Table 3. Altered Proteins between Tg and NTg Mouse Livers for 16 Months[a]

peak number	Tg/NTg ratio	protein name	molecular mass (Da)	GI number[b]
		Down-Regulated		
		respiration		
4	0.41	Hemoglobin, β adult major chain	15738	gi|31982300
67	0.69	Hemoglobin β	15653	gi|229301
53	0.70	α-globin	15076	gi|49902
108	0.70	Quininoid dihydropteridine reductase	25554	gi|21312520
		protein synthesis		
101	0.54	Regucalcin	33385	gi|6677739
		defense		
16	0.69	SOD	15955	gi|201006
65	0.64	Manganese superoxide dismutase	24662	gi|53450
40	0.53	Thioredoxin 1	11668	gi|6755911
63	0.72	Glutathione peroxidase (GSHPx-1) (Cellular glutathione peroxidase)	22268	gi|121666
75	0.49*	Glycine *N*-methyltransferase	32712	gi|15679953
79	0.61	Glutathione S-transferase, mu 1	25953	gi|61402231
83	0.69	BHMT	44992	gi|62533211
95	0.61	Glutathione *S*-transferase, α 3	25344	gi|31981724
97	0.51	Chain B, Glutathione *S*-Transferase Yfyf Cys 47-Carboxymethylated Class Pi, Free Enzyme	23350	gi|2624496
		fatty acid metabolism (containing β-oxidation)		
36	0.69	Fatty acid-binding protein, hepatic (fragment)	10173	gi|90485
82	0.58	Acetyl-Coenzyme A acyltransferase 2 (mitochondrial 3-oxoacyl-Coenzyme A thiolase)	41831	gi|20810027
107	0.70	Acetyl-Coenzyme A acyltransferase 1	43926	gi|18700004
85	0.32*	Hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), β subunit (HADHB)	51353	gi|13542763
102	0.58	Peroxisomal acyl-CoA oxidase	74608	gi|2253380
		apoptosis		
3	0.69	Eukaryotic translation elongation factor 1 α 1	50140	gi|13278382
8	0.58*	Ribosomal protein S29, isoform 1	6672	gi|22267962
		glycolytic system		
61	0.79	Fructose-bisphosphate aldolase B	39548	gi|15723269
98	0.42	Enolase 1, α non-neuron	47095	gi|12963491
99	0.58	Enolase 1, α non-neuron	47095	gi|12963491
		metabolism		
32	0.46**	Cystatin B	11039	gi|6681071
68	0.80	Carbonic anhydrase 3	29348	gi|31982861
72	0.53	Carbonic anhydrase 3	29348	gi|31982861
109	0.30**	PREDICTED: Carbamoyl-phosphate synthetase 1 (CPS1)	165705	gi|51705066
84	0.50	Argininosuccinate synthetase	46555	gi|6996911
		signal transduction		
87	0.65	Electron transferring flavoprotein, α polypeptide	35018	gi|13097375
		amino acid synthesis		
80	0.39	4-Hydroxyphenylpyruvate dioxygenase	45054	gi|849053
		other		
38	0.54	Histidine triad nucleotide binding protein 1	13768	gi|33468857
49	0.71	Peptidylprolyl isomerase A	17960	gi|71051228
64	0.69	Nit protein 2	30483	gi|12963555
86	0.64	γ-actin	40992	gi|809561
96	0.50	Unknown (protein for IMAGE:6414729)	50209	gi|53734652
103	0.70	Sorbitol dehydrogenase precursor	40066	gi|1009706
106	0.68	Heat-responsive protein	18462	gi|1255116
48	0.59	Unnamed protein product	65586	gi|12859782
		Up-Regulated		
		respiration		
37	1.33	α-globin	15076	gi|49900
		fatty acid metabolism (containing β-oxidation)		
35	1.60	Fatty acid-binding protein, hepatic (fragment)	10173	gi|90485
		other		
17	1.30	ND***		
33	1.34	Unnamed protein product	57807, 58587, 57007, 52653	gi|12852157, gi|26345440, gi|2634914, gi|26349459

[a] Peak numbers correspond to those in Figure 1. Asterisks indicate significant differences (two-tailed Student's *t* test, $*P \leq 0.05$, $**P \leq 0.01$). ***ND, not detected. [b] GI number is simply a series of digits that are assigned consecutively to each sequence record processed by NCBI. The GI system of sequence identifiers runs parallel to the accession.version system, which was implemented by GenBank, EMBL, and DDBJ in February 1999. Therefore, if the protein sequence changes in any way, it will receive a new GI number. (http://www.ncbi.nlm.nih.gov/Sitemap/samplerecord.html#ProteinIDB).

Differential Profiling. Differential profiling analysis was performed using liver tissue from HCV core gene transgenic and non-transgenic mice as model samples to evaluate the feasibility of the FD-LC–MS/MS method for clinical proteomics. To investigate the differential expression of proteins in transgenic and non-transgenic mice, the heights of the peaks corresponding to specific retention times were compared for each month of age, with 106 altered proteins observed. The differentially expressed proteins were classified by age, regulation and function (see Tables 1–3). Tg/NTg ratios over 1.2 were defined as up-regulated, and those below 0.8 were defined as down-regulated. Many proteins were up- or down-regulated during the progression of HCV-associated liver disease. Fifteen proteins were significantly altered in their levels of protein contents (Figure 2). At the age of 6 months, there were fewer down-regulated proteins than up-regulated (9 vs 19 proteins). In contrast, many kinds of proteins were different between transgenic- and non-transgenic mice at 12 months, with 11 proteins being down-regulated and 65 being up-regulated. At 16 months, there were more down-regulated proteins than in any other months (39 proteins), but only a small minority (four) of proteins were up-regulated.

The remarkable decrease in major urinary protein (MUP) and eukaryotic translation elongation factor 1 α 1 (EF-1α1) seen in Figure 2a represents an early event in the progression of HCV-associated liver disease (at 6 months). MUP has been known as a negative tumor marker.[23] Suppression of EF-1α1 expression prevents the induction of apoptosis, with the regulation reflected in an antiapoptotic mode.[24] Although one of the α-globin peaks (peak no. 52) decreased significantly, the other three peaks of α-globin (peak nos. 27, 29, and 37) tended to increase (see Table 1). The expression of α-globin has been shown to be up-regulated in apoptotic stimuli.[25] Therefore, the phenomenon might be considered a trend in apoptosis at this stage. Another observation made at the age of 6 months was the up-regulation of enzymes related to β-oxidation.

At 12 months of age, proteins related to respiration, the electron-transfer system, and defense against reactive oxygen species (ROS) were significantly up-regulated (Figure 2b). Moreover, a majority of proteins involved in respiration, protein synthesis, defense, apoptosis, the glycolytic system, and metabolism were more up-regulated than the changes observed at 6 months (Table 2).

Finally, at 16 months, proteins related to defense, β-oxidation, and apoptosis significantly decreased. Cystatin B[26] and carbamoyl-phosphate synthetase 1 (CPS1)[27] are known to be down-regulated in tumor and/or carcinoma and exhibited a significant decrease with the proposed method (Figure 2c). It was also established that various biological functions such as respiration, protein synthesis, defense, and metabolism tended to decline (Table 3).

As a whole, the investigation of the differential expression of proteins in transgenic and non-transgenic mice revealed that many proteins related to biological functions such as respiration, protein synthesis, defense, β-oxidation, and apoptosis fluctuate during the progression of chronic hepatitis C. These changes may reflect a gross effect derived from the loss of liver function in the various stages of chronic hepatitis in HCV infection.

Additionally, these data support, from the viewpoint of proteomics, the former results obtained from morphological and biochemical observation.[19–21] For example, previous reports suggested that HCV core protein might affect a specific pathway in the lipid metabolism.[19,21] In fact, the core protein has a specific effect on lipid metabolism; fat droplets are formed and accumulate in the liver, leading to steatosis. An analysis of the composition of these lipid droplets determined that the concentration of carbon 18 monosaturated (C18:1) fatty acids, such as oleic and vaccenic acid, significantly increased in the livers of transgenic mice as well as in chronic hepatitis C patients.[21] In the present study, hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein) β subunit (HADHB), which catalyzes fatty-acid metabolism, significantly decreased after 16 months (Figure 2). However, at 12 months, other enzymes associated with β-oxidation tended to increase (Table 2: peak nos. 7 and 82). In addition, up-regulation of ATP synthase led to an increase in the synthesis and metabolism of fatty acid at 12 months (Figure 2). Furthermore, acetaldehyde dehydrogenase (ALDH), which catalyzes the acetaldehyde metabolism, tended to be up-regulated in the same month (Figure 2). The metabolic reactions of fatty acid and acetaldehyde generate $NADH_2^+$, and the overexpression then causes suppression of both metabolisms. Hence, these results suggest that the fatty-acid metabolism may become milder and resulted from the multiple protein changes related to β-oxidation, ATP synthase, and acetaldehyde metabolism with the progression of HCV-associated liver disease.

Previous reports also suggested that HCV core protein might alter the oxidant/antioxidant state in the liver.[20] The reports demonstrated that there is no significant difference in the levels of lipid peroxidation at 3 and 12 months of age, resulting in cellular and tissue damage by ROS. In contrast, after 16 months, the peroxidation and hydrogen peroxide levels increased remarkably and the levels of total and reduced glutathione, which plays an important role as an antioxidant, decreased. While, our results demonstrate that enzymes related to the antioxidant effect, such as betaine-homocysteine methyltransferase (BHMT) and Cu/Zn-superoxide dismutase (SOD), were up-regulated in transgenic mice at 12 months (Table 2: defense). Subsequently, up to 16 months, a decrease in BHMT and glycine *N*-methyltransferase related to the methylation cycle was observed (Figure 2). The decrease in these enzymes led to a deficiency of adenosylmethionine, impairing mitochondrial function and generating oxidative stress in the liver.[28,30] It has recently been shown that a chronic deficiency of adenosylmethionine in the liver results in the spontaneous progression of steatohepatitis and HCC.[31] In addition, the down-regulation of glycine *N*-methyltransferase would inhibit the synthesis of glutathione resulting in a shift to the oxidizing state, thereby reducing cell proliferation and increasing apoptosis.[32] Therefore, the observed expression of antioxidants might reflect direct oxidative stress status; although at 12 months the up-regulated antioxidants protected against oxidative stress, the oxidative stress might become dominant by the deficiency of antioxidants among the progression of liver disease. Also, these results, derived from both studies, strongly suggest that HCV core protein induces ROS in an age-dependent manner. After 16 months, a biochemical[20] and proteomic analysis revealed a lack of glutathione, suggesting that supplying glutathione might be more effective than SOD in the progression of HCC in the late stage. Although a further animal experiment should be required for reliable clarification of the hepatocarcinogenesis mechanism, the proposed method was demonstrated to be extremely

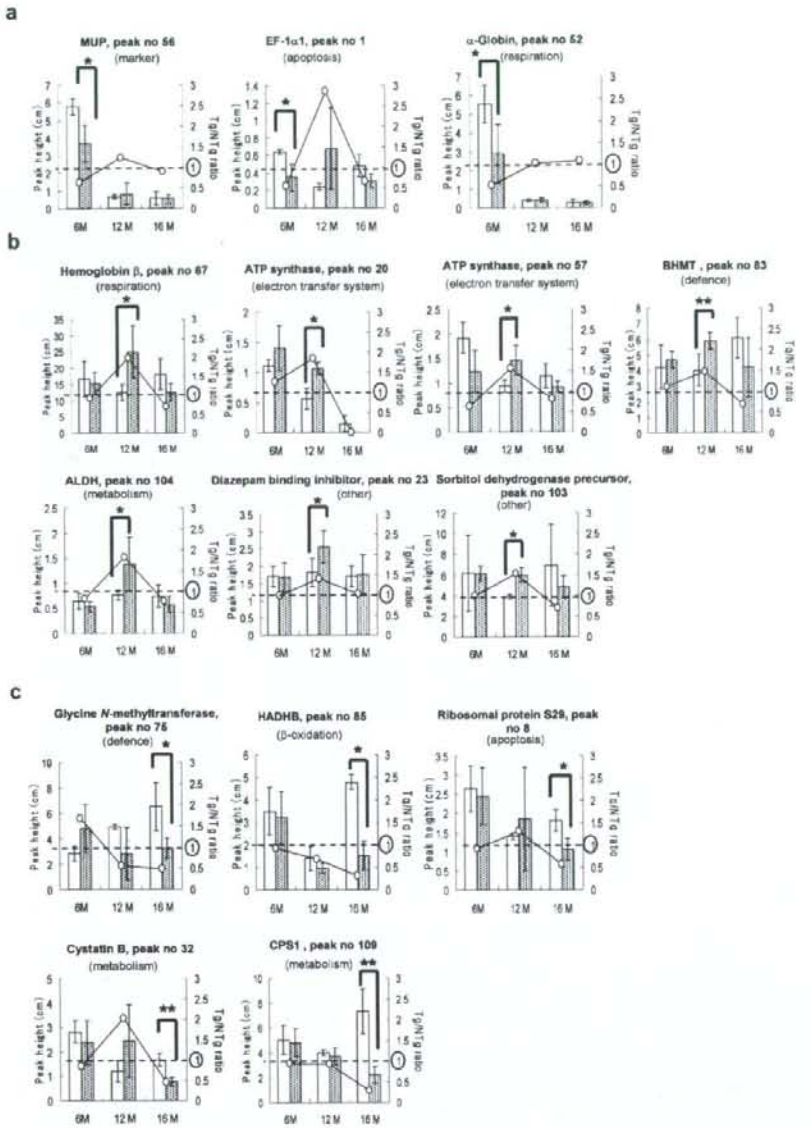

Figure 2. Comparison of peak heights between transgenic (Tg; gray bar) and non-transgenic (NTg; white bar) mice, and the Tg-to-NTg ratio (open circle) from 6 months (6M) to 16 months (16M). Significantly altered proteins are seen at 6M (a), 12M (b), and 16M (c). Peak numbers correspond to those in Figure 1. Mean values ± SD are plotted. Asterisks indicate significant differences (two-tailed Student's *t* test of all data points, $*P \leq 0.05$, $**P \leq 0.01$).

useful for understanding biotransformation from the viewpoint of proteomics. Also, the data obtained in this experiment could support the understanding of hepatocarcinogenesis with HCV infection in terms of proteomics in addition to the morphological and biochemical observations mentioned above.

Conclusions

The proposed method demonstrated for the first time the existence of several event-marker proteins at the three progression stages of hepatocarcinogenesis in transgenic mice. It should be stressed that the FD-LC−MS/MS method would also

be worthwhile for clinical proteomics analysis, as a supplement to gel- and LC-based methods.

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Review

Hepatitis C as a systemic disease: virus and host immunologic responses underlie hepatic and extrahepatic manifestations

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Key words: extrahepatic legion, HCV, immunoglobulin

Introduction

Hepatitis C virus (HCV) causes liver diseases. Approximately 2 million people in Japan and approximately 170 million people worldwide are infected with HCV, and they often suffer from chronic hepatitis, followed by hepatic cirrhosis, leading to hepatic cancer. It was determined relatively soon after the discovery of HCV that HCV infection does not involve the liver only. Other than hepatitis, many complicating diseases of the organs and tissues other than the liver, referred to as extrahepatic lesions, occur in association with HCV infection (Table 1). This review provides an overview of typical extrahepatic lesions associated with hepatitis C.

Cryoglobulinemia

Cryoglobulins are abnormal immunoglobulins that solidify into white deposits at 4°C and liquefy at 37°C.[1] The etiology of cryoglobulinemia in HCV infection has not yet been clarified. However, the involvement of apoptosis suppression by B lymphocytes, which produce monoclonal IgM, induced by the association of *bcl-2* and *IgJ(H)* as a result of the translocation of chromosome t(14:18), is suspected. Intrahepatic growth of CD5- and CD81-positive B lymphocytes has been observed, suggesting monoclonal IgM induction as a possible cause.[7]

Cryoglobulins are classified into three types, namely, monoclonal cryoglobulins (type I), polyclonal cryoglobulins (type III), and mixed cryoglobulins (type II). Cryoglobulinemia associated with HCV infection mainly involves the mixed type. More specifically, it involves monoclonal IgM and polyclonal IgG antibodies having rheumatoid factor activity.[8,9]

The clinical symptoms of essential mixed cryoglobulinemia (EMC) include purpura, arthralgia, and renal impairments.[10] Renal impairments are particularly known for showing membranoproliferative glomerulonephritis histologically and progressing to renal insufficiency.[11] Approximately 80% of EMC patients are infected with HCV.[12] When the high-sensitivity gel diffusion method is used, cryoglobulins are detected in 70% of patients chronically infected with HCV.[13] Many patients with HCV-associated cryoglobulinemia show subclinical symptoms, but the incidence of EMC is highest as an extrahepatic complication of hepatitis C.

Interferon (IFN) therapy has been used for HCV-associated cryoglobulinemia.[14] Misiani et al.[15] reported that, following the administration of IFN to 25 patients with HCV-associated cryoglobulinemia, cryoglobulinemia symptoms improved in 15 patients after the start of treatment but that the symptoms recurred after treatment ended. The combination of IFN and ribavirin has become standard therapy for chronic hepatitis C. It has also been used to treat HCV-associated cryoglobulinemia, with particular efficacy expected in patients for whom IFN monotherapy is ineffective. Zuckerman et al.[16] reported that the administration of both IFN and ribavirin to nine EMC patients who had not responded to IFN monotherapy alleviated cryoglobulinemia in all and improved clinical symptoms in seven of the nine patients.

In addition, for patients with severe cryoglobulinemia, antiviral therapy based on IFN and combination therapy with a steroid or an immunosuppressant are considered effective.[17] Other treatment strategies, including plasma exchange therapy[17] and splenectomy,[18]

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Table 1. Extrahepatic manifestations of chronic hepatitis C

Complication	Pathogenesis	Prevalence of HCV antibody (%)	Treatment with antiviral drug	References
Cryoglobulinemia	Apoptosis suppression of B lymphocytes. monoclonal IgM production caused by translocation of chromosome t(14:18)	50–90	Interferon Pegylated Interferon plus ribavirin	1–17
Renal impairment	Accumulation of an immune complex formed by monoclonal or polyclonal IgM-κ with rheumatoid factor activity produced by HCV-infected B lymphocytes in the glomerular vascular endothelium and mesangium	10–60	Interferon Pegylated Interferon plus ribavirin	18–26
Myocardial impairment	Involvement of host immunologic responses to HCV, particularly human MHC class II antigen	6–10	Not reported	27–31
Porphyria cutanea tarda	Reduced activity of uroporphybrinogen decarboxylase associated with an excessive deposition of iron in the liver induced by HCV infection	60–100	Interferon	32–37
Sjögren's syndrome	Involvement of host immunologic responses to HCV	0–45	Not reported	38–43
Lichen planus	Involvement of HCV-specific T cells	0–65	Interferon	44–63
Oral cancer	Unknown	70–100 (HCV-RNA)	Not reported	64–65
Diabetes mellitus	Involvement of insulin resistance and insulin secretory deficiency. Disruption of tyrosine phosphorylation of IRS-1. Involvement of TNF-α	50	Not reported	66–77
Malignant lymphoma	Involvement of *myc* gene mutation in some cryoglobulinemia patients	0–33	Interferon Pegylated Interferon plus ribavirin	78–94
Autoimmune thyroid disease	Involvement of LKM1	10	Not reported	95–102
Idiopathic interstitial pneumonitis	Involvement of activated T lymphocytes and eosinophils	28	Not reported	103–107
Mooren's ulcer	Unknown	Unknown	Not reported	108–114

HCV, hepatitis C virus; MHC, major histocompatibility; IRS, insulin receptor substrate; TNF, tumor necrosis factor; LKM1, liver/kidney microsomal antibody 1

have also been attempted, and future development of these strategies is promising.

Renal impairments

Reported renal impairments associated with HCV infection include membranoproliferative glomerulonephritis, membranous nephropathy, mesangial proliferative glomerulonephritis, Henoch-Schönlein purpura nephritis, and tubulointerstitial nephritis.[19]

Membranoproliferative glomerulonephritis, in particular, is considered a typical example of hepatic disease involving renal impairment associated with HCV and is referred to as HCV-associated nephritis. In 1993, Johnson et al.[11] first reported on eight patients with HCV infection complicated by membranoproliferative glomerulonephritis.[11] The incidence of HCV-associated nephritis developing as a complication of hepatitis C has not been confirmed. In a study of 188 autopsied cases of chronic hepatitis C, Arase et al.[20] reported that 11.2% of patients exhibited membranoproliferative glomerulonephritis, 2.7% membranous nephropathy, and 17.6% mesangial proliferative glomerulonephritis. The pathogenic mechanism underlying HCV-associated nephritis is considered to be the accumulation of an immunocomplex formed by monoclonal or polyclonal IgM-κ with rheumatoid factor activity produced by HCV-infected

peripheral blood B lymphocytes in the glomerular vascular endothelium and mesangium.[21]

Histopathological features of HCV-associated nephritis are similar to those of typical membranoproliferative glomerulonephritis type I, but the former sometimes show cryoglobulin deposition.[22] In essential cryoglobulinemia and nephrotic syndrome with a rheumatoid factor, HCV-associated nephritis is suspected; therefore, the presence or absence of HCV infection should be determined.

IFN therapy has been reported to be efficacious for HCV-associated nephritis.[23,24] Johnson et al.[23] reported that the administration of IFN to 14 patients with HCV-associated nephritis improved proteinuria, but they observed a relapse of nephritis in association with HCV reexpression after the end of IFN therapy in many patients.[23] Recently, IFN and ribavirin combination therapy, which shows a low relapse rate, has been tested.[25,26] Sabry et al.[26] reported on the effectiveness of IFN and ribavirin combination therapy administered to 16 patients with HCV-associated nephritis for whom IFN monotherapy had proved ineffective; a follow-up study is awaited. Steroid and cyclophosphamide have been used for immunosuppression therapy, but satisfactory results using an immunosuppressant alone have not yet been obtained.[27] Because patients with HCV-associated nephritis have been reported to have a poor prognosis,[11] early establishment of a therapeutic procedure based mainly on IFN and ribavirin combination therapy is desirable.

Myocardial impairments

Myocardial impairments for which a causal relationship with HCV infection has been suspected to date include dilated cardiomyopathy, hypertrophic cardiomyopathy, arrhythmogenic right ventricular dysplasia cardiomyopathy, and chronic myocarditis.[28–30]

A study by Matsumori[28] observed positivity for serum anti-HCV antibody in 6.3% (42/663) of patients with hypertrophic cardiomyopathy and in 10.6% (74/697) of patients with dilated cardiomyopathy. These positivity rates were higher than the rate (2.4%) observed among age-matched Japanese blood donors.[28] Positive- and negative-strand HCV RNAs were detected in cardiac muscle samples of these patients, indicating potential intramyocardial HCV multiplication.[29,30] HCV RNA has also been detected in cardiac muscle samples of patients with arrhythmogenic right ventricular dysplasia cardiomyopathy and chronic myocarditis, indicating that HCV potentially plays an important role in the onset of myocardial impairments.[32]

With regard to the cause of myocardial impairments associated with HCV, the involvement of host immunologic responses to HCV, particularly that of the human major histocompatibility (MHC) class II antigen, has been suggested.[30] There are many patients with normal liver enzyme levels among hepatitis C patients with a concomitant myocardial impairment.[28] No established therapy is currently available, but the use of IFN-based antiviral therapy should be considered.

Porphyria cutanea tarda

Porphyria cutanea tarda is an acquired condition in which patients exhibit solar photosensitivity and hepatic damage owing to decreased activity of uroporphyrinogen decarboxylase in the liver.[33] The involvement of alcohol, excess iron, and medications for hepatic impairments in porphyria cutanea tarda was previously considered. However, because HCV infection has been observed in 60%–100% of cases of porphyria cutanea tarda, the involvement of HCV infection in the pathogenesis of porphyria cutanea tarda is suspected.[34]

The mechanism underlying the pathogenesis of porphyria cutanea tarda associated with HCV infection has not yet been clarified. It is assumed, however, that porphyria cutanea tarda results from reduced uroporphyrinogen decarboxylase activity associated with excessive deposition of iron in the liver as a result of HCV infection.[34]

The efficacy of IFN therapy for the treatment of porphyria cutanea tarda has been demonstrated, in addition to avoidance of sun exposure, abstention from alcoholic beverages, and blood letting. Okano et al.[35] reported that IFN therapy given to porphyria cutanea tarda patients with HCV infection led to transaminase normalization, HCV RNA disappearance, and normalization of porphyrin and ferritin levels with improvement of clinical symptoms, including vesicle formation and hypertrichosis. These results demonstrate the efficacy of IFN therapy for porphyria cutanea tarda.

Sjögren's syndrome

Sjögren's syndrome is an aggregate of symptoms characterized by insufficient tear production by the lacrimal glands and insufficient saliva production by the salivary glands because of exocrine lymphocyte infiltration, causing dryness of the eyes and mouth. Patients with Sjögren's syndrome are classified roughly into two groups, those exhibiting only dryness and those exhibiting both dryness and connective tissue disease symptoms such as arthralgia.[39]

An association of Sjögren's syndrome with viral infection has been reported for some time, and 0%–45% of Sjögren's syndrome patients test positive for

anti-HCV antibody.[40] Differences in the anti-HCV antibody positivity rate are attributed to regional differences in the HCV infection rate. Koike et al.[41] verified that transgenic mice with the 1b HCV envelope genotype developed sialadenitis resembling Sjögren's syndrome. Takamatsu et al.[42] detected HCV RNA in salivary gland tissue from anti-HCV antibody-positive patients with Sjögren's syndrome by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Arreita et al.[43] performed in situ hybridization of 19 salivary gland tissue samples obtained from eight anti-HCV antibody-positive patients and 11 anti-HCV antibody-negative patients with chronic sialoadenitis or Sjögren's syndrome, and detected HCV RNA in all salivary gland tissue samples from the anti-HCV antibody-positive patients. Moreover, the HCV-infected salivary gland epithelium showed viral multiplication.[43] These reports indicate that HCV plays some role in the development of sialoadenitis in Sjögren's syndrome, but it has not yet been determined whether HCV itself or immunologic responses to HCV infection induce sialoadenitis.

Current therapies for Sjögren's syndrome mainly aim to alleviate the symptoms. Artificial lacrimal fluid and artificial saliva are used to alleviate dryness, and a nonsteroidal anti-inflammatory drug or a steroid is administered for treatment of fever and articular symptoms.[39] There are no reports regarding the efficacy of IFN therapy for HCV-associated sialadenitis,[44] and it is necessary to establish a treatment protocol in the future on the basis of accumulated case reports.

Lichen planus

Lichen planus is an inflammatory disease associated with abnormal chronic dermal and intraoral keratinization of unknown etiology. The assumed causes of lichen planus include viral or bacterial infection, immunologic responses, circulatory disorder, allergy, mental stress, abnormal autonomic function, medication, and glucose metabolism disorder.[45,46]

There are many reports of a relationship between lichen planus and HCV infection, but the anti-HCV antibody positivity rate in lichen planus shows marked regional differences, ranging from 0% to 65%.[47-53] HCV reproduction in the skin and oral mucosal epithelium has been examined by in situ hybridization and RT-PCR analysis.[54-56] HCV-specific T cells are reported to be associated with the pathogenesis of lichen planus,[57] but its pathogenesis is not associated with HCV level, genotype, or pathologic severity.[58,59]

The intravenous administration of a glycyrrhizinate preparation has been demonstrated to have efficacy for treatment of HCV-associated lichen planus.[60] Antiviral therapy based on IFN has also been attempted recently and has been reported to be effective,[61] but other investigators have reported that IFN is a lichen-planus-inducing factor[62] or that it can be aggravating factor.[63] No definite conclusion on the effectiveness of IFN against lichen planus is possible. Nagao et al.[64] reported that when intraoral lichen planus lesions in chronic hepatitis C patients administered IFN were observed over time, no macroscopic changes were observed in the lesions 1 year after the end of IFN administration, but that macroscopic and histological improvements were observed 3 or more years after the end of IFN administration. They also assumed that, since positive-strand HCV RNA was detected in the oral mucous membrane of some patients despite the demonstration of histological recovery from lichen planus following IFN therapy, host immunologic responses to HCV infection were related to the development of oral lichen planus.[64] The early establishment of a treatment procedure for lichen planus is desired, because lichen planus is also considered to be a precancerous condition.[45,46]

Oral cancer

A relationship between HCV infection and oral cancer was first reported by Nagao et al.[65] They showed that the HCV infection rate was higher in oral cancer patients than in esophageal, gastric, or colorectal cancer patients.[65] The HCV infection rate has also been found to be higher in patients with cervical squamous cell carcinoma than in controls.[66] When HCV-RNA was examined in cancer tissues from 17 oral cancer patients by RT-PCR analysis, positive-strand HCV RNA was detected in all anti-HCV antibody-positive patients and negative-strand HCV RNA was detected in 71.4% of the anti-HCV antibody-positive patients.[55] These findings interestingly indicate the possibility of HCV multiplication in cancer tissue. No definite conclusion has been arrived at regarding the relationship between oral lichen planus and oral cancer. However, because lichen planus is considered precancerous, as mentioned above, oral examination is also important for patients with chronic hepatitis C.

Diabetes mellitus

In 1994, Allison et al.[67] reported a relationship between HCV-associated cirrhosis and diabetes mellitus, because the rate of diabetes mellitus complication in patients with both cirrhosis and HCV infection was 50%, which is much higher than that (9%) in patients with cirrhosis but without HCV infection. A large-scale epidemiologic survey showed that the rate of non-insulin-dependent

diabetes mellitus occurring as a complication of chronic hepatic diseases associated with HCV infection was higher than that of other chronic hepatic diseases, and that anti-HCV antibody-positive patients aged 40 years or more had a 3.77-fold higher risk of becoming diabetic than anti-HCV antibody-negative patients.[68] In addition, it has been demonstrated that complication by diabetes mellitus is both a risk factor for hepatocellular carcinoma[69] and a prognostic factor in cirrhosis patients.[70] These reports suggest a correlation between HCV infection and type 2 diabetes. Increased insulin resistance and insulin secretory deficiency are considered to be highly involved in the pathogenesis of type 2 diabetes.[71] Petit et al.[72] reported that insulin resistance increases even in chronic hepatitis C patients with slight hepatic impairment and that the index of impairment (HOMA-IR) correlates with the severity of the liver tissue disorder. Tumor necrosis factor (TNF)-α, which closely correlates with hepatic inflammation and fibrillation in chronic hepatitis C,[73] is considered to enhance glucose uptake in peripheral tissue and to promote gluconeogenesis in the liver, leading to the induction of insulin resistance.[74] Shintani et al.[75] confirmed that in transgenic mice with the 1b HCV core genotype, tyrosyl phosphorylation of the insulin receptor substrate 1 in the insulin signal transduction pathway is disrupted and that this disruption causes gluconeogenesis inhibition by insulin in the liver, leading to the induction of marked insulin resistance. These transgenic mice exhibited a high anti-TNF-α antibody level, and insulin resistance was improved by the administration of an anti-TNF-α antibody. These results indicate a close relationship between HCV infection and the pathogenesis of diabetes mellitus. The relationship between HCV infection and hepatocyte fat modification has also attracted attention.[76] Moriya et al.[77] suggested the possible direct involvement of HCV core protein in hepatocyte fat modification, because they observed hepatocyte fat deposits in transgenic mice expressing the HCV core gene. In summary, the above-described findings strongly indicate that hepatitis C has the characteristics of a metabolic disease, and nutritional management is also considered important in the treatment of chronic hepatitis C.

Malignant lymphoma

HCV reproduces in lymphocytes, and studies of a short-term HCV culture system using lymphocytes have been reported.[78,79] Infected lymphocytes may undergo malignant transformation, leading to the development of malignant lymphoma. HCV infection is considered to be associated with the development of malignant lymphoma, particularly in association with the pathogenesis of non-Hodgkin B-cell lymphoma, and many reports suggest a relationship between HCV infection and malignant lymphoma.[80–88] It has been assumed that some cryoglobulinemia patients develop non-Hodgkin B-cell lymphoma in association with *myc* gene mutation.[89] The anti-HCV antibody positivity rates in patients with non-Hodgkin B-cell lymphoma range from 0% to 33%.[80–88] These differences in HCV antibody positivity rates are considered to relate to regional differences in the HCV infection rate. The HCV antibody prevalence tends to be higher in Japan and Italy but lower in Britain and Canada. Studies indicating a relationship between HCV infection and malignant lymphoma have been reported by Ferri et al.[90] and De Vita et al.[91] Ferri et al.[90] reported that 14 of 500 patients with chronic hepatitis C were complicated with non-Hodgkin B-cell lymphoma, and they detected HCV RNA in peripheral blood lymphocytes in all of these patients. De Vita et al.[91] detected positive-strand and negative-strand HCV RNAs in the parotid glands of patients with parotid non-Hodgkin B-cell lymphoma associated with HCV infection, and confirmed the presence of HCV in the parotid gland by in situ hybridization.[91] As shown by these findings, many patients with HCV-associated non-Hodgkin B-cell lymphoma show involvement of extranodal sites such as the liver and salivary glands.[92]

Treatment of HCV-associated malignant lymphoma is similar to that of HCV-associated non-Hodgkin B-cell lymphoma; however, recently, IFN monotherapy or IFN and ribavirin combination therapy have been reported to be effective.[93–96] Vallisa et al.[96] reported that administration of both pegylated IFN and ribavirin to 13 patients with HCV-associated non-Hodgkin B-cell lymphoma achieved a complete response in seven of these patients. It is interesting that IFN-based antiviral therapy has been demonstrated to be useful for malignant lymphoma associated with HCV-associated non-Hodgkin B-cell lymphoma in addition to conventional chemotherapy.

Autoimmune thyroid disease

The relationship between HCV infection and thyroid disease has been analyzed in many studies,[97,100] and a causal relationship between HCV infection and autoimmune thyroid disease has been particularly suggested.[98–100] Antonelli et al.[98] assessed the incidence of thyroid dysfunction in 630 chronic hepatitis C patients without cirrhosis or hepatocellular carcinoma who had not been treated with IFN by recruiting 389 patients from an iodine-deficient area, 268 patients from an iodine-sufficient area, and 86 patients with chronic hepatitis B aged 40 years or older as study subjects. The chronic hepatitis C patients exhibited a higher thyroid-

stimulating hormone level and lower free thyroxine and triiodothyronine levels than the controls. In addition, the chronic hepatitis C patients exhibited hypothyroidism and tended to have antithyroglobulin antibodies and anti-thyroid peroxidase antibodies. These findings suggest a relationship between HCV infection and thyroid disorder.[98] A possible relationship between HCV infection and thyroid cancer has also attracted attention recently.[99] The mechanism underlying the pathogenesis of thyroid disease associated with HCV infection has not yet been elucidated, but a relationship with liver/kidney microsomal antibody type 1 has been suggested.[99] Many patients with thyroid disorder caused by HCV infection are asymptomatic, requiring no special treatment. Thyroid disorder is also known to be an adverse reaction to IFN-α therapy for chronic hepatitis C.[99,101-104] Thyroid hypofunction caused by the administration of IFN-α is usually transient, and the patient recovers spontaneously after the end of the therapy. Hence, discontinuation of IFN-α is not required in many cases.[103]

Idiopathic interstitial pneumonitis

Recently, viral infection has been suggested to be a cause of idiopathic interstitial pneumonitis.[104] With regard to the relationship between HCV infection and idiopathic interstitial pneumonitis, Ueda et al.[106] reported in 1992 that the anti-HCV antibody positivity rate in 66 patients with idiopathic interstitial pneumonitis determined by enzyme-linked immunosorbent assay was 28.8%, which was significantly higher than that in 9464 normal subjects serving as controls.[106] It has not yet been clarified how HCV infection is associated with the pathogenesis of idiopathic interstitial pneumonitis. Kubo et al.[107] suggested that activated T lymphocytes and eosinophils are related to the pathogenesis of idiopathic interstitial pneumonitis associated with HCV infection, because they observed increased activated T-lymphocyte and eosinophil counts in the bronchoalveolar fluid of 13 chronic hepatitis C patients, despite their having the same total cell counts as normal subjects. On the other hand, studies disagree regarding the relationship between HCV infection and idiopathic interstitial pneumonitis,[108] and in-depth studies of this issue are expected. Idiopathic interstitial pneumonitis is also reported to be an adverse reaction to IFN therapy in chronic hepatitis C patients.[109] Such patients often have a high pretreatment KL-6 level, and the potential of their developing idiopathic interstitial pneumonitis is suggested. Recovery from IFN therapy-induced idiopathic interstitial pneumonitis is achieved by the discontinuation of the therapy,[109] but steroid administration is required in some cases.

Rheumatoid arthritis

HCV-associated rheumatoid arthritis complicated by cryoglobulinemia or Sjögren's syndrome has been reported.[10,39] For further information, please refer to the cited references.

Mooren's ulcer

Mooren's ulcer is a progressive ulcer associated with congestion and pain around the cornea.[110] HCV infection has been suggested to contribute to the development of this disease.[111-113] The effectiveness of IFN therapy for HCV-associated Mooren's ulcer has been reported,[110,111] but the exacerbation of ocular pain following the discontinuation of IFN therapy has also been observed; hence, caution is required.[111] Systemic corticoid administration has also been reported to be effective.[112] However, other investigators reported a negative correlation between HCV infection and Mooren's ulcer.[114-116] It is hoped that further detailed studies will clarify this issue.

Conclusion

It is necessary to consider possible complications associated with extrahepatic diseases in the treatment of HCV-infected patients.

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

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

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

Introduction

Hepatitis C virus (HCV) infects approximately 1.8 million people in Japan alone and as many as 200 million over the world and induces liver disease ranging from chronic hepatitis through cirrhosis to hepatocellular carcinoma (HCC) [1, 2]. It has been noticed soon after the discovery that the infection with HCV does not exclusively involve the liver. In fact, type II cryoglobulinemia [3] and membranoproliferative glomerulonephritis [4] frequently occur in patients infected with HCV. Furthermore, strong associations of HCV infection with Sjogren's syndrome [5] and lichen planus [6] have been noted, which is verified in the animal model [7]. In addition, the relation between HCV infection and B cell lymphoma has attracted attention especially in Europe [8].

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

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

HCV Infection and Diabetes

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

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

HCV Infection Induces Insulin Resistance

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

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

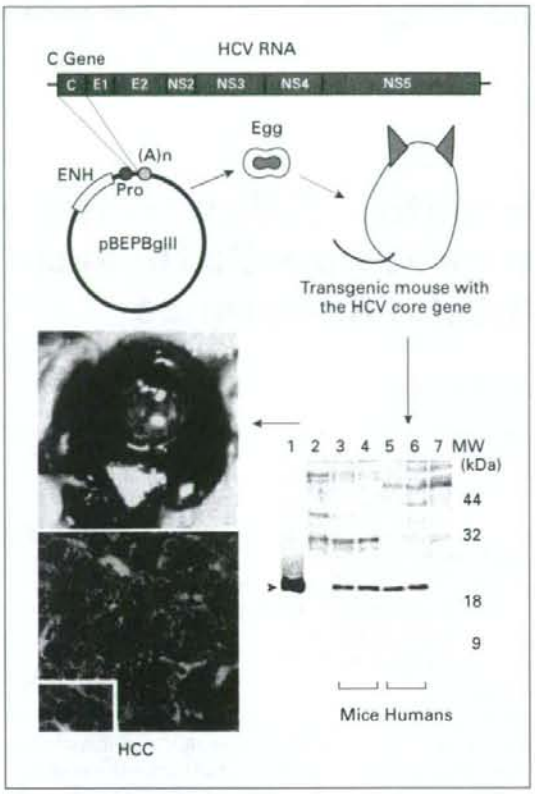

Fig. 1. Expression of HCV core gene in transgenic mouse. It carries the core gene of HCV genotype 1b alone and expresses the core protein of expected size in the liver, at levels similar to those in human patients. Mice eventually develop HCC later in their lives.

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

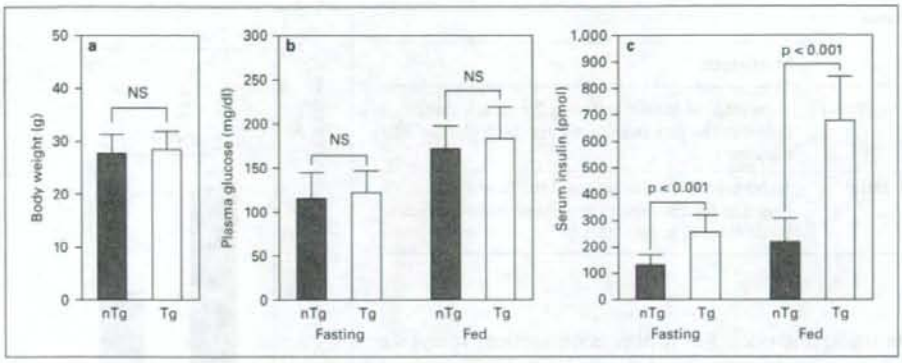

Fig. 2. Altered homeostasis of glucose in mice transgenic for the HCV core gene. Body weight of 2-month-old mice (**a**), plasma glucose levels in fasting or fed mice (**b**) and serum insulin levels in fasting or fed mice (**c**) are shown. Values represent means ± SE. NS = Not significant statistically;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

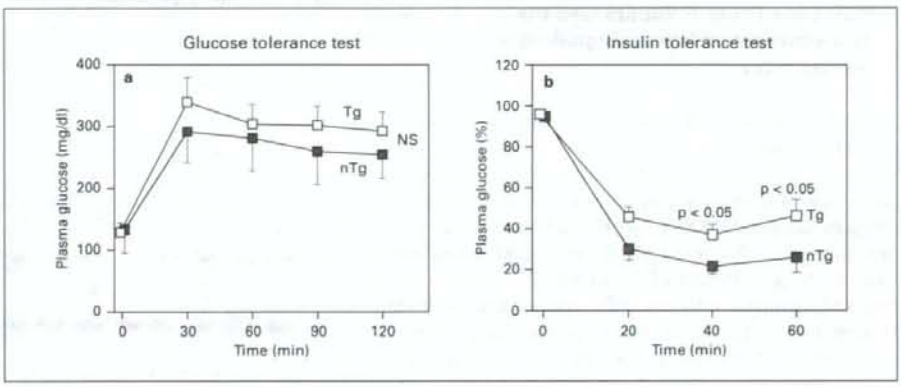

Fig. 3. Insulin resistance in transgenic mice. Glucose tolerance in mice after overnight fasting (**a**). *D*-Glucose (1 g/kg body weight) was given intraperitoneally to conscious mice, and plasma glucose levels were determined at time points indicated. **b** Insulin tolerance in mice fasted overnight.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

By what mechanism does insulin resistance arise in this animal model? The insulin resistance is considered to involve two factors, namely central and peripheral insulin resistances (table 1) [21]. The hyperinsulinemic-euglycemic clamp method was employed to differentiate between them. In this method, hepatic glucose production (HGP) is calculated on the basis of amounts of glucose required to keep plasma glucose levels within a certain range at serum insulin levels higher than physiological ones. In normal control mice, HPG was suppressed by 60% by the administration of insulin, in contrast to core-transgenic mice in which there was no appreciable suppression of HGP by insulin (fig. 4). These results indicate a hepatic (central) origin of the insulin resistance

Table 1. Two types of insulin resistance

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

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

HCV Core Protein Suppresses the Transduction of Insulin Signaling in Hepatocytes

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

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

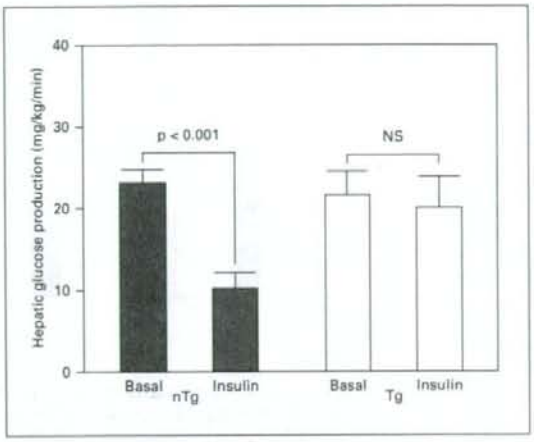

Fig. 4. Characterization of glucose metabolism in transgenic mice. Glucose production in the liver was calculated using the hyperinsulinemic-euglycemic clamp method. NS = Not significant statistically; nTg = nontransgenic mice; Tg = transgenic mice.

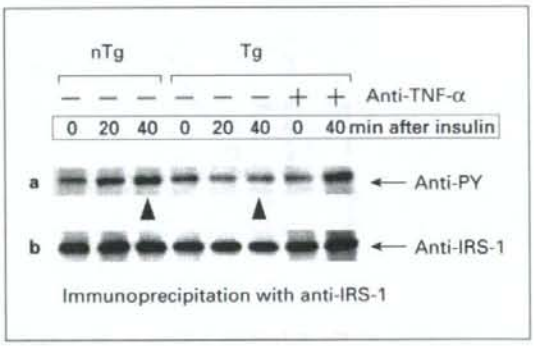

Fig. 5. Phosphorylation of tyrosine in IRS-1 in response to insulin stimulation. Liver tissues from control mice, transgenic mice with or without anti-TNF-α antibody treatment, were analyzed before and 20 as well as 40 min after administration of insulin. Samples were subjected to immunoprecipitation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

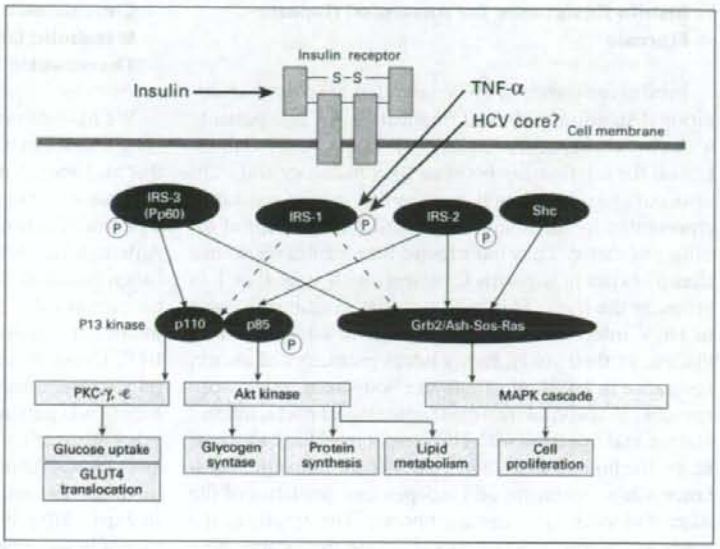

Fig. 6. A proposed mechanism for insulin resistance in HCV infection. HCV itself or elevated levels of cytokines such as TNF-α may inhibit tyrosine phosphorylation of IRS-1 in the liver, suppress intracellular transduction of insulin signal and lead to insulin resistance. PKC = Protein kinase C; MAPK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

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

Insulin Resistance in Patients with Chronic Hepatitis C

Concurrently with our report in experimental systems, Aytug et al. [23] investigated insulin signaling in biopsied liver specimens from patients with chronic hepatitis C. Specifically, they evaluated changes in IRS-1, IRS-2 and phosphatidyl inositol (PI)3 kinase levels in the liver of patients. With insulin stimulation of biopsied liver samples, insulin receptor proteins and IRS-1 increased, while phosphorylation of tyrosines in IRS-1 decreased to one half of the baseline value, along with a diminished activity for PI3 kinase associated with IRS-1, in patients with chronic hepatitis C. The authors went on to propose a possibility for disturbed transduction of the insulin signaling pathway in the liver to induce insulin resistance in patients with chronic hepatitis C [23]. Their report is quite intriguing in that it opens up the way for evaluating an association between HCV infection and insulin resistance in clinical samples at the molecular level.

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

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

Insulin Resistance for Advanced Hepatic Fibrosis

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

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

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

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