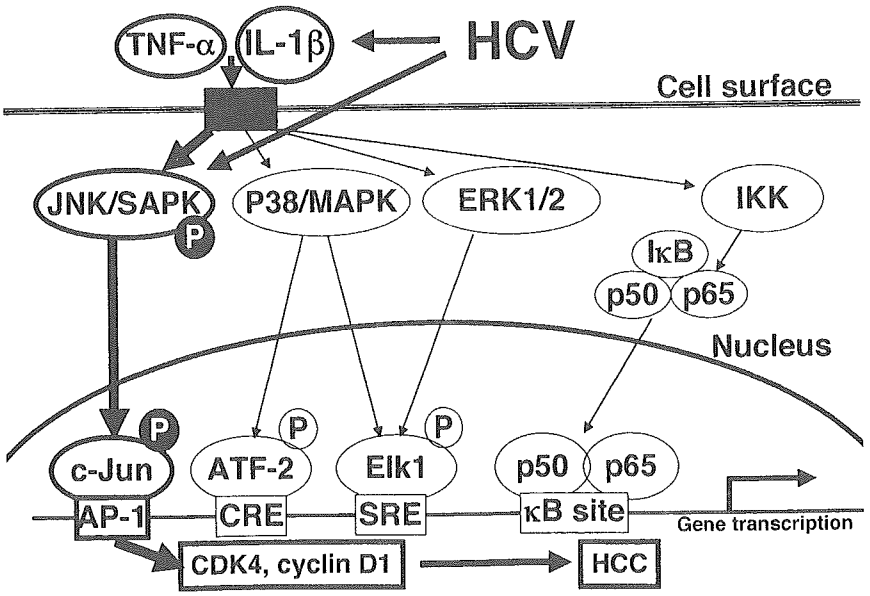

Fig. 4. *TNF*, tumor necrosis factor; *IL*, interleukin

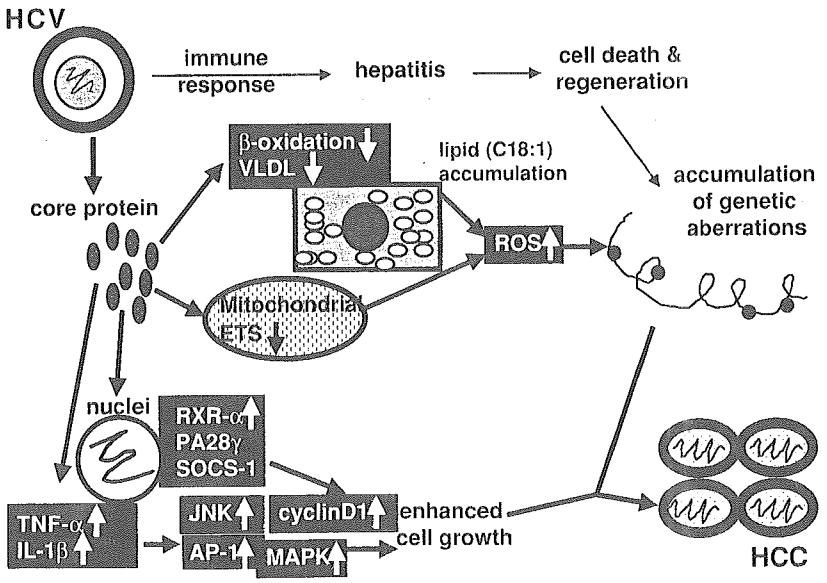

Fig. 5. Molecular pathogenesis of liver disease in HCV infection. The induction of oxidative stress, together with hepatic steatosis induced by the HCV core protein could play a pivotal role in the development of HCC. Alterations in the expressions of cellular genes, such as *TNF-α* or *SOCS-1*, and alterations in the intracellular signaling pathways, including c-Jun N terminal kinase (*JNK*), could be co-accelerators of hepatocarcinogenesis in HCV infection. The activation of intracellular signaling pathway has not been found in nonalcoholic steatohepatitis (NASH), while induction of oxidative stress may be common in the pathogenesis of both hepatitis C and NASH. *HCC*, hepatocellular carcinoma; *TNF-α*, tumor necrosis factor-α; *SOCS-1*, suppressor of cytokine signaling-1; *VLDL*, very low density lipoprotein; *ROS*, reactive oxygen species; *RXR-α*, retinoid X receptor; *PA28γ*, proteasome activator; *AP1*, activator protein

Though not yet completely elucidated, the pathogenesis of HCC in HCV infection has been substantially understood by the analysis of animal models.[23,24] For instance, in the MAPK intracellular signaling system, c-Jun N-terminal kinase (JNK) is activated in the liver by HCV. Downstream of JNK, a transcription factor, AP1, and cell-cycle molecules, CDK4 and cyclin D1, are subsequently activated, conferring a proliferative advantage to hepatocytes (Fig. 4).[16,23] Such activation of cellular gene expression and signaling systems has not yet been identified for NASH. The overproduction of ROS, together with the presence of hepatic steatosis, may be a common pathway to hepatocarcinogenesis in both hepatitis C and NASH (Fig. 5, upper half). However, the alterations in cellular gene expression and/or intracellular signaling systems occur only in hepatitis C in the presence of the viral protein(s), putting chronic hepatitis C onto the fast track to the development of HCC (Fig. 5, lower half). This aspect, observed in HCV infection, is distinct from NASH, which may explain the difference in the incidence of HCC in the two conditions. Based on the analogous metabolic pathways of hepatitis C and NASH, but taking into account the differences cellular gene expression and/or intracellular signaling systems,

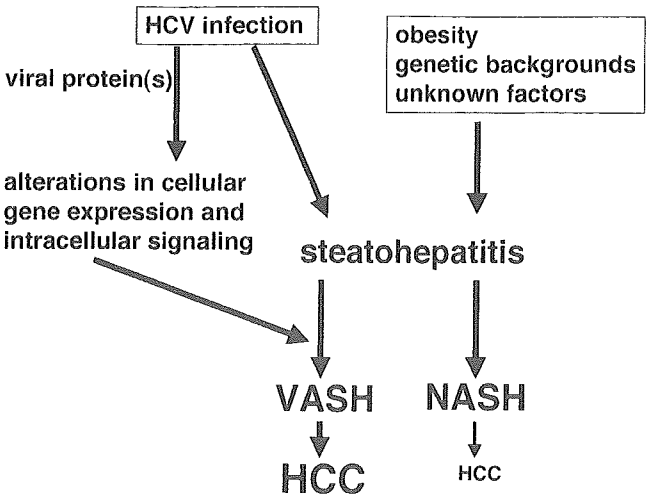

Fig. 6. Virus-associated steatohepatitis (*VASH*). HCV infection and NASH show a similar phenotype, steatohepatitis. However, in HCV infection, the presence of the viral proteins; in particular, the core protein of HCV, confers alterations in cellular gene expression and intracellular signaling systems to hepatocytes, leading to the high incidence of hepatocellular carcinoma (*HCC*). *HCV*, hepatitis C virus; *NASH*, nonalcoholic steatohepatitis

which are induced by the viral protein, we would like to propose the term "virus-associated steatohepatitis (VASH)" for hepatitis C (Fig. 6).

Perspectives for therapeutic strategies

We have demonstrated that HCV per se induces insulin resistance in an animal model. A high-fat diet and obesity superimposed on HCV infection lead to overt diabetes. In view of the progression of chronic hepatitis C being accelerated by insulin resistance, insulin resistance would naturally influence the development of HCC. Although an association between NASH and HCC has not been established, investigation of such an association needs to be pursued energetically, in view of the histological homology of NASH to chronic hepatitis C. Drugs for improving glucose metabolism and reducing insulin resistance need to be kept in mind in the treatment of hepatitis C patients who have failed to respond to antiviral treatment, because such drugs may well prevent the progression of fibrosis and the development of HCC in such patients. The traditional "high-protein and high-calorie" diet, advocated for chronic hepatitis patients in Japan, especially post-World War II, is obviously detrimental, except in some patients with advanced cirrhosis. Because hepatitis C is an infectious disease, the eradication of the virus is, naturally, the most efficient way to cure the disease. However, nearly one-half of chronic hepatitis C patients who were treated with interferon/ribavirin combination therapy did not achieve eradication of the virus.[25] Therefore, besides anti-viral treatment for HCV, consultations with hepatitis C patients on their dietary habits should include recommendations for iron restriction,[26] as well as weight control, because a high-calorie intake is likely to accelerate hepatic fibrosis by aggravating insulin resistance in these patients.

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Hepatitis C as a metabolic disease: Implication for the pathogenesis of NASH

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Abstract

In addition to the link with development of hepatocellular carcinoma (HCC), hepatitis C virus (HCV) infection is associated with several extrahepatic manifestations such as essential mixed cryoglobulinemia, porphyria cutanea tarda or Sjögren's syndrome. A role of hepatic steatosis in the pathogenesis of chronic hepatitis C has also been known, implying hepatitis C as a metabolic disease. In addition, recent epidemiological studies have suggested a linkage between type 2 diabetes and chronic HCV infection. However, the presence of additional factors in patients, such as obesity, aging or cirrhosis, prevents the establishment of a definite relationship between HCV infection and these two conditions, lipid metabolism disturbance and diabetes. In addition to the data indicating the presence of dyslipidemia and diabetes or insulin resistance in our cohort of chronic hepatitis C patients, we found a series of evidence showing the association between the conditions and HCV infection in mouse models that are transgenic for the HCV genes.

In patients with chronic hepatitis C, a significant decrease in the serum levels of total cholesterol and apolipoproteins C2 and C3 was observed compared to those with chronic hepatitis B that were comparable in liver function. In an animal model, C18:1 mono-unsaturated fatty acids were significantly increased in the liver from HCV core gene transgenic mice, which was similarly observed in the liver from human hepatitis C patients. Thus, a disturbance in lipid metabolism was observed in both humans and an HCV mouse model, supporting that it is a specific event in HCV infection.

A significant increase in the value of an indicator for homeostasis model assessment of insulin resistance (HOMA-IR), was observed in patients with chronic hepatitis C, even at the very early stage of chronic hepatitis. In the animal model, a marked insulin resistance was exhibited from a very young age in HCV core gene transgenic mice. Insulin resistance observed in the core gene transgenic mice was chiefly due to the shortage of insulin action on the suppression of glucose production in the liver. Thus, the ability of insulin to lower the plasma glucose level in the HCV transgenic mice was impaired, as observed in chronic hepatitis C patients. These results provide a direct experimental evidence for the contribution of HCV in the development of insulin resistance in human HCV infection, which finally leads to the development of type 2 diabetes.

Insulin resistance may be a critical factor in the pathogenesis of chronic hepatitis C as recently suggested in non-alcoholic steatohepatitis (NASH), along with impairment in lipid metabolism. Our results would provide a clue for further understanding of pathobiology of HCV infection, and may provide an implication for the pathogenesis of NASH.

Keywords: Diabetes; Hepatitis C virus; Insulin resistance; NASH; Hepatocarcinogenesis; Transgenic mouse

1. Introduction

Approximately 1.8 million people in Japan and 200 million people in the world are chronically infected with hepatitis C virus (HCV). Chronic HCV infection may lead to cirrhosis and hepatocellular carcinoma (HCC), thereby being a worldwide problem both in medical and socio-economical aspects [1]. In addition, chronic HCV infection is a multifaceted disease, which is associated with numerous clinical manifestations, such as type II mixed cryoglobulinemia, porphyria cutanea tarda and membranoproliferative glomerulonephritis [2]. Furthermore, a strong association of HCV infection with Sjögren's syndrome and lichen

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planus have been noted, which is validated in the animal model [3].

In addition, recently, there have been increasing lines of evidence to indicate metabolic disturbances in HCV infection, which would influence the pathogenesis of chronic hepatitis C. The discovery of HCV in 1989 enabled the comparison between chronic hepatitis C and the other chronic hepatitis, resulting in repeated reports that steatosis is significantly associated with chronic hepatitis C [4,5]. Steatosis in HCV infection is reproduced in animal models [6] or cultured cells [7], strengthening a pathologic role of HCV in it. Furthermore, patients infected with HCV have abnormalities in serum lipids, such as hypocholesterolemia or abnormal levels of apolipoproteins in serum [8,9]; they are corrected in sustained virological responders to antiviral treatment [9]. Thus, the association between HCV infection and disturbance in lipid metabolism has become increasingly strong both in patients and experimental systems including animals. Finally, patients with chronic hepatitis C accompanied by severe steatosis develop hepatic fibrosis more rapidly [10]. Thus, abnormal lipid metabolism in HCV infection would be deeply involved in the pathogenesis of hepatitis C.

2. Diabetes may also be a manifestation of HCV infection

The next character appearing as a metabolic aspect of HCV infection is type 2 diabetes. In 1994, Allison et al. [11] reported an epidemiological link between diabetes and HCV infection, but in a cirrhotic cohort. There was little impact, however, in view of well-known impaired glucose tolerance in advanced chronic liver disease. Several reports followed along this line from the same group and others. The trend to accept a positive association between diabetes and HCV infection seems to have been triggered by the population-based study in the United States [12], in which a solid association was found between them. The association between diabetes and HCV infection, however, is blemished by factors such as the development of cirrhosis, obesity and ageing common in patients with hepatitis C; they would make it difficult to prove this association as real. Hence, there is a need to evaluate the association using experimental systems.

3. HCV induces insulin resistance in vivo

We used mice transgenic for the HCV core gene [6,13] to assess the association between HCV infection and diabetes. These mice carry the core gene of genotype 1b HCV, and express HCV core protein of an expected size in the liver, in levels comparable to those in patients with chronic hepatitis C (Fig. 1).

They develop HCC late in life [13]. These transgenic mice were maintained and fed together with their normal littermates, and the glucose metabolism was studied [14]. Although the core gene transgenic mice did not develop overt diabetes, they had markedly elevated serum levels of insulin. Plasma glucose levels were somewhat higher in transgenic mice than their normal littermates control mice with no significant difference between them. In contrast, serum insulin levels were significantly higher in transgenic than normal control mice in both fasting and fed conditions (Fig. 2).

Since such a combination of normal glucose levels and hyperinsulinemia points to the insulin resistance, then, we conducted glucose and insulin resistance. The core gene transgenic mice exhibited glucose levels a little higher than normal littermates without any significant differences between them. In insulin resistance tests, glucose levels were significantly higher in transgenic than normal control mice both 40 and 60 min after injection with insulin (Fig. 3). These

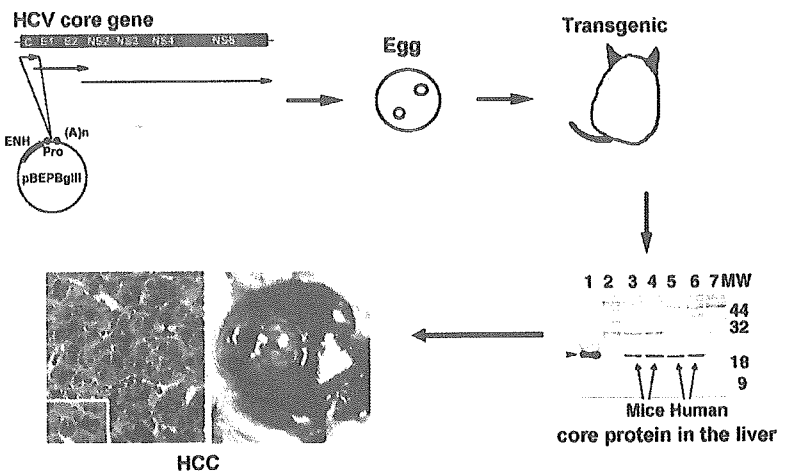

Fig. 1. Hepatitis C virus core gene transgenic mouse. These mice carry the core gene alone of genotype 1b HCV and express the core protein of an expected size in the liver, at levels comparable to those in human chronic hepatitis C patients. The mice eventually develop HCC late in life.

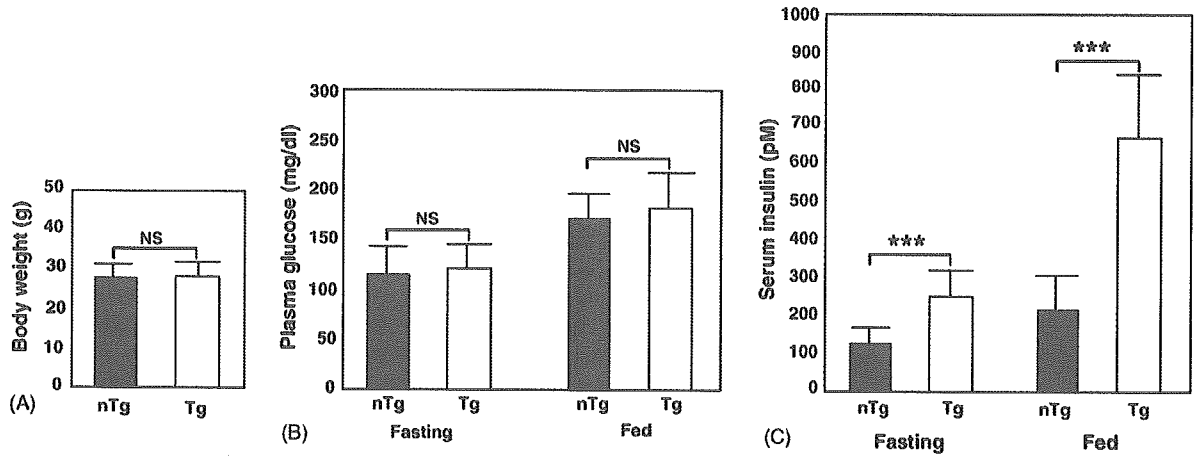

Fig. 2. Altered glucose homeostasis in hepatitis C virus core gene transgenic mice: (A) body weight of 2-month-old mice; (B) plasma glucose levels in fasting or fed mice; (C) serum insulin levels in fasting or fed mice. The insulin level was significantly higher in the core gene transgenic mice than in control mice. Values are mean ± S.E.; ***$p < 0.001$; NS, statistically not significant; nTg, nontransgenic mice; Tg, transgenic mice.

results indicate the presence of insulin resistance in the core gene transgenic mice. Since only the HCV core gene had been incorporated into these transgenic mice, the core protein of HCV would be able to induce insulin resistance in vivo.

By what mechanism, then, insulin resistance observed in the animal model would arise? The insulin resistance is considered to involve two factors. They are the central and peripheral insulin resistances (Table 1) [15]. The hyperinsulinemic–euglycemic clamp method was employed for differentiating between them. In this method, hepatic glucose production (HGP) is calculated on the basis of amounts of glucose required for keeping plasma glucose levels within a certain range at serum insulin levels higher than physiological ones. In normal control mice, HPG was suppressed by 60% by the administration of insulin, in contrast to core gene transgenic mice, in which there was only marginal suppression of HGP by insulin. These results indicate a hepatic (central) origin of insulin resistance in the transgenic mice. In further confirmation of this, uptake of glucose into the muscle was determined. There was no difference in the uptake in response to administration of insulin between transgenic

Table 1
Insulin resistance

1. Peripheral insulin resistance: a shortage of insulin action in the muscle (deficit in the insulin-induced glucose uptake into the muscles)
2. Central insulin resistance: a shortage of insulin action in the liver (deficit in the insulin-induced suppression of glucose production in the liver)

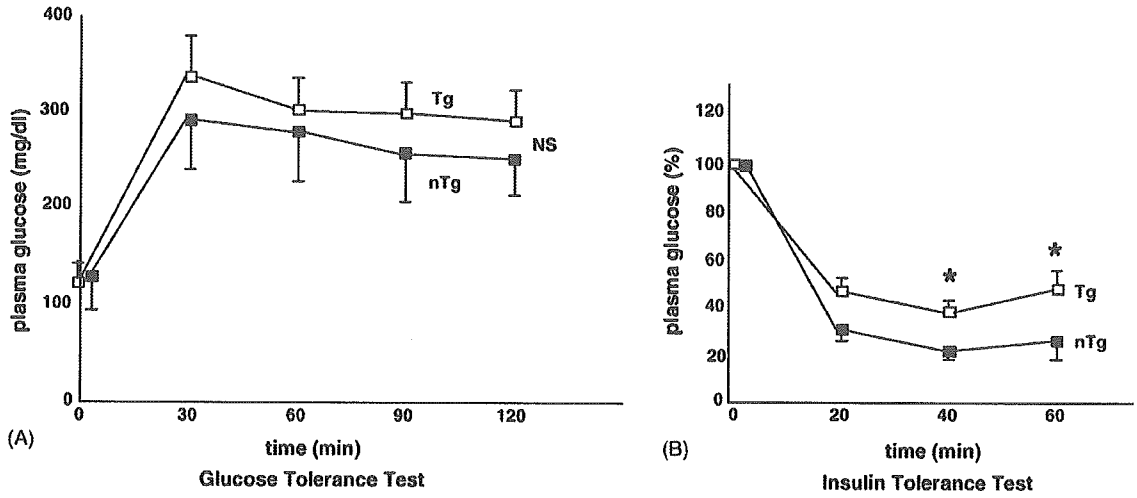

Fig. 3. Insulin resistance in the core gene transgenic mice: (A) Glucose tolerance test. Animals were fasted overnight. D-Glucose (1 g/kg body weight) was administered by i.p. injection to conscious mice, and plasma glucose levels were determined at the time points indicated; (B) Insulin tolerance test. Human insulin (1 U/kg body weight) was administered by i.p. injection to fasted conscious mice and glucose concentrations were determined. Values were normalized to the baseline glucose concentration at the time of insulin administration. Values are mean ± S.E.; *$p < 0.05$; NS, statistically not significant; nTg, nontransgenic mice; Tg, transgenic mice.

and normal control mice. The insulin resistance in mice transgenic for the HCV core gene, therefore, is central and hepatic.

4. The mechanism underlying insulin resistance in HCV infection

Next, we evaluated how insulin resistance emerges in the mouse model. For this purpose, liver homogenate was immunoblotted with anti-phosphotyrosine and anti-phosphserine 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. After the administration of insulin, however, the phosphorylation of tyrosines in IRS-1 increased in normal but not transgenic mice. Obtained results suggested disturbance in tyrosine phosphorylation as one of the factors for insulin resistance in the liver. There were no differences in phosphorylation of serines in IRS-1 or tyrosines in IRS-2 between transgenic and normal control mice. Overall, they provided experimental evidence for development of insulin resistance by the presence of HCV in the liver that would disturb the transduction of insulin signaling in hepatocytes (Fig. 4). There remains a possibility for the HCV core protein that would 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 core gene transgenic mice, only TNF-α and IL-1β levels have been found increased [16]. For the purpose of evaluating the role of TNF-α in insulin resistance in transgenic mice, therefore, serum insulin was determined and insulin resistance test performed in them after they had received anti-TNF-α intraperitoneally. Pretreatment with anti-TNF-α partially restored insulin sensitivity in the core gene transgenic mice. Albeit a direct anti-insulin activity of the core protein cannot be excluded, high levels of TNF-α in the liver would be one of the factors for induction of insulin resistance in this mouse model.

5. Insulin resistance as a risk factor for progression of hepatic fibrosis

Insulin resistance in HCV infection may have an additional significant clinical implication. In 260 patients with chronic hepatitis C, Hui et al. [17] have tried to establish the relationship between liver histology and indicators of glucose metabolism as well as insulin resistance represented by homeostasis model assessment of insulin resistance (HOMA-IR). 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. HOMA-IR was a significant and independent predictor for the stage and velocity of hepatic fibrosis. The results of their study are important, because they implicate a role of hyperinsulinemia, and insulin resistance by inference, in promoting the progression of hepatic fibrosis. Insulin has been proven for an aggravating factor not only in atherosclerosis but also systemic inflammation and fibrosis. The liver would not be an exception in this respect.

6. Similarities and differences between hepatitis C and NASH: implication for the pathogenesis of NASH

We have demonstrated that HCV per se induces insulin resistance in the animal model. High-fat diet and obesity superimposed on it lead to overt diabetes [14]. In view of the progression of chronic hepatitis C accelerated by insulin resistance, insulin resistance would naturally influence the development of HCC. Although the association has not been

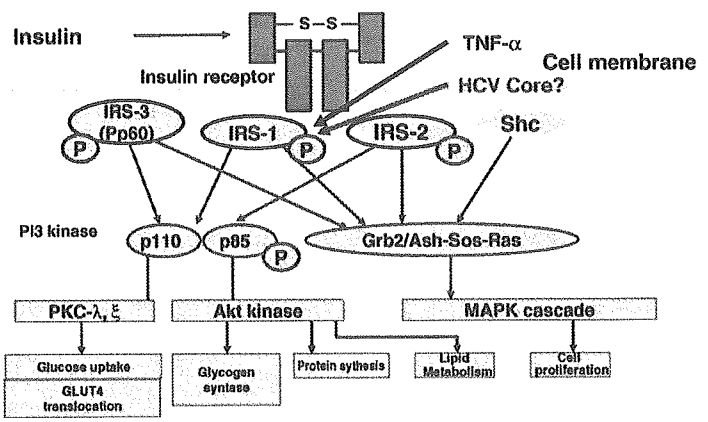

Fig. 4. Insulin resistance and HCV infection, HCV core protein or elevated intrahepatic TNF-α inhibits tyrosine phosphorylation of IRS-1 in the liver, suppresses insulin intracellular signal transduction and leads to insulin resistance. PKC, protein kinase C; PI3-kinase, phosphatidyl inositol 3 kinase; MAPK, mitogen-activated protein kinase; IRS, insulin receptor substrate.

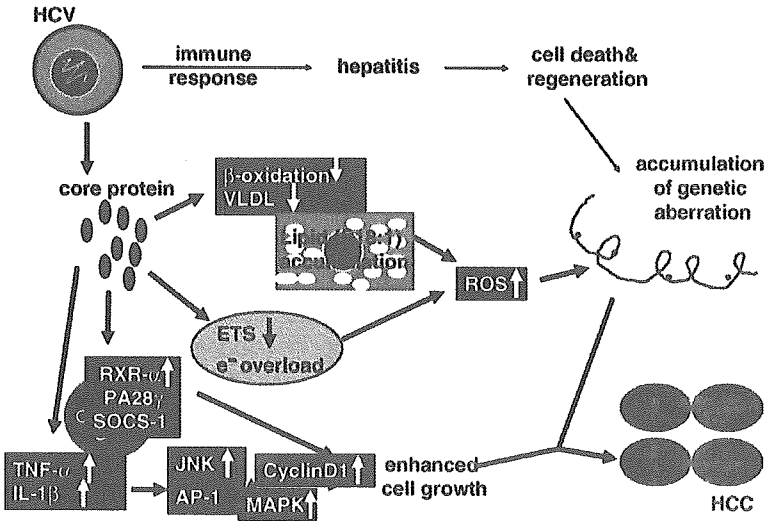

Fig. 5. Molecular hepatocarcinogenesis in HCV infection. Oxidative stress together with hepatic steatosis induced by the HCV core protein would play a pivotal role in the development of HCC. Alterations in cellular gene expressions, such as TNF-α or SOCS-1, and those in the intracellular signaling pathways 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-α, tumor necrosis factor-α; 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-β-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-α, are considered to be critical in the pathogenesis of these conditions. TNF-α levels are increased in patients with hepatitis C and are implicated in insulin resistance. Single nucleotide polymorphism in TNF-α 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).

Table 2
Comparison of hepatitis C and NASH

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

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 HASH should be investigated. The

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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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.

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

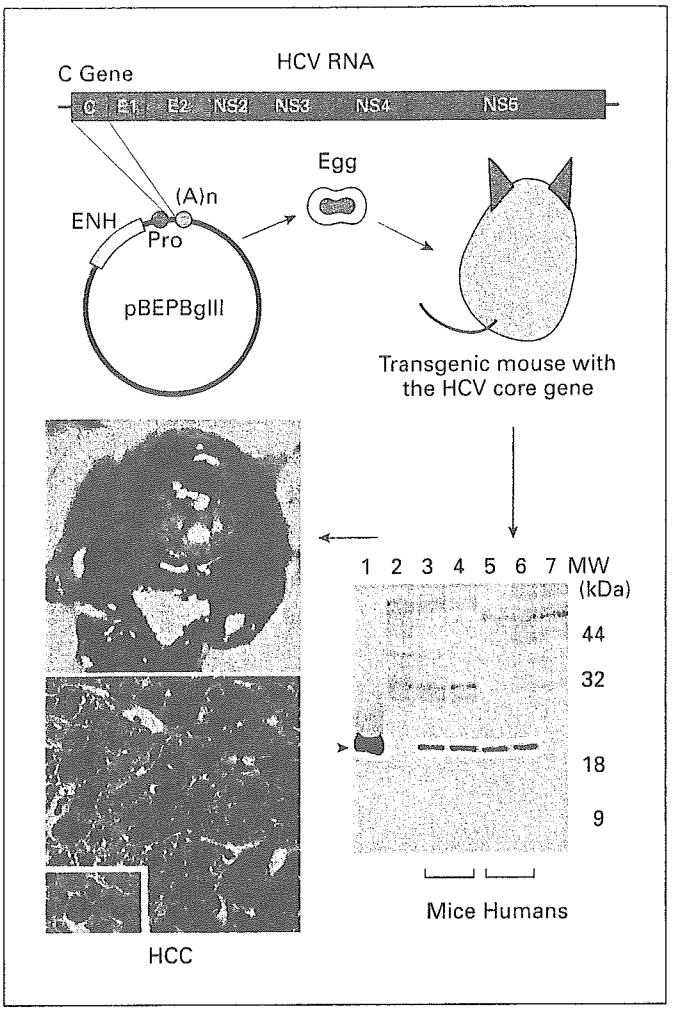

Fig. 1. Expression of HCV core gene in transgenic mouse. It carries the core gene of HCV genotype 1b alone and expresses the core protein of expected size in the liver, at levels similar to those in human patients. Mice eventually develop HCC later in their lives.

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.

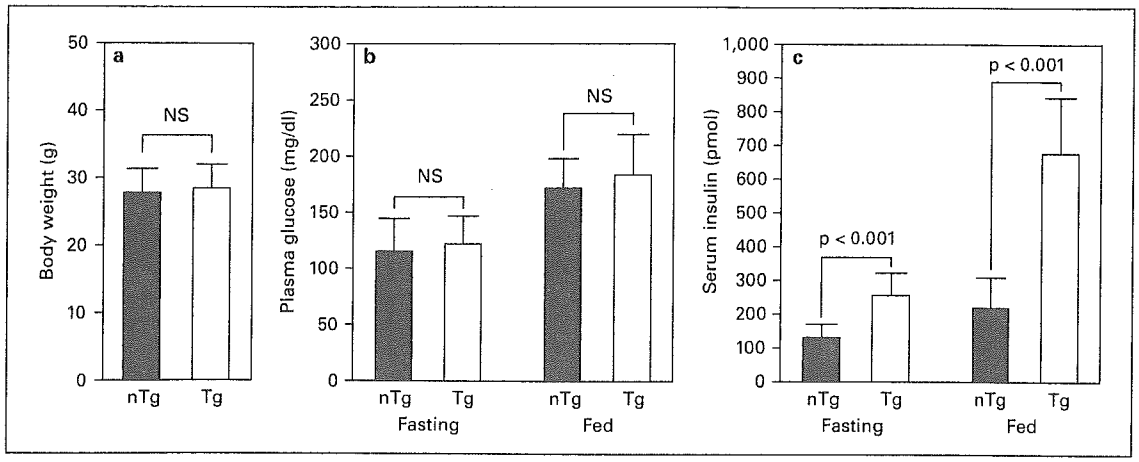

Fig. 2. Altered homeostasis of glucose in mice transgenic for the HCV core gene. Body weight of 2-month-old mice (**a**), plasma glucose levels in fasting or fed mice (**b**) and serum insulin levels in fasting or fed mice (**c**) are shown. Values represent means ± SE. NS = Not significant statistically;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

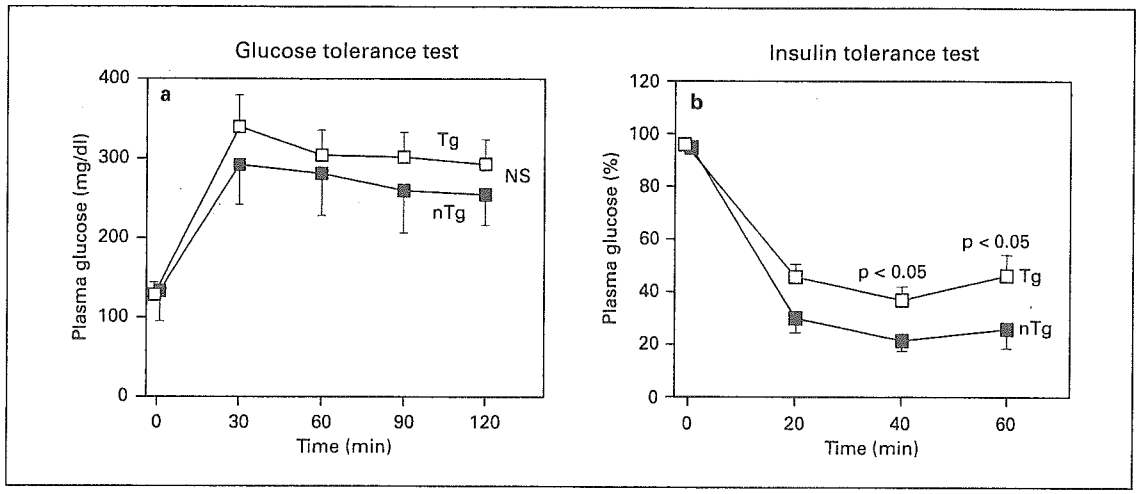

Fig. 3. Insulin resistance in transgenic mice. Glucose tolerance in mice after overnight fasting (**a**). *D*-Glucose (1 g/kg body weight) was given intraperitoneally to conscious mice, and plasma glucose levels were determined at time points indicated. **b** Insulin tolerance in mice fasted overnight. 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 glucose required to keep plasma glucose levels within a certain range at serum insulin levels higher than physiological ones. In normal control mice, HPG 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-α and IL-1β have been found with an increased expression [22]. Therefore, for the purpose of evaluating the role of

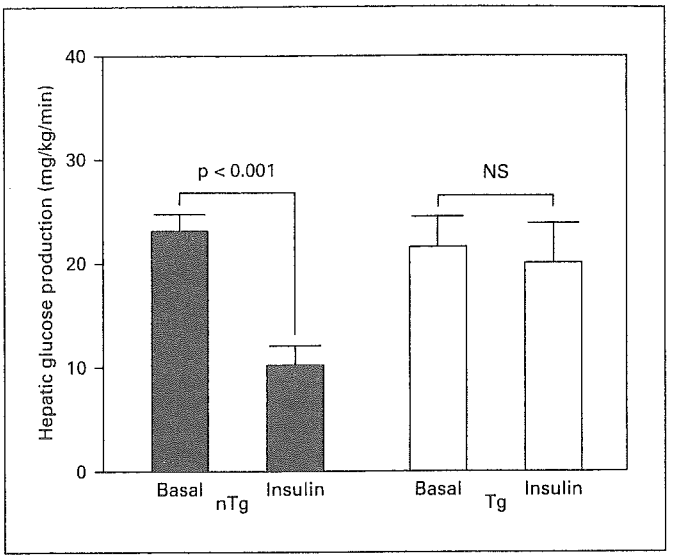

Fig. 4. Characterization of glucose metabolism in transgenic mice. Glucose production in the liver was calculated using the hyperinsulinemic-euglycemic clamp method. NS = Not significant statistically; nTg = nontransgenic mice; Tg = transgenic mice.

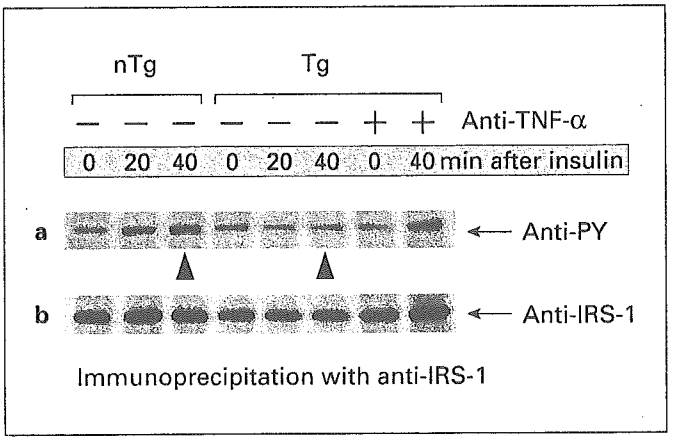

Fig. 5. Phosphorylation of tyrosine in IRS-1 in response to insulin stimulation. Liver tissues from control mice, transgenic mice with or without anti-TNF-α antibody treatment, were analyzed before and 20 as well as 40 min after administration of insulin. Samples were subjected to immunoprecipitation 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-α 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.

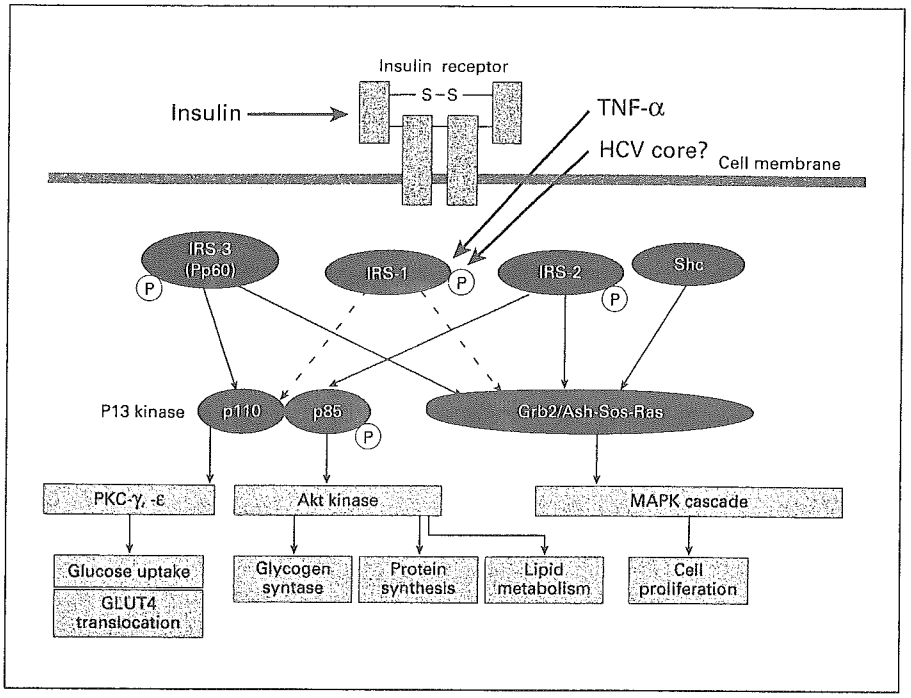

Fig. 6. A proposed mechanism for insulin resistance in HCV infection. HCV itself or elevated levels of cytokines such as TNF-α may inhibit tyrosine phosphorylation of IRS-1 in the liver, suppress intracellular transduction of insulin signal and lead to insulin resistance. PKC = Protein kinase C; MAPK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

TNF-α in insulin resistance in transgenic mice, serum insulin was determined and an insulin tolerance test performed after they had received anti-TNF-α intraperitoneally. Pretreatment with anti-TNF-α 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-α 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 signaling 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, nonresponse 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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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.[1] 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%.[2] Knowledge about the mechanism 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.[1,3,4] 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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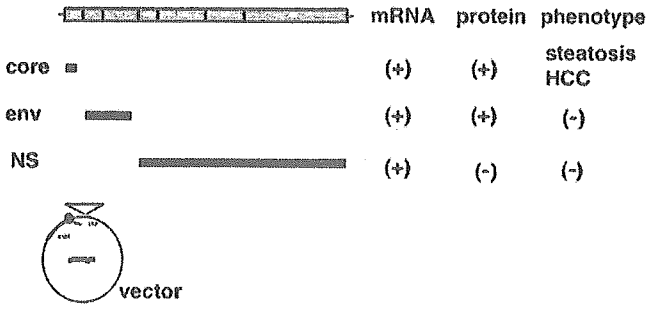

Figure 1. HCV gene transgenic mouse lines. Among the 3 different transgenic mouse lines established, only the transgenic mice carrying the HCV core gene develop HCC after an early phase with hepatic steatosis in 2 independent lineages. The mice transgenic for the envelope (*env*) genes or nonstructural (*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.[5,6] The background of the mouse lines is a C57BL/6 strain, which is known for a rare spontaneous occurrence of HCC.[7] 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).[6] The envelope gene transgenic mice do not develop HCC, despite high expression levels of both E1 and E2 proteins.[8,9] 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.[10] 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.[6] 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.[6] 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.[11] 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.[6,12] 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.[13,14] 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–α and interleukin-1β[19]
2. Activation of MAPK pathway and enhancement of AP-1 activation[19,21]
3. Overproduction of oxidative stress or reactive oxygen species in the absence of inflammation[13]
4. Synergy of HCV core and alcohol in inducing oxidative stress and activating MAPK[13,21]
5. Interaction of HCV core and retinoid X receptor–α and peroxisome proliferator activated receptor–α[20]
6. Induction of insulin resistance[17]
7. Development of steatosis by inhibiting microsomal triglyceride transfer protein activity[5,14,24]
8. Interaction of HCV core and proteasome activator PA28γ[25]
9. Inhibition of suppressor of cytokine signaling–1[26]

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.[13,15] The function of the electron transfer system of the mitochondrion is suggested in association with the presence of the HCV core protein.[16] Hepatic steatosis in hepatitis C might work as fuel for oxidative stress overproduction.[14,17,18]

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–α and interleukin-1β have been found to be transcriptionally activated.[19] The core protein has also been found to interact with some cellular proteins, such as retinoid X receptor–α, that play pivotal roles in cell proliferation and metabolism.[20] 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.[19,21] Far downstream, both the mRNA and protein levels of cyclin D1 and CDK4 are increased. Thus, the HCV core protein modulates the intracellular signaling 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[22] 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[22] for the hepatocarcinogenesis in HCV infection. We do allow multistages in the induction of all

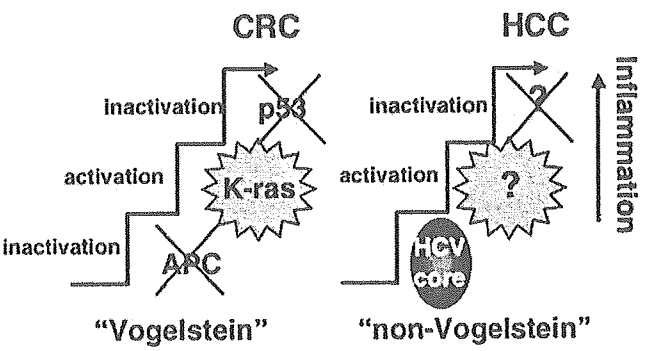

Figure 2. Mechanism of HCV-associated hepatocarcinogenesis. Multiple steps are required in the induction of all cancers; it would be mandatory for hepatocarcinogenesis that genetic mutations accumulate in hepatocytes. However, in HCV infection, some of these steps might be skipped in the development 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.[23] 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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