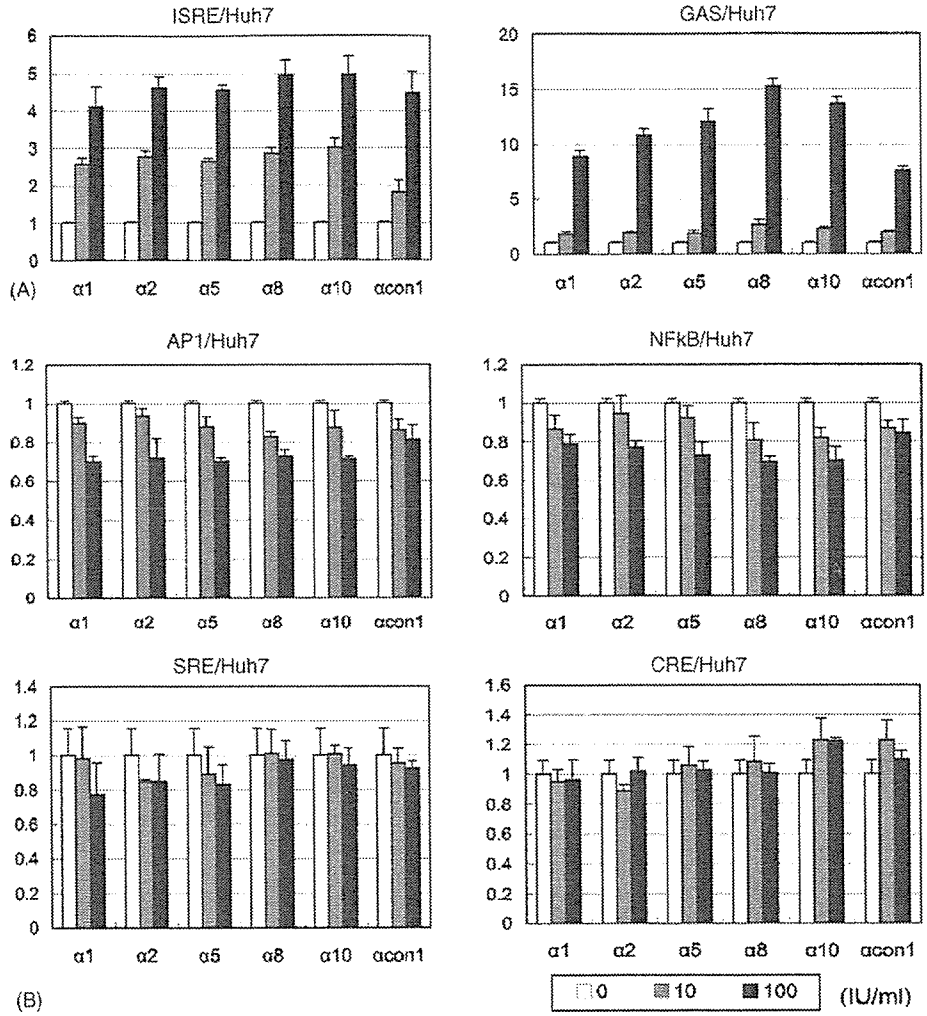


Fig. 2. ISRE, GAS, AP1, NF-kappa B, CRE and SRE reporter assay of Huh7 cells. ISRE-, GAS-, AP1-, NF-kappa B-, CRE-, or SRE-Luc reporter plasmids was transfected into Huh7 with pRL-CMV as a control. Twenty-four hours after transfection, the medium was supplemented with the IFN-alpha subtypes indicated. Dual luciferase assays were done at 6 h after the addition of IFNs. Bars indicate relative reporter activities as percents of IFN-negative controls. Error bars indicate mean ± S.D.

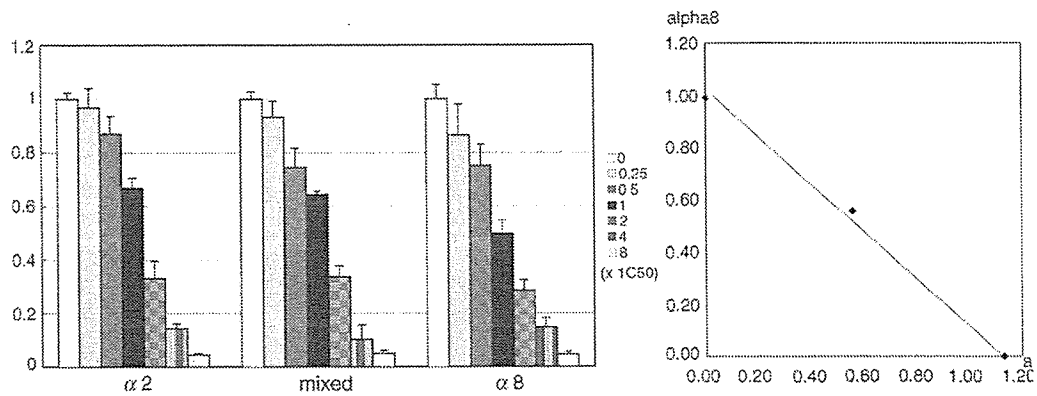


Fig. 3. Analyses of synergism between IFN subtypes 2 and 8: (A) IFNs; IFN-alpha2, -alpha8, mixed, dose modified by IC50 are added to Huh/Rep-Feo. Luciferase activities of Huh/Rep-Feo were measured 48 h after the addition of IFNs. (B) Graphical representation of the isobologram analysis. IC50s were calculated from the values in panel A. Each fractional IC50 for IFN-alpha2 and -alpha8 was plotted on *X*- and *Y*-axes, respectively. The FIC plot for the IFN-alpha2 and -alpha8 combinations of 1:1 falls on a theoretical line of additivity that is drawn between the IS50 plot for each IFN alone, indicating the effects of combination of IFN-alpha2 and -alpha8 is additive.

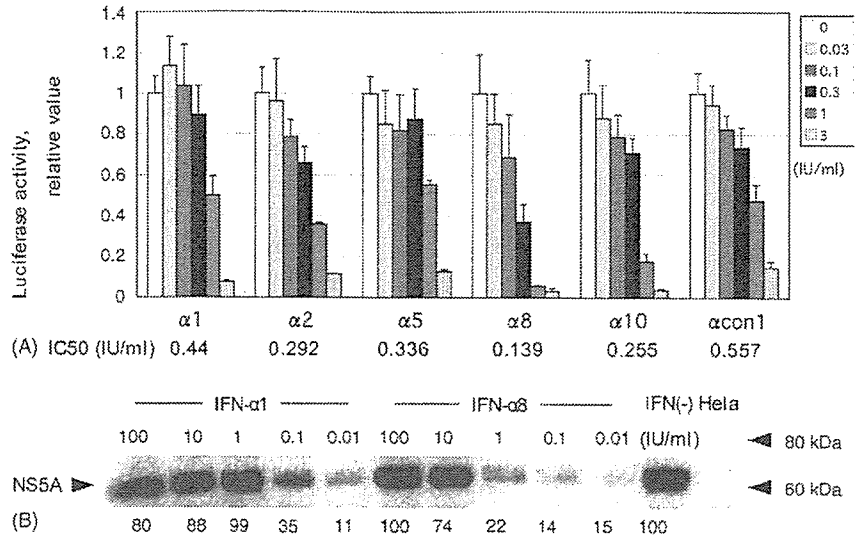


Fig. 4. Analysis of antiviral effect of the IFN-alpha subtypes using HCV replicon HeLa cells: (A) Luciferase activities of HeLa/Rep-Reo were cultured in the presence of indicated concentration of IFN subtypes. Luciferase activities were measured at 48 h after culture. Values indicate relative luciferase activities as percents of IFN-negative controls. Error bars indicate mean ± S.D. (B) Western blotting. HeLa/Rep-Reo cells were treated with indicated concentrations (numbers on the top) of IFN-alpha1 and -alpha8. Total cell lysate was harvested after 48 h, and Western blotting was done using primary antibody directed against NS5A. Numbers on the bottom indicate densitometric values displayed as percents of an IFN-negative control.

combination on intracellular HCV-RNA replication is additive.

### 3.4. Analysis of antiviral effect of the IFN-alpha subtypes using HCV replicon HeLa cells

Different type of cells may respond to IFN in a different manner possibly depending on the expression profiles of interferon receptors or cellular factors that mediate IFN signal transduction. To investigate the effects of IFN subtypes in a non-hepatocyte cell line, we used HeLa cells expressing chimeric luciferase reporter HCV replicon, HeLa/Rep-Reo. Treatment of HeLa/Rep-Reo cells with IFN subtypes suppressed expression of HCV replicon in dose-dependent manner (Fig. 4A). The IC50 for each IFN subtypes were 0.44 IU/ml (147 pg/ml) for alpha1, 0.292 IU/ml (4.06 pg/ml) for alpha2, 0.336 IU/ml (8.4 pg/ml) for alpha5, 0.139 IU/ml (0.479 pg/ml) for alpha8, 0.255 IU/ml (5.20 pg/ml) for

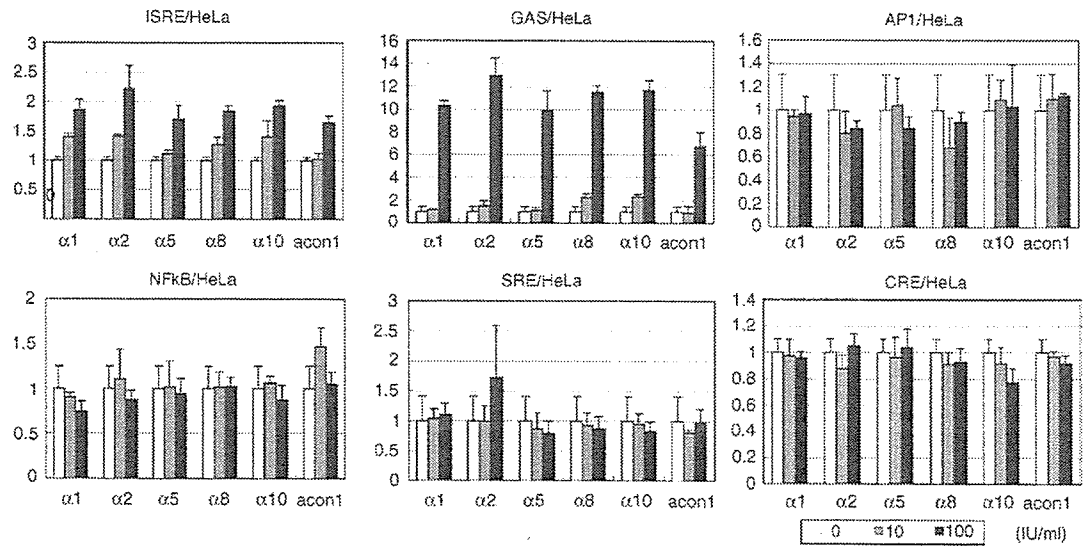


Fig. 5. Reporter assay of HeLa cells. ISRE-, GAS-, AP1-, NF-kappa B-, CRE-, and SRE-Luc reporter plasmids were, respectively, transfected into HeLa cells with pRL-CMV as a control. Twenty-four hours after transfection, IFN subtypes were added onto the medium. Dual luciferase assays were done at 6 h after the addition of IFNs. Bars indicate relative reporter activities as percents of IFN-negative controls. Error bars indicate mean ± S.D.

alpha10, and 0.557 IU/ml (0.271 pg/ml) for alpha con1, respectively. Similarly to the Huh7 cells, IFN-alpha8 was the strongest to suppress expression of HCV replicon. On the other hand, IFN-alpha5 and alpha con1 showed weaker antiviral effects on HCV replicon in HeLa cells than in Huh7 cells. Western blotting of HeLa/Rep-Reo cells treated with IFNs-alpha1 and alpha8 showed dose-dependent suppression of HCV replication, and differential activities of IFN subtypes, which were comparable to that of luciferase activities (Fig. 4B).

Reporter assays were performed by transfecting plasmids expressing ISRE-, GAS-, AP1-, NF-kappa B-, CRE-, and SRE-luciferase reporters into HeLa cells. The ISRE- and GAS-luciferase constructs responded to treatment with IFN subtypes similarly to that in Huh7 cells (Fig. 5). There was no significant difference in induction velocity of ISRE and GAS reporter activities between different IFN subtypes as were seen in Huh7 cells. IFN treatment showed no significant effects on AP1, NF-kappa B, CRE, and SRE activities on HeLa cells.

### 3.5. Analyses of IFN receptors expression

It is possible that the differences in expression levels of the cell-surface IFN receptors may associate with the response to IFN. We then analyzed expression of the respective subunits of type I IFN receptor mRNAs of Huh7 and HeLa cells by RT-PCR. Type I IFN receptor, IFNAR, is constituted by two subunits; 110 kilo-dalton (kDa) alpha subunit (IFNAR-1), and a 102 kDa beta subunit (IFNAR-2). IFNAR-2 has three isoforms that are translated from alternatively spliced mRNA transcripts; a 40 kDa soluble form of IFNAR-2a, a 55 kDa short form of IFNAR-2b and a 102 KDa long form of IFNAR-2c [34–36]; IFNAR-2c is the authentic beta subunit that is functionally active and coexpressed with IFNAR-1 (Fig. 6A). An RT-PCR analysis of IFN receptors showed that both cell lines expressed IFNAR-1 and IFNAR-2 (Fig. 6B). Although the relative expression levels of IFNAR-2a was slightly higher in Huh7 cells that in HeLa cells, There were no apparent differences in the expression level of the major subunits, IFNAR-1 and IFNAR-2c between Huh7 and HeLa cells.

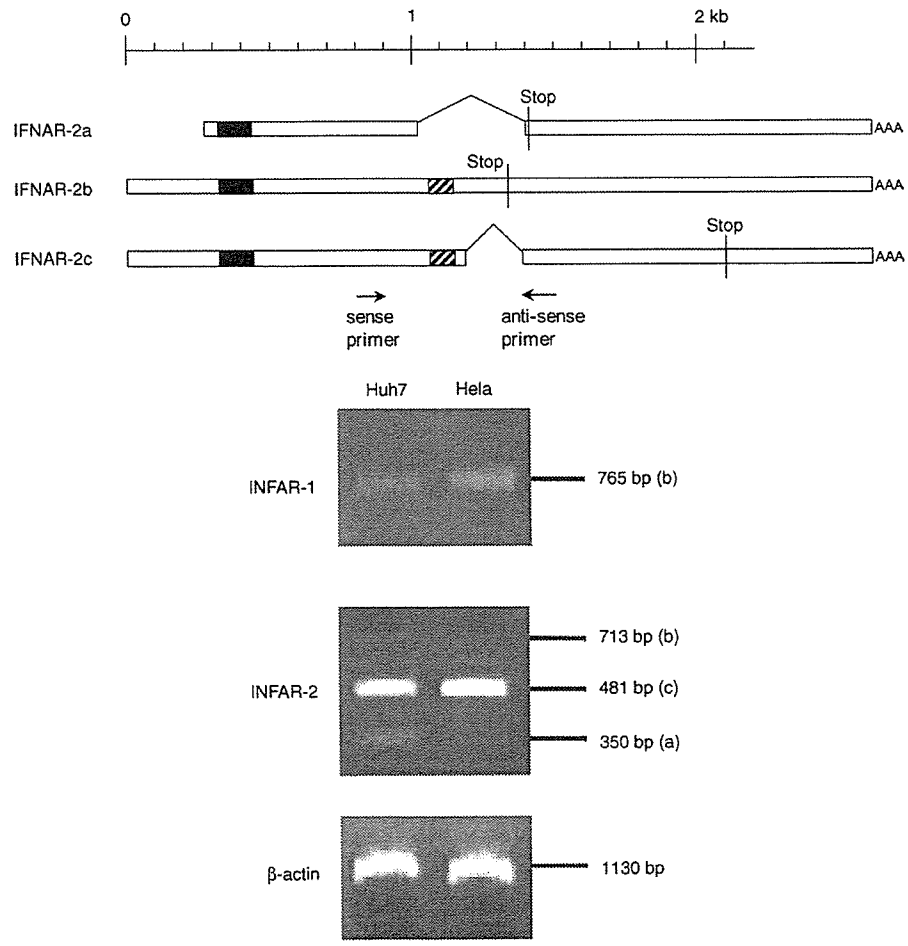


Fig. 6. IFN receptor expression: (A) structures of 3 IFNAR2 mRNA isoforms. Closed boxes indicate the leader peptide domains. The striped boxes indicate the transmembrane domain. Stop codons are indicated by vertical bars. Position of the sense and antisense primers are shown at the bottom. (B). Expression of IFN-alpha receptors in Huh7 and HeLa cells were evaluated by RT-PCR. The top panel: IFNAR-1 PCR-amplified DNA of 765 bp. The middle panel: IFNAR-2 PCR products, (a) 350 bp as IFNAR-2a, (b) 713 bp as IFNAR-2b, and (c) 481 bp as IFNAR-2c in size, respectively. The bottom panel: beta actin DNA.

## 4. Discussion

In this study, we used two cell lines that support expression of HCV replicon, in which the level of the viral genomic replication can by readily monitored by luciferase reporter assay. We showed that the five IFN-alpha subtypes have different activities to suppress expression of HCV replicon (Figs. 1 and 3). Using two IFN titers standardized in IU/ml and in pg/ml, IFN-alpha8 had the strongest antiviral effect on replicon, while IFN-alpha1 had the weakest effect in both titers. These finding are consistent with those reported by Foster et al. that IFN-alpha8 had the greatest antiviral activity in cells of three human tumor cell lines challenged with murine encephalomyelitis virus [17]. On the other hand, the reporter assay showed that activation of ISRE-dependent promoter, which is the primary signal transduction pathway, showed very similar results between Huh7 and HeLa cells, while the ISRE activities in neither of the cell lines correlated with the anti-HCV activities of the IFN subtypes (Figs. 2 and 4). GAS reporter activity, which bound by phosphorylated STAT1 homodimer, showed similar activation between each IFN subtypes. Other reporter assays, NF-kappa B, CRE, and SRE, showed no activation by the IFNs. These findings suggest that the divergent action of IFN subtypes may be independent of the classical JAK-STAT pathway.

Beside the classical JAK1-STAT1 and -2 pathway, type I IFN activates alternative signaling pathways. JAK2 mediates activity of IFNs as well as JAK1. As for STAT family, dimmers of STAT1:1, STAT3:3, STAT1:3, STAT5:5, and a heterodimer CrkL:STAT5 have been reported to be formed during the IFN-alpha signaling [18,19]. Furthermore, IFN-alpha treatment of cells activates expression of various genes that modulate virus infection and replication in JAK-STAT-independent manner; those include the insulin receptor substrate family, CrkL adaptor, protooncogene Vav, PKC-delta, p38 kinase, ERK 1/2, and PI-3 kinase, although the targets for these signaling pathways have not been well understood (reviewed in [18,19]).

Actions of IFN-alpha is initiated by binding the type I IFN receptors. It has been suggested that biologic activities of different IFN-alpha subtypes correlate with their respective binding affinities to the cells used [37]. Although we have not tested the cell-binding affinity of the IFN subtypes onto their receptor, activation of ISRE promoter, which is triggered by the receptor binding of IFN, did not correlate with their antiviral activities. Furthermore, analyses of IFN receptors by RT-PCR did not find differences in expression profiles the type I IFN receptor subunits, IFNAR1 and three isoforms of IFNAR2 [34–36] in Huh7 and HeLa cells that support expression of HCV replicon (Fig. 6B), suggesting that the differential effects of IFN subtypes may not be due to different expression profiles of their receptor subunits. Alternatively, binding of IFNs onto their receptor might recruit unidentified subunits or adaptor molecules that may activate aberrant signal transduction pathways.

Most studies on actions of the IFN subtypes focused only on the effects of the individual subtypes, and very little is known about their effects in combination [38–40]. On the other hand, because of the existence of multiple IFN subtypes, mutual interactions between the subtypes may be involved in the cellular responses, although these interaction between IFN subtypes are not well understood. Greiner et al. had reported IFN-alpha1 competes with IFN-alpha2 for binding to its receptor [16]. Our previous study also demonstrated additive and antagonistic effect of IFN-alpha subtypes, for instance, IFN-alpha2 and alpha8 had synergistic antiviral effect against VSV virus in HepG2 cells. [29] In our present study, we could not find such synergistic effects of IFN-alpha2 and alpha8 subtypes on cellular HCV replication (Fig. 3B). The result suggests that effects of IFN subtypes and their combination may show different effects depending on the target pathogens.

IFN-alpha con1 is a recombinant IFN that has consensus amino acid sequence of multiple IFN-alpha subtypes on its receptor-binding domain. The IFN-alpha con1 shows greater antiviral activity against HCV replication than the individual IFN-alpha subtypes in vitro [41] as well as in vivo [42]. In our present study, IFN con1 was moderately effective to suppress HCV replication with the IC50 of close to that of IFN-alpha5. However, activation of ISRE and GAS by IFN-alpha con1 seemed to be slight weaker, compared to the other IFN subtypes. Although it might be due to the different definition of units from that of the other IFN-alpha subtypes [30,41], the reportedly strong biological activity of IFN-alpha con1 might also involve pathway other than the Jak-STAT pathway.

Our present results using HCV replicon system have shown that IFN-alpha8 was the strongest to suppress HCV replication among 5 IFN-alpha subtypes 1, 2, 5, 8, and 10. Among clinically used IFN-alpha preparations, natural IFN-alpha preparations contain substantial amounts of IFN-alpha8 [29]. The differential activity shown in this study might direct a spotlight to the drugs, and might propose a hint for more effective IFN drugs used alone or in combination with ribavirin. Taken togather, IFN-alpha8 showed the strongest suppressive effect on in vitro HCV replication. The discrepancy between cellular ISRE responses and the anti-HCV effect implies other pathways other than IFN-activated JAK-STAT pathway. Further investigation of their differential antiviral actions may help elucidating the IFN-mediated cellular defense mechanisms against virus infection.

## Acknowledgements

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

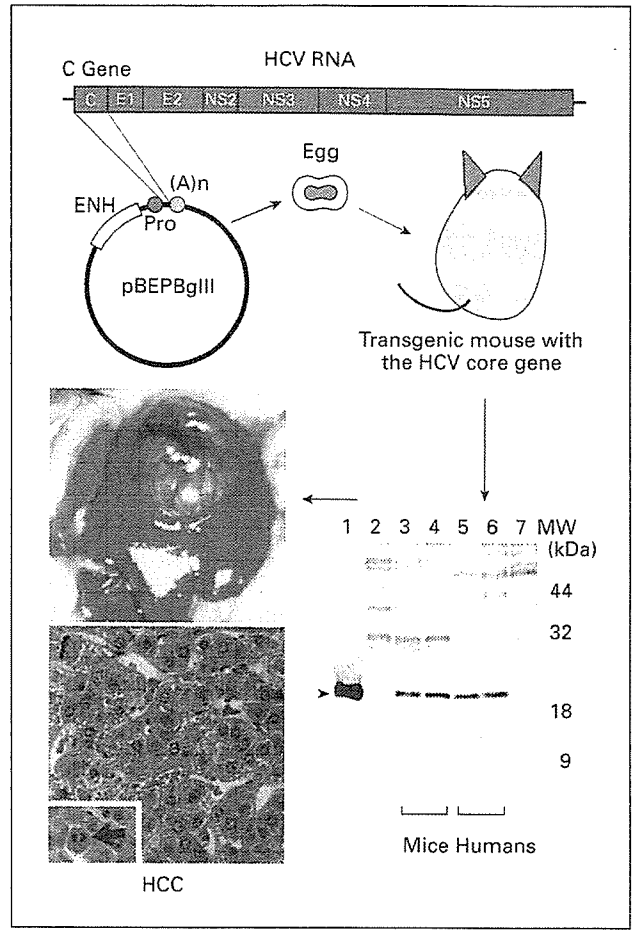


**Fig. 1.** Expression of HCV core gene in transgenic mouse. It carries the core gene of HCV genotype 1b alone and expresses the core protein of expected size in the liver, at levels similar to those in human patients. Mice eventually develop HCC later in their lives.

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.

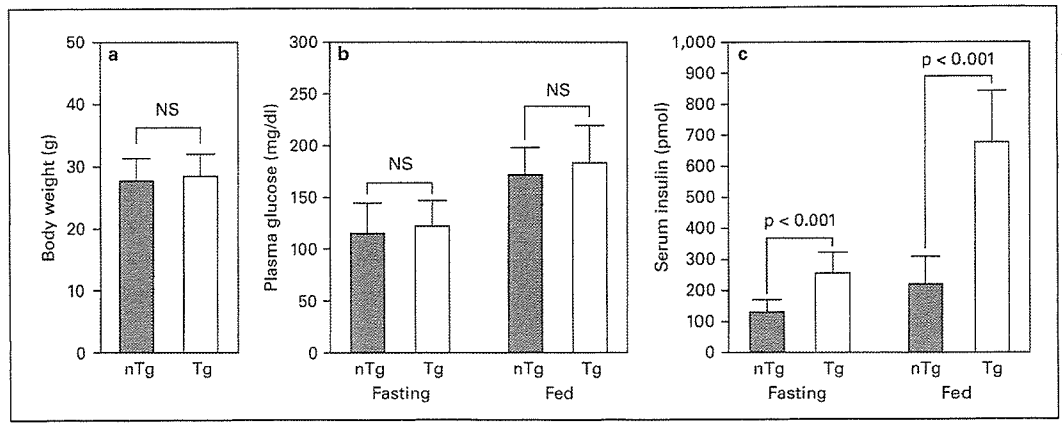


**Fig. 2.** Altered homeostasis of glucose in mice transgenic for the HCV core gene. Body weight of 2-month-old mice (**a**), plasma glucose levels in fasting or fed mice (**b**) and serum insulin levels in fasting or fed mice (**c**) are shown. Values represent means ± SE. NS = Not significant statistically;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

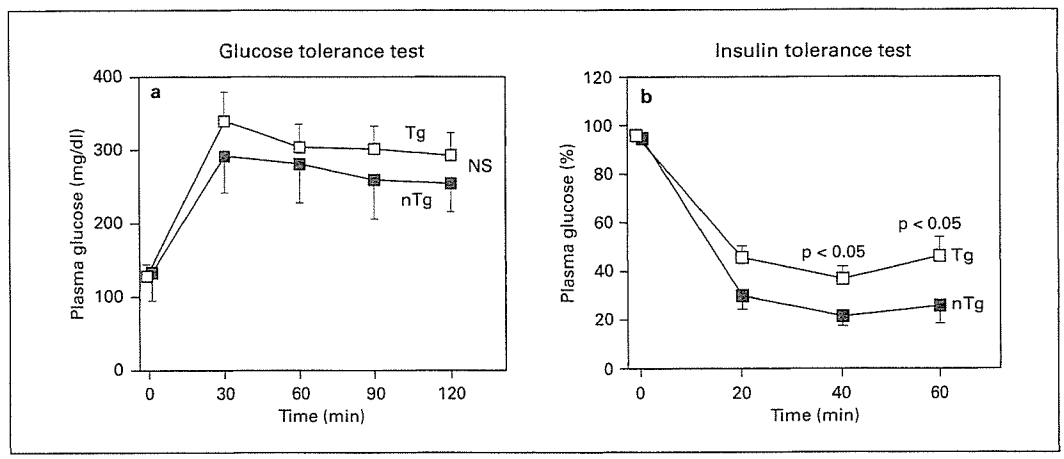


**Fig. 3.** Insulin resistance in transgenic mice. Glucose tolerance in mice after overnight fasting (**a**). *D*-Glucose (1 g/kg body weight) was given intraperitoneally to conscious mice, and plasma glucose levels were determined at time points indicated. **b** Insulin tolerance in mice fasted overnight. 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

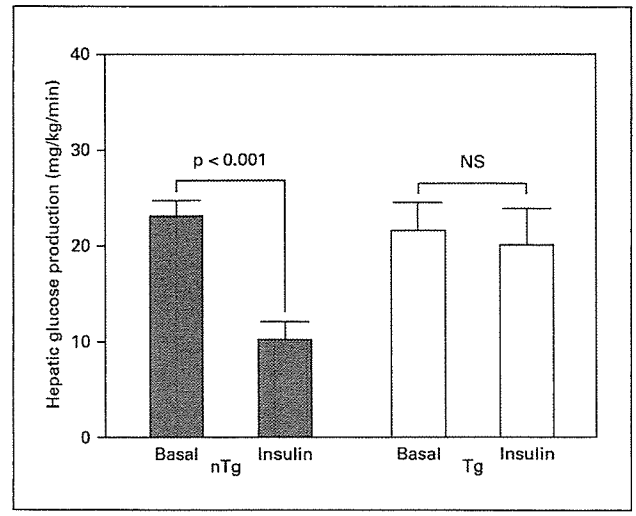


**Fig. 4.** Characterization of glucose metabolism in transgenic mice. Glucose production in the liver was calculated using the hyperinsulinemic-euglycemic clamp method. NS = Not significant statistically; nTg = nontransgenic mice; Tg = transgenic mice.

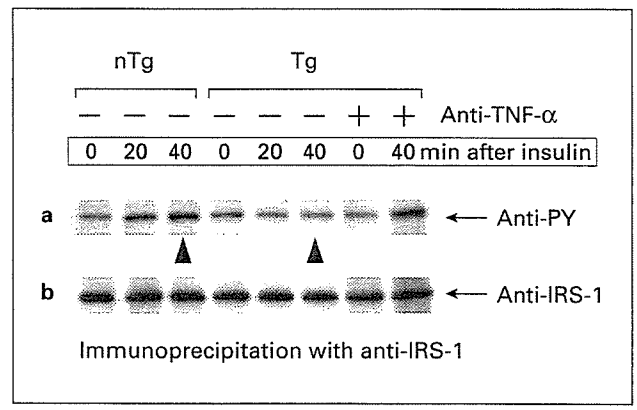


**Fig. 5.** Phosphorylation of tyrosine in IRS-1 in response to insulin stimulation. Liver tissues from control mice, transgenic mice with or without anti-TNF-α antibody treatment, were analyzed before and 20 as well as 40 min after administration of insulin. Samples were subjected to immunoprecipitation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

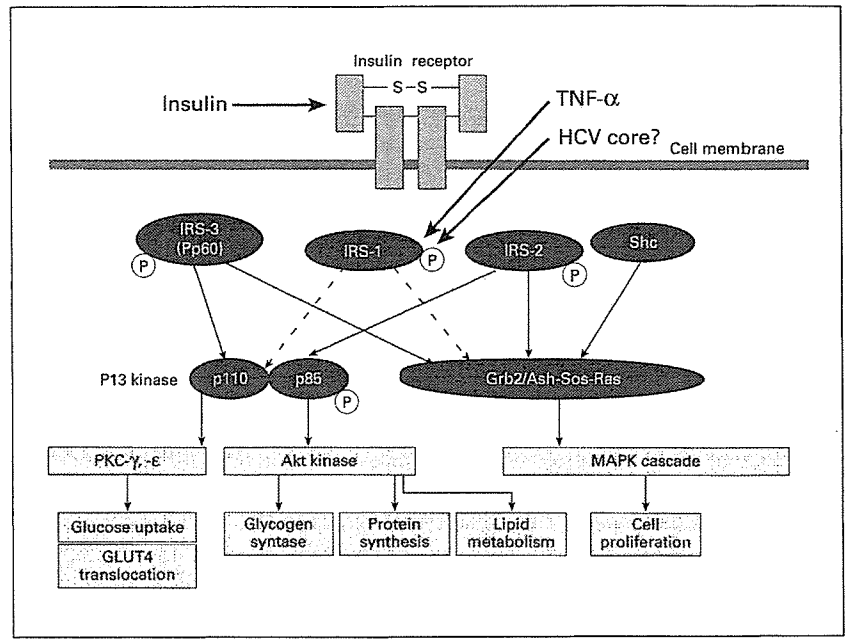


**Fig. 6.** A proposed mechanism for insulin resistance in HCV infection. HCV itself or elevated levels of cytokines such as TNF-α may inhibit tyrosine phosphorylation of IRS-1 in the liver, suppress intracellular transduction of insulin signal and lead to insulin resistance. PKC = Protein kinase C;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



*Editorial*

# Oxidative stress and apoptosis in hepatitis C: the core issue

Article on page 257
**Hepatitis C virus core protein inhibits deoxycholic acid-mediated apoptosis despite generating mitochondrial reactive oxygen species**
HARA Y, HINO K, OKUDA M, et al.

Extra- as well as intracellular stimuli elicit a wide range of responses, such as cell proliferation, differentiation, survival and apoptosis, via the regulation of intracellular signaling. Recent studies have revealed that stress-responsive signal transduction pathways are stringently regulated by the intracellular redox state.[1] The redox state of the cell is determined by the delicate balance between the levels of oxidizing and reducing equivalents, including reactive oxygen species (ROS) and endogenous antioxidants. The production of ROS, a representative of oxidative stress, fluctuates in response to alterations in both external and internal environments and, in turn, triggers specific signaling cascades, such as mitogen-activated protein kinases, which determine cell survival or death. Thus, ROS are profoundly involved in cell death or apoptosis.

In the liver, ROS are also key cytotoxic and signaling mediators in the pathophysiology of liver diseases, including viral hepatitis, in which hepatocytes and resident and infiltrating phagocytes can generate ROS. While ROS are able to cause cell death through massive lipid peroxidation, they also act to modulate signal transduction pathways by affecting redox-sensitive enzymes, transcription factors, and organelles, including mitochondria and endoplasmic reticulum. ROS, thus, directly regulate apoptotic and necrotic cell death.[2] In addition, ROS have indirect effects on the pathophysiology of cell death by supporting protease activity via inactivation of antiproteases.

In hepatitis C virus (HCV) infection, both ROS and apoptosis are closely involved in the process of progressive liver diseases from chronic hepatitis to cirrhosis and hepatocellular carcinoma (HCC).[3–7] ROS are assumed to play a major role in the pathogenesis of chronic hepatitis, which is characterized by continual cell death followed by regeneration. In this condition, the viral proteins of HCV, the core and nonstructural (NS) 5A proteins, have been shown to play a role in inducing ROS as well as in modulating apoptosis of hepatocytes.

The HCV genome comprises the genes of four structural proteins and six nonstructural proteins, and at least two of these viral proteins have been reported to cause oxidative stress in cells. The core protein, a structural protein, has been found to have various actions, including the induction of oxidative stress and the accumulation of lipids, in experimental studies using cultured cells and transgenic mice.[7,8] Experiments using mice transgenic for the core gene showed increased ROS production, increased intrahepatic catalase activity, a decreased intrahepatic glutathione (GSH) level, and a decreased GSH/GSH-GSSG (dimeric oxidized glutathione) ratio, indicating inhibition of antioxidation effects, although there was neither an increase in the serum alanine aminotransferase (ALT) level nor a histological finding of hepatitis.[7] Increased levels of intrahepatic peroxide lipids in the core gene transgenic mice with aging, compared with levels in control mice, also indicate an increase in oxidative stress. One possible mechanism underlying oxidative stress induction by the core protein is mitochondrial damage. Morphological abnormalities of the mitochondria have been observed in core gene transgenic mouse liver,[7] and increased ROS production caused by damage to the mitochondrial electron transport system has been noted in core protein-expressing cells.[8] Mitochondrial DNA, which has no protective proteins such as histone, is susceptible to damage by ROS. Mitochondrial DNA damage in the core gene transgenic mice appeared when they were as young as 3 months old. This mitochondrial damage disrupts the synthesis of proteins constituting the electron transport system complex and might also increase oxidative stress caused by damage to the electron transport system.

*Reprint requests to:* K. Koike

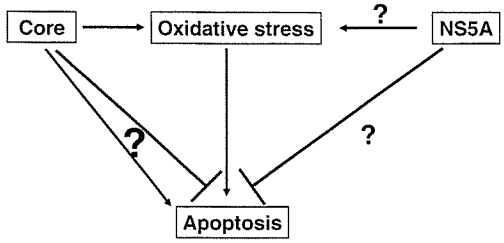


**Fig. 1.** Relationships among oxidative stress, apoptosis, and HCV proteins

A study using a cell culture system demonstrated that the NS5A protein also causes oxidative stress. NS5A induces the endoplasmic reticulum to release calcium by causing stress to the endoplasmic reticulum, and this release leads to increased ROS production in the mitochondria.[9] Although the effect of NS5A has not yet been confirmed by other study groups, HCV has the direct action of increasing intracellular ROS production via its proteins, separate from the induction of oxidative stress as a result of inflammation caused by viral infection (Fig. 1). A report that oxidative stress is also observed in HCV carriers with a normal ALT level[10] indicates that it is induced directly, without any mediating inflammatory reactions being necessary.

In contrast to the production of ROS by the core protein, which is now quite evident, the role of HCV core protein in apoptosis is rather controversial. Regarding the HCV-induced apoptotic mechanism, the HCV core protein may have a regulatory function in modulating apoptosis, either by enhancing or inhibiting it. In particular, the core protein exhibits both proapoptotic and antiapoptotic actions, depending on experimental conditions and the type of cells used,[11–14] whereas both the NS3 and the NS5A proteins have antiapoptotic effects (Fig. 1).[15] Modulation of apoptosis may involve binding of the core protein to the intracellular signal transducing portion of death receptors such as TNF-α, Fas, or lymphotoxin-αβ. Thus, HCV proteins may modulate hepatocyte apoptosis by indirect rather than by direct mechanisms. The real role of the core protein in the apoptotic process is, thus, not defined yet.

In the current issue of *Journal of Gastroenterology*, Hara et al.[16] tried to elucidate this core issue of HCV pathogenesis by separating the two properties of the HCV core protein with cultured cells, Huh-7 and HeLa. They confirmed that the core protein induced ROS, which was followed by activation of the scavenging system and insults to the cellular DNA, as shown previously.[6] In the study by Hara et al.,[16] the core protein inhibited the proapoptotic action of deoxycholic acid (DCA), which is known to cause both ROS production and apoptosis. Thus, the core protein seems to act to oppose the proapoptotic function of ROS, which ROS are also induced by the core protein itself. Such apparently opposing actions of the core protein, the production of ROS and the inhibition of apoptosis, might well explain the mode of hepatocarcinogenesis in HCV infection: hepatocytes with ROS-induced DNA damage may evade apoptosis by another effect of the core protein that inhibits apoptosis. Such a mechanism, similar to one previously postulated by other researchers,[3] in which both ROS production and mitogen-activated protein kinase activation are ascribed to the core protein, may clarify how cells with DNA damage can survive and develop into buds of HCC. Regrettably, the current study was done using DCA as an agent to induce both apoptosis and ROS, making it difficult to interpret the authentic role of the core protein in the execution of such biological functions. As noted above, the effect of the core protein in apoptosis varies depending on the system used. Therefore, further studies using different systems may be necessary to bring a conclusion to the core issue of HCV-induced pathogenesis associated with the multipotential HCV core protein.

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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.


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

## Contents



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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 ($^{\bullet}O_2^-$), hydrogen peroxide ($H_2O_2$) and the hydroxyl radical ($HO^{\bullet}$). ROS are mainly produced from $^{\bullet}O_2^-$ and converted into stable $H_2O_2$ through dismutation reaction. $H_2O_2$ is converted into highly reactive $HO^{\bullet}$ 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 β-carotene. Reactive oxygen elimination enzymes include superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase. SOD is induced by oxidative stress and dismutates $^{\bullet}O_2^-$ to $H_2O_2$ 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 $H_2O_2$ into water

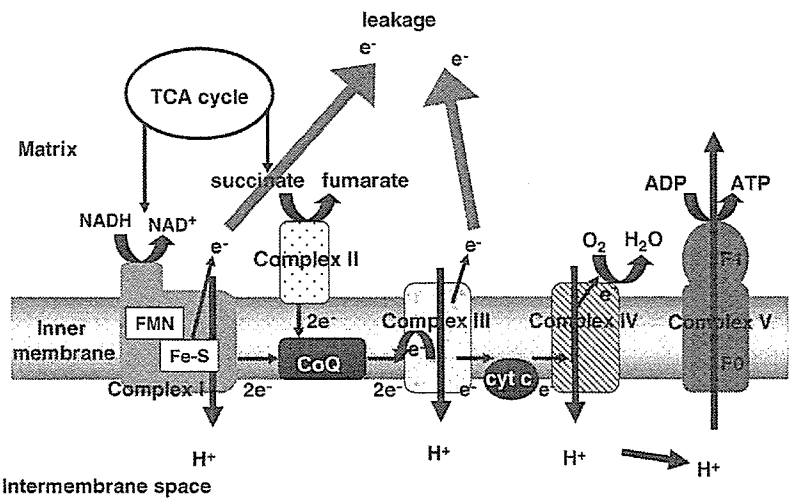


Fig. 1. The electron transfer system (ETS) of the mitochondrion. Most of the oxygen, consumed by mammalian cells, is converted to water via the mitochondrial ETS. However, up to 5% of the electrons entering the mitochondrial ETS can become uncoupled and singly leak out onto oxygen to form superoxide. 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.

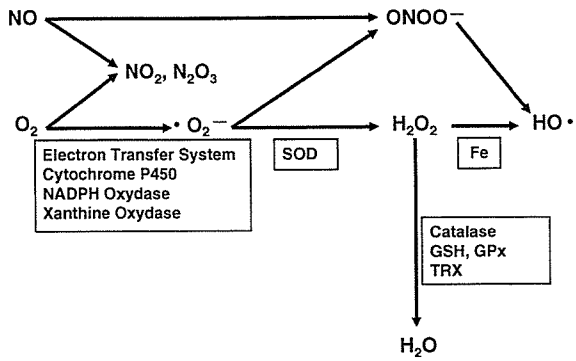


Fig. 2. Generation and scavenging of oxidative stress. SOD, super-oxide dismutase; GSH, reduced glutathione; GPx, glutathione peroxidase; TRX, thioredoxin.

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.

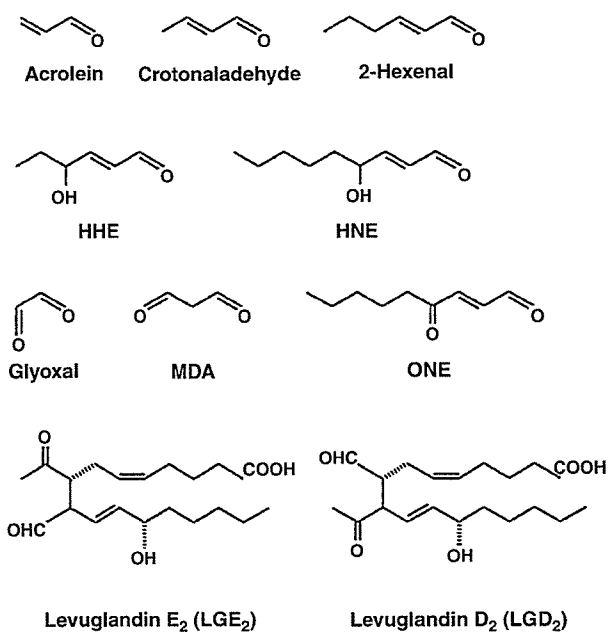


Fig. 3. Representative aldehyde species generated via lipid peroxidation reaction.

## 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-α and IL-1β 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-α and IFN-γ [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-κB 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 $O_2^-$ (reactive nitrogen species, RNS). Upon reaction to $O_2^-$, 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 antioxidation 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

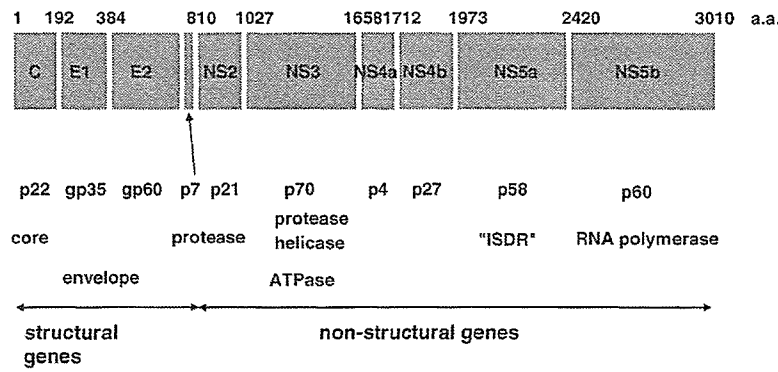


Fig. 4. Structure of hepatitis C virus genome. The genome of HCV consists of two parts, structural and non-structural regions. The former comprises the core and envelope regions, and the latter consists of NS2 to NS5a, which regions chiefly code enzymes necessary for viral replication. NS, non-structural; 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 β-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 liproproteins (VLDLs) [42]. All these actions could cause hepatic steatosis. In the liver of non-alcoholic steatohepatitis (NASH) patients, it was reported that β-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 exsanguination 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