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(和文論文は省略した)

研究成果刊行物の別刷乃至アブストラクト

（前掲一覧表のうちの一部を収載）

HCV

Kato T, Date T, Miyamoto M, Sugiyama M, Tanaka Y, Orito E, Ohno T, Sugihara K, Hasegawa I, Fujiwara K, Ito K, Ozasa A, Mizokami M, Wakita T.

Detection of anti-hepatitis C virus effects of interferon and ribavirin by a sensitive replicon system.

J Clin Microbiol. 2005 Nov;43(11):5679-84.

Although combination therapy with interferon and ribavirin has improved the treatment for chronic hepatitis C virus (HCV) infection, the detailed anti-HCV effect of ribavirin in clinical concentrations remains uncertain. To detect the anti-HCV effect of ribavirin in lower concentrations, a sensitive and accurate assay system was developed using the reporter replicon system with an HCV genotype 2a subgenomic replicon (clone JFH-1) that exhibits robust replication in various cell lines. This reporter replicon was generated by introducing the luciferase reporter gene (instead of the neomycin resistance gene) into the subgenomic JFH-1 replicon. To assess the replication of this reporter replicon, luciferase activity was measured serially up to day 3 after transient transfection of Huh7 cells. The luciferase activity increased exponentially over the time course of the experiment. After adjustment for transfection efficiency and transfected cell viability, the impacts of interferon and ribavirin were determined. The administration of interferon and ribavirin resulted in dose-dependent suppression of replicon RNA replications. The 50% inhibitory concentration of interferon and ribavirin was 1.80 IU/ml and 3.70 microg/ml, respectively. In clinical concentrations, replications were reduced to 0.09% and 53.74% by interferon (100 IU/ml) and ribavirin (3 microg/ml), respectively. Combination use of ribavirin and interferon enhanced the anti-HCV effect of interferon by 1.46- to 1.62-fold. In conclusion, we developed an accurate and sensitive replicon system, and the antivirus effect of interferon and ribavirin was easily detected within their clinical concentrations by this replicon system. This system will provide a powerful tool for screening new antiviral compounds against HCV.

Kato N, Nakamura T, Dansako H, Namba K, Abe K, Nozaki A, Naka K, Ikeda M, Shimotohno K.

Genetic variation and dynamics of hepatitis C virus replicons in long-term cell culture.

J Gen Virol. 2005 Mar;86(Pt 3):645-56.

Hepatitis C virus (HCV) genomic sequences are known to vary widely among HCV strains, but to date there have been few reports on the genetic variations and dynamics of HCV in an experimental system of HCV replication. In this study, a genetic analysis of HCV replicons obtained in long-term culture of two HCV replicon cells (50-1 and 1B-2R1), which were established from two HCV strains, 1B-1 and 1B-2, respectively, was performed. One person cultured 50-1 cells for 18 months, and two people independently cultured 50-1 cells for 12 months. 1B-2R1 cells were also cultured for 12 months. The whole nucleotide sequences of the three independent replicon RNA clones obtained at several time points were determined. It was observed that genetic mutations in both replicons accumulated in a time-dependent manner, and that the mutation rates of both replicons were approximately 3.0 x 10(-3) base substitutions/site/year. The genetic diversity of both replicons was also enlarged in a time-dependent manner. The colony formation assay by transfection of total RNAs isolated from both replicon cells at different time points into naive HuH-7 cells revealed that the genetic mutations accumulating with time in both replicons apparently improved colony formation efficiency. Taken together, these results suggest that the HCV replicon system is useful for the analysis of evolutionary dynamics and variations of HCV. Using this replicon cell culture system, it was demonstrated further that neither ribavirin nor its derivative mizoribine accelerated the mutation rate or the increase in the genetic diversity of HCV replicon.

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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

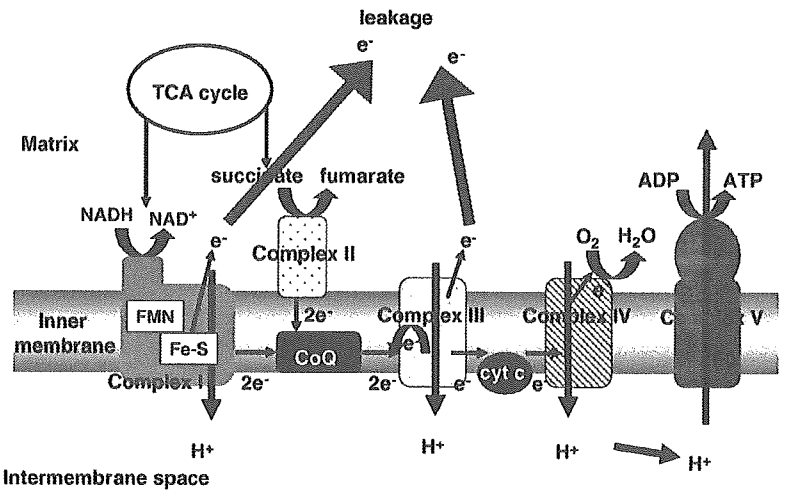

Fig. 1. The electron transfer system (ETS) of the mitochondrion. Most of the oxygen, consumed by mammalian cells, is converted to water via the mitochondrial ETS. However, up to 5% of the electrons entering the mitochondrial ETS can become uncoupled and singly leak out onto oxygen to form superoxide.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

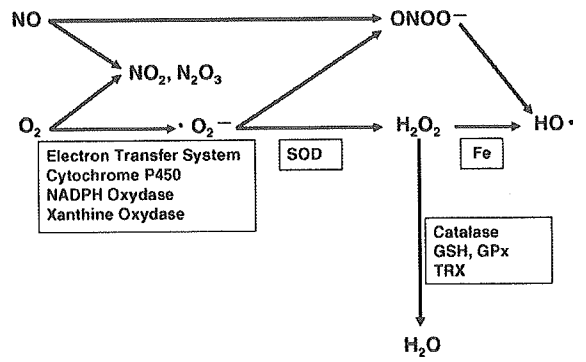

Fig. 2. Generation and scavenging of oxidative stress. SOD, super-oxide dismutase; GSH, reduced glutathione; GPx, glutathione peroxidase; TRX, thioredoxin.

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.

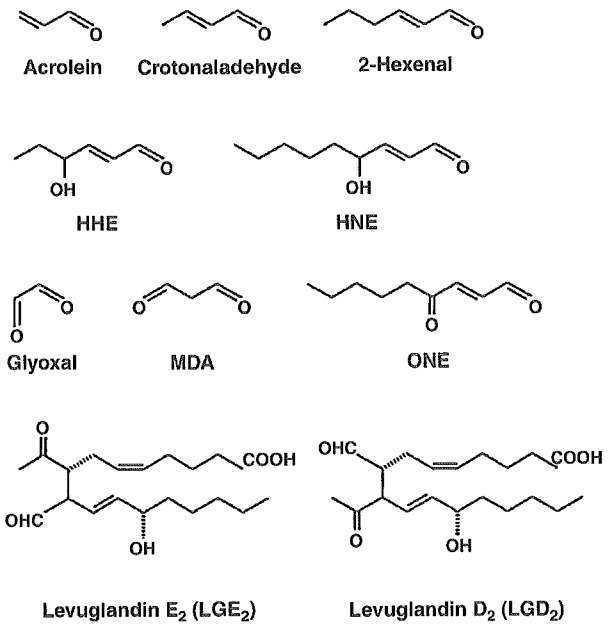

Fig. 3. Representative aldehyde species generated via lipid peroxidation reaction.

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 nonstructural 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

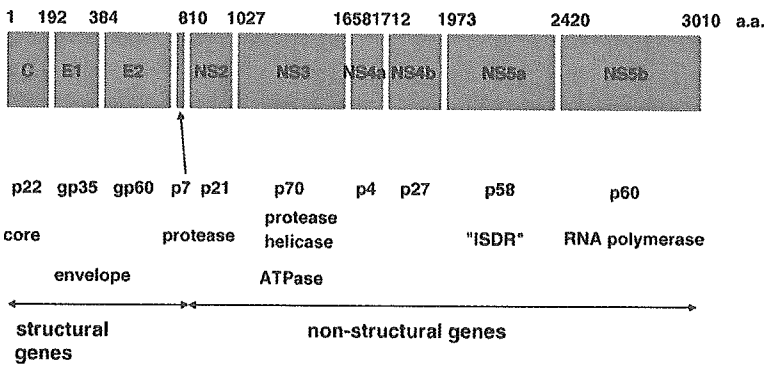

Fig. 4. Structure of hepatitis C virus genome. The genome of HCV consists of two parts, structural and non-structural regions. The former comprises the core and envelope regions, and the latter consists of NS2 to NS5a, which regions chiefly code enzymes necessary for viral replication. NS, non-structural; 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• (Fenton reaction) [50,51]. HO• 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

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

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

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

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

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

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

10. Conclusions

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

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

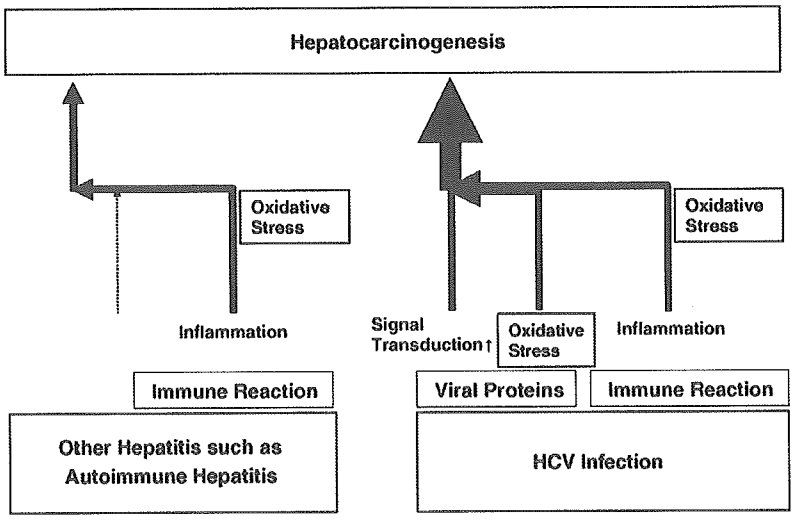

Fig. 5. Oxidative stress and hepatocarcinogenesis in various types of hepatitis (hypothesis). Oxidative stress is generated in all types of hepatitis via inflammation accompanied by continual cell death and regeneration. In HCV infection, HCV itself causes the production of oxidative stress in a synergy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

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Review

Metabolic aspects of hepatitis C viral infection: steatohepatitis resembling but distinct from NASH

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Although the target of hepatitis C virus (HCV) infection is the liver, it has become progressively more evident that HCV can induce diseases in numerous organs. Recently, much attention has been drawn to metabolic disorders in HCV infection. Initially, hepatic steatosis and disturbances in lipid metabolism were found to be characteristic of HCV infection, and, subsequently, a correlation was noted between HCV infection and diabetes. It is now evident that HCV, by itself, can induce insulin resistance by way of disturbing the intracellular signaling pathway of insulin by the function of HCV core protein. Insulin resistance, caused by HCV infection, evolves to type 2 diabetes when superimposed on a high-fat diet and obesity. The fact that HCV infection induces insulin resistance by the virus itself may influence the progression of chronic hepatitis and open up novel therapeutic approaches. When hepatitis C is compared with nonalcoholic steatohepatitis (NASH), there are a number of similarities and several differences. From the metabolic aspect, hepatitis C resembles NASH in numerous features, such as the presence of steatosis, serum dyslipidemia, and oxidative stress in the liver, suggesting that hepatitis C is a steatohepatitis. In contrast, there are noticeable differences between hepatitis C and NASH, in that HCV modulates cellular gene expression and intracellular signal transduction, including the activation of mitogen-activated protein (MAP) kinase and transcription factor activator protein (AP)-1, while such details have not been noted for NASH. This difference may explain the markedly higher incidence of HCC development in chronic hepatitis C compared with that in NASH. HCV infection needs to be viewed not only as a liver disease but also as a metabolic disease, and this viewpoint could open up a novel way to the molecular understanding of the pathogenesis of hepatitis C, as a virus-associated steatohepatitis (VASH).

Key words: diabetes, hepatitis C virus, insulin resistance, steatohepatitis, hepatocarcinogenesis, lipid metabolism

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 from the medical and socioeconomic 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 (Table 1).[2] Furthermore, strong associations of HCV infection with Sjögren's syndrome and lichen planus have been noted, which have been validated in an animal model.[3]

Steatosis and HCV infection

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 a comparison between chronic hepatitis C and other types of chronic hepatitis, resulting in repeated reports that steatosis was significantly associated with chronic hepatitis C.[4,5] Steatosis in HCV infection is reproduced in animal models[6] and cultured cells,[7]

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strengthening the idea of a pathologic role of HCV in steatosis. Furthermore, patients infected with HCV have abnormalities in serum lipids, such as hypocholesterolemia or abnormal levels of apolipoproteins in serum;[8,9] these abnormalities are corrected in sustained virological responders to antiviral treatment.[9] Thus, the association shown between HCV infection and disturbances in lipid metabolism has become increasingly stronger both in patients and in experimental systems, including animals. Further, patients with chronic hepatitis C accompanied by severe steatosis develop hepatic fibrosis more rapidly than those without steatosis.[10] Thus, abnormal lipid metabolism in HCV infection could be deeply involved in the pathogenesis of hepatitis C.

Diabetes may also be a manifestation of HCV infection

Another 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. This report made little impact, however, in view of the 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 a 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 confounded by factors such as the development of cirrhosis, obesity, and older age, which are common in patients with hepatitis C; these factors could make it difficult to prove this association to be real. Hence, there is a need to evaluate the association, using experimental systems.

Table 1. Hepatitis C as a multifaceted disease

Hepatitis, cirrhosis and, eventually, HCC
Mixed cryoglobulinemia
MPGN
Sjögren's syndrome
Lichen planus
B-cell lymphoma
Disturbance in lipid metabolism
Diabetes or insulin resistance

HCV infection 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 in their normal control littermates, but there was

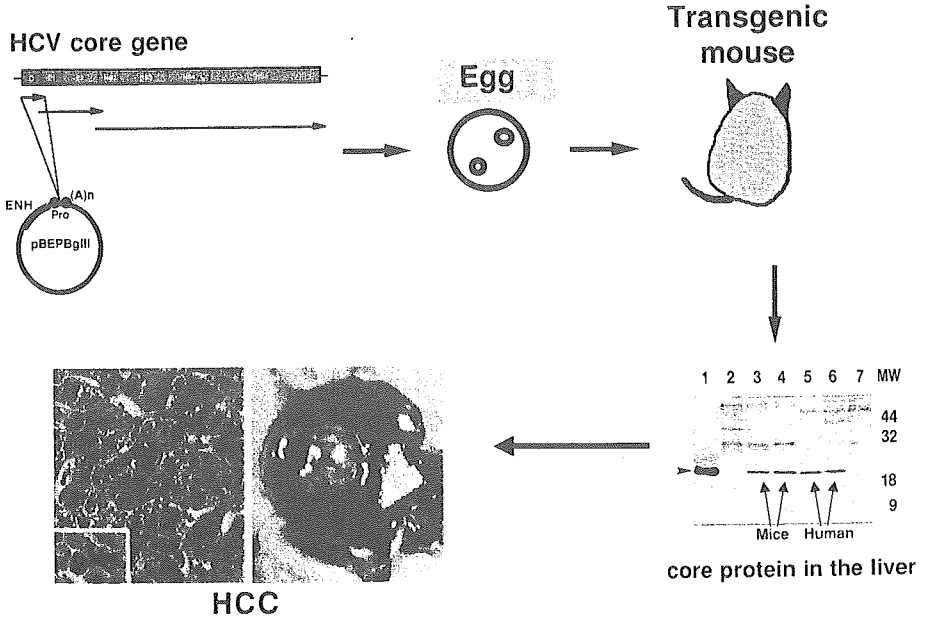

Fig. 1. Mouse model of hepatitis C virus (*HCV*)-induced liver pathogenesis. HCV core gene transgenic mice carry the core gene, alone, of genotype 1b HCV and express the core protein of an expected size in the liver, at levels comparable to those in human patients with chronic hepatitis C. The mice eventually develop hepatocellular carcinoma (*HCC*) late in life. *ENH*, enhancer; *Pro*, promoter; *A(n)*, polyadenylation signal

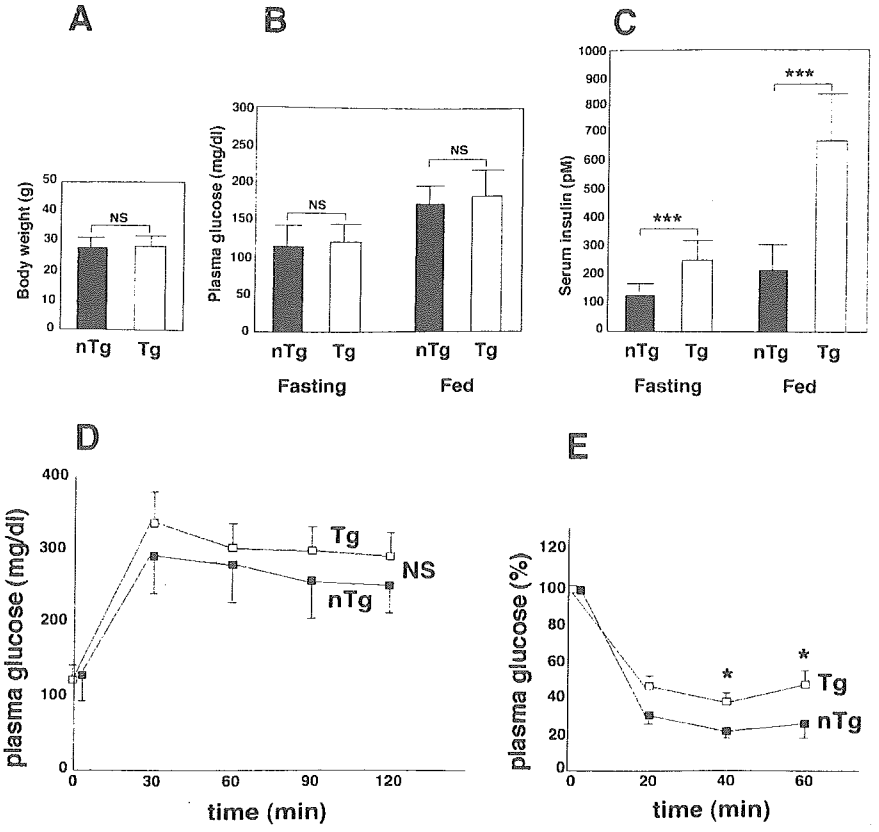

Fig. 2A–E. Altered glucose homeostasis in HCV core gene transgenic mice. **A** Body weights of 2-month-old mice. **B** Plasma glucose levels in fasting and fed mice. **C** Serum insulin levels in fasting and fed mice. The insulin level was significantly higher in the core gene transgenic mice than in control mice. **D** 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. **E** 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 means ± SE, $*P < 0.05$; $***P < 0.001$, *NS*, statistically not significant; *nTg*, nontransgenic mice; *Tg*, transgenic mice

Table 2. Types of insulin resistance

Peripheral insulin resistance	A shortage of insulin action in the muscle (deficit in the insulin-induced glucose uptake into the muscles)
Central insulin resistance	A shortage of insulin action in the liver (deficit in the insulin-induced suppression of glucose production in the liver)

no significant difference between them (Fig. 2B). In contrast, serum insulin levels were significantly higher in transgenic than in normal control mice in both the fasting and fed conditions (Fig. 2C). Because such a combination of normal glucose levels and hyperinsulinemia points to insulin resistance, we conducted tests to determine glucose levels and insulin resistance. The core gene transgenic mice exhibited glucose levels a little higher than those of their normal littermates, but without any significant differences between them (Fig. 2D). In the insulin resistance tests, glucose levels were significantly higher in the transgenic than in the normal control mice, both 40 and 60 min after injection with insulin (Fig. 2E). These results indicate the presence of insulin resistance in the core gene transgenic mice. Because 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, would the insulin resistance observed in this animal model arise? Insulin resistance is considered to involve two factors: central and peripheral insulin resistances (Table 2).[15] The hyperinsulinemic-euglycemic clamp method was employed for differentiating between these factors. In this method, hepatic glucose production (HGP) is calculated on the basis of the amount of glucose required for keeping plasma glucose levels within a certain range at serum insulin levels higher than physiological ones. In the normal control mice, HPG was suppressed by 60% by the administration of insulin, in contrast to findings in the 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. For further confirmation of this, uptake of glucose into the muscle was determined. There was no difference in this uptake in response to the administration of insulin between the transgenic

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

The mechanism underlying insulin resistance in HCV infection

Next, we evaluated how insulin resistance emerged in our mouse model. For this purpose, liver homogenate was immunoblotted with anti-phosphotyrosine and anti-phosphoserine antibodies after insulin receptor substrate (IRS)-1 and IRS-2 had been immunoprecipitated. Tyrosines in IRS-1 were weakly phosphorylated in both the 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 the normal, but not in the transgenic mice. The obtained results suggested a disturbance in tyrosine phosphorylation as one of the factors for insulin resistance in the liver. There were no differences in the phosphorylation of serines in IRS-1 or tyrosines in IRS-2 between the transgenic and normal control mice. Overall, these results provided experimental evidence for the development of insulin resistance induced by the presence of HCV in the liver, which would disturb the transduction of insulin signaling in hepatocytes (Fig. 3). There remains a possibility that the HCV core protein could directly prohibit the phosphorylation of tyrosines. Alternatively, this protein may inhibit tyrosine phosphorylation via certain cytokines.

In our extensive search for the expression of cytokines in the liver of the HCV core gene transgenic mice, only tumor necrosis factor (TNF)-α and interleukin (IL)-1β levels were found to be increased.[16] For the purpose of evaluating the role of TNF-α in insulin resistance in transgenic mice, therefore, serum insulin was determined and an insulin resistance test was performed in them after they had received anti-TNF-α intraperitoneally. Pretreatment with anti-TNF-α partially restored insulin sensitivity in the HCV core gene transgenic mice. Although direct anti-insulin activity of the core protein cannot be excluded, high levels of TNF-α in the liver could be one of the factors involved in the induction of insulin resistance in this mouse model.

Pathogenesis of insulin resistance in hepatitis C patients

Simultaneously with our report of experimental systems, Aytug et al.[17] 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 livers of the patients. With insulin stimulation of the biopsied liver samples, insulin-receptor proteins and IRS-1 increased, while the phosphorylation of tyrosines in IRS-1 decreased to one-half the baseline value, along with diminished activity for PI3-kinase associated with IRS-1. The results reported by Aytug et al.[17] coincide with ours, in terms of analyzing the mechanism of insulin resistance in our experimental system in mice. Both our findings and theirs implicate the impaired tyrosine phosphorylation in IRS-1 in the induction of insulin resistance by HCV infection. It struck us as a surprise, in a sense, that the mechanism of insulin resistance induced by HCV infection showed agreement between

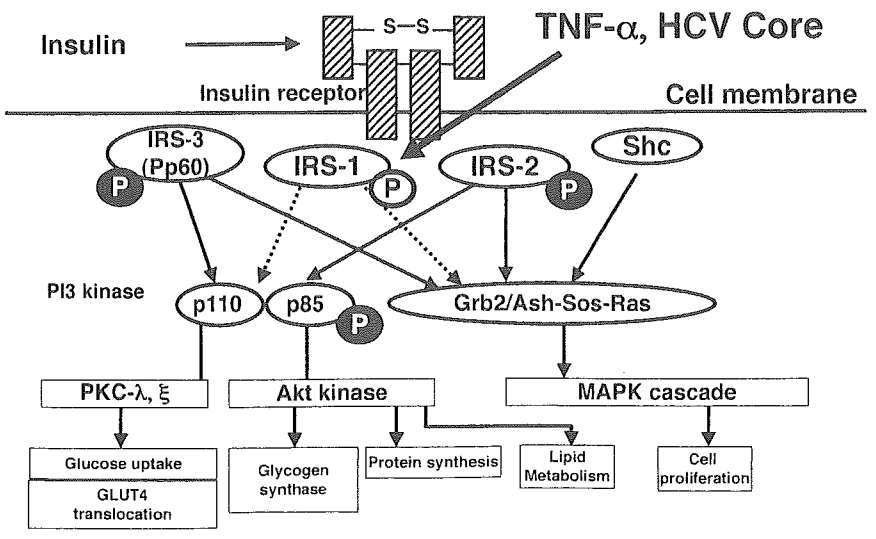

Fig. 3. Insulin resistance and HCV infection. HCV core protein or elevated intrahepatic tumor necrosis factor-α (*TNF-α*) inhibits tyrosine phosphorylation of insulin receptor substrate (*IRS*)-1 in the liver, suppresses insulin intracellular signal transduction, and leads to insulin resistance. *PKC*,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

clinical samples and experimental animals, although hepatic IRS-2 was reported to be preferred to IRS-1 for a role in the development of insulin resistance in earlier studies.[18] HCV infection could be peculiar, in that IRS-1 is more deeply involved than IRS-2 in the induction of hepatic insulin resistance. Although our data strongly indicate a hepatic characteristic of insulin resistance in HCV infection, they by no means exclude the roles of other factors in the induction of this resistance. There is little expression of the HCV core gene in the muscles of our animal model; it is not known if HCV infects muscle cells in patients with chronic hepatitis C. Factors not intrinsic to the liver would have to be evaluated to sort this out, including mitochondria dysfunction being involved in the induction of insulin resistance.[19]

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.[20] tried to establish a relationship between liver histology and indicators of glucose metabolism, as well as insulin resistance, represented by the homeostasis model assessment of insulin resistance (HOMA-IR). They found that insulin resistance already existed in hepatitis C patients with stage 0 or stage 1 fibrosis of 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 progression 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 to be an aggravating factor not only in atherosclerosis but also in systemic inflammation and fibrosis. The liver would not be an exception in this respect.

Similarities and differences between hepatitis C and nonalcoholic steatohepatitis (NASH): hepatitis C could be a virus-associated steatohepatitis

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.[14] In view of the progression of chronic hepatitis C accelerated by insulin resistance,[20] insulin resistance would naturally influence the development of HCC. Although the association has not yet been shown to be definite between NASH and the development of HCC, it needs to be pursued energetically, in view of the 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 3). Steatosis, which is one of the definitions of NASH, is a characteristic trait of chronic hepatitis C.[4–6,13] Disturbances in lipid metabolism are present in both conditions, although the phenotypes may be distinct: hypo-β-lipoproteinemia in hepatitis C vs hyperlipidemia in NASH. As described above, insulin resistance often arises in chronic hepatitis C, and it is also a feature frequently observed in NASH; indeed insulin resistance is considered to be a basis for the pathogenesis of NASH.[21] Some cytokines, such as TNF-α, are considered to be critical in the pathogenesis of both conditions. TNF-α levels are increased in patients with chronic hepatitis C and are implicated in insulin resistance. TNF-α is also implicated in the pathogenesis of NASH.[21] The overproduction of oxidative stress or reactive oxygen species (ROS) plays a pivotal role in the progression of hepatitis and the development of HCC in both hepatitis C and NASH: in a mouse model of HCV infection, ROS were overproduced in the liver in the absence of inflammation, contributing, at least in part, to the development of HCC.[13,19,22] Presumably associated with ROS overproduction, a functional abnormality in the mitochondrion is suggested in the pathogenesis of liver diseases, including HCC, in both hepatitis C and NASH. In an HCV mouse model, a functional disorder of the electron transfer system of the mitochondrion was implicated as the origin of ROS overproduction (Table 3).

HCC develops in both chronic hepatitis C and NASH. However, an association between NASH and HCC is not yet conclusive, while there is a well-established connection of HCC with HCV infection.[1,20] Nevertheless, HCC does develop in patients with NASH, although the reported rate of occurrence varies. Hence, the mechanism underlying hepatocarcinogenesis in NASH awaits further investigation. The analogy between chronic hepatitis C and NASH, as described above, may be a clue to solve puzzles in the pathogenesis of NASH, including hepatocarcinogenesis.

Table 3. 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?