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BASIC–LIVER, PANCREAS, AND BILIARY TRACT

Hepatitis C Virus Infection and Diabetes: Direct Involvement of the Virus in the Development of Insulin Resistance

YOSHIZUMI SHINTANI,* HAJIME FUJIE,* HIDEYUKI MIYOSHI,* TAKEYA TSUTSUMI,* KAZUHISA TSUKAMOTO,† SATOSHI KIMURA,* KYOJI MORIYA,* and KAZUHIKO KOIKE*
Departments of *Internal Medicine and †Metabolic Diseases, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

See editorial on page 917.

Background & Aims: Epidemiological studies have suggested a linkage between type 2 diabetes and chronic hepatitis C virus (HCV) infection. However, the presence of additional factors such as obesity, aging, or cirrhosis prevents the establishment of a definite relationship between these 2 conditions. *Methods:* A mouse model transgenic for the HCV core gene was used. *Results:* In the glucose tolerance test, plasma glucose levels were higher at all time points including in the fasting state in the core gene transgenic mice than in control mice, although the difference was not statistically significant. In contrast, the transgenic mice exhibited a marked insulin resistance as revealed by the insulin tolerance test, as well as significantly higher basal serum insulin levels. Feeding with a high-fat diet led to the development of overt diabetes in the transgenic mice but not in control mice. A high level of tumor necrosis factor-α, which has been also observed in human chronic hepatitis C patients, was considered to be one of the bases of insulin resistance in the transgenic mice, which acts by disturbing tyrosine phosphorylation of insulin receptor substrate-1. Moreover, administration of an anti-tumor necrosis factor-α antibody restored insulin sensitivity. *Conclusions:* The ability of insulin to lower the plasma glucose level in the HCV transgenic mice was impaired, as observed in chronic hepatitis C patients. These results provide a direct experimental evidence for the contribution of HCV in the development of insulin resistance in human HCV infection, which finally leads to the development of type 2 diabetes.

Approximately 200 million people are chronically infected with hepatitis C virus (HCV) in the world. Chronic HCV infection may lead to cirrhosis and hepatocellular carcinoma, thereby being a worldwide problem both in medical and socioeconomical aspects.[1,2] In addition, chronic HCV infection is a multifaceted disease, which is associated with numerous clinical manifestations, such as essential mixed cryoglobulinemia, porphyria cutanea tarda, and membranoproliferative glomerulonephritis.[3] Recent epidemiological studies have added another clinical condition, type 2 diabetes, to a spectrum of HCV-associated diseases.[4–7] However, the establishment of a definite causative relationship between HCV infection and diabetes is hampered by the presence of other factors such as obesity, aging, or liver injury in patients with chronic HCV infection.

Type 2 diabetes is a complex, multisystem disease with a pathophysiology that includes a defect in insulin secretion, increased hepatic glucose production, and resistance to the action of insulin, all of which contribute to the development of overt hyperglycemia.[8,9] Although the precise mechanisms whereby these factors interact to produce glucose intolerance and diabetes are uncertain, it has been suggested that the final common pathway responsible for the development of type 2 diabetes is the failure of the pancreatic β-cells to compensate for the insulin resistance. Hyperinsulinemia in the fasting state is observed relatively early in type 2 diabetes, but it is considered to be a secondary response that compensates for the insulin resistance.[8,9] Overt diabetes occurs over time when pancreatic β-cells bearing the burden of increased insulin secretion fail to compensate for the insulin resistance.

In this study, to elucidate the role of HCV in a possible association between diabetes and HCV infection, transgenic mice that carry the core gene of HCV[10,11] were analyzed. We found that these mice developed insulin resistance. An addition of a high-calorie diet led to the development of type 2 diabetes by dis-

Abbreviations used in this paper: EDL, extensor digitorum longus; ELISA, enzyme-linked immunosorbent assay; FPG, fasting plasma glucose; HCV, hepatitis C virus; IRS, insulin receptor substrate; JNK, c-Jun N-terminal kinase; TNF-α, tumor necrosis factor-α.

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rupting the balance between insulin resistance and secretion.

Materials and Methods

Transgenic Mice

The production of HCV core gene transgenic mice has been described previously.[11] Briefly, the core gene from HCV of genotype 1b, which is placed downstream of a transcriptional regulatory region from the hepatitis B virus, was introduced into C57BL/6 mouse embryos (Clea Japan, Tokyo, Japan). The mice were cared for according to institutional guidelines, fed an ordinary chow diet (Funabashi Farms, Funabashi, Japan), and maintained in a specific pathogen-free state. At an indicated time, the mice were fed a high-fat diet (Oriental Yeast Co., Ltd., Tokyo, Japan) for up to 2 months. Caloric content of food was 4.70 kcal/g for high-fat diet and 3.56 kcal/g for ordinary diet. The high-fat diet contains 18.5% protein, 22.1% fat (4.7% vegetable fat and 17.4% animal fat), 5.4% ash, 2.5% fiber, 6.5% moisture, and 45.0% carbohydrate, and the ordinary diet contains 22.4% protein, 5.7% fat, 6.6% ash, 3.1% fiber, 7.7% moisture, and 54.5% carbohydrate. Because there is a sex preference in the development of liver lesion in the transgenic mice, we used only male mice that were heterozygously transgenic for the core gene, and as controls we used nontransgenic litter mates of the transgenic mice. Transgenic mice carrying the HCV envelope genes under the same regulatory region as that in the core gene transgenic mice were also used as controls.[12] At least 5 mice were used in each experiment and the data were subjected to statistical analysis.

Glucose Tolerance Test

The mice were fasted for >16 hours before the study. D-Glucose (1g/kg body weight) was administered by intraperitoneally (IP) injection to conscious mice. Blood was drawn at different time points from the orbital sinus, and plasma glucose concentrations were measured by using an automatic biochemical analyzer DRI-CHEM 3000V (Fuji Film, Tokyo, Japan). The levels of serum insulin were determined by radioimmunoassay (BIOTRAK; Amersham Pharmacia Biotech, Piscataway, NJ) with rat insulin as a standard.

Insulin Tolerance Test

The mice were fed freely and then fasted during the study period. Human insulin (1 U/kg body weight) (Humulin; Novo Nordisk, Denmark) was administered by IP injection to fasted conscious mice, and glucose concentrations were determined at the time points indicated. Values were normalized to the baseline glucose concentration at the administration of insulin.

Morphometric Analysis

Sections of the pancreas were prepared and evaluated for morphometry after H&E staining or immunostaining. Relative islet area and islet number were determined with an image analyzer (QUE-2; Olympus Optical Co., Tokyo, Japan).

Enzyme-Linked Immunosorbent Assay

ELISA for mouse tumor necrosis factor (TNF)-α was performed using a commercially available mouse TNF-α ELISA kit (BioSource International, Camarillo, CA). Samples were prepared as reported previously.[13] Briefly, the liver of transgenic and control mice were lysed with a buffer containing 1% Tween 80, 10 mmol/L Tris-HCl [pH 7.4], 1 mmol/L EDTA, 0.05% sodium azide, 2 mmol/L PMSF, and the Protease Inhibitor Cocktail (Complete; Roche Molecular Biochemicals, Indianapolis, IN) and homogenized on ice for 20 seconds. The homogenates were centrifuged at 11,000 × *g* for 10 minutes at 4° C, and the supernatants were collected and assayed. ELISA was performed in triplicate for each sample. The concentrations of the cytokines in the liver were normalized by determining the amount of total protein in each sample using the BCA Protein Assay Kit (Pierce, Rockford, IL).

Immunoprecipitation and Western Blotting

For immunoprecipitation studies, liver tissues were homogenized in lysis buffer (10 mmol/L Tris-HCl at pH 7.5, 150 mmol/L NaCl, 10 mmol/L sodium pyrophosphate, 1.0 mmol/L β-glycerophosphate, 1.0 mmol/L sodium orthovanadate [Na_3VO_4], 50 mmol/L sodium fluoride [NaF], the Protease Inhibitor Cocktail [Complete, Roche Molecular Biochemicals], and 1.0% Triton X-100), and homogenates were precipitated with an anti–insulin receptor substrate (IRS)-1 or anti–IRS-2 rabbit polyclonal antibody (UBI, Lake Placid, NY) and then with Sepharose 4B beads (Amersham Biosciences). Resulting pellets were washed 3 times and then subjected to Western blotting. Pellets were resuspended in Western sample buffer (5% β-mercaptoethanol, 2% sodium dodecyl sulfate, 62.5 mmol/L Tris-HCl, 1 mmol/L EDTA, 10% glycerol), and then subjected to 2%–15% gradient sodium dodecyl sulfate/PAGE (PAG Mini "DAIICHI" 2/15 (13W), Daiichi Diagnostics, Tokyo, Japan), and electrotransferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA). The filter was then reacted with antiphosphorylated tyrosine (Santa Cruz Biotechnology Inc., Santa Cruz, CA), antiphosphorylated serine (Cell Signaling Technology, Inc., Beverly, MA), anti–IRS-1 or anti–IRS-2 mouse monoclonal antibody (BD Biosciences, Lexington, KY), followed by immunostaining with secondary biotinylated IgG (Vector Labs, Inc., Burlingame, CA) and visualization using an ECL kit (Amersham Intl., Buckinghamshire, UK).[14]

Hyperinsulinemic-Euglycemic Clamp

Mice underwent a hyperinsulinemic-euglycemic clamp using D-[3-^{3}H]glucose (NEN Life Science, Boston, MA) to measure the rate of glucose appearance and hepatic glucose production (HGP) as described previously.[15] Three days after jugular catheter placement, a hyperinsulinemic-euglycemic clamp was conducted with a continuous infusion of human

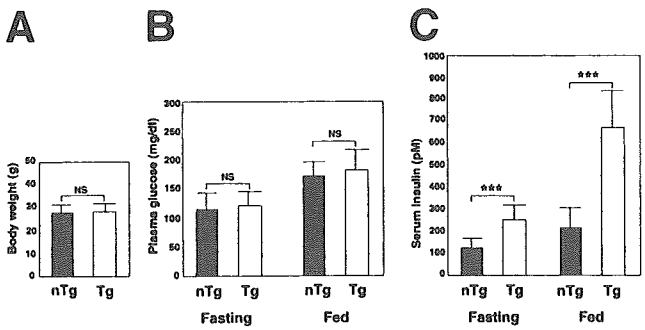

Figure 1. Altered glucose homeostasis in hepatitis C virus core gene transgenic mice. (*A*) Body weight of 2-month-old mice (n = 10 in each group). (*B*) Plasma glucose levels in fasting or fed mice (n = 10 in each group). (*C*) Serum insulin levels in fasting or fed mice (n = 10 in each group). The insulin level was significantly higher in the core gene transgenic mice than in control mice. Values are mean ± standard error; $***P < 0.001$; NS, statistically not significant; nTg, nontransgenic mice; Tg, transgenic mice.

insulin to raise serum insulin within a physiological range. Blood samples were drawn at intervals for the immediate measurement of blood glucose concentration, and 20% glucose was infused at variable rates to maintain blood glucose at ca. 125 mg/dL. All infusions were done using microdialysis pumps (KD Scientific Inc., Boston, MA). The rate of glucose appearance (mg/kg per minute), which equals the rate of total body glucose utilization during steady state, was calculated as the ratio of the rate of infusion of [3-^{3}H]glucose and the steady state plasma [^{3}H-]glucose specific activity. HGP (mg/kg/min) during clamps was determined by subtracting the glucose infusion rate from the rate of glucose appearance.

Glucose Uptake by Skeletal Muscle

The extensor digitorum longus (EDL) or soleus muscle was excised from 2-month-old mice and exposed to insulin at the indicated concentrations. 2-Deoxyglucose uptake was determined as described previously.[16]

Treatment With Anti-TNF-α Antibody

To suppress TNF-α, a dose of 200 μg/mouse of neutralizing hamster monoclonal antibody (TN3-19.12, Santa Cruz Biotechnology Inc.) was administered by IP injection on days 1 and 4, and plasma glucose and insulin levels were determined at day 5.[17]

Statistical Analysis

The results are expressed as means ± standard error. The significance of the difference in means was determined by Student *t* test or Mann–Whitney *U* test whenever appropriate. $P < 0.05$ was considered significant.

Results

Hyperinsulinemia and Insulin Resistance in Transgenic Mice

At the age between 1 and 12 months, there was no significant difference in body weight between the core gene transgenic mice and control mice. Figure 1*A* shows body weight of 2-month-old mice. Fasting plasma glucose (FPG) levels were slightly elevated in the core gene transgenic mice compared with control mice, but the difference was not significant ($P = 0.79$, Figure 1*B*). In contrast, there was a marked increase in the level of serum insulin in the core gene transgenic mice than control mice ($P < 0.001$, Figure 1*C*). Hyperinsulinemia was observed in the core gene transgenic mice as early as 1 month old. These findings suggest that decreased responsiveness to the hormone may have resulted in compensatory hyperinsulinemia. Administration of glucose to 2-month-old core gene transgenic mice revealed mild glucose intolerance compared with control mice of the same age, but the difference was not statistically significant at any time points measured (Figure 2*A*). HCV envelope gene transgenic mice of the same age, in which the envelope genes were expressed under the same transcriptional regulatory region as the core gene transgenic mice, did not manifest hyperinsulinemia or elevated FPG levels, indicating that not the transcriptional regulatory region used but the expressed gene itself is essential in this phenotype.

The insulin tolerance test conducted at the age of 2 months revealed that the reduction in plasma glucose concentration after IP insulin injection was impaired in the core gene transgenic mice, displaying higher plasma glucose levels than those in control mice at all time points measured (Figure 2*B*). At 40 and 60 minutes, the difference was statistically significant between transgenic

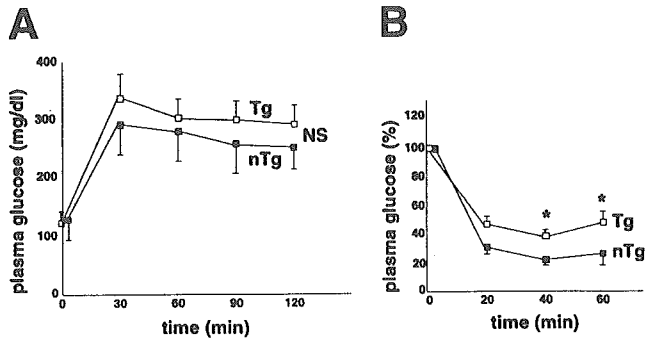

Figure 2. Insulin resistance in the core gene transgenic mice. (*A*) Glucose tolerance test (n = 5 in each group). Animals were fasted overnight (>16 hours). D-Glucose (1 g/kg body weight) was administered by IP injection to conscious mice, and plasma glucose levels were determined at the time points indicated. (*B*) Insulin tolerance test (n = 5 in each group). Human insulin (1 U/kg body weight) was administered by IP injection to fasted conscious mice and glucose concentrations were determined. Values were normalized to the baseline glucose concentration at the time of insulin administration. Values are mean ± standard error; $*P < 0.05$; NS, statistically not significant; nTg, nontransgenic mice; Tg, transgenic mice.

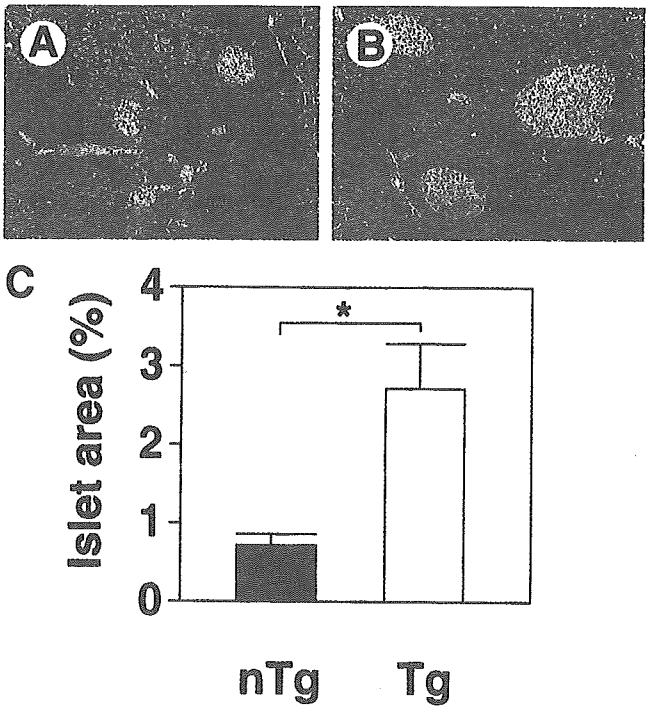

Figure 3. Analysis of pancreatic islet mass in the core gene transgenic and control mice. (*A* and *B*) Morphology of representative islets (H&E staining) from normal control mice (*A*) or the core gene transgenic mice (*B*). (*C*) Relative islet area, expressed as a percentage of the total stained pancreatic section, for control mice (nTg) and the core gene transgenic mice (Tg) (n = 10 in each group). Values are mean ± standard error; $*P < 0.05$.

and control mice (39.6 ± 1.3 vs. 24.4 ± 1.1 and 43.7 ± 2.1 vs. 26.4 ± 2.3, $P < 0.05$). These data are consistent with a defect in the actions of insulin on glucose disposal and/or production in the core gene transgenic mice.

Morphology of Pancreatic Islet Cells

Because a critical factor contributing to whether insulin resistance progresses to diabetes is the capacity of the pancreatic β-cells to respond to increased demands for insulin secretion, we evaluated the morphology of pancreatic islet cells by histologic examination. In the pancreas of HCV core gene transgenic mice, an approximately 3-fold increase in islet mass was observed (Figure 3, $P < 0.05$), which is consistent with β-cell compensation to insulin resistance. There was no infiltration of inflammatory cells within or surrounding the islets.

Feeding Transgenic Mice a High-Fat Diet Leads to Overt Diabetes

Thus, an insulin resistance is present but no apparent glucose intolerance (overt diabetes) in the HCV core gene transgenic mice. This is probably because of the genetic background of C57BL/6 mice, which has been shown to maintain either normal or mildly elevated glucose levels despite insulin resistance.[18] To determine whether a high-fat diet exacerbates the prediabetic phenotype, 2-month-old HCV core gene transgenic mice were fed a high-fat diet for up to 8 weeks. Both the transgenic and control mice showed a similar increase (about 30%) in body weight (Figure 4*A*). After 8 weeks on this diet, 100% (10 out of 10) of the transgenic mice exhibited casual (fed) plasma glucose levels >250 mg/dL, whereas none of the 10 control mice fed the same diet exhibited levels >250 mg/dL (325.0 ± 66.6 vs. 179.0 ± 17.4 mg/dL, $P < 0.01$, Figure 4*B*). Insulin levels were significantly higher in the core gene transgenic mice than in control mice both at fasting and fed state (Figure 4*C*, $P < 0.01$ and $P < 0.001$). In control mice, serum insulin levels in high-fat diet state were significantly higher than those in normal diet state at fed state (Figures 1*C* and 4*C*, $P < 0.01$). Although FPG levels were not significantly different between the transgenic and control mice, these results indicate that feeding a high-fat diet leads to the development of overt diabetes in a mouse model for HCV infection. Body weight gain, particularly with high levels of lipid, may trigger the process leading to overt diabetes in an insulin resistance model mouse with compensatory hyperplasia of islet cells.

Insulin Resistance in the Core Gene Transgenic Mice Is Chiefly Caused by Hepatic Insulin Resistance

We then investigated the mechanism of insulin resistance in the core gene transgenic mice. There was no

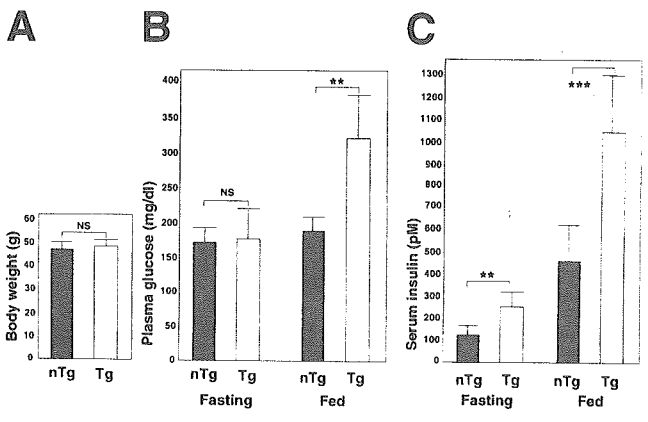

Figure 4. Body weight and glucose homeostasis after a high-fat diet. Control and transgenic mice were fed a high-fat diet for 8 weeks; thereafter, body weight and blood parameters were determined. (*A*) Body weight at the end of the high-fat diet (n = 10 in each group). (*B*) Plasma glucose levels determined in a fasting or fed state (n = 10 in each group). (*C*) Serum insulin levels in a fasting or fed state (n = 10 in each group). Values are mean ± standard error; NS, statistically not significant; $**P < 0.01$; $***P < 0.001$; nTg, nontransgenic mice; Tg, transgenic mice.

significant difference in body weight between the transgenic and control mice as already shown in Figure 1*A*. After the age of 3 months, the core gene transgenic mice developed hepatic steatosis, which is known to be one of the causes of insulin resistance in humans.[19] However, in 1-month-old mouse livers that were used in the analysis of insulin resistance, no hepatic steatosis was noted. No difference was observed in the levels of free fatty acids in the sera between the transgenic and control mice (0.56 ± 0.33 vs. 0.50 ± 0.21 mmol/L, n = 7 in each group, P = 0.65).

Then, we explored the role of the liver in pathogenesis of insulin resistance in the core gene transgenic mice. To directly measure HGP, the hyperinsulinemic-euglycemic clamp technique was conducted as described in Materials and Methods. The core gene transgenic mice showed a normal or slightly lower rate of HGP during the basal period as compared with control mice (Figure 5*A*). Although insulin infusion during the clamp suppressed HGP by 60% in the control mice, insulin induced little effect on HGP of the core gene mice (Figure 5*A*). This is consistent with the notion that insulin resistance in the core gene transgenic mice is chiefly depending on the shortage of insulin action on the liver.

To study the involvement of muscles in the development of insulin resistance in the core gene transgenic mice, we then examined whether or not insulin-stimulated glucose uptake is impaired in the skeletal muscles. The extensor digitorum longus muscle (EDL) from 2-month-old core gene transgenic and control mice were excised and exposed to insulin at the intermediate (0.30 nmol/L) and maximal (10.0 nmol/L) concentrations. There was no significant difference in 2-deoxyglucose uptake in the EDL muscle between the core gene transgenic mice and control mice at either insulin concentration (Figure 5*B*, at 0.30 nmol/L, P = 0.23 and at 10.0 nmol/L, P = 0.76). As another representative muscle that differs from EDL in metabolic properties, the soleus muscle was examined in the same manner as EDL. 2-Deoxyglucose uptake by the soleus muscle was not significantly different between the core gene transgenic and control mice (Figure 5*C*, at 0.30 nmol/L, P = 0.49 and at 10.0 nmol/L, P = 0.49). Thus, in the core gene transgenic mice, contribution of the peripheral skeletal muscle in the development of insulin resistance is negligible. This is in agreement with the observation that the core protein was exclusively present in the liver as detected by Western blotting,[20] which was confirmed by a sensitive enzyme immunoassay (Tsutsumi T. et al., unpublished data, December 2002).[21]

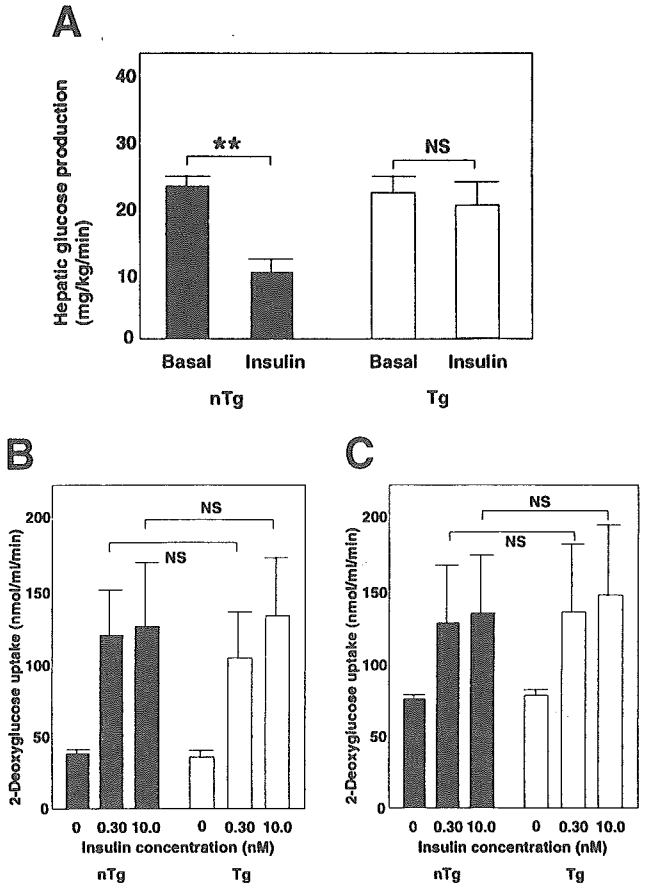

Figure 5. Characterization of glucose metabolism in the core gene transgenic mice. (*A*) Hyperinsulinemic-euglycemic clamp. Hepatic glucose production was calculated using hyperinsulinemic-euglycemic clamp. There was a failure of insulin to suppress hepatic glucose production in the core gene transgenic mice (n = 5 in each group). (*B* and *C*) Glucose uptake by the muscle after insulin stimulation. The extensor digitorum longus muscle (*A*) or soleus muscle (*B*) of 2-month-old mice were excised and exposed to insulin at intermediate (0.30 nmol/L) and maximal (10.0 nmol/L) concentrations. 2-Deoxyglucose uptake was determined as described in the Materials and Methods section (n = 8 in each group). Values are mean ± standard error; NS, statistically not significant; nTg, nontransgenic mice; Tg, transgenic mice.

Elevated TNF-α Level and Altered Tyrosine Phosphorylation of Insulin Receptor Substrate-1 in the Liver and Insulin Resistance

We have noted an increase in TNF-α levels in the liver of HCV core gene transgenic mice,[13] which has also been documented in the sera of human hepatitis C patients.[22–25] On the other hand, TNF-α has been shown to induce insulin resistance in experimental animals and cultured cells.[26–29] Therefore, we next determined the protein expression level of TNF-α by ELISA in the liver of these mice that were used in the current study. The TNF-α levels in the liver of 2-month-old transgenic mice were 702.2 ± 283.3 pg/mg protein and 313.5 ±

113.6 pg/mg protein in that of 2-month-old control mice (n = 10 in each group, $P < 0.001$). Thus, the levels of TNF-α exhibited a more than 2-fold increase in the HCV core gene transgenic mice compared with the control mice, which may be associated with insulin resistance.

Suppression of tyrosine phosphorylation of IRS-1 and -2 is one of the mechanisms by which a high level of TNF-α causes insulin resistance.[29–31] We, therefore, examined the suppression of tyrosine phosphorylation of IRS-1 in response to insulin action in the core gene transgenic mice. Twenty minutes after the administration of human insulin (1 U/kg body weight), when the plasma glucose levels decreased (Figure 2*B*), IRS-1 in the liver of control mice exhibited a marked phosphorylation of its tyrosine. In contrast, phosphorylation level of tyrosine in IRS-1 in the liver of core gene transgenic mice manifested apparently no increase compared with the basal level after the administration of insulin (Figure 6). In contrast, there was no difference in the time course of tyrosine phosphorylation of IRS-2 between the core gene transgenic and control mice (data not shown). These results indicate that a suppression of tyrosine phosphorylation of IRS-1, that is, a suppression of the insulin action in the liver, is at least one of the mechanisms of insulin resistance in HCV transgenic mice, whereas pathways other than IRS-1 may also be involved.

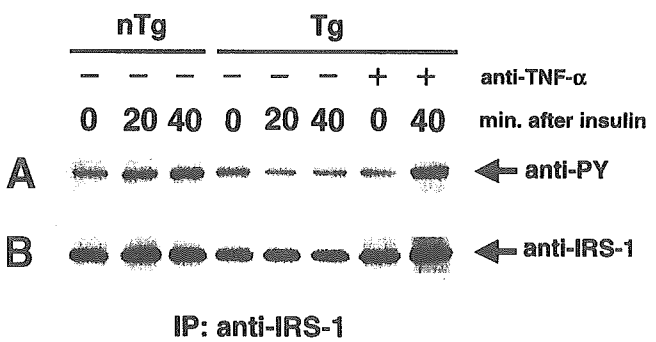

Figure 6. Phosphorylation of tyrosine in IRS-1 in response to insulin stimulation. Liver tissues from control mice and core gene transgenic mice with or without anti–TNF-α antibody treatment were analyzed before and 20 and 40 minutes after insulin administration. The samples were subjected to immunoprecipitation with anti-IRS-1 antibody and subsequently immunoblotted with antibodies as indicated. Experiments were performed in triplicate, and a representative picture is shown. (*A*) Immunoblot with antiphosphotyrosine antibody. There was no augmentation of phosphorylation of tyrosine in IRS-1 after insulin stimulation in the core gene transgenic mice, whereas tyrosine phosphorylation was markedly enhanced in control mice. Insulin-stimulated tyrosine phosphorylation was restored 40 minutes after anti–TNF-α antibody treatment. (*B*) Immunoblotting with anti-IRS-1 antibody as a control of IRS-1 load. nTg, nontransgenic mice; Tg, transgenic mice; anti-PY, antiphosphotyrosine antibody; anti-PS, antiphosphoserine antibody. IP, immunoprecipitation.

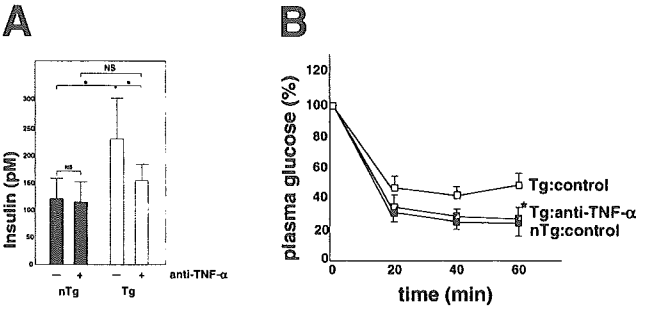

Figure 7. Serum insulin levels and insulin tolerance test after anti–TNF-α antibody treatment. (*A*) Serum insulin levels were determined in the fasting state with or without anti–TNF-α antibody treatment as described in the Materials and Methods section. Insulin levels decreased significantly after anti–TNF-α antibody treatment in the core gene transgenic mice (n = 5 in each group). (*B*) Insulin tolerance test (n = 5 in each group). Human insulin was administered by IP injection to fasted conscious mice and glucose concentrations were determined 24 hours after the second administration of anti–TNF-α antibody. As control, mice were injected with hamster IgG instead of anti–TNF-α antibody. Values were normalized to the baseline glucose concentration at the time of insulin administration. Values are mean ± standard error; $*P < 0.05$ when compared with Tg control; nTg, nontransgenic mice; Tg, transgenic mice.

The c-Jun N-terminal kinase (JNK) pathway has been shown to mediate the inhibitory effect of TNF-α on insulin action through the phosphorylation of serine in IRS-1.[32,33] Because an activation of the JNK pathway was observed in the liver of core gene transgenic mice,[13] phosphorylation of serine residues in IRS-1 was examined using antiphosphorylated serine monoclonal antibodies (Ser^{307} and Ser^{612}). However, there was no difference in the time course of serine phosphorylation after insulin stimulation between the core gene transgenic and control mice (data not shown).

Blockade of TNF-α Action Restores Insulin Sensitivity

Then the anti–TNF-α antibody was administered to block the in vivo activity of TNF-α in mice as described in the Materials and Methods section.[17] Twenty-four hours after the second administration of the anti–TNF-α antibody (200 μg/mouse), serum insulin levels in transgenic mice became significantly lower than the baseline (Figure 7*A*, 230.8 ± 70.7 vs. 153.6 ± 17.4 pmol/L, $P < 0.05$). Serum insulin levels in control mice also decreased, but there was no significant difference from the baseline (123.3 ± 36.1 vs. 112.0 ± 39.7 pmol/L, $P = 0.25$). Levels of FPG also decreased, but the difference from the baseline was not statistically significant. The insulin tolerance test conducted 24 hours after the second administration of anti–TNF-α antibody is shown in Figure 7*B*. As expected from serum insulin levels, anti–TNF-α antibody treatment restored the sen-

sitivity of the core gene transgenic mice to insulin activity. At this time point, phosphorylation of tyrosine in IRS-1 in the liver of transgenic mice in response to insulin action was restored to a similar level to that in control mice (Figure 6*A*, 40 minutes after insulin administration). These results strongly support the notion that the increased level of TNF-α is one of the bases for insulin resistance in the HCV core gene transgenic mice.

Taken together, these data indicate that the presence of the HCV core protein in the liver, at a level similar to that in human chronic hepatitis C patients,[21] confers insulin resistance to the mice by affecting the liver, by disturbing the insulin-induced suppression of hepatic glucose production.[34,35]

Discussion

Since Allison et al.[4] reported an association between HCV infection and diabetes, evidence has been accumulating connecting these 2 conditions. In such studies, HCV infection has a significantly stronger association with diabetes than hepatitis B viral infection.[4–7] The variables other than HCV infection that are associated with diabetes are cirrhosis, male sex,[5] and aging.[6] In addition to these clinic-based, case-control studies, Mehta et al.[7] have reported the result of investigation at population level. In this cross-sectional national survey, persons 40 years or older with HCV infection were more than 3 times more likely to have type 2 diabetes than those without HCV infection. Thus, the association of HCV infection with diabetes has become closer as shown by epidemiological studies. However, there are some difficulties in establishing a definite relationship between HCV infection and diabetes on the basis of epidemiological studies; in patients, there are other numerous factors perturbing the verification of the definite relationship, such as obesity, aging, or particularly advanced liver injuries. Moreover, the biological mechanism underlying diabetes or insulin resistance in HCV infection is unknown. In vitro or cultured cell studies have a very limited utility for the study of insulin resistance or diabetes because insulin resistance is a condition that involves multiple organs, such as the skeletal muscles and liver. Thus, the use of good experimental animal model systems may be useful both in establishing a definite relationship between diabetes and HCV infection and in elucidating the role of HCV in the development of insulin resistance.

In the current study, the HCV core gene transgenic mice exhibited insulin resistance as early as 1-month old, despite an apparent absence of glucose intolerance. Development of insulin resistance without any liver injury[10,11] or excessive body weight gain, as shown in the current study, clearly indicates that infection of HCV per se is a cause of the development of insulin resistance. Although only the core protein is expressed in these mice instead of HCV replication in humans, the fact that the intrahepatic core protein levels are similar between the core gene transgenic mice and chronic hepatitis C patients[20] warrants extrapolating the result into hepatitis C patients. Certainly, dispersion in the intrahepatic core protein levels in human chronic hepatitis C patients compared with the constant amount of the core protein must be taken into account. The occurrence of insulin resistance in the core gene transgenic mice as early as 1-month old also excluded the possibility that aging is a cause of insulin resistance. Nonetheless, aging could be an aggravating factor for insulin resistance. Thus, the current analysis shows a definite causal relationship between HCV infection and the development of insulin resistance.

Our earlier studies have shown the development of hepatic steatosis in these HCV core gene transgenic mice after the age of 3 months.[11] However, insulin resistance invariably preceded the occurrence of hepatic steatosis, indicating that insulin resistance is not a consequence of hepatic steatosis in these mice. Certainly, it is possible that insulin resistance in the core gene transgenic mice may be affected or aggravated after the occurrence of hepatic steatosis. On the other hand, insulin resistance may be one of the factors that cause hepatic steatosis,[19] whereas the impairment of very-low-density lipoprotein (VLDL) secretion from the liver and hypo-β-oxidation of fatty acids are considered to be the bases of development of hepatic steatosis in the core gene transgenic mice.[21,36]

The general mechanism underlying insulin resistance is not precisely understood and is considered to be multifactorial.[8,9,37,38] Chiefly, it involves glucose consumption by the skeletal muscle and glucose production in the liver. Our current analysis revealed a failure of insulin in the suppression of HPG in the liver and an absence of suppression of glucose uptake by the muscles in the core gene transgenic mice. Combined, these results indicate the insulin resistance in the core gene transgenic mice is chiefly due to hepatic insulin resistance. An elevated intrahepatic TNF-α level plays one of the roles in causing insulin resistance through suppressing insulin-induced tyrosine phosphorylation of IRS-1. It should be noted that TNF-α levels are invariably elevated in the sera of patients with HCV infection.[22] Moreover, restoration of insulin sensitivity after anti–TNF-α antibody administration strongly supports the notion that TNF-α

is, at least in this animal model, a major factor for the development of insulin resistance in HCV infection. Taken together, insulin resistance in the core gene transgenic mice mainly depends on suppression of the inhibitory effect of insulin on hepatic glucose production. This is consistent with the observation that the core protein is present only in the liver but absent in the skeletal muscle of the core gene transgenic mice (Tsutsumi T., unpublished data, December 2002).[21] Impairment in other undetermined pathways may also be responsible for the development of insulin resistance in HCV infection.

Insulin resistance alone does not always lead to the development of overt diabetes in humans or murine models. Particularly, in the models with the C57/BL6 strain,[18] hyperplasia of the islets of Langerhans in the pancreas compensates for insulin resistance by secreting higher amounts of insulin. Along with a gain in body weight by being fed a high-calorie diet, the core gene transgenic mice but no control mice developed overt diabetes, showing that obesity is a risk factor for diabetes as observed in patients or as shown in animal models for diabetes unrelated to HCV infection.[37,38] This observation would suggest that HCV infection confers insulin resistance and additional factors such as obesity, aging, or possibly inflammation may contribute to the complete development of overt diabetes. The effect of high-fat diet on control C57BL/6 mice may be milder in the current study compared with a previous study.[39] However, there was a substantial increase in FPG levels in high-fat-diet-fed control mice compared with normal-diet-fed control mice (Figures 1*B* and 4*B*). In addition, at fed-state, serum insulin levels in high-fat-diet-fed control mice were significantly increased compared with those in normal-diet-fed control mice (Figures 1*B* and 4*B*). It is unclear why plasma glucose levels were not very high at fed-state in control mice, but one possible explanation is the lower calorie content in the current study than those in the previous report: 4.70 kcal/g for our high-fat diet vs. 5.55 kcal/g for high-calorie diet in the previous study. A shorter duration of high-fat diet than the previous study (2 months vs. 6 months) may be another possible explanation.[39] Such a mild elevation in plasma glucose levels in high-fat-diet-fed C57BL/6 mice as the one observed in our study has also been described in previous studies.[40]

In conclusion, the HCV core protein induces insulin resistance in transgenic mice without gain in body weight at young age. These results indicate a direct involvement of HCV per se in the pathogenesis of diabetes in patients with HCV infection and provide a molecular basis for insulin resistance in such a condition.

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Address requests for reprints to: Kazuhiko Koike, M.D., Department of Infectious Diseases, Internal Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. e-mail: kkoike-tky@umin.ac.jp; fax: (81) 3-5800-8807.

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Y.S. and H.F. contributed equally to this work.

臨　床

HIV・HCV重複感染症の現状

塚田訓久，小池和彦*
東京大学医学部附属病院　感染症内科（*科長）

重複感染の疫学

2003年末の時点で，全世界で約4,000万人がヒト免疫不全ウイルス(human immunodeficiency virus；HIV)に感染していると推測されている[1]．国内での感染者数も年々増加しており，非加熱凝固因子製剤などの血液製剤投与による感染例(血友病例を代表とするいわゆる「薬害エイズ」例：以下血友病例)を除いて，2003年末までの累計で9,000人近くが報告されている(厚生労働省エイズ動向委員会)．感染に気付いていない例も含めると，さらに多くの感染者が存在すると考えられる．

HIVは血液・体液を介して感染するため，同様の経路で感染するC型肝炎ウイルス(hepatitis C virus；HCV)との重複感染が生じ得る．海外からの報告では，HIV感染者のうちかなりの割合がHCVに重複感染しているとされる(2000年のGreubらの報告[2]では37.2%，2002年のSulkowskiらの報告[3]では44.6%など)．ただし，2003年までに国立国際医療センターエイズ治療・研究開発センター(AIDS Clinical Center；ACC)を受診した症例に関する筆者の検討(2003年エイズ学会報告)では，国内のHIV感染者のHCV重複感染率は，血友病例を除けば5%程度であり，海外と比較して低い．

国内で重複感染率が低い真の理由を知ることは難しいが，HIV感染の主要な経路(血液製剤による感染を除く)は性行為によるものと静注薬物濫用に伴うものであり，一般に前者でのHCV感染リスクは低く，後者ではきわめて高い．厚生労働省エイズ動向委員会の報告によれば，報告された平成15年度の新規感染者の8割以上が性行為による感染者であり，静注薬物濫用によるものは1%未満と考えられていることから，静注薬物濫用の頻度の差が重複感染率の差に影響していると推測できる．

血友病例においては，HIVに感染している例のほとんどがHCVにも重複感染しており，総数においても国内でのHIV・HCV重複感染例のかなりの割合を占めると考えられる．

重複感染の問題点

HIV・HCV重複感染の問題点を考える際は，HIVの重複感染がHCV感染症に与える影響と，HCVの重複感染がHIV感染症に与える影響に分けて考える必要がある(表1)．

HIVの重複感染がHCV感染症に与える影

表 1. 重複感染の相互作用

HIV 重複感染の HCV 感染症への影響	HCV 重複感染の HIV 感染症への影響
・肝線維化の進行が速くなる. ・インターフェロン療法の成績が低下する. ・抗 HIV 薬の長期毒性がリバビリンにより増強される可能性がある.	・HIV 感染症の進行には影響しない(これに反対する意見もある). ・抗 HIV 薬による肝機能障害がより高頻度に出現する.

響としては，①HIV の重複感染により肝線維化の進行が速くなる[4]，②HIV の重複感染例では，HCV 単独感染例と比較してインターフェロン(IFN)療法の有効性が低い，が挙げられる.

HCV の重複感染が HIV 感染症に与える影響については，重複感染例で AIDS 指標疾患の発症率・死亡率がより高いとする報告もときにみられるが，影響しないとする意見が現在の主流である．ただし，HCV 重複感染例では，HIV 単独感染例と比較して抗 HIV 薬による肝障害がより高頻度に出現することが知られている.

最近では，抗 HIV 療法の進歩に伴い，日和見疾患のために死亡する HIV 感染者数は減少している．とくに血友病例は，いわゆる「非加熱製剤」が流通していた時期，すなわち 1980 年代までに HIV・HCV に重複感染しており，感染から約 20 年を経過していることから，C 型肝炎・肝硬変が生命予後を規定する最大の因子となりつつある.

重複感染例の現状

筆者による 2003 年現在 ACC に通院中の重複感染例の検討では，血友病例以外の症例で，①HIV 感染症のコントロールはより良好で，②肝機能も良好に保たれている例が多かった．この理由として，血友病例以外の症例の多くが 1997 年頃，すなわち HIV 感染症に対する 3 剤併用療法(highly active anti-retroviral therapy；HAART)が広く行われるようになった時期以降の HIV 感染例であり，最初から有効な抗 HIV 療法を行うことが可能であったこと，HCV 感染からの期間が血友病例と比較して短いことが考えられる．非代償性肝硬変と判断される例は血友病例で 7 例，それ以外の例で 2 例であったが，後者の 2 例はいずれも 50 歳以上であり，HIV に感染したことが推定される時期よりも前に HCV に感染していたことが疑われ，HCV の感染経路と HIV の感染経路が同一であるか否かについても不明であった.

2001 年エイズ学会における矢崎らの報告によれば，1997 年以降に ACC で死亡した症例を検討した結果，血友病例の半数(6 例中 3 例)が肝硬変・肝細胞癌による死亡であり，残り 3 例中 2 例でも進行性肝疾患の所見がみられたのに対し，それ以外の原因による HIV 感染例(HCV 重複感染例とは限らない)の死亡原因は，そのほとんど(12 例中 11 例)が日和見疾患による死亡(いわゆる「エイズ死」)であった．このことから，少なくとも血友病例においては，日和見疾患より C 型肝炎関連疾患が現在より大きな問題であると考えることができるだろう.

重複感染の治療：HIV

HIV 感染症の治療の目標は，抗 HIV 薬を使用して体内での HIV の増殖を可能な限り抑制し，これにより HIV による細胞性免疫システムの破壊を抑制することである．この目標自体は，HIV・HCV 重複感染例においても変わらない.

現在のHIV感染症の標準的治療は，3剤以上の抗HIV薬を組み合わせて行う，いわゆるHAARTである．現在使用可能な抗HIV薬はおおまかに，核酸系逆転写酵素阻害薬(nucleoside reverse transcriptase inhibitor；NRTI)，非核酸系逆転写酵素阻害薬(non-nucleoside reverse transcriptase inhibitor；NNRTI)，プロテアーゼ阻害薬(protease inhibitor；PI)の3系統に分類され，通常は二種類のNRTIに1種類のNNRTIあるいはPIを組み合わせた3剤が選択される(詳細は成書および最新のガイドラインを参照のこと)．

重複感染例におけるHIV感染症治療で考慮すべき点として，前述のように薬剤性肝障害がより高頻度に発生することが挙げられる．肝障害が理由で有効であるはずの抗HIV療法を継続できない例も存在することから，これは大きな問題である．2003年のPuotiらの報告[5]によれば，IFNによるC型肝炎の先行治療によって，HAARTによる肝障害の発生を抑えることが可能であった．これは，この肝障害に細胞性免疫能の改善によりそれまで見かけ上沈静化していたC型肝炎が顕在化する，いわゆる「免疫再構築症候群」が関与している可能性を示唆する．

重複感染の治療：HCV

HCVを体内から排除できる可能性のある治療法として，現時点で広く利用できるのはIFN投与のみであり，リバビリンの併用によりその治療効果は増強される．

2000年のLandauらの報告[6]以降，HIV・HCV重複感染例においてもリバビリン併用療法の報告が相次いでおり，現在では主流となっている．ただしその治療成績については，重複感染例においてもリバビリン併用によりIFN単独投与による治療と比較して治療成績は改善されるものの，HCV単独感染例との比較では重複感染例で治療成績が劣るとするものが多い．我が国では欧米諸国と比較してIFN投与量が多くリバビリン投与量が少なめであるという違いがあるが，治療成績についてはおおむねこのような傾向を示す．HCV単独感染例より劣る治療成績の理由として，HIV感染による細胞性免疫能の障害や，(一部関係するが)高いHCV-RNA量などが考えられている．

HIV・HCV重複感染例における併用療法の問題点として，①血球減少などIFNによる副作用がHCV単独感染例と比較して強く出る可能性がある，②IFN使用中はCD4陽性Tリンパ球数が低下する(ただしこれは必ずしも日和見疾患の増加とは関連しないとされる)，③NRTIとリバビリンの併用によりNRTIの副作用(ミトコンドリア障害によるとされるもの)が増強される可能性がある，などが挙げられる．

現在ではPEG-IFNの導入など新たな試みが行われており，その治療成績が注目される．ただし我が国の現状として，現在でもHCVによる活動性肝炎が残存している血友病例には，過去にIFN投与による治療を試みたものの副作用のため完遂できなかった例，過去の治療が失敗に終わった例も含まれており，このような症例で今後再度のIFN投与を行った場合の成績はより劣ることが予想される．このような症例に対しては，必要に応じて対症的に肝庇護療法を行っているのが実情であるが，肝線維化の進行は免れない．

重複感染例に対する肝移植：背景

非代償性肝硬変に至った症例では，内科的治療による肝機能の改善は期待できない．腹水・食道静脈瘤・血小板減少など一般的な肝硬変の合併症に加え，HIV重複感染例においては肝予備能の低下のために抗HIV薬の使用に制限が生じることが大きな問題である．また，肝予備能が保たれていても，抗HIV薬の肝障害が強く現れるために有効なHAARTを行うことが

できず，結果として免疫不全が進行する症例もみられる．

肝硬変に対する根治療法は，現時点では肝移植のみである．HCV 抗体陽性例に対する肝移植は世界中で広く行われているが，HIV・HCV 重複感染例においては HCV 単独感染例と比較して，以下に述べるように，より多くの考慮すべき点があり，脳死ドナーの絶対的な不足もあって，ごく限られた施設でしか行われていないのが現状である．我が国においては，2001 年に我々の施設で重複感染例に対する世界初の生体部分肝移植術[7]が行われて以来，本稿執筆時点の 2004 年 6 月までに 5 例の同様の手術が行われている．

重複感染例に対する肝移植：問題点

肝移植後には移植片拒絶の予防・治療のため免疫抑制剤やステロイドが投与され，細胞性免疫が低下した状態にある．このため，移植後にはサイトメガロウイルス(cytomegalovirus；CMV)感染症をはじめとする各種の日和見疾患のハイリスク状態となるが，HIV 感染症の合併によりこのリスクはさらに増大すると考えられる．通常の HIV 感染症診療においては，CD4 陽性 T リンパ球数を免疫能の指標として日和見感染症の検索・予防内服を行うが，移植後においては必ずしも CD4 陽性 T リンパ球数が免疫状態と比例しないため，予防内服については明確な基準がない状態である．実際に我々の施設においても，重複感染例の肝移植術後に CMV 感染症を発症し，最終的に小腸病変からの出血により死亡した例を経験しているが，この例では CD4 陽性 T リンパ球数が比較的高い値を示していた時点ですでに CMV 抗原血症が遷延していた．

肝移植後の C 型肝炎の再発は必発であるが，これに対しては術後 IFN とリバビリンの併用療法が行われる．症例数が少ないためいまだその成績を評価できる段階にはないが，肝移植後においても通常の C 型肝炎治療時と同様 HIV 重複感染例では HCV 単独感染例と比較して効果が低い可能性がある．肝移植後に C 型肝炎が再燃した場合には，HIV の重複感染によりその進行は速くなることが予想される．

重複感染例に対する肝移植：移植後の HIV 感染症治療

肝移植後の HIV 感染症治療に関して大きな問題となるのは，①抗 HIV 薬の肝障害，②抗 HIV 薬と免疫抑制剤の相互作用の 2 点である．とくに生体肝移植の場合には，脳死全肝移植と異なり，術後数週間の間に移植片の容積が急速に増加するが，この期間中に抗 HIV 薬を投与することによる影響はいまだ不明である．また，とくに PI と免疫抑制剤との相互作用は強く，併用によりタクロリムス(tacrolimus)やシクロスポリン(cyclosporine)の血中濃度が数十倍にも上昇するため，免疫抑制剤の血中濃度の微調節を必要とする術後急性期の投与は，可能な限り避けたい．しかし，逆に HAART 開始を遅らせることにより HIV 感染症が進行した場合，日和見疾患を発症するリスクが高くなる．症例ごとに経過が大きく異なるため，明確な基準を作ることは将来的にも難しいことが予想されるが，術後しばらくの間は HAART を行うことができないという前提で(その間の HIV 感染症の進行も視野に入れた上で)，手術適応を判断する必要があるだろう．

術後の抗 HIV 薬選択に関しては，通常の HAART の際の薬剤選択と同様，耐性のない薬剤から 3 種類を選択することが原則となる．しかし実際には，血友病例では過去に長期間の薬剤投与歴を有するものが多く，単剤投与の繰り返しにより複数の薬剤に耐性を獲得している場合も珍しくない．また，外科的合併症などさまざまな理由により，一時的に抗 HIV 薬の投与を中止せざるを得ない場合があり，これにより HIV が耐性を獲得してしまう可能性もある．過

去に耐性を獲得していない薬剤を中心に最も有効と考えられる治療薬を組み合わせることになるが，周術期の合併症(腎機能障害・貧血など)により，術前に予定していた薬剤を使用できなくなる可能性があることに注意が必要である．術後に使用できる有効と考えられる抗HIV薬が存在しない場合は，新規薬剤の開発状況と合わせ移植自体の適応を再検討する必要があるだろう．

このように，重複感染例に対する肝移植についてはその長期成績も含めいまだ不明な点もあるが，実際に移植が成功した例においては，生命予後は勿論のこと，その生活の質の改善には著しいものがあり，IFNが無効で将来的に非代償性肝硬変に至ることが予想される例では，治療法の一つとして考慮すべきものと考える．

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Review

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

KAZUHIKO KOIKE and KYOJI MORIYA

Department of Infectious Diseases, Internal Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan

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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Reprint requests to: K. Koike

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

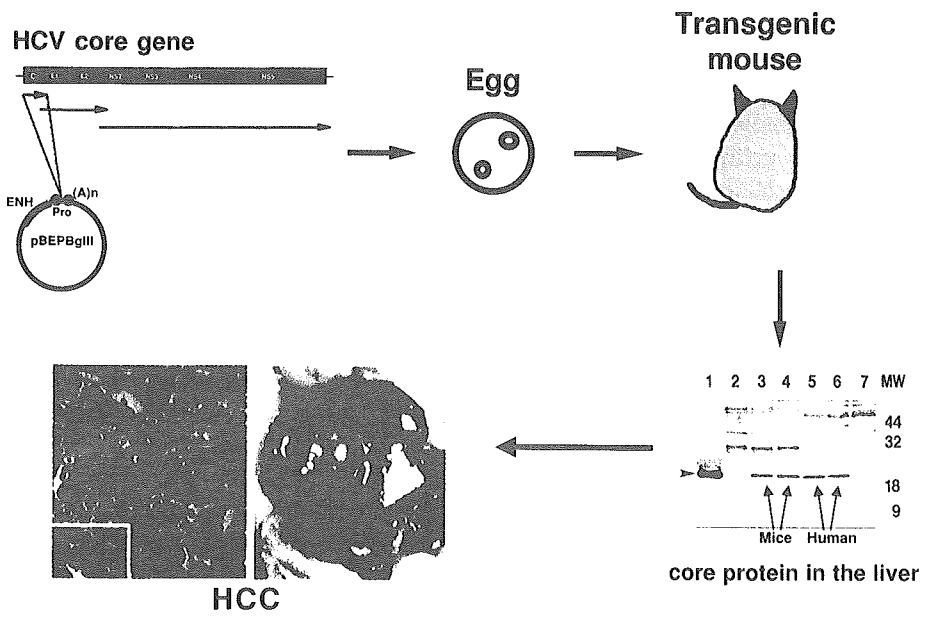

Fig. 1. Mouse model of hepatitis C virus (*HCV*)-induced liver pathogenesis. HCV core gene transgenic mice carry the core gene, alone, of genotype 1b HCV and express the core protein of an expected size in the liver, at levels comparable to those in human patients with chronic hepatitis C. The mice eventually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

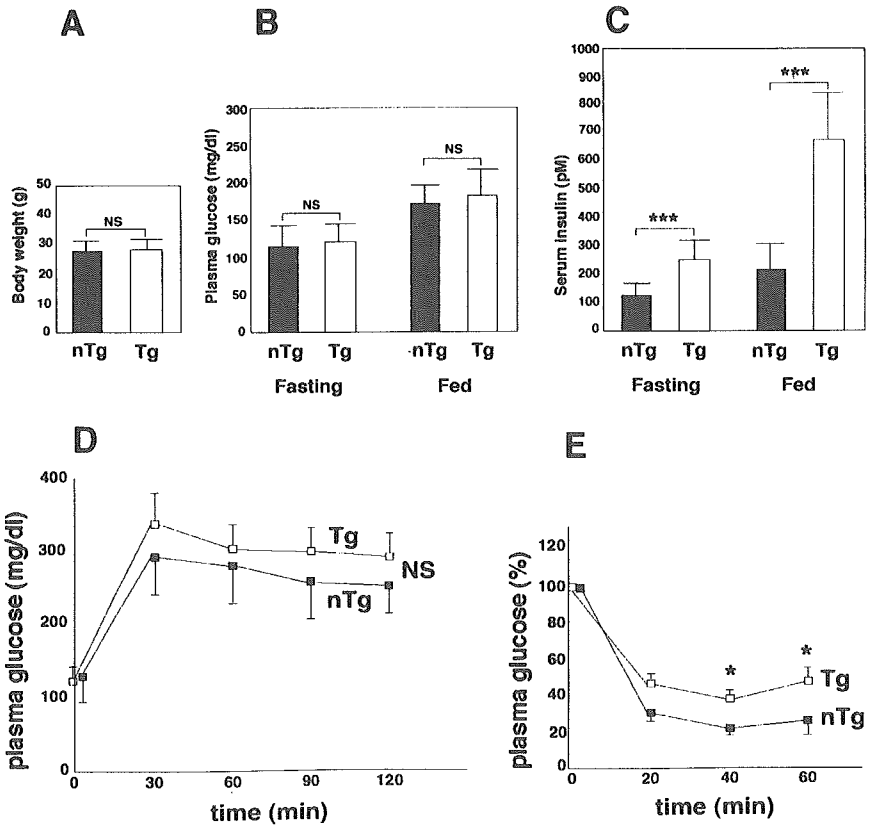

Fig. 2A–E. Altered glucose homeostasis in HCV core gene transgenic mice. **A** Body weights of 2-month-old mice. **B** Plasma glucose levels in fasting and fed mice. **C** Serum insulin levels in fasting and fed mice. The insulin level was significantly higher in the core gene transgenic mice than in 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

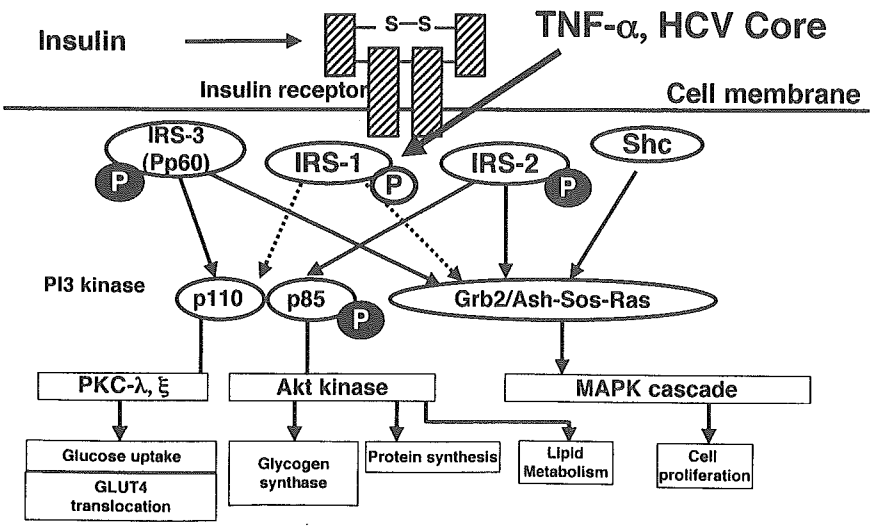

Fig. 3. Insulin resistance and HCV infection. HCV core protein or elevated intrahepatic tumor necrosis factor-α (*TNF-α*) inhibits tyrosine phosphorylation of insulin receptor substrate (*IRS*)-1 in the liver, suppresses insulin intracellular signal transduction, and leads to insulin resistance. *PKC*,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