Table VI. Twelve years after residents were diagnosed with chronic hepatitis C in 1990.

1990	n		2002	n
CH-C	61	→	CH-C	16
		→	CH-C post IFN (CR)	1
		→	LC-C	5
Anti-HCV (+), HCV RNA (+), HBsAg (-)		→	LC-C+HCC	3
		→	CH-C+HCC	2
		→	Deceased	12
			Cause of death	
			LC (2)	
			HCC (3)	
			Other than liver disease (7)	
		→	Unknown liver disease due to non-participation in the follow-up survey	22

CH-C, chronic hepatitis C; LC-C, HCV-related liver cirrhosis; HCC, hepatocellular carcinoma.

Table VII. Twelve years after residents were diagnosed with HCV-related liver cirrhosis in 1990.

1990	n		2002	n
LC-C	6	→	LC-C	1
		→	Deceased (cause of death: HCC)	4
Anti-HCV (+), HCV RNA (+), HBsAg (-)		→	Unknown liver disease due to non-participation in the follow-up survey	1
LC-C+HCC	1	→	Deceased (cause of death: HCC)	1

LC-C, HCV-related liver cirrhosis; HCC, hepatocellular carcinoma.

diagnosed with chronic hepatitis C (positive anti-HCV, positive HCV RNA, negative HBsAg, and abnormal liver function data) in 1990. Of the 61 persons diagnosed with chronic hepatitis C in 1990, the diagnosis of liver disease in 2002 was: chronic hepatitis C (n=16), chronic hepatitis C post-IFN (n=1), liver cirrhosis (n=5), HCC (5), deceased (n=12), and unknown liver disease due to non-participation in the follow-up survey (n=22). Among the 12 deceased, 2 died from HCV-related liver cirrhosis, and 3 died from HCC. Table VII shows the distribution of the diagnosis of liver disease in 2002 among the persons diagnosed with HCV-related liver cirrhosis or HCC (positive anti-HCV, positive HCV RNA, negative HBsAg, and abnormal liver function data) in 1990. The person diagnosed with HCV-related liver cirrhosis with HCC in 1990 died of HCC.

Figs. 5 and 6 show the outcome of 216 persons with liver diseases (excludes HBV carriers) and 6 persons with persistent HBV infection, respectively, identified among the inhabitants in the 12-year follow-up. The residents for whom we could not analyze the long-term prognosis of liver disease were deleted from these numbers. Approximately half of sustained HCV carriers, such as asymptomatic healthy carrier, chronic hepatitis, liver cirrhosis, and HCC, had an advanced stage of disease in the 12-year follow-up. Just 1 (2.6%) out of 39 inhabitants with chronic hepatitis C recovered from sustained HCV infection due to IFN treatment. Two (1.3%) of the 143 inhabitants without HCV infection (alcoholic liver disease or fatty liver, 16 inhabitants; normal, 127 inhabitants) became infected with HCV infection (alcoholic liver disease or fatty liver, 16 inhabitants; normal, 127 inhabitants).

In 1990, 10 persons had negative anti-HCV and positive HBsAg. Three persons diagnosed as HBV-related asymptomatic healthy carriers in 1990 remained the same in 2002. The remaining person with HBV-related liver cirrhosis had died from HCC.

Three persons had both positive anti-HCV and HBsAg in 1990. One who was an HBV-related asymptomatic healthy carrier and had a past history of HCV infection in 1990 was

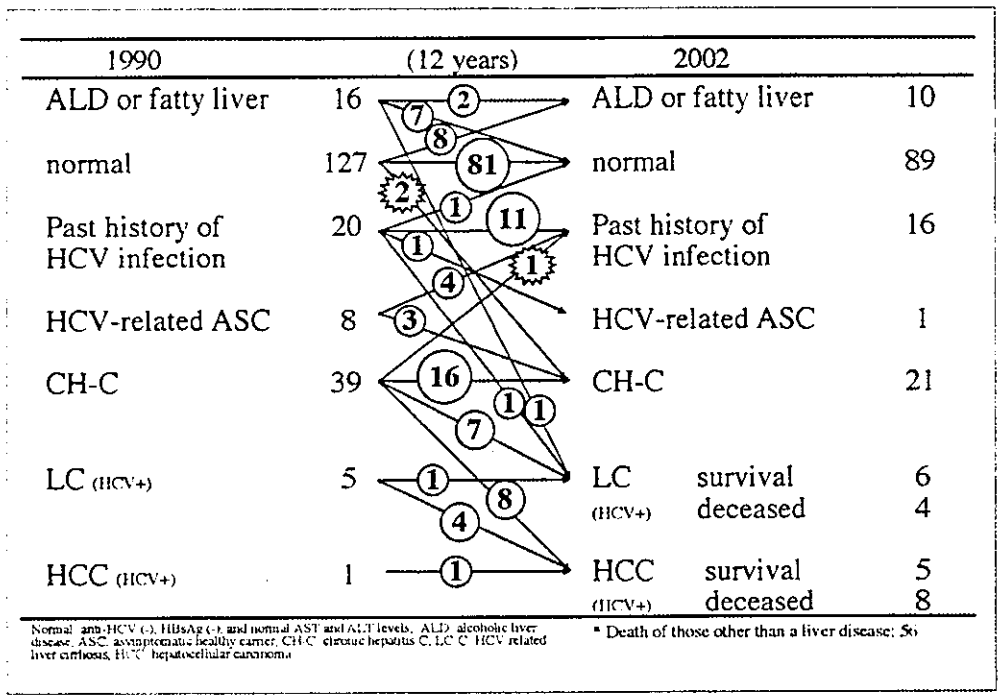

Figure 5. Outcome of 216 persons with liver disease (HBV carriers are omitted) identified among the inhabitants a follow-up after 12 years.

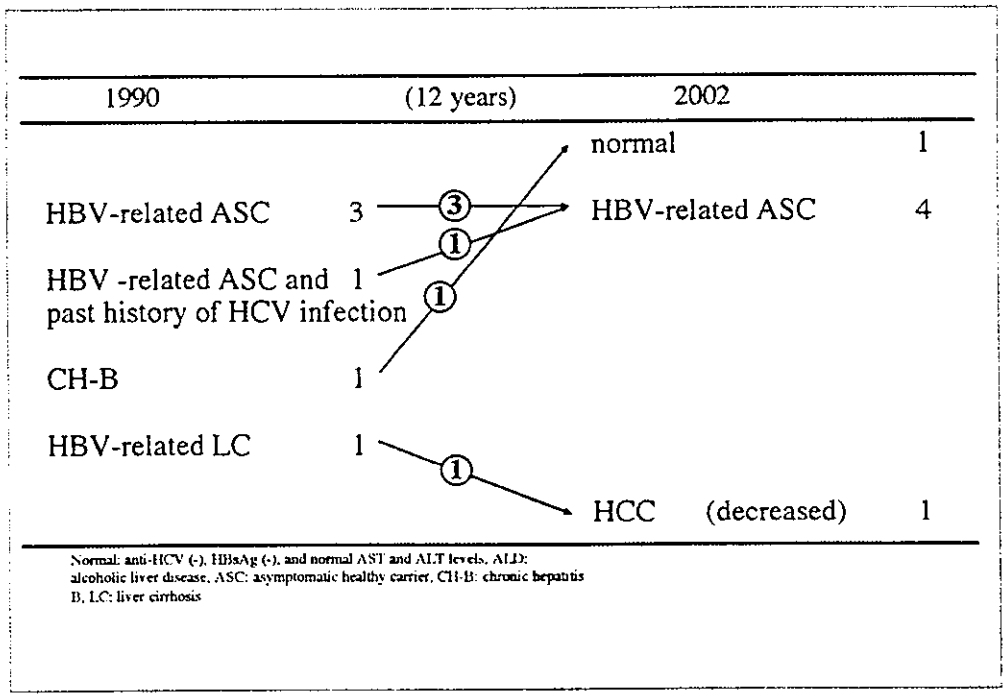

Figure 6. Outcome of 6 HBV carriers identified among the inhabitants in a follow-up after 12 years.

also an HBV-related asymptomatic healthy carrier in 2002. The other person who was an HBV- and HCV-related asymptomatic healthy carrier as well as the remaining person with chronic hepatitis B and C in 1990 had unknown liver disease in 2002.

Discussion

Hepatitis C is a global health problem caused by HCV infection. However, there is a great geographic difference in the prevalence of HCV infection. Memon and Memon reviewed the literature on the epidemiology of HCV infection from 1991 to 2000 (14). In healthy volunteer blood donors, the incidence of anti-HCV is 0.05% in Germany (15), 0.01% in Northern Ireland (16), 0.03% in Italy (17), 0.088% in Scotland (18), 0.35% in the UK (19), 0.17-0.5% in USA (20-22), 0.78% in Australia (23), 0.93-1.2% in Spain (24,25), 0.19-2.2% in Japan (26-29), and up to 24.8% in Egypt (30). The incidence of infection as determined by anti-HCV in healthcare professionals dealing with blood and blood

products is ever higher in each country (31-41). Furthermore, certain groups of individuals such as intravenous drug users have an increased risk of acquiring HCV infection irrespective of their geographical location (14). However, in Japan, it had been reported that there were some areas where the general incidence of HCV infection is extremely high in persons who are not intravenous drug users (7-11,42-47). In Japan the incidence of anti-HCV in local residents is 19.7% (403/2,046) in H village in Fukuoka prefecture (42), 14.1% (158/1,122) in Iki Island in Nagasaki prefecture (43), 17.7% (77/435) in Area-O of town T in Akita prefecture (44), 20.7% (493/2,382) in N city in Yamagata prefecture (45), 22% (671/3,117) in the southern part of Japan (46), 23.6% (120/509) in H town in Fukuoka prefecture (7), and 28.5% (82/288) in Y town in the Setonaikai sea (47). The most likely reason for the high prevalence of HCV infection in these areas is considered to be iatrogenic infection through insufficient sterilization of needles and/or syringes for treatment in the same clinic (7,42,44).

We conducted a follow-up survey after 12 years for the 509 inhabitants who were examined for liver disease in an HCV hyperendemic area (7). In terms of the area (H town), we previously reported that medical treatment was considered to be a causative route of HCV transmission (8), that most HCV carriers died from HCC or liver cirrhosis (11), and that the prevalence of various extrahepatic manifestations in HCV carriers was higher than in those without HCV (12,13). As shown in Fig. 5, after 12 years, of the 39 residents with chronic hepatitis C, 7 suffered from liver cirrhosis and 8 developed HCC. That is, in 12 years, the incidence of HCC that originated from chronic hepatitis C was 20.5% (1.7% annual rate). Four out of 5 persons with liver cirrhosis who were diagnosed in 1990 had died from HCC during these 12 years. In other words, the incidence of HCC that originated from liver cirrhosis was 80% (6.7% annual rate). The 1.7% annual rate of HCC developing from chronic hepatitis C and the 6.7% annual rate of HCC developing from liver cirrhosis in H town's general population closely resemble the rate of HCC that develops in HCV-infected patients who consulted the hospital, which has been reported up to the present time. The incidence of HCC that originates from chronic hepatitis C and liver cirrhosis in examination of patients who consulted the hospital is reported to be 2 and 7-8% respectively, in Japan (3,4). Ikeda *et al* reported that the appearance rate of HCC in 349 patients only with positive anti-HCV was 21.5% in the 5th year, 53.2% in the 10th year, and 75.2% in the 15th year (4). This is the first report of the natural course of HCV infection in 'the general population'.

Of the 69 persons who died, the mortality rate caused by HCC or liver cirrhosis was 44 and 53%, respectively, among 25 persons with positive anti-HCV, and 19 with positive HCV RNA. The HCV or HBV carriers had significantly higher mortality rates from liver cirrhosis and HCC than those who were not carriers ($P<0.00001$). We reported that the ALT value and HCV RNA were associated with deaths due to HCC or liver cirrhosis by multivariate analyses (11). In other words, eradicating persistent HCV infection or normalizing the ALT value would lead to controlling the development of HCC.

On the other hand, during the 12-year observation period, 2 (1.4%) of the 143 inhabitants without HCV infection became infected with HCV. The appearance rate of new HCV-infected residents was 0.12%/year in this area. In our previous report of the same subjects from 1990 to 1995, the seroconversion rate of HCV was 0.28%/year (8). Of 287 subjects negative at the first examination in 1990, 4 became positive by 1995 (8). Medical treatment is considered the causative route of HCV transmission. We consider that not only medical treatment for HCV carriers but education of medical staff who work in H town, such as doctors, dentists, nurses, and dialysis staff, is required in order to prevent new infection and to acquire correct knowledge of liver disease.

Recently, IFN therapy in patients with chronic hepatitis and liver cirrhosis was shown to be associated with a reduced incidence of HCC (48). Yoshida *et al* reported on data from a large surveillance program of patients with chronic hepatitis C (49). In multivariate analysis, IFN therapy was associated with a reduced risk for HCC compared with untreated controls (adjusted risk ratio, 0.516).

We prospectively studied 509 consecutive residents in a HCV hyperendemic area of Japan for 12 years. In conclusion, the early detection and treatment for HCC should be carried out as HCV carriers are aging. As Yoshizawa reported (2), persistent HCV carriers should receive aggressive IFN therapy to eradicate HCV, or anti-inflammatory therapies to suppress the development of HCC, or HCC should be diagnosed at the early stage for early treatment and prolonged life span. We should eradicate of HCC as a national goal.

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Analysis of approach to therapy for chronic liver disease in an HCV hyperendemic area of Japan

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Abstract

In 1990, we conducted an epidemiological study of 509 residents in a hepatitis C virus (HCV) hyperendemic area in Japan. The purpose of the present study was to examine the approach to therapy for liver diseases accompanied by HCV and hepatitis B virus (HBV) infections among the surviving residents in the town after 12 years. Fifty-three with HCV or HBV infections among 385 people who resided in the town were clearly analyzed in 2002. The number of persons diagnosed with the liver diseases was as follows: HCV-related asymptomatic healthy carrier (1), past history of HCV infection (15), chronic hepatitis C (22), HCV-related liver cirrhosis (6), HCV-related hepatocellular carcinoma (HCC) (5), and HBV-related asymptomatic healthy carrier (4). HCC was detected in residents who did not have periodic regular hospital checkups. Only 19% of the 53 inhabitants consulted liver medical specialists, and 75% (3/4) who received interferon therapy received treatment from a liver medical specialist. It is necessary to provide continuous medical treatment to HCV carriers, minimizing difference in treatment quality in different medical institution. An efficient HCV medical checkup and a program of subsequence health management are important problems to be solved for improved health care.

Keywords: Hepatitis C virus (HCV); Hyperendemic area; HCV carrier; Epidemiology; Hepatocellular carcinoma (HCC); Liver medical specialist

1. Introduction

Hepatitis C is a global health problem caused by infection with the hepatitis C virus (HCV). It is estimated that about 170 million people worldwide are infected with HCV [1]. HCV carriers in Japan are presumed to number 2 million people [2,3]. The growing incidence of HCC is expected to reach a plateau around the year 2015, and then to start to decrease according to the study of Yoshizawa [2]. However, there are many persons who are not aware that they are infected, some of whom will advance to liver cirrhosis or hepatocellular carcinoma (HCC) [4,5], and this has become an important social concern. Therefore, the following are now implemented as emergency measures by hepatitis C in the Ministry of Health, Labour and Welfare in Japan: (i) substantial dissemination of education and instructions for consulting; (ii) implementation of a hepatitis virus examination which utilizes the organization of the present health checkup; (iii) research and development of medical treatments, and maintenance of the medical-examination organization; and (iv) prevention and blocking the route of infection. The latter measure is urgent.

In our country, as part of a 5-year program from 2002 to 2007, candidates who reach certain turning points during every 5-year period from age 40–70, or those in whom abnormalities in liver function were pointed out during the basic health checkup according to the Health and Medical Service Law for the Aged will undergo examination for HCV and hepatitis B virus (HBV) infection.

Since 1990, we have conducted health screenings of the residents of "H town" (adult population: 7389), Fukuoka prefecture in northern Kyushu, Japan, which is known for

Abbreviations: HCV, hepatitis C virus; HBV, hepatitis B virus; anti-HCV, antibodies to HCV; HBsAg, hepatitis B virus surface antigen; HCC, hepatocellular carcinoma; IFN, interferon

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its high prevalence of liver diseases. We previously reported that the town had a high prevalence of HCV carriers, and that HCV infection was the principal cause of liver disorders [6–12]. The prevalence of anti-HCV antibodies (anti-HCV) among the local residents of "H town" in 1990 was 23.6% (120/509) [6].

In 1990, 509 randomly selected subjects participated in the study for examination of liver diseases accompanied by HCV or HBV infections. In the present study, we analyzed the approach therapy for HCV-or HBV-infected liver disease among the surviving residents after 12 years in the same town.

2. Subjects and methods

2.1. Subjects

As shown in Fig. 1, of 509 subjects, 69 people had died, and 55 people had moved out to other regions. Thus, just 385 of the original 509 people resided in H town in May 2002. Of these 139 inhabitants agreed to participate in the medical follow-up survey, 26 did not agree to participate, and the remaining 220 inhabitants did not declare their intention either way in 2002. Of the surviving 139 inhabitants, 35 were infected with HCV and 4 with HBV. In these 39 inhabitants with HCV or HBV infection, the following items were questioned: present health condition, continuous regular hospital checkups, medical treatment received in the past 12 years in the hospital, the name of the family doctor, and the kind of medicine taken. The 39 subjects were examined in liver function tests, and for antibodies to anti-HCV, serum HCV RNA, hepatitis B virus surface antigen (HBsAg), and ultrasonographic examination. Only 14 residents of the surviving 220 inhabitants who did not declare their intention to participate in 2002 stated by telephone the current condition of their liver disease and their primary physician treating this disease. We then asked the primary physicians about the treatment for each of these resident's liver disease, the diagnosis of liver disease, and the latest data. Consequently, we analyzed the details of liver disease in 53 inhabitants positive for anti-HCV or HBsAg in 2002.

Informed consent was obtained from all residents after the purpose and methods of the study were explained.

2.2. Serological and abdominal ultrasonic echo examination

Sera from 39 residents were provided for the following liver function tests: serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), gammaglutamyl transpeptidase (γ-GTP), lactate dehydrogenase (LDH), total bilirubin (T.Bil), total protein (TP), albumin (Alb) and gamma-globulin (γ-glob.). Sera were also examined for the presence or absence of HCV or HBV infection. Anti-HCV was measured by a chemiluminescent enzyme immunoassay (CLEIA) kit (Lumipulse II HCV, Fujirebio Inc., Tokyo, Japan). HCV RNA in the sera was detected using the

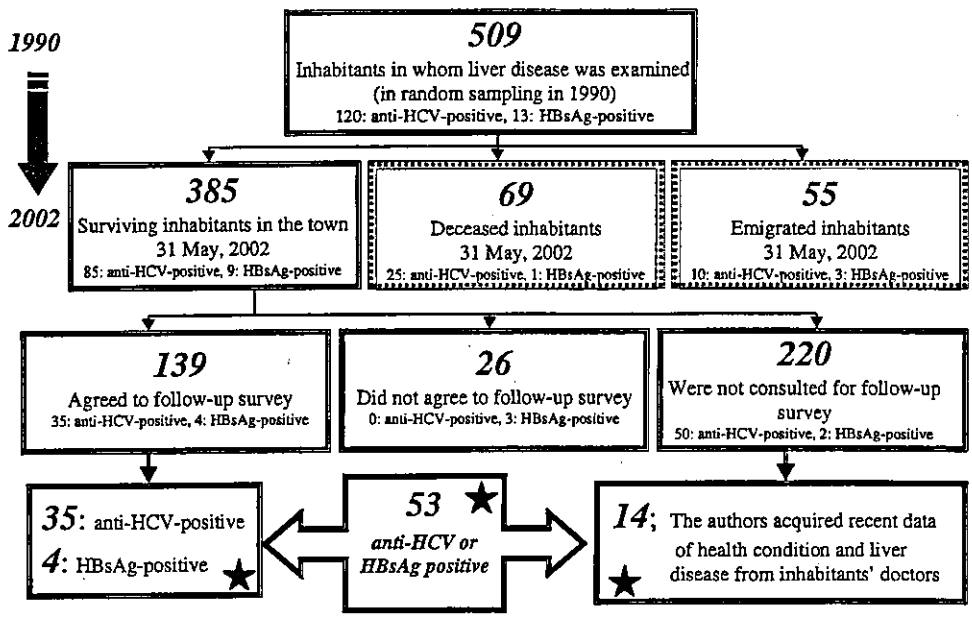

Fig. 1. Diagram for pursuing the prognostic investigation of 509 inhabitants. Of the 509 subjects, 69 people had died, and 55 people had moved out to other regions by 31 May 2002. Thus, just 385 of the original persons investigated in 1990 resided in H town in May 2002. Of these, 139 persons agreed to participate in the medical follow-up survey, but 26 did not agree. The remaining 220 inhabitants did not declare their intention either way. These 139 subjects were interviewed in person by two trained interviewers, and were their liver disease examined. Among the 220, we could ask their primary physicians about treatment for 14 of them liver disease, diagnosis of liver disease, and the latest data. Consequently, we analyzed the details of liver disease in 53 inhabitants positive for anti-HCV or HBsAg in 2002.

Amplicor HCV test (Nippon Roche, Tokyo, Japan). HCV RNA was quantified by Roche Amplicor Monitor assay. HBsAg was assayed by a chemiluminescent immunoassay (CLIA) kit (ARCHITECT™, HBsAg QT, Dainabot Co. Ltd., Tokyo, Japan). Ultrasonographic examination in subjects with abnormalities of liver function tests and who were anti-HCV- or HBsAg-positive was performed in all subjects in order to investigate the shape of the liver and lesions occupying the hepatic space. Computed tomography and liver biopsy were performed in some subjects.

3. Results

We clearly analyzed the treatment of liver diseases accompanied by HCV or HBV infection in 53 surviving inhabitants in 2002. As shown in Table 1, there were 49 inhabitants with HCV infection and 4 with HBV. The diagnoses of HCV-related liver diseases were: asymptomatic healthy carrier (1 case), past history of HCV infection (15), chronic hepatitis C (CH-C) (22), HCV-related liver cirrhosis (LC) (6), HCC with CH-C (2) and HCC with LC (3). The diagnoses of HBV-related liver disease were: asymptomatic healthy carrier (4). Three of 22 with CH-C were treated with interferon (IFN). Effect of IFN was sustained virologic response (1 case), non responder (1), and currently undergoing IFN therapy (1). One of six with HCV-related LC was treated with IFN. IFN effect was judged as non responder. Table 1 shows the treatment of liver disease in the 53 inhabitants. Ten subjects (18.9%, 10/53) visited the hospital regularly to consult the liver medical specialist. Here a "liver medical specialist" means a doctor authorized by The Japan Society of Hepatology. The treatments were distributed as follows: regular follow-up at least every 3 or 6 months by blood test or abdomen ultrasonographic examination (32 subjects), medication for liver disease other than IFN treatment, such as glycyrrhizin and ursodeoxycholic acid (10), IFN treatment (4), treatment of HCC (3), and untreated (4). Of four residents who did not visit the hospital periodically, one HCC was observed in a 76-year-old woman, in spite of having been advised that they were HCV carriers in 1990. Moreover, the other resident with liver disease accompanied by HCV infection who did not consult with the liver medical specialist also developed HCC (a 65-year-old man).

On the other hand, there were three residents who were undergoing medical treatment of HCC. One of these is a 68-year-old woman, who developed HCC in 1996, and underwent percutaneous ethanol injection therapy (PEIT) for a total of 12 times as well as therapy. The other is a 76-year-old woman, who received surgical treatment for HCC in 1993, underwent PEIT and percutaneous radiofrequency ablation (PRF) in 1999, and had received chemotherapy by transcatheter arterial embolization (TAE) in 2002. One person of the last group is an 82-year-old woman, who had received PEIT treatment in 2000.

Table 2 shows the characteristics of 22 persons with chronic hepatitis C and 6 with HCV-related liver cirrhosis. This table gives more details of the residents with hepatitis C and liver cirrhosis in Table 1. Age, sex, AST and ALT values, the level of HCV RNA (KIU/ml), and other complications are shown for each resident. Three of the four inhabitants (75%) treated with IFN consulted liver medical specialists. These results reaffirm the significance of cooperation in the medical treatment of liver disease between the primary physician and the liver medical specialist.

Fig. 2 shows the diagnosis of liver diseases in 1990 in the 49 inhabitants with accompanied by HCV infection in 2002. Most HCV carriers showed an advanced stage of disease. Just one of 27 inhabitants with chronic hepatitis C recovered from sustained HCV infection by IFN treatment. Two of the inhabitants surveyed became infected with HCV between 1990 and 2002.

Table 1
Treatment of liver diseases in 53 inhabitants positive for anti-HCV or HBsAg

Case (number of patients)	Follow-up (32)	Treatment of liver disease without IFN (10)	History of IFN (4)	History of treatment for HCC (3)	No treatment (4)
HCV-related ASC (1)	●				
Past history of HCV infection (15)	✱●●●●● ●●				◎
CH-C (22)	✱✱●●●●	●●●●●	✱✱●		◎◎
LC-C (6)	●	●●●●	✱		
CH-C + HCC (2)				●	◎
LC-C + HCC (3)		●		✱✱	
HBV-related ASC (4)	●●●●				

ASC: asymptomatic healthy carrier; CH-C: chronic hepatitis C; LC-C: HCV-related liver cirrhosis; HCC: hepatocellular carcinoma; (✱) hepatologist; (●) no hepatologist involved.

Table 2
Characteristics of 22 persons with chronic hepatitis C and 6 HCV-infected persons with live cirrhosis

	Follow-up (32)	Treatment of liver disease without IFN (10)	History of IFN (4)	No treatment (4)
	Age (sex, AST/ALT [IU/l], HCV RNA level [KIU/ml], the other)	Age (sex, AST/ALT [IU/l], HCV RNA level [KIU/ml])	Age (sex, AST/ALT [IU/l], HCV RNA level [KIU/ml], IFN effect)	Age (sex, AST/ALT [IU/l], HCV RNA level [KIU/ml])
CH-C (22)	(1) 67 (F, 33/32, 930) #[a] (2) 66 (F, 24/31, 194) #[a]	(1) 50 (F, 30/28, 573) (2) 75 (F, 27/13, 260)	(1) 70 (M, 48/22, 352, NR) #[a] (2) 65 (M, 41/34, HCV RNA negative, SVR) #[a]	(1) 65 (F, 71/99, 423) (2) 52 (F, 90/115, >850)
	(3) 72 (M, 40/40, ND, liver meta. For rectal cancer	(3) 74 (M, 45/31, 379)	(3) 57 (M, 67/59, undergoing IFN + ribavirin treatment)	
	(4) 81 (M, 15/12, ND	(4) 67 (F, 37/37, 114)		
	(5) 84 (M, 38/22, >850)	(5) 66 (F, 27/30, 696)		
	(6) 35 (F, 25/30, 48)			
	(7) 66 (F, 27/66, ND)			
	(8) 47 (F, 27/43, 520)			
	(9) 89 (F, 29/17, 733)			
	(10) 77 (F, 38/35, 270)			
	(11) 51 (F, 22/20, >850)			
	(12) 56 (F, 30/26, 166)			
LC-C (6)	1. 75 (F, 37/15, ND, terminal bladder cancer)	(1) 86 (M, 79/70, ND)	(1) 53 (M, 55/57, >850, NR) #[a]	
		(2) 69 (F, 23/27, 366)		
		(3) 57 (F, 49/55, 201)		
		(4) 68 (M, 101/78, 558)		

CH-C: chronic hepatitis C; LC-C: HCV-related liver cirrhosis; ND: not done; SVR: sustained virologic response; NR: non responder.
[a] Hepatologist.

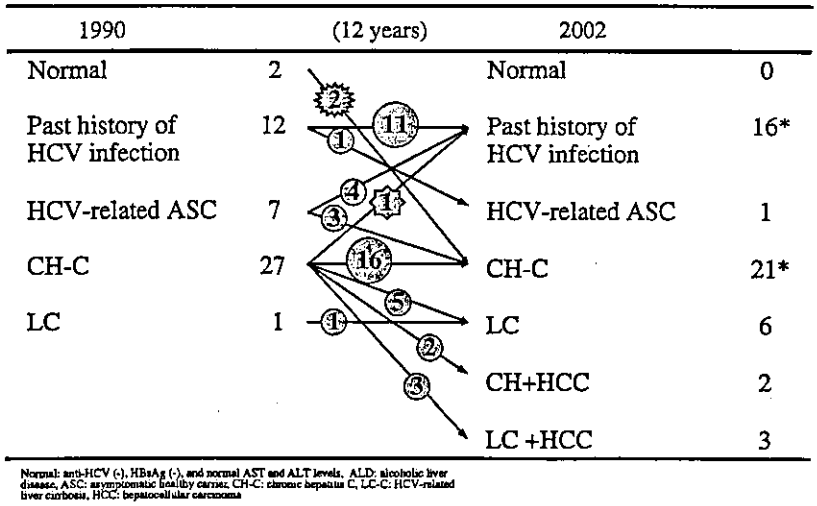

Fig. 2. The diagnosis off liver disease 12 years ago in 49 inhabitants with liver diseases accompanied by HCV infection in 2002. Among the 22 with chronic hepatitis C indicated in Table 1, one person had recovered from HCV infection due to IFN treatment. Therefore, he was counted under "past history of HCV infection". Most HCV carriers had an advanced stage of disease. A newly infected person is denoted by O, and IFN therapy with eradication of HCV is denoted by O.

4. Discussion

We previously reported studies of "H town", a hyperendemic area of HCV infection [6–12]. In the present report, we have analyzed the details of liver disease of 53 inhabitants positive for anti-HCV or HBsAg in 2002 through a follow-up survey after 12 years of the original 509 inhabitants in whom liver disease examined in an HCV hyperendemic area in 1990. HCC was detected in residents who did not visit the hospital regularly for 1 year or more, in spite of being advised of their HCV infection in 1990. Only 19% who did visit the hospital regularly consulted liver medical specialists, and 75% of residents with IFN treatment history received medical treatment by the liver medical

specialist. Though some of the inhabitants who underwent a treatment of liver disease without IFN treatment and a follow-up shown in Table 2 had indication to undergo an IFN treatment, few of them received explanations about an IFN treatment. Some of residents suffered from the medical institution as hepatitis C or liver cirrhosis might be able to cure HCV infection, if they are treated with IFN treatment at the younger age. The present study suggests that the cooperation is essential in the medical treatment of liver disease between the primary physician and the liver medical specialist.

IFN treatment is administered as the standard therapy for chronic hepatitis accompanied by HCV infection. Recently, the anti-viral drug ribavirin has been used in combination with IFN and has resulted in significantly improved responses: current studies show a 28–66% sustained response after 48 weeks of treatment [13,14]. The side-effects of the combination therapy are, however, "universal, significant, and possibly serious." Ribavirin frequently causes hemolytic anemia leading to necessary dose reductions and is a known teratogen [15]. For the remaining patients who cannot be treated with IFN, glycyrrhizin is often used. In Japan, glycyrrhizin has been an accepted treatment of chronic hepatitis for over 20 years [16]. Glycyrrhizin is known to prevent the development of HCC [17]. The purpose of the medical checkup in the high-risk group infected with HCV is the followings three items, as Yoshizawa has advocated [2]: (i) IFN therapy for eradication of HCV so that the patient is no longer at high risk for HCC; (ii) conservative (anti-inflammatory) therapies, such as glycyrrhizin, ursodeoxycholic acid, and phlebotomy, for postponing the development of HCC; and (iii) diagnosis of HCC at an early stage for early treatment and prolonged lifespan.

By telephone interview, we were able to acquire the reasons why some of the residents did not have medical checkups and did not visit the hospital regularly, in spite of being recognized as infected with HCV in 1990. These reasons were: the high cost of medical care, the absence of liver disease, to keep the condition a secret, "too busyness", etc. While we emphasize consultation and medical examinations for HCV carriers, we must not forget efforts to provide accurate information of liver diseases to the general population or to trained medical workers. Since 2003 an HCV carrier discovered newly by an examination in the H town has been obliged to undergo a precision examination at a designated hospital holding a full-time hepatologist. Moreover, the letter of introductions and the report of health guidance from the municipality to a medical institution, the result report and the request letter of health guidance from a medical institution to that municipality were made. Now, based on the flow chart of HCV examination, hospitals of a primary medical institution and a second medical institution cooperate together.

In conclusion, it is necessary to continuously provide medical treatment to recognized cases of HCV carriers, minimizing difference in treatment quality in different medical institution. An efficient HCV medical checkup and a program of subsequent health management are important problems to be solved for improved health care.

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Gastrointestinal, Hepatobiliary and Pancreatic Pathology

Hepatitis C Virus Down-Regulates Insulin Receptor Substrates 1 and 2 through Up-Regulation of Suppressor of Cytokine Signaling 3

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The pathogenesis of hepatitis C virus (HCV)-associated insulin resistance remains unclear. Therefore, we investigated mechanisms for HCV-associated insulin resistance. Homeostasis model assessment for insulin resistance was increased in patients with HCV infection. An increase in fasting insulin levels was associated with the presence of serum HCV core, the severity of hepatic fibrosis and a decrease in expression of insulin receptor substrate (IRS) 1 and IRS2, central molecules of the insulin-signaling cascade, in patients with HCV infection. Down-regulation of IRS1 and IRS2 was also seen in HCV core-transgenic mice livers and HCV core-transfected human hepatoma cells. Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal, a potent proteosomal proteolysis inhibitor, blocked down-regulation of IRS1 and IRS2 in HCV core-transfected hepatoma cells. In human hepatoma cells, HCV core up-regulated suppressor of cytokine signaling (SOCS) 3 and caused ubiquitination of IRS1 and IRS2. HCV core-induced down-regulation of IRS1 and IRS2 was not seen in SOCS3$^{-/-}$ mouse embryonic fibroblast cells. Furthermore, HCV core suppressed insulin-induced phosphorylation of p85 subunit of phosphatidylinositol 3-kinase and Akt, activation of 6-phosphofructo-2-kinase, and glucose uptake. In conclusion, HCV infection changes a subset of hepatic molecules regulating glucose metabolism. A possible mechanism is that HCV core-induced SOCS3 promotes proteosomal degradation of IRS1 and IRS2 through ubiquitination. *(Am J Pathol 2004, 165:1499–1508)*

Chronic liver diseases are associated with glucose intolerance called hepatogenous diabetes.[1] Glucose intolerance impairs sustained response rate to anti-viral therapy in patients with chronic hepatitis C virus (HCV) infection[2] and is a risk factor for development of hepatocellular carcinoma[3] as well as long-term survival in patients with cirrhosis.[4] Several epidemiological studies have revealed an association between HCV infection and type 2 diabetes mellitus (DM) in cirrhotic patients.[5–13] Case-cohort analysis confirms an increased risk for type 2 DM in cirrhotic patients with HCV infection.[14] Cirrhotic patients with HCV infection are twice as likely to have type 2 DM than patients with hepatitis B virus (HBV) infection.[6,7,12] Thus, epidemiological data show that HCV infection antedates type 2 DM. It is, however, difficult to prove that HCV itself triggers glucose intolerance in patients with liver cirrhosis. Various factors such as reduced glucose uptake,[15] porto-systemic shunting,[16] and impaired glucagon metabolism[17] are also involved in glucose metabolism in patients with liver cirrhosis. Although glucose intolerance may occur even in the early stage of HCV infection, changes in glucose metabolism in noncirrhotic patients are not evident. To ascertain if HCV infection

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directly causes glucose intolerance, changes in glucose metabolism in noncirrhotic patients with various hepatobiliary disorders were investigated.

The liver plays a major role in regulation of glucose metabolism because it is the main source of endogenous glucose and the major site involved in insulin metabolism.[18,19] Thus, hepatic factors may be involved in HCV-associated glucose intolerance. However, the pathogenic mechanisms for HCV-associated glucose intolerance remain unclear. Insulin exerts many biological effects through insulin receptor substrate (IRS) 1 and IRS2. Disruption of IRS1 results in insulin resistance, but not DM, because of compensatory hyperinsulinemia.[20,21] Disruption of IRS2 results in severe DM because of insulin resistance and disturbance of insulin secretion.[22] Thus, IRS1 and IRS2 are the molecules that augment the specificity of the insulin-signaling cascade and play a central role in insulin-mediated glucose metabolism.

HCV chronically infects hepatocytes. HCV may escape from the host immune response by suppressing cytokine signaling. We recently showed that HCV core up-regulates suppressor of cytokine signaling (SOCS) 3 expression.[23] Although SOCS3 is known to be a negative regulator for cytokine signaling such as interleukin-6, growth hormone, and interferon-α, the role of SOCS3 on HCV-associated glucose intolerance has never been investigated. The aims of this study were to investigate changes in glucose metabolism in noncirrhotic patients with various hepatobiliary disorders and the molecular mechanisms for HCV-associated glucose intolerance.

Materials and Methods

Materials

All reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan) unless otherwise indicated. Affinity-purified polyclonal rabbit anti-SOCS3 antibody was generated against synthetic peptide SKFPAAGMSRPLDTSLRL (Immuno-Biological Laboratories, Gunma, Japan).

Patients

A total of 357 patients with chronic hepatitis C (n = 158), chronic hepatitis B (n = 54), autoimmune hepatitis (AIH) (n = 36), fatty liver (n = 40), primary biliary cirrhosis (PBC) (n = 49), or histologically normal livers (CON; n = 20) were studied retrospectively during the period from January 1997 to August 2003 at Kurume University Hospital. All of the patients were untreated and hospitalized for diagnostic liver biopsy. All of the diagnoses were based on clinical, serological, and histological evidence. Domestic data were collected at the time of liver biopsy including age, sex, and alcohol use. Body mass index (BMI) was calculated as body weight in kg divided by the square of height in meters (kg/m^2). Some liver diseases such as AIH and PBC show gender differences, and it is also possible that HCV infection affects BMI. Therefore, age, sex, BMI, and biochemical parameters were not matched among the groups to reduce selection bias. Patients with other causes of liver disease, in particular those known to be involved in the pathogenesis of diabetes such as hemochromatosis or alcoholic liver disease (on the basis of histology or a history of excessive alcohol consumption) were excluded, as were those who had been taking corticosteroids or with a history of, or evidence of, pancreatitis or a pancreatic tumor. The study protocol was approved by the institutional review board, and informed consent for participation in the study was obtained from each subject. None of the patients was institutionalized.

Laboratory Determinations

Venous blood samples were taken in the morning after a 12-hour overnight fast. Plasma glucose levels were measured by a glucose oxidase method. Serum insulin levels were measured by using a sandwich enzyme immunoassay kit (Eiken Chemical, Tokyo, Japan). β-Cell function and insulin resistance were calculated on the basis of fasting levels of plasma glucose and insulin, according to the homeostasis model assessment (HOMA) method.[24] The formulas for the HOMA model are as follows: β-cell function (HOMA-β) = fasting insulin (μU/ml) $\times$ 360/(fasting glucose (mg/dl) $-$ 63); insulin resistance (HOMA-IR) = fasting glucose (mg/dl) $\times$ fasting insulin (μU/ml)/405.

Determination of HCV Genotype and Measurement of HCV Core

HCV genotype was determined by polymerase chain reaction with type-specific primers and HCV genotypes were classified according to classification system of Simmonds and colleagues.[25] Unselected serum samples (n = 58) were assayed for HCV core by using a newly developed HCV core antigen enzyme-linked immunosorbent assay test system (Ortho-Clinical Diagnostics K.K., Tokyo, Japan) as previously described.[26] This assay has high stability and reproducibility under all conditions and the detection limit is 44 fmol/L.

Histological Data

For each patient, a liver biopsy specimen was fixed in 10% formalin buffer and stained with hematoxylin and eosin. Liver biopsy specimens were evaluated by a single experienced pathologist who was unaware of the patients' clinical and laboratory data. The specimens were scored according to the METAVIR scoring system, which is suited for evaluation of chronic hepatitis C.[27] Activity was graded according to the intensity of necroinflammatory lesions: 0 , no activity; 1, mild activity; 2, moderate activity; and 3, severe activity. The stage of fibrosis was scored as follows: 0, no fibrosis; 1, portal fibrosis without septa; 2, portal fibrosis with few septa; 3, portal fibrosis with many septa; and 4, cirrhosis.

Immunohistochemistry

Paraffin-embedded liver sections from patients with HCV infection were deparaffinized and subjected to immunohistochemical staining using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) with an anti-human IRS1 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or an anti-human IRS2 polyclonal antibody (Santa Cruz Biotechnology), and developed with 3,3'-diaminobenzidine. The primary antibodies for IRS1 and IRS2 were used at a 1:100 dilution. The specificity of IRS1 and IRS2 staining was confirmed by immunization using an excess amount of the N-terminal peptide of IRS1 and IRS2.

Immunoblotting

Immunoblotting was performed as previously described[28,29] using antibodies against the following: IRS1, IRS2, insulin receptor (Chemicon, Temecula, CA), SOCS-3, Myc (Santa Cruz Biotechnology), phospho-(Tyr) p85 subunit of phosphatidylinositol 3-kinase (PI3K; Cell Signaling Technologies, Beverly, MA), phospho-(Ser 473)-Akt (Cell Signaling Technologies), signal transducer and activation of transcription (STAT) 5 (Santa Cruz Biotechnology), or ubiquitin (Santa Cruz Biotechnology). Equal amounts of protein (40 μg) from liver homogenates or cell extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 7.5% acrylamide gel. The resolved proteins were transferred electrophoretically onto polyvinylidene difluoride membranes (Amersham Int., Buckinghamshire, UK). The membranes were incubated with the primary antibodies indicated in each figure, and were subsequently incubated by the secondary antibodies: a horseradish peroxidase-conjugated goat anti-mouse IgG (Amersham Int.) and a horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham Int.). The membranes were then incubated with chemiluminescence reagents (ECL kit; Amersham Int.) and immediately exposed on radiograph film. In the experiment for ubiquitination of IRS1 and IRS2 (Figure 4, b and c), cell extracts were immunoprecipitated with anti-IRS1 or anti-IRS2 antibodies and then immunoblotted with anti-ubiquitin antibody as previously described.[23]

Core and HCV cDNA

The HCV core region (573 nucleotides) was amplified by reverse transcriptase- polymerase chain reaction, using HCV RNA as a template extracted from the serum of a patient with HCV (genotype Ib) infection. The nucleotide sequence is 98% identical and the amino acid sequence is 100% identical to those of HCV strain MD7-1.[30] The HCV core region was subcloned into expression vector pcDNA3 with the NH_2-terminal Myc tag. Expression of all HCV proteins, including the core, envelope proteins (E1and E2), and nonstructural proteins (NS1, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) were confirmed by immunoblotting in our previous study.[23]

HCV Core-Transgenic Mice

The transgenic mouse was generated using a construct carrying the HCV core cDNA (genotype 1b) fused to the promoter of the HBV X gene.[23] Transgenic mice were developed using conventional methods (C57BL/6 × DBA/2). F1 mice were used to obtain fertilized eggs and those founder mice were mated with C57BL/6 mice for more than five generations. The expression of HCV core was expressed in various tissues including brain, heart, lung, kidney, thymus, and liver. All animal experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the University of Kurume Institutional Animal Care and Use Committee.

Cells and Transfection

HepG2 cells derived from a human hepatoblastoma and retaining many of the differentiated features of mature hepatocytes,[31] Huh 7 and HLF cells derived from hepatocellular carcinomas,[32] and primary mouse embryonic fibroblast (MEF) cells from heterozygous ($SOCS3^{-/-}$) SOCS3 knockout mice and from wild-type ($SOCS3^{+/+}$) mice were cultured in Dulbecco's modified Eagle's medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% dialyzed fetal bovine serum (Life Technologies, Gaithersburg, MD). The expression vector carrying the HCV core region was transfected using synthetic liposomes (Lipofectamine 2000; Life Technologies) in Opti-MEM I (Life Technologies) as previously described.[33,34] Cell extracts for each experiment were prepared 24 hours after transfection. In some experiments, 10 μmol/L of carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG132; Peptide Institute, Osaka, Japan), a proteosomal proteolysis inhibitor, was mixed with cDNA of Myc-tagged HCV core and incubated for 1 hour. To examine the effects of HCV core on insulin signaling, stable transfectants of HCV core in the HLF were used.

Assay for 6-Phosphofructo-2-Kinase (EC 2.7.1.1; Fru 6-P,2-Kinase)

The activity of Fru 6-P,2-kinase was assayed by measuring formation of fructose 2,6-bisphosphate as described previously.[35]

Statistical Analysis

All data are expressed as mean ± SD. Differences between two groups were analyzed using the Mann-Whitney *U*-test. Statistical comparisons among multiple groups were performed by analysis of variance followed by Scheffé's post hoc test using StatView Power PC version for Macintosh (version 5.1; SAS Institute, Cary, NC). P values <0.05 were considered significant.

Table 1. Characteristics of All Patients

	Control	CH-C	CH-B	AIH	Fatty liver	PBC
Number	20	158	54	36	40	49
Age (yr)	52 ± 10	53 ± 8	42 ± 9	50 ± 11	44 ± 7	51 ± 8
Sex						
Female	12 (60.0%)	63 (39.9%)	29 (53.7%)	31 (86.1%)	23 (57.5%)	41 (83.7%)
Male	8 (40.0%)	95 (60.1%)	25 (46.3%)	5 (13.9%)	17 (42.5%)	8 (16.3%)
Body mass index (kg/m^2)	22.3 ± 1.9	22.8 ± 2.0	22.0 ± 1.5	22.2 ± 1.8	23.2 ± 3.0	22.5 ± 1.8
Aspartate aminotransferase (U/l)	25 ± 16	70 ± 29	86 ± 57	71 ± 39	35 ± 37	23 ± 12
Alanine aminotransaminase (U/l)	28 ± 18	77 ± 36	94 ± 56	85 ± 50	43 ± 33	33 ± 13
Albumin (g/dl)	3.8 ± 0.3	3.8 ± 0.3	3.7 ± 0.3	3.7 ± 0.4	4.0 ± 0.4	3.7 ± 0.3
Total bilirubin (mg/dl)	0.6 ± 0.2	0.7 ± 0.2	0.6 ± 0.3	1.0 ± 0.8	0.6 ± 0.2	0.7 ± 0.3

Note. Data are expressed as mean ± SD or number of patients. All patients were Japanese. All the diagnoses are based on clinical, serological, and histological evidences.
CH-C, chronic hepatitis C; CH-B, chronic hepatitis B; AIH, autoimmune hepatitis; PBC, primary biliary cirrhosis.

Results

Characteristics of All Patients

We enrolled 337 patients with noncirrhotic chronic hepatobiliary diseases and 20 controls (histologically normal liver). Clinical and laboratory data for these patients are summarized in Table 1. All patients were Japanese. Patients with HBV infection and fatty liver were younger than the other groups and females constituted more than 80% of the group of patients with AIH and PBC. Serum aspartate aminotransferase and alanine aminotransferase levels were increased in all of the groups except for controls. There was no significant difference in BMI among all of the groups. Serum albumin levels and bilirubin levels were normal in all of the groups.

Changes in Glucose Metabolism in Patients with Various Chronic Liver Diseases

Fasting glucose levels were within normal range in all of the groups and showed no significant differences among the groups. However, fasting insulin levels were ~1.5 times higher in patients with HCV infection compared to the other groups (Figure 1, a and b). β-Cell function and insulin resistance were evaluated by HOMA-β and HOMA-IR, respectively. HOMA-β levels were increased in patients with HCV infection compared to controls (Figure 1c). HOMA-IR levels were significantly higher in patients with HCV infection compared to the other groups (Figure 1d).

The Involvement of Virological Factors in HCV-Associated Hyperinsulinemia

All serum samples from patients with HCV infection were HCV-RNA-positive. HCV genotypes were classified according to the classification system of Simmonds and colleagues.[25] Genotype 1a and 3 were not found in any sample. Three samples were excluded in this analysis because of mixed HCV genotypes or undetermined HCV genotype. There was no significant difference in fasting insulin levels among different HCV genotypes (Figure 2a). On the other hand, in patients with >44 fmol/L of HCV core, fasting insulin levels were significantly elevated compared to patients with undetectable levels (<44 fmol/L) of HCV core (Figure 2b).

Histological Parameters and Fasting Insulin Levels

Liver specimens were evaluated according to the METAVIR system.[27] There was no significant difference in fasting insulin levels among different activities

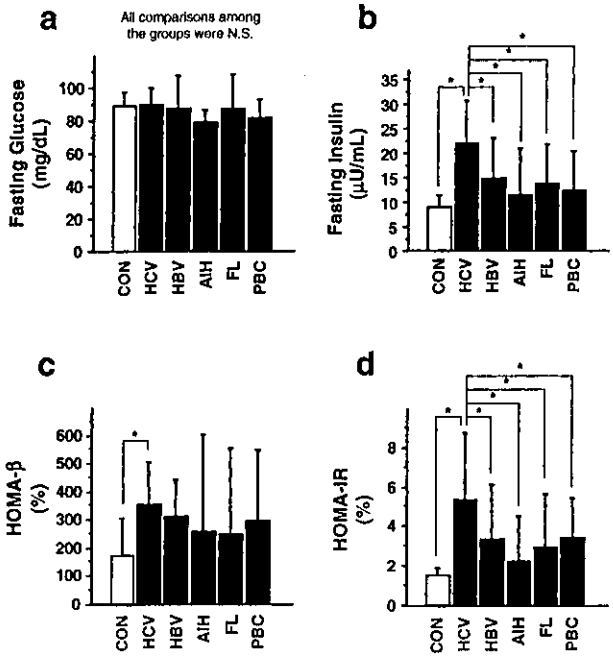

Figure 1. Fasting glucose and insulin levels, β-cell function, and insulin resistance in patients with various chronic liver diseases. Fasting plasma glucose (**a**) and fasting serum insulin (**b**) were measured. HOMA-β (**c**) and HOMA-IR (**d**) were calculated (see Materials and Methods). Values were expressed as mean ± SD. The comparisons between the groups were made using analysis of variance with Scheffé's post hoc test. N.S., not significant. *, $P < 0.05$. CON, histologically normal livers as controls ($n = 20$); HCV, chronic hepatitis C virus infection ($n = 158$); HBV, chronic hepatitis B virus infection ($n = 54$); AIH, autoimmune hepatitis ($n = 36$); FL, fatty liver ($n = 40$); PBC, primary biliary cirrhosis ($n = 49$).

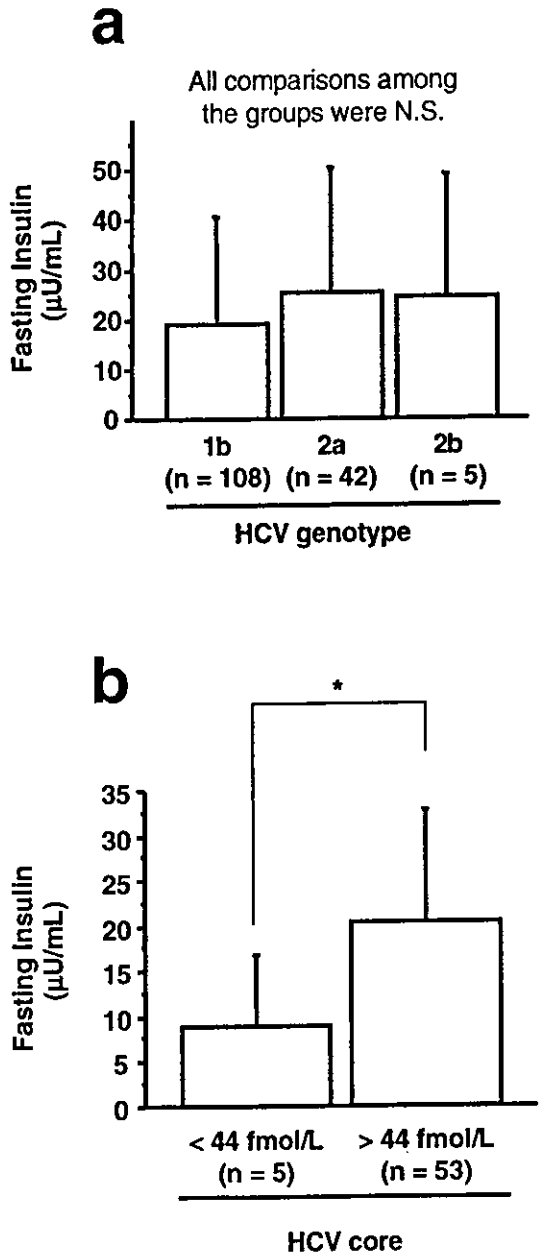

Figure 2. The involvement of virological factors in HCV-associated hyperinsulinemia. **a:** HCV genotypes and fasting insulin levels. Three cases that showed mixed or undetermined HCV genotypes were excluded. Values were expressed as mean ± SD. The comparisons between the groups were made using analysis of variance with Scheffé's post hoc test. N.S., not significant. **b:** HCV core and fasting insulin levels. Values were expressed as mean ± SD. The comparison between the two groups was made using the Mann-Whitney *U*-test. *, $P < 0.05$.

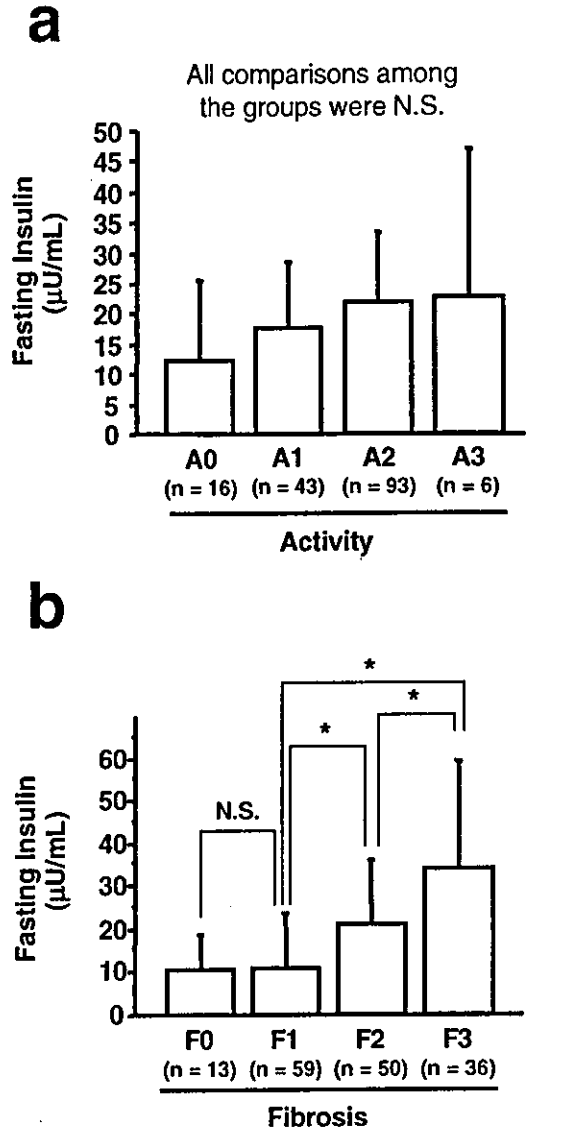

Figure 3. Histological parameters and fasting insulin levels in patients with HCV infection. The liver specimens were evaluated according to the METAVIR system (see Materials and Methods). **a:** Activity and fasting insulin levels. **b:** Fibrosis and fasting insulin levels. Values were expressed as mean ± SD. The comparisons between groups were made using analysis of variance with Scheffé's post hoc test. N.S., not significant. *, $P < 0.05$.

(Figure 3a). Although no significant difference was seen in fasting insulin levels between F0 and F1, fasting insulin levels were significantly increased in F2 compared to those of F0 and F1. Fasting insulin levels in F3 were elevated more than those of F0, F1, and F2 (Figure 3b). A similar relationship was also found between HOMA-IR levels and the degree of hepatic fibrosis (data not shown).

Protein Expression Levels of IRS1 and IRS2 in the Liver from Patients with HCV Infection and HCV Core-Tg Mice

The protein expression levels of IRS1 and IRS2 in liver samples from controls and patients with HCV infection were examined by immunostaining and immunoblotting. In control livers, immunostaining demonstrated that periportal hepatocytes, rather than perivenular hepatocytes, highly expressed both IRS1 and IRS2, showing lobular heterogeneity of IRS1 and IRS2 expression (Figure 4a). Decreased IRS1 and IRS2 expression levels along with progression of hepatic fibrosis were seen in periportal hepatocytes. Immunoblotting showed that IRS1 and IRS2 expression levels decreased with the progression of hepatic fibrosis in livers from patients with HCV infection

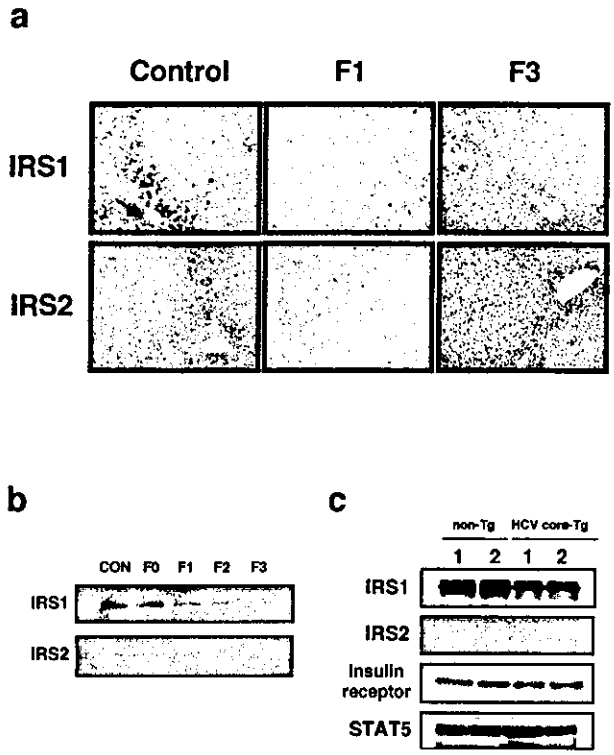

Figure 4. Protein expression levels of IRS1 and IRS2 in livers from patients with HCV infection and HCV core-transgenic (Tg) mice. **a:** Immunostaining for IRS1 and IRS2. IRS1 (**top column**) and IRS2 (**bottom column**) staining of liver sections from control (**left column**), F1 (**middle column**), and F3 (**right column**). Expression of IRS1 and IRS2 were visualized by 3,3'-diaminobenzidine (brown). **Arrow** indicates portal vein. **b:** Immunoblotting for IRS1 and IRS2. Proteins (40 μg) in liver extracts from control and patients with HCV infection were immunoblotted with anti-IRS1 antibodies (**top column**) or anti-IRS2 antibodies (**bottom column**). **c:** Hepatic IRS1 and IRS2 expression in HCV core-Tg mice. Non-Tg littermates and HCV core Tg-mice livers (8 weeks old) were subjected to immunoblotting for IRS1, IRS2, insulin receptor, and STAT5 (as a reference protein). These experiments were repeated three times and representative immunoblotting and immunostaining images are shown. Original magnifications, ×400.

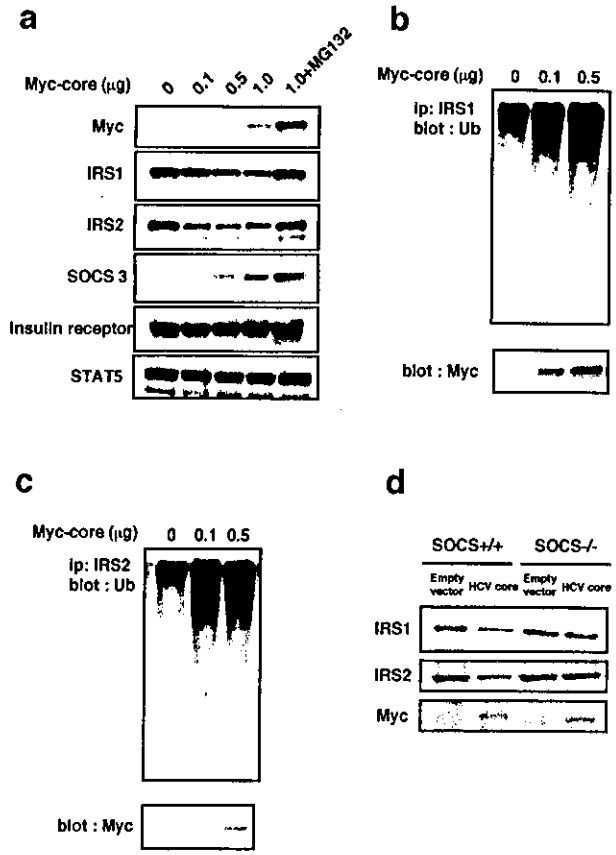

Figure 5. The effect of HCV core on insulin signaling molecules. **a:** The effect of HCV core on IRS1, IRS2, SOCS3, and insulin receptor expression. Myc-tagged HCV core was transiently expressed in HepG2 cells. Twenty-four hours after transfection the cell extracts were immunoblotted with the indicated antibodies. MG132 (10 μmol/L) was added with cDNA of Myc-tagged HCV core and incubated for 1 hour. STAT5 was used as a reference protein. **b** and **c:** The identification of ubiquitinated IRS1 and IRS2. Whole-cell extracts (40 μg of crude extract) were subjected to immunoprecipitation with IRS1 or IRS2 and followed by immunoblotting using anti-ubiquitin monoclonal antibody. **d:** The effect of HCV core on IRS1 and IRS2 expression in SOCS3$^{-/-}$ MEF cells. Twenty-four hours after transfection of HCV core, MEF cell extracts were immunoblotted with anti-IRS1 antibodies or anti-IRS2 antibodies. Representative immunoblotting images from three separate experiments are shown.

(Figure 4b), confirming results of immunostaining. We developed HCV core-Tg mice in which the HCV core protein is expressed ubiquitously. The HCV core-Tg mouse is an informative animal model for studying the pathogenesis of HCV infection.[36,37] In two independent HCV core-Tg mice, decreased expression of hepatic IRS1 and IRS2, but not of insulin receptor, were also demonstrated compared to two independent wild-type littermates (Figure 4c).

The Effect of HCV Core on IRS1, IRS2, SOCS3, and Insulin Receptor Expression

The effects of HCV core on IRS1, IRS2, SOCS3, and insulin receptor expression were examined in HepG2 and Huh7 cells prepared by transient transfection with Myc-tagged HCV core and HLF cells with stable transfection of Myc-tagged HCV core. Expression of Myc-tagged HCV core was confirmed by immunoblotting for Myc in both cells. HCV core dose dependently decreased IRS1 and IRS2 expression in HepG2 cells (Figure 5a). In contrast, SOCS3 expression was dose dependently increased by transient transfection with Myc-tagged HCV core in HepG2 cells. No changes in insulin receptor expression and STAT5 (used as a reference protein) were seen in HepG2 cells transfected with Myc-tagged HCV-core (Figure 5a). We also analyzed the effects of a proteosomal proteolysis inhibitor, MG132. The treatment with MG132 caused an increase in expression levels of IRS1 and IRS2 (Figure 5a). Similar results were obtained in Huh7 cells with transient transfection of HCV core and HLF cells with stable transfection of HCV core (data not shown).

Role of Ubiquitination in the Regulation of IRS1 and IRS2 Expression

To investigate the involvement of ubiquitination in down-regulation of IRS1 and IRS2 in HepG2 cells transfected with HCV core, whole-cell extracts were immunoprecipitated with anti-IRS1 or anti-IRS2 antibodies and immunoblotted with anti-ubiquitin monoclonal antibodies. HCV

core caused an accumulation of ubiquitin-conjugated IRS1 (Figure 5b) and IRS2 (Figure 5c). Expression of Myc-tagged HCV core was confirmed by immunoblotting for Myc (Figure 5, b and c).

Role of SOCS3 in the Regulation of IRS1 and IRS2 Expression

Because SOCS3$^{-/-}$ mice display embryonic lethality, we examined the association between SOCS3 and regulation of IRS1 and IRS2 by using SOCS3$^{-/-}$ MEF cells. HCV core down-regulated IRS1 and IRS2 in SOCS3$^{+/+}$ MEF cells. On the other hand, HCV core did not cause down-regulation of IRS1 and IRS2 in SOCS3$^{-/-}$ MEF cells (Figure 5d).

The Effects of HCV Core on Insulin Signaling

HLF cells with stable transfection of HCV core were treated with insulin (100 ng/ml) from 0 minutes to 60 minutes and phosphorylation of p85 subunit of PI3K and Akt were determined. Insulin-induced phosphorylation of p85 subunit of PI3K and Akt was observed in HLF cells transfected with empty vector. On the other hand, HCV core decreased phosphorylation of p85 subunit of PI3K and Akt at the base line (0 minutes) and inhibited insulin-induced phosphorylation of p85 subunit of PI3K and Akt (Figure 6a). STAT5 protein (reference protein) levels were unchanged by insulin stimulation. Insulin activated Fru 6-P, 2-kinase, a downstream of Akt signal and one of the potent regulators of glycolysis, and decreased medium glucose levels (Figure 6, b and c). HCV core suppresses insulin-induced activation of Fru 6-P, 2-kinase and decreases in medium glucose levels (Figure 6, b and c).

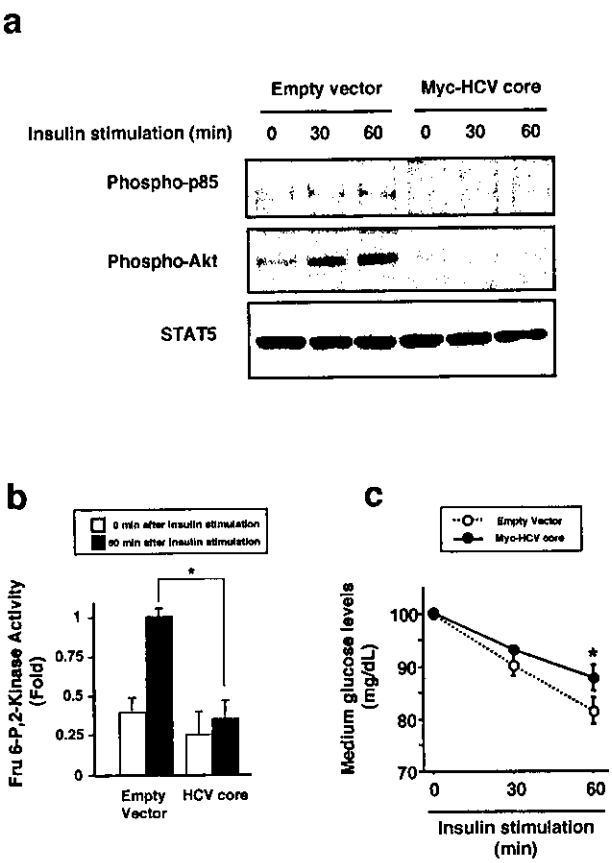

Figure 6. The effects of HCV-core on insulin signaling. HLF cells with transfection of empty vector or Myc-HCV core were incubated in the presence of insulin (100 ng/ml) for 60 minutes. **a:** The effect of HCV-core on insulin-induced phosphorylation of p85 subunit of phosphatidylinositol 3-kinase (PI3K) and Akt. Whole-cell lysates were subjected to immunoblotting for phospho-p85 subunit of PI3K and phospho-Akt. STAT5 was used as reference protein. Representative immunoblotting images from three separate experiments are shown. **b:** The effect of HCV-core on insulin-induced activation of Fru 6-P, 2-kinase. Sixty minutes after insulin stimulation, Fru 6-P, 2-kinase activity in whole-cell lysates was assayed. Values were expressed as mean ± SD. The comparison between the two groups was made using the Mann-Whitney *U*-test. *, $P < 0.05$. **c:** The effect of HCV-core on insulin-induced activation of glucose uptake. Glucose levels in culture medium were measured at 0, 30, and 60 minutes after insulin stimulation. Values were expressed as mean ± SD. The comparisons between groups were made using analysis of variance with Scheffé's post hoc test. N.S., not significant. *, $P < 0.05$.

Discussion

In this study, we showed that more severe insulin resistance was present in noncirrhotic patients with HCV infection than in patients with other hepatobiliary diseases. Insulin resistance was associated with the presence of serum HCV core, the severity of hepatic fibrosis and decreased expression of hepatic IRS1 and IRS2 in patients with HCV infection. HCV core down-regulated the expression of IRS1 and IRS2 in human hepatoma cell lines as well as in whole animals. These findings suggest that HCV causes changes in specific hepatic molecules regulating glucose metabolism and results in severe insulin resistance. A possible mechanism is that HCV core-induced SOCS3 promotes proteosomal degradation of IRS1 and IRS2 through ubiquitination.

Although fasting glucose levels were similar among all of the groups, fasting insulin and HOMA-IR levels, a indicator of insulin resistance, were significantly increased in patients with HCV infection compared to the other hepatobiliary disorders. β-Cell function evaluated by HOMA-β was also increased in patients with HCV infection compared to controls. These findings indicate that HCV infection induced insulin resistance and fasting glucose levels were compensated by hyperinsulinemia. Mangia and colleagues[38] reported no association between HCV infection and DM in noncirrhotic patients and that the prevalence of DM in noncirrhotic patients is comparable to the expected prevalence in the general population. These results are not in accord with our results and this discrepancy may be explained by different evaluation methods for glucose intolerance. They evaluated glucose intolerance by fasting glucose levels and only patients who had >126 mg/dl of glucose levels were considered as abnormal glucose metabolizers. Because fasting glucose levels are compensated by hyperinsulinemia (Figure 1, a and b), cryptic changes in glucose metabolism can be evaluated by measuring fasting insulin or HOMA-IR levels.[24] In our own study, we provide convincing evidence that more severe insulin resistance is present in noncirrhotic patients with HCV infection than

in patients with other hepatobiliary disorders. Unique mechanisms may underlie HCV-associated severe insulin resistance.

HCV genotype 2a is specifically linked with extrahepatic manifestations such as cryoglobulinemia and benign monoclonal gammopathy.[39,40] Mason and colleagues[6] also reported an association between genotype 2a and DM, whereas no association was found between fasting insulin levels and HCV genotypes in our study. The limited number of patients may prevent drawing definite conclusions in both studies. HCV core can modulate cell signaling.[23] Therefore, we investigated the relationship between HCV core and fasting insulin levels. In patients with undetectable levels of HCV core, fasting insulin levels were within the normal range. In contrast, in patients with detectable levels of HCV core, fasting insulin levels were increased. Thus, HCV core seems to play a crucial role in HCV-associated insulin resistance.

Then, we examined an association between histological parameters and insulin resistance. Although no significant association was found between activity and insulin resistance, serum insulin levels and HOMA-IR levels were significantly increased with the severity of hepatic fibrosis. These data are in good agreement with recent report by Hui and colleagues.[13] HOMA-IR is independently associated with an increased rate of fibrosis progression.[13,41] Insulin stimulates hepatic stellate cells to proliferate and secrete extracellular matrix.[42] Thus, it appears that insulin resistance contributes to fibrotic progression in patients with HCV infection. To innovate therapies for prevention of fibrotic progression, it is important to investigate the molecular mechanisms for HCV-induced insulin resistance.

IRS1 and IRS2 act as important mediators of insulin action and down-regulation of hepatic IRS1 and IRS2 results in an increase in hepatic insulin resistance. Knockout of the IRS1 gene induces insulin resistance and subsequent compensatory hyperinsulinemia.[20] Knockout of the IRS2 gene causes severe diabetes as a consequence of insulin resistance and disturbance of insulin secretion.[22] We showed that decrease in expression of IRS1 and IRS2 was associated with the progression of hepatic fibrosis. Down-regulation of IRS1 and IRS2 was also seen in livers from HCV core-Tg mice. These data suggest that down-regulation of IRS1 and IRS2 is responsible for compensatory hyperinsulinemia and progression of hepatic fibrosis.

The effects of HCV core on the expression of IRS1 and IRS2 were investigated by simplified *in vitro* experiments. HCV is a positive-strand RNA virus consisting of a putative structure (core, E1, E2/p7) and at least six nonstructural proteins (NS2, NS3, NS4A, NS5A, NS5B). HCV core is implicated in cellular transformation.[37] In this study, HCV core decreased expression of IRS1 and IRS2 in human hepatoma cell lines. These *in vitro* findings add weight to the results of our human studies.

One of the negative modulation mechanisms of IRS1 and IRS2 is proteosomal degradation.[43] On the other hand, we previously reported that HCV core induced SOCS3 in mouse fibroblast NIH 3T3 cells.[23] In the current study, HCV core-induced SOCS3 was also validated in HepG2 cells as well as Huh7 cells and HLF cells. The SOCS family of proteins has similar structural characteristics referred to collectively as a "SOCS box," a unique NH_2-terminal domain of variable length, a central Src-homology 2 domain, and a COOH-terminal, with this structural resemblance reflecting functional similarities among SOCS proteins. The SOCS box acts as an adaptor to facilitate the ubiquitination of signaling proteins and their subsequent targeting to the proteosome by complexing with Elongins B and C.[43,44] These facts and our findings led to the assumption that HCV core-induced SOCS3 promoted proteosomal degradation of IRS1 and IRS2 through ubiquitination. To test this hypothesis, HCV core-transfected cells were incubated with MG132, a potent proteosomal proteolysis inhibitor. MG132 blocked HCV core-induced decrease of IRS1 and IRS2 in HepG2. Ubiquitination of IRS1 and IRS2 was increased by transfection of HCV core. Moreover, HCV core did not cause down-regulation of IRS1 and IRS2 in SOCS3$^{-/-}$ MEF cells. All of these data support our hypothesis. Transient overexpression of SOCS3 in mouse liver induces fasting hyperglycemia, and fasting hyperinsulinemia. These changes are returned to normal as SOCS3 expression subsided.[43] Thus, studies in whole animals lend added credence to our hypothesis that HCV core-induced SOCS3 promotes proteosomal degradation of IRS1 and IRS2 through ubiquitination.

To verify the biological significance of these studies on IRS1 and IRS2, the effects of HCV core on insulin signaling were examined. Insulin phosphorylates the p85 subunit of PI3K and Akt, which are downstream components of IRS in liver.[45] Akt activates Fru 6-P,2-kinase, one of the key enzymes of glycolysis, and glucose uptake.[46] HCV core inhibited insulin-induced phosphorylation of p85 subunit of PI3K and Akt, activation of Fru 6-P,2-kinase, and glucose uptake. Thus, HCV core-transfected HLF cells were resistant to insulin stimulation compared with empty vector-transfected HLF cells, suggesting biological significance of HCV core-induced down-regulation of IRS1 and IRS2.

Recently, Aytug and colleagues[47] studied changes in the upstream insulin-signaling molecules in the liver specimens obtained from patients with chronic HCV infection and showed impairments of insulin-stimulated PI3K activity and Akt phosphorylation, which were in good agreements with our findings. However, our data for IRS1 differed from those reported by Aytug and colleagues.[47] They showed increased IRS1 expression with reduction in tyrosine phosphorylation. Although the reason for this discrepancy remains unclear, one possibility is that their HCV-infected patients show higher BMI values than those of ours. Obesity is a well-recognized risk factor for the development of insulin resistance. Adipocytes secrete a large number of factors with diverse functions. Tumor necrosis factor-α and free fatty acids are secreted by adipocytes and are known to impair insulin signaling by reducing IRS1 tyrosine phosphorylation.[48,49] Increased IRS1 expression may be for an adaptation to reduction of IRS1 tyrosine phosphorylation. Moreover, leptin, which is also secreted by adipocytes, is involved in the development of HCV-associated insulin

resistance.[50] Thus, there may exist several molecular mechanisms for HCV-associated insulin resistance.

In conclusion, more severe insulin resistance was present in noncirrhotic patients with HCV infection than in patients with other hepatobiliary diseases. We uncovered a unique mechanism of HCV-associated insulin resistance. HCV core-induced SOCS3 may promote proteosomal degradation of IRS1 and IRS2 through ubiquitination.

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