reports show the close relationships of chronic hepatitis C with iron metabolism and oxidative stress.

## 8. Interactions with alcohol

Alcohol metabolism plays an important role in ROS production. Mainly alcohol dehydrogenase (ADH) in the cytosol and CYP2E1 (microsomal ethanol-oxidizing system) in microsomes are responsible for alcohol metabolism in the liver. When alcohol dehydrogenase oxidizes ethanol to acetaldehyde, the reduction from  $NAD^+$  to NADH simultaneously occurs. NADH accumulation causes stress on the mitochondrial electron transfer system, leading to an increased production of ROS [59]. NADH also inhibits xanthine dehydrogenase activity, and xanthine is thereby oxidized by xanthine oxidase with the production of ROS [60]. CYP2E1 is induced by chronic alcohol intake and ROS are produced when CYP2E1 oxidizes ethanol to acetaldehyde [52,61].

There is no significant difference in hepatic peroxide level between core gene transgenic mice at 3–6-months old and control transgenic mice, but hepatic peroxide level significantly increases following the administration of a low dose of alcohol in the core gene transgenic mice [2]. ROS production increases upon glutathione reduction in HepG2 cells, with the co-expression of the core protein and CYP2D1, the latter of which is induced by alcohol [62]. These findings show that the core protein and alcohol in combination increase oxidative stress. Indeed, it was reported that alcohol intake plays a role in promoting the progression of chronic hepatitis C [63,64] and that increased levels of oxidative stress markers such as HNE and lipid hydroperoxide also support these findings [65]. From the viewpoint of oxidative stress also, HCV infection and alcohol intake are both considered to promote hepatic impairment.

## 9. Hepatocarcinogenesis and oxidative stress

It has been demonstrated that oxidative stress plays a key role in carcinogenesis [66,67]. Animal experiments using hepatocarcinogenesis models with the administration of a chemical substance (diethyl-nitrosamine, peroxisome proliferators) and with the administration of a choline-deficient

amino acid diet also indicates the involvement of oxidative stress [68–72]. In Long Evans Cinnamon (LEC) rats, an animal model that spontaneously develops heritable hepatitis and HCC caused by an abnormal copper accumulation, a congenitally decreased glutathione peroxidase expression level was reported, and the close relationship between oxidative stress and hepatocarcinogenesis was indicated [73].

The epidemiological relationship between HCV infection and HCC is evident [74,75], but the mechanism underlying this relationship has not been fully elucidated yet. Among postulated hypotheses on the mechanism of HCV-associated hepatocarcinogenesis, that of the involvement of the viral protein, in particular, the core protein of HCV is attractive: HCC develops in core gene transgenic mice, and carcinogenesis starts with well-differentiated carcinoma with an excessively high fat content, similar to hepatocarcinogenesis in human chronic hepatitis C, and poorly differentiated carcinoma with a low fat content develops in the form of “nodules in nodules” [76]. Because oxidative stress is increased in the core gene transgenic mice as mentioned above, it is assumed that oxidative stress plays an important role in hepatocarcinogenesis in chronic hepatitis C. Because the development of HCC is also observed in transgenic mice carrying the full-length HCV protein gene, the non-structural protein may have an additive effect to the effect of the structural proteins including the core protein, contributing to hepatocarcinogenesis [77]. NS5A, which was also reported to induce ROS production [4], may also contribute to hepatocarcinogenesis, although ROS induction by NS5A is not unequivocally confirmed yet.

Mitochondrial DNA has no potent protective proteins such as histone and is near the electron transport system, the major ROS production site. Hence, it is 10 to 15 times more susceptible to mutation caused by ROS than nuclear DNA [28,29]. In an investigation of mitochondrial DNA mutation in the human normal liver, both cancerous and non-cancerous liver tissues in patients with HCC showed very high incidences of DNA mutations [78]; thus, a relationship between oxidative stress persistence and hepatocarcinogenesis is suggested.

In the core protein expression system in the hepatic tissue and cultured cells of core gene transgenic mice, the activation of transcription factor AP-1 via mitogen-activated protein (MAP) kinase was observed [79–83]. The activation of the transcription factors AP-1, NF- $\kappa$ B, and signal transducer and activator of transcription (STAT) 3 by NS5A were also reported [4,84]. The activation of these transcription factors may facilitate cell proliferation, contributing to tumorigenic transformation.

It was also reported that ROS facilitate apoptosis via c-Jun N-terminal kinase (JNK)/p38 MAP kinase or by directly attacking the mitochondria. Apoptosis is a protective mechanism of the host against viral infection and carcinogenesis. Some reports stated that the core protein facilitates apoptosis [85–88], whereas other reports stated that the core protein inhibits apoptosis [89–92]; thus, no fixed view has yet been established. If it indeed inhibits apoptosis, it is assumed that this inhibition proceeds by maintaining oxidative stress and

that the core protein has a beneficial effect against carcinogenesis and persistent viral infection.

In HCV infection, viral proteins such as the core protein and, possibly, NS5A protein induce oxidative stress, intracellular signaling, and transcription factors, which are not reflected in blood ALT level, contributing to the progression of carcinogenesis. Carcinogenesis, however, is slow as is observed in humans and core gene transgenic mice, the latter of which developed HCC in the latter half of their life. Recently, Okanoue et al. reported a long-term follow-up study of subjects with persistent HCV infection who had persistently normal ALT levels (PNAL) [93]. In their study, serum thioredoxin levels were not elevated in those with PNAL compared to those with chronic hepatitis. This may apparently seem contradictory to the results of our above-mentioned animal model studies. However, we should realize that anti-oxidant system is also instrumental in the liver. In these relatively younger people with PNAL than those with CH [93], active anti-oxidant system may erase the apparent elevation of ROS. Such a phenomenon was described in a mouse model by Moriya et al. [2], in which ROS was apparently normal in young core gene transgenic mice with the activation of catalase and reduction of GSH. Clinically, the presence of inflammation is thought to facilitate the process of hepatocarcinogenesis.

## 10. Conclusions

A very close pathological relationship between oxidative stress and HCV infection is observed, as shown by the above overview of relevant publications and discussion. The causes of oxidative stress in HCV infection are considered to include various factors such as mitochondrial damage, endoplasmic reticulum stress, iron accumulation, and lipid accumulation in the liver. Various study results demonstrated that even only viral proteins, mainly the HCV core protein, cause oxidative stress. When inflammation via immunoreactions to viral infection is added to oxidative stress, ROS production is expected to further increase, leading to a state in which the anti-oxidation system cannot cope with. In this sense, inflammation in chronic hepatitis C is considered to be qualitatively different from inflammation observed in other types of hepatitis such as autoimmune hepatitis or hepatitis B [94] (Fig. 5). As a treatment of chronic hepatitis C, the eradication of the virus is ideal. If it is not possible, however, the control of factors that exacerbate oxidative stress, such as inflammation via immune reaction and alcohol, and the relief of oxidative stress by the iron removal therapy and the administration of an anti-oxidation agent are considered to delay the progression of chronic hepatitis.

The development of such new anti-oxidation agents is being awaited. In further studies on the development of new therapies for hepatitis C and control methods for hepatocarcinogenesis in the future, the importance of those focusing on oxidative stress is expected to markedly increase.

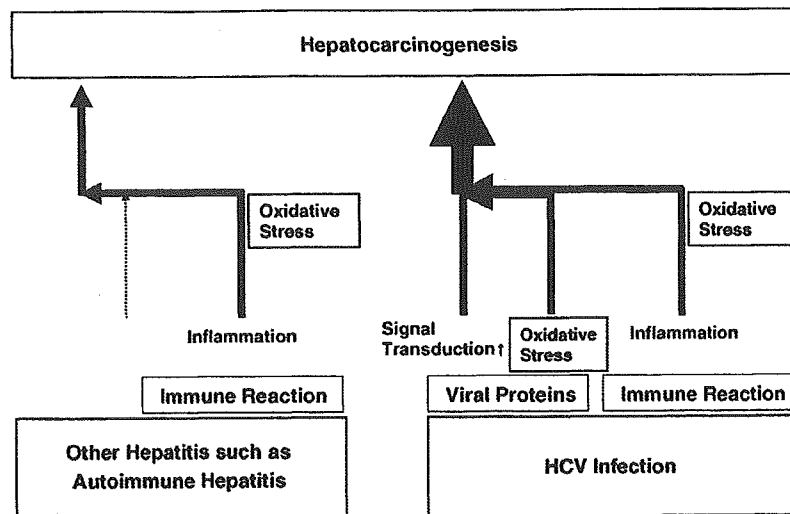


Fig. 5. Oxidative stress and hepatocarcinogenesis in various types of hepatitis (hypothesis). Oxidative stress is generated in all types of hepatitis via inflammation accompanied by continual cell death and regeneration. In HCV infection, HCV itself causes the production of oxidative stress in a synergy with inflammation. In this sense, the quality of “inflammation” in HCV infection may be different from that in other types of hepatitis. Additional impact of HCV proteins on the intracellular signal transduction would provoke the development of HCC. These may explain the conspicuous properties of HCC development.

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