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Molecular epidemiology of hepatitis A virus in metropolitan areas in Japan

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Background. Transmission routes of hepatitis A virus (HAV) in Japan have changed. The present study investigated changes of transmission routes in relation to genetic drift. ***Methods.*** All 60 patients who were admitted between 1993 and 2003 with a diagnosis of hepatitis A were retrospectively analyzed. Nucleotide sequences of the VP1/2A region of the HAV recovered from their sera were determined. ***Results.*** The suspected transmission routes were household contact, 19 (31%); food or waterborne, 16 (27%); homosexual activity, 11 (18%); international travel, 4 (7%); and unknown 10. (17%). The 11 patients presumably infected through homosexual activity were found exclusively in 1998 and 1999. The proportion of patients exposed through homosexual behavior and household contact was higher in those 2 years than in other years. Nucleotide sequences could be determined for 58 patients. Fifty-seven of the 58 sequences belonged to genotype IA HAV, with less than 10% nucleotide diversity. Of the 27 sequences isolated during 1998 and 1999, 25 had an identical nucleotide sequence regardless of the suspected transmission route. In contrast, sequences obtained in the other years differed from one another. A phylogenetic tree constructed from sequences recovered from patients without a history of travel abroad showed several clusters. ***Conclusions.*** Our results suggest that (1) HAV acquired through homosexual activity may be transmitted to nonhomosexual individuals; (2) hepatitis A in metropolitan areas in Japan is caused mainly by sporadic infection with genotype IA HAV; and (3) several subtypes of genotype IA HAV are endemic in Japan.

Key words: hepatitis A virus, epidemiology, transmission route, sexuality, Japan

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Introduction

Hepatitis A virus (HAV) is one of the major causes of viral hepatitis and a worldwide problem. The annual incidence of hepatitis A is 1.5 million cases of clinical disease, and the true incidence, including subclinical disease, may be much higher.[1] Fulminant hepatic failure is a complication for some patients with clinical disease.[2] Therefore, preventing the spread of HAV is an important issue.

Good sanitation and a sterilized water supply are essential for the prevention of hepatitis A. In developing countries with a high incidence of hepatitis A, the main transmission route of hepatitis A is the fecal–oral route caused by poor sanitation, which increases the chance of ingesting contaminated food or water.[2,3] In contrast, in developed countries with good sanitation and a sterilized water supply, such as North America, Western Europe, Australia, and New Zealand, the incidence of hepatitis A is low and transmission is caused by personal contact with an infected person, homosexual activity, or transfusion of contaminated blood products, in addition to ingestion of contaminated food or water.[2–5]

The incidence of hepatitis A in Japan has markedly decreased recently. National surveillance of HAV in Japan has shown that more than 90% of people over 65 but fewer than 10% of people under 34 are positive for anti-HAV.[6] The difference can probably be attributed to changes in sanitation. If this hypothesis is true, then the transmission route of HAV in Japan may have changed with time. Studying changes in HAV transmission routes in Japan may therefore elucidate the influence of sanitation on transmission routes.

Molecular epidemiological approaches may also be useful for studying transmission routes. Studies from European countries have shown that several clusters of viral strains from various genotypes prevail in those countries.[7,8] The heterogeneity of isolated strains

suggests multiple transmission routes. Information on transmission routes is, however, not available in these reports. Sequential molecular epidemiological studies linked to transmission routes may elucidate native strains in Japan and provide new information for the control of this disease.

The aim of this study was to understand both clinical and molecular epidemiology of HAV infection in Japan.

Methods

Patients

Sixty patients admitted to our institutions between 1993 and 2003 who were diagnosed with hepatitis A were analyzed retrospectively. The patients comprised 39 men (65%) and 21 women (35%), and their median age was 34.0 years (range, 22–55 years). The diagnosis of hepatitis A was based on a high titer serum IgM anti-HA level with acute liver injury. Coinfection with hepatitis B virus, hepatitis C virus, or other hepatotropic viruses was excluded by serological testing. Serum samples were available from all patients on admission. Fifty-eight of the 60 samples were positive for HAV RNA by reverse transcription (RT)-nested polymerase chain reaction (PCR) with the protocol outlined below. None of the 58 patients had fulminant hepatic failure. Intrahepatic cholestasis was a complication in one patient. The other 57 patients underwent a noncomplicated and self-limited clinical course.

Informed consent was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethics committees of our institutions.

Detection of hepatitis A viral RNA in serum

RNA was extracted from sera using the acid guanidinium-phenol-chloroform method.[9] In brief, 100 µl of sample was mixed with 300 µl of solution D (guanidinium solution), 60 µl of chloroform, and 40 µl of NaOAc (pH 5.2) and precipitated with 1 ml of ethanol. The RNA pellet was washed twice with 70% ethanol and dissolved in 25 µl of RNase-free distilled water.

For reverse transcription, 1 µl of RNA solution, extracted from 100 µl of sera using the acid guanidinium-phenol-chloroform method and dissolved in 25 µl of RNase-free distilled water, was heat-denatured at 68°C for 10 min. It was chilled rapidly on ice and mixed with 4 µl of 1.5 mM $MgCl_2$ solution, 2 µl of 10× RNA PCR buffer (100 mM Tris-HCl, pH 8.3; 500 mM KCl), 8.5 µl of RNase-free distilled H_2O, 2 µl of dNTP mixture (10 mM dATP, dCTP, dGTP, dTTP), 1 µl of random 9-mers (5′-NNNNNNNNN-3′), 0.5 µl of RNase inhibitor (Takara-Shuzo, Kyoto, Japan), and 1 µl of reverse transcriptase (Takara-Shuzo). After incubation at 30°C for 10 min, reverse transcription reaction was carried out at 42°C for 30 min, followed by inactivation at 95°C for 5 min.

In the first PCR, 5 µl of the 20 µl cDNA solution was used. The first PCR was performed in 50 µl of reaction mixture containing 1.0 µM each of outer sense primer (5′-GGTTTCTATTCAGATTGCAAATTA-3′ nt. 2891–2914) and antisense primer (5′-AGTAAAAACT CCAGCATCCATTTC-3′ nt. 3398–3375), 200 µM of each dNTP, 5 µl of 10× PCR buffer (100 mM Tris-HCl, pH 8.3; 500 mM KCl; 15 mM $MgCl_2$; 0.01% (w/v) gelatin), and 2.5 U of *Ex Taq* polymerase (Takara) with proofreading activity. The amplification conditions were 94°C for 16 min followed by 40 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min.

One microliter of the first PCR product was used for the second PCR. The reaction mixture contained 1.0 µM each of inner sense primer (5′-TTGCAAATTACAAT CATTCTG-3′ nt. 2905–2925) and inner antisense primer (5′-TTCAAGAGTCCACACACTTCT-3′ nt. 3377–3367), 5 µl of 10× PCR buffer, 35 µl of RNase-free dH_2O, 5 µl of dNTP mixture (2 mM dATP, dCTP, dGTP, dTTP), and 0.5 µl of amplitaq gold (Roche Diagnostics, Branchburg, NJ, USA). The amplification conditions for the second PCR were the same as those of the first PCR. The second PCR products were analyzed by 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized by UV transillumination. Standard precautions to avoid contamination were taken during PCR, with a negative control serum included in each run.

Sequencing of PCR products

Amplification products were purified on Wizard PCR Preps DNA purification resin (Promega, Madison, WI, USA), and sequenced bidirectionally with the Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, CA, USA) using the above PCR primers. Sequencing was performed on an automated DNA sequencer ABI 377 (PE Applied Biosystems).

The nucleotide sequences of HAV isolates from the patients were compared with those of seven reference HAV strains retrieved from the DDBJ/EMBL/GenBank databases, representing each of the seven major genotypes (I–VII). Phylogenetic trees were constructed with the Mega program version 2.1 using the Kimura two-parameter matrix and the neighbor-joining method.[10] To confirm the reliability of the phylogenetic tree analysis, bootstrap resampling and reconstruction were carried out 500 times.

Statistical analysis

Data were analyzed by a χ-squared test. P values less than 0.05 were regarded as statistically significant.

Results

Transmission routes

Table 1 shows the numbers of patients and transmission routes. An epidemic of hepatitis A among homosexuals was reported in metropolitan areas in Japan between 1998 and 1999.[11,12] Our results showed that more patients were admitted during that period. The increase was caused not only by patients involved in homosexual activity but also by individuals without that risk factor.

The suspected transmission routes for the patients were as follows: household contact, 19 (31%); food or water, 16 (27%); homosexual activity, 11 (18%); international travel, 4 (7%); and unknown, 10 (17%). In 1998 and 1999, the suspected transmission routes were as follows: household contact, 13 (45%); homosexual activity, 11 (38%); food or water, 1 (3%); international travel, 1 (3%); and unknown, 3 (11%). The proportion of cases associated with homosexuality and household contact was higher in those 2 years than in the other years (homosexuality-associated, $P = 0.0006$; household contact-associated, $P = 0.034$). Figure 1 shows the time of onset for all patients over the 2 years. The times of onset for those 2 years were from July 1998 to July 1999 for homosexual patients and from February 1998 to September 1999 for nonhomosexual patients. After excluding two patients with different sequences, the onset of nonhomosexual patients varied from August 1998 to September 1999. The periods of transmission were similar between homosexual and nonhomosexual groups.

Sequence analysis of HAV RNA

The sequence between nucleotides 3024 and 3191 of the VP1/2A region was determined for the 58 patients. Many nucleotide sequences were closely related to HAS-15, a representative HAV genotype IA strain. Twenty-five of the 27 viral sequences recovered during 1998–1999 were identical. This sequence is identical to IMSTU, which is prevalent among homosexuals in metropolitan areas.[12] As shown in Table 2, all sequences except one showed more than 90% identity with the reference sequences.

We then performed a phylogenetic analysis of the region between nucleotides 3024 and 3191 and classified the virus strains (Fig. 2). Bootstrap analysis to evaluate the statistical reliability of the phylogenetic tree revealed 500/500 (100%) reliability. All strains belonged to genotype IA, except for one that belonged to genotype IIIA. The patient harboring the genotype IIIA virus had a history of travel to Africa 1 month before admission.

Table 1. Numbers of patients and routes of transmission

	1993–1997	1998–1999	2000–2004	Total
Household contact	4 (29%)	13 (45%)*	2 (12%)	19 (31%)
Food or waterborne	4 (29%)	1 (3%)	11 (64%)	16 (27%)
Homosexual activity	0	11 (38%)**	0	11 (18%)
Foreign travel	1 (7%)	1 (3%)	2 (12%)	4 (7%)
Unknown	5 (35%)	3 (11%)	2 (12%)	10 (17%)
Total	14	29	17	60

* $P = 0.034$, ** $P = 0.0006$; χ-squared test

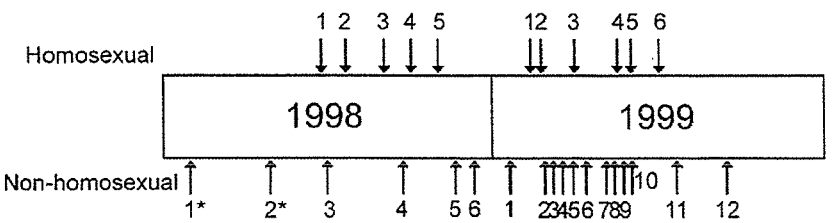

Fig. 1. Time of first visit of patients with hepatitis A in 1998 and 1999. *Arrows* in the upper part show the times of visit of homosexual patients. *Arrows* in the lower part show those of nonhomosexual patients. All except two (cases 1 and 2) had the same sequences. The period of transmission was similar for homosexual and nonhomosexual groups. * shows the sequences that were different

Table 2. Homology with recovered sequences and representative strains (HAS-15 and IMSTU)

Homology with IMSTU			Homology with HAS-15		
	Nucleic acid	Amino acid		Nucleic acid	Amino acid
HAS-15	95.7	98.2	IMSTU	95.7	98.2
1993	97.6	100	1993	94.4	98.2
1994	77.1	89.1	1994	73.8	87.3
1995	95.7–98.8	98.2–100	1995	93.8–94.4	96.4–98.2
1996	97.6	100	1996	94.4	98.0
1997	95.7–98.8	96.4–100	1997	92.4–94.4	94.5–98.2
1998	95.7–100	98.2–100	1998	93.7–95.7	96.4–98.2
1999	100	100	1999	95.7	98.2
2000	90.4–95.7	92.7–98.2	2000	86.8–92.4	90.9–96.4
2001	93.7–97.6	96.4	2001	92.4–94.4	94.5–98.2
2002	93.1–96.3	100	2002	92.4–96.3	94.5–98.2
2003	96.7	100	2003	95.0	98.2

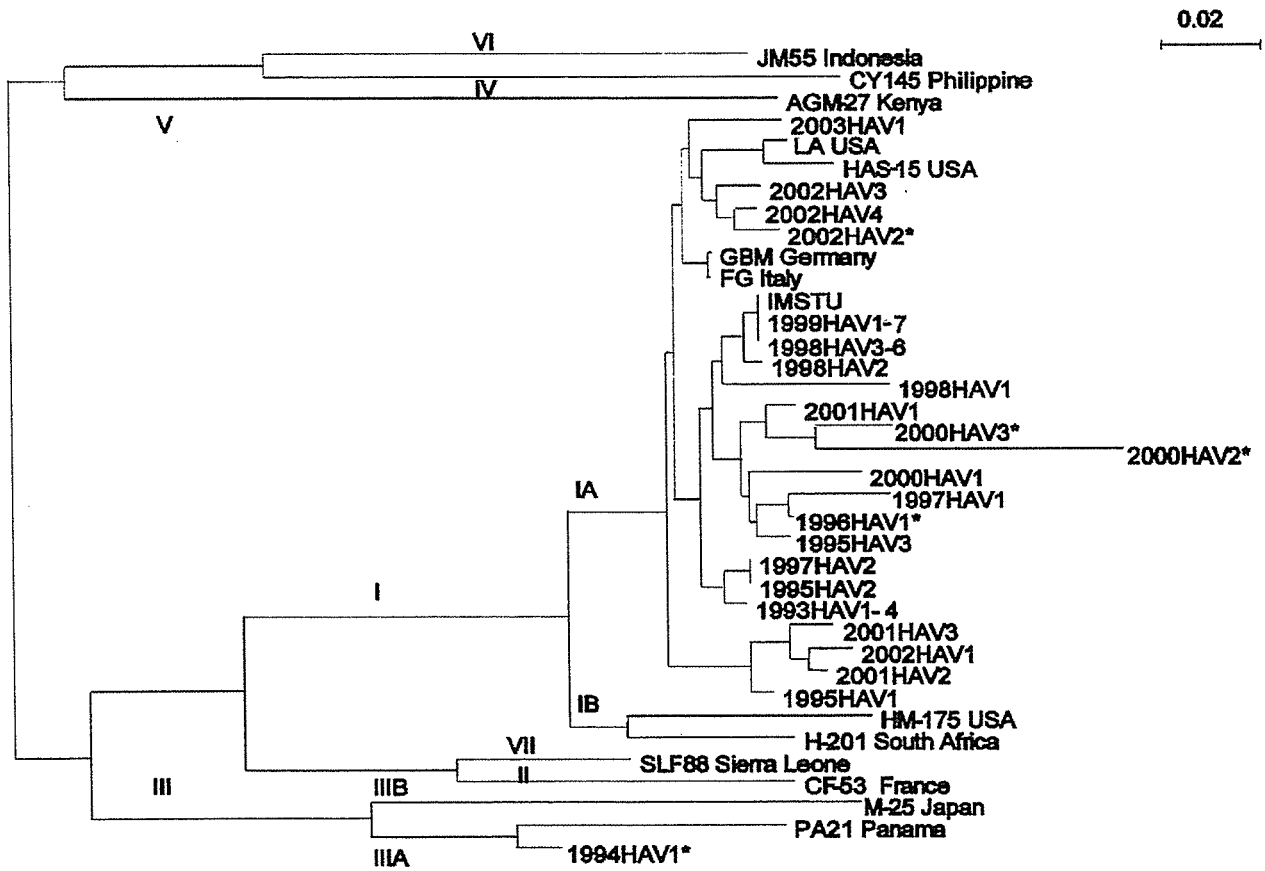

Fig. 2. A phylogenetic tree constructed for RNA sequences located in the VP1/2A region of hepatitis A virus (HAV) genomes reported previously. Accession numbers are shown for the isolates that have been deposited in the DDBJ/EMBL/GenBank databases. Many nucleotide sequences were close to that of HAS-15, a representative HAV genotype IA strain. Twenty-five of 27 viral sequences recovered during 1998–1999 were identical to IMSTU. *shows the sequences that were acquired abroad

Discussion

In this study, the most frequent transmission route was personal contact, which is also the case in the United States (www.cdc.gov/ncidod/diseases/hepatitis/resource/PDFs/hep_surveillance_60.pdf). The next most frequent cause was contaminated food, which is different from the United States. Additionally, the

proportion of patients whose hepatitis was caused by contaminated food was highest in recent years. As mentioned above, anti-HAV prevalence, which may reflect poor sanitation, has decreased in Japan. Therefore, the high incidence of hepatitis A with food/water as a transmission route may not result from poor sanitation. The reason for the high percentage is presumably related to diet. Japanese people often eat raw fish or shellfish, which increases the chances of transmission. Indeed an outbreak caused by eating raw oysters has been reported.[13] In other words, the transmission routes of HAV in Japan are different from those in other developed countries irrespective of improved sanitation.

The molecular epidemiological study showed interesting results. The sequences recovered in the years other than 1998 and 1999 were heterogeneous (Fig. 1). Furthermore, the phylogenetic analysis showed several clusters of genotype IA HAV strains, after excluding cases presumably acquired abroad. This suggests that several subgroups of genotype IA HAV strains are endemic in Japan and cause sporadic hepatitis. A large-scale epidemiological study may be useful for testing this hypothesis.

In contrast, an epidemic caused by homosexual activity was observed in 1998 and 1999.[11,12] The same nucleotide sequences were detected among patients for more than 1 year (Fig. 1), which suggests that the same strain was transmitted secondarily. Among 24 patients whose sera harbored the same HAV sequences in this period, 11 were presumably infected through homosexual activity. Among the other patients, four were infected through close person-to-person contact (heterosexual activity, familial transmission, or transmission in day-care facilities), while the other four had no relevant history. These findings suggest that homosexual persons can transmit HAV to nonhomosexual persons through close contact or heterosexual activity.

It is interesting that 24 separate sequences in 1998 and 1999 were identical to the IMSTU recovered from ten patients in another institution. A serum sample recovered 2 months before the epidemic had a similar sequence, with 99.4% homology. Because a homosexual patient may have sexual contact with multiple partners within that community, the 34 patients may have been infected by the same strain. Therefore, our results suggest that the rate of mutation in this region of 168 base pairs is probably very low. A previous study has shown that the mutation rate of HAV within a person is very low;[14] our results are consistent with that observation.

A previous report has shown that sequences recovered in Japan in the early 1990s are variable and belong to multiple genotypes.[14] Our results are contrary to those of that study. This suggests that improvements in sanitation decrease both the number of patients and viral heterogeneity. The endemicity in low-HAV-prevalence countries may be caused by highly related viral strains. A report from the United States showing that most patients infected in a community-wide outbreak were infected by the same strain supports this hypothesis.[15]

In developed countries, a substantial number of patients with hepatitis A acquired HAV through homosexual activity (www.cdc.gov/nciod/diseases/hepatitis/h96surve.htm). Recent studies using PCR analysis have shown that the fecal excretion of HAV continues even after recovery,[16] which suggests that hepatitis A patients may transmit the virus even after recovery. This is in accordance with the fact that positivity for anti-HAV among homosexual people is very high.[17–19] Therefore, people who engage in homosexual activity should be considered for HAV vaccination regardless of human immunodeficiency virus coinfection.

To conclude, recent hepatitis A in metropolitan areas is caused predominantly by sporadic infection by genotype IA HAV. Homosexual activity may cause an HAV epidemic among not only homosexuals but also heterosexuals, and involve homogeneous viral strains.

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References

1. Hepatitis A vaccines. Wkly Epidemiol Rec 2000;75:38–44.
2. Cuthbert JA. Hepatitis A: old and new. Clin Microbiol Rev 2001;14:38–58.
3. Gust ID. Epidemiological patterns of hepatitis A in different parts of the world. Vaccine 1992;10 Suppl 1:S56–8.
4. Dentinger CM, Bower WA, Nainan OV, et al. An outbreak of hepatitis A associated with green onions. J Infect Dis 2001;183:1273–6.
5. Hepatitis A among persons with hemophilia who received clotting factor concentrate—United States, September–December 1995. MMWR Morb Mortal Wkly Rep 1996;45:29–32.
6. Kiyohara T, Satoh T, Yamamoto H, Totsuka A, Moritsugu Y. The latest seroepidemiological pattern of hepatitis A in Japan. Jpn J Med Sci Biol 1997;50:123–31.
7. Bruisten SM, van Steenbergen JE, Pijl AS, Niesters HG, van Doornum GJ, Coutinho RA. Molecular epidemiology of hepatitis A virus in Amsterdam, the Netherlands. J Med Virol 2001;63:88–95.
8. Tallo T, Norder H, Tefanova V, Ott K, Ustina V, Prukk T, et al. Sequential changes in hepatitis A virus genotype distribution in Estonia during 1994 to 2001. J Med Virol 2003;70:187–93.
9. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156–9.
10. Sugita S, Yoshioka Y, Itamura S, Kanegae Y, Oguchi K, Gojobori T, et al. Molecular evolution of hemagglutinin genes of H1N1 swine and human influenza A viruses. J Mol Evol 1991;32:16–23.
11. Baba M, Oka H, Asayama M, Yoshinaga S, Yamashita H, Tachikawa N, et al. Acute hepatitis outbreak in Tokyo caused by

hepatitis A virus of common origin transmitted through oral and sexual routes. Jpn J Infect Dis 2000;53:127–9.
12. Ishida T, Nakamura T, Ajisawa A, Negishi M, Kashiyama T, Takechi A, et al. Outbreak of hepatitis A virus infection among HIV-1 seropositive men who had sex with men. Jpn J Infect Dis 1999;52:131–2.
13. Ohara H, Naruto H, Watanabe W, Ebisawa I. An outbreak of hepatitis A caused by consumption of raw oysters. J Hyg (Lond) 1983;91:163–5.
14. Robertson BH, Jansen RW, Khanna B, Totsuka A, Nainan OV, Siegl G, et al. Genetic relatedness of hepatitis A virus strains recovered from different geographical regions. J Gen Virol 1992; 73(Pt 6):1365–77.
15. Robertson BH, Averhoff F, Cromeans TL, Han X, Khoprasert B, Nainan OV, et al. Genetic relatedness of hepatitis A virus isolates during a community-wide outbreak. J Med Virol 2000;62:144–50.
16. Yotsuyanagi H, Koike K, Yasuda K, Moriya K, Shintani Y, Fujie H, et al. Prolonged fecal excretion of hepatitis A virus in adult patients with hepatitis A as determined by polymerase chain reaction. Hepatology 1996;24:10–3.
17. Villano SA, Nelson KE, Vlahov D, Purcell RH, Saah AJ, Thomas DL. Hepatitis A among homosexual men and injection drug users: more evidence for vaccination. Clin Infect Dis 1997;25:726–8.
18. Diamond C, Thiede H, Perdue T, Secura GM, Valleroy L, Mackellar D, et al. Viral hepatitis among young men who have sex with men: prevalence of infection, risk behaviors, and vaccination. Sex Transm Dis 2003;30:425–32.
19. Koff RS. Seroepidemiology of hepatitis A in the United States. J Infect Dis 1995;171 Suppl 1:S19–23.

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Original Article

Prevalence of coinfection with human immunodeficiency virus and hepatitis C virus in Japan

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People with human immunodeficiency virus (HIV) infection are frequently infected with hepatitis C virus (HCV), because of the common transmission routes. Since the dissemination of hyperactive antiretrovirus therapy (HAART), the morbidity and mortality associated with HIV infection have declined. However, the reduction in mortality due to opportunistic infection has made HCV-associated liver diseases the leading cause of mortality in Western countries. A similar situation is assumed in Japan, but the status of coinfection with HIV and HCV is unclear. We conducted a nationwide survey to determine the prevalence of coinfection with HIV and HCV by distributing a questionnaire to the hospitals in the HIV/AIDS Network of Japan. Among 4877 patients reported to be HIV-positive, 935 (19.2%) were also positive for the anti-HCV antibody. Most (84.1%) of the patients coinfected with HIV and HCV were recipients of blood products. These data, for the first time, show the current status of coinfection with HIV and HCV in Japan. A detailed analysis of the progression and severity of liver diseases in the coinfected patients is expected.

Key words: coinfection, hepatitis C, HIV, liver disease

INTRODUCTION

HEPATITIS C VIRUS (HCV) infection and human immunodeficiency virus (HIV) infection are major public health problems worldwide. In the USA, the estimated prevalence of the anti-HCV antibody is 1.8%, with 2.7 million people having HCV-RNA detected in their blood, indicative of ongoing HCV infection.[1] The prevalence of HIV is <1%, and the virus is estimated to have infected approximately 800 000 people.[2] Because of the common transmission routes, that is, parenteral ones, many people with HIV infection are also infected with HCV.[3] Before the introduction of hyperactive antiretroviral treatment (HAART) in 1996, most people with HIV infection died of HIV-associated opportunistic infections such as *Pneumocystis carinii* (currently called *P. jiroveci*) pneumonia and cytomegaloviral infection. Since the dissemination of HAART, the morbidity and mortality associated with HIV infection have declined. However, the reduction in mortality due to opportunistic infection has made patients coinfected with HIV and HCV faced with the menace of progressive liver diseases due to HCV infection in the United States and Europe.[4,5]

Coinfection with HIV has been shown to increase the HCV load in HCV infection,[6] being a negative prognostic factor for clearance of HCV in anti-HCV therapy using interferon.[7,8] It also accelerates the development of cirrhosis and, eventually, hepatocellular carcinoma. Although still controversial, coinfection with HIV and HCV yields a more rapid progression to acquired immunodeficiency syndrome (AIDS) in some cases.[9,10] Importantly, coinfection with HIV and HCV will increase the morbidity and mortality of HIV-infected patients also in Japan, where the prevalence of HIV infection is increasing in a linear fashion, exceptionally among developed countries.[11] There are more than 10 000 HIV-positive people in Japan as of the end of 2004, according to the AIDS National Survey in Japan,[12] and approximately 1.8 million chronic HCV carriers, according to the estimation by the Ministry of Health, Labor and Welfare (MHLW) of Japan. However, unfortunately, the prevalence of coinfection with HIV and HCV in Japan has been unclarified to date. Therefore, we conducted a nationwide study by distributing an

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email-based questionnaire to the hospitals in the HIV/AIDS Network of Japan.

METHODS

IN THE QUESTIONNAIRE, the following information was obtained from hospitals regarding the number of patients who visited the hospitals at least once between January and December 2003: (1) the number of HIV-positive patients; (2) the number of anti-HCV-positive patients among (1); (3) the number of HCV-RNA-positive patients among (2); (4) the number of HIV-positive patients who contracted HIV from blood products; (5) the number of anti-HCV-positive patients among (4); (6) the number of HCV-RNA-positive patients among (5); (7) the number of HIV-positive patients among men who have sex with men (MSM); (8) the number of anti-HCV-positive patients among (7); (9) the number of HCV-RNA-positive patients among (8); (10) the number of HIV-positive patients who contracted HIV through intravenous drug use; (11) the number of anti-HCV-positive patients among (10); (12) the number of HCV-RNA-positive patients among (11); (13) the number of HIV-positive patients whose transmission routes were classified as 'others'; (14) the number of anti-HCV-positive patients among (13); and (15) the number of HCV-RNA-positive patients among (14).

The questionnaire was sent to the 366 hospitals in the HIV/AIDS Network of Japan by email. When emails were returned with a failure of delivery, the questionnaire was forwarded by post. Answers were mostly returned by email, and in some cases by fax. The list of the hospitals in the HIV/AIDS Network of Japan can be browsed at: http://www.acc.go.jp/mLhw/mLhw_frame.htm.

RESULTS

THE QUESTIONNAIRE WAS sent to all 366 hospitals that were on the list of hospitals in the HIV/AIDS Network of Japan in January 2004. One hundred and seventy-six hospitals (48.1%) responded within the indicated period. A collection rate of 47.8% may appear rather low, particularly considering the number of reported HIV-positive people, 10 000, in 2004 according to the statistics of the MHLW of Japan.[12] However, not all the HIV-positive cases are visiting hospitals, and answers to the questionnaire were obtained from most of the major hospitals in the HIV/AIDS Network in big cities around Japan. These factors suggest that not all but a majority of HIV-positive patients in Japan were enrolled in the study.

Table 1 Number of hospitals categorized by the number of patients infected with HIV and those coinfected with HIV and HCV

No. of HIV(+)/HCV(+)	No. of HIV(+)				
	0	1–19	20–49	50+	Total
0	43	52	5	1	101
1–9	0	45	9	3	57
10+	0	2	4	12	18
Total	43	99	18	16	176

There were one or more HIV-positive patients in 133 of 176 (75.6%) hospitals; there were no HIV-positive patients in the remaining 43 hospitals (Table 1). Eighteen of 176 (10.2%) hospitals had 20–49 HIV-positive patients, and 16 (9.1%) hospitals had 50 or more HIV-positive patients. On the other hand, there were one or more patients who were coinfected with HIV and HCV in 75 (42.6%) of 176 hospitals, and there were 10 or more HIV/HCV coinfected patients in 18 (10.2%) hospitals. HIV/HCV coinfected patients were concentrated in specific hospitals in big cities around Japan. In particular, in the Kanto area, HIV/HCV coinfected patients were concentrated in the HIV/AIDS Network hospitals in the Tokyo city area (Fig. 1). Of the 16 hospitals with 50 or more HIV-positive patients and of the 18 hospitals with 10 or more HIV/HCV coinfected patients, 12 were the same hospitals (Table 1). Hospitals with 10 or more HIV/HCV coinfected patients, but with less than 50 HIV-positive patients had the characteristic that most HIV-positive patients contracted HIV from blood products.

In total, 4877 patients were reported to be HIV-positive. Among these, 935 (19.2%) were positive for anti-HCV (Table 2). Of these 935 patients, 780 were HCV-RNA-positive, although it should be noted that not all the patients underwent HCV-RNA testing.

HCV prevalence when fractionated by routes of transmission was as follows. Among 811 HIV-positive patients who contracted HIV from blood products such as unheated concentrated coagulation factors, 786 (96.9%) were anti-HCV-antibody-positive. Of 20 intravenous drug users, nine (45.0%) were anti-HCV-antibody-positive. Among 2730 HIV-positive patients who were MSM (men who have sex with men), 114 (4.2%) were anti-HCV positive. In the remaining 1316 HIV-positive patients whose routes of HIV transmission

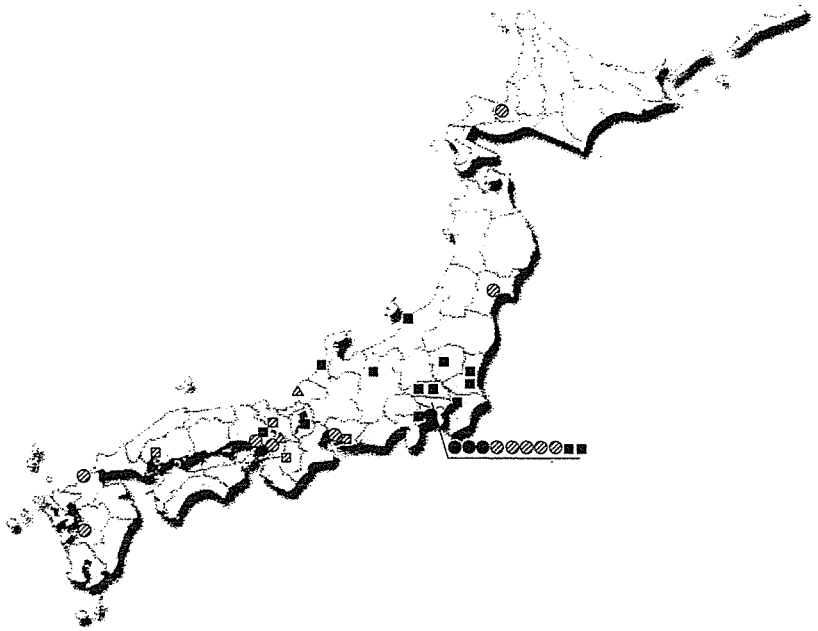

Figure 1 Nationwide distribution of hospitals in the HIV/AIDS Network of Japan that a number of HIV-positive or HIV/HCV coinfected patients are visiting regularly. Note that in the Kanto area, HIV/HCV coinfected patients were concentrated in the HIV/AIDS Network hospitals in the Tokyo city area. (△) hospitals with 1–19 HIV-positive patients; (□) hospitals with 20–49 HIV-positive patients; (○) hospitals with 50+ HIV-positive patients. Hatched figures: hospitals with 10 or more HIV/HCV coinfected patients. Closed figures: hospitals with less than 10 HIV/HCV coinfected patients. For easier visual comprehension, hospitals with 19 or less HIV-positive patients and 9 or less HIV/HCV coinfected patients are omitted from the figure.

were classified as "others", most of whom contracted HIV heterosexually, 26 (2.0%) were anti-HCV-antibody-positive. On the other hand, in HIV/HCV coinfected patients, 786 (84.1%) of 935 patients were recipients of blood products. Thus, the majority of HIV/HCV coinfected patients in Japan are those who contracted HIV, and most likely also HCV, from blood products.

DISCUSSION

ACCORDING TO THE statistics of the MHLW of Japan, the number of reported HIV-positive people was just over 10 000 in 2004.[12] The total number of HIV-positive patients in the current study is approximately half of that. By a simple calculation, there would be about 1900 HIV/HCV coinfected patients in Japan. However, because HIV-positive patients who contracted HIV from blood products are almost all registered in Japan and most of them should have been enrolled in this survey, the number of HIV/HCV coinfected patients is likely smaller than 1900. It is regrettable that not all the patients underwent HCV-RNA testing, but it is unavoidable in this type of questionnaire-based study. In some cases, the existence of a positive anti-HCV antibody indicates a memory of a remote HCV infection.

Almost all of the patients who contracted HIV through blood products were also anti-HCV-antibody-positive, suggesting that both viruses were transmitted through the same route. In MSM patients who were HIV-positive, approximately 4% were anti-HCV-antibody-positive, which is about threefold higher than the prevalence of HCV in Japan.[13] In people aging from 40 to 50 years old in the general Japanese population, whose ages are similar to those of the MSM patients in the current study, the prevalence of HCV is less than 0.5%.[13] Therefore, an HCV prevalence of 4% in MSM

Table 2 Prevalence of HCV infection in HIV-positive patients

Routes of transmission	No. of patients	Anti-HCV-positive	HCV-RNA-positive†
Blood products	811	786 (96.9%)	667
MSM‡	2730	114 (4.2%)	98
Drug addicts	20	9 (45.0%)	8
Others (heterosexual etc.)	1316	26 (2.0%)	7
Total	4877	935 (19.2%)	780

†Not all patients were subjected to HCV-RNA test. ‡MSM, men who have sex with men.

HIV-positive patients is quite high, suggesting the same route of the transmission of HIV and HCV, and a more intensive exposure to HCV or more susceptibility to HCV in these HIV-positive patients. Similarly, an HCV prevalence of 1.4% in heterosexually transmitted HIV-positive patients is higher than that of the general Japanese population of the same age.

To establish measures that decrease the morbidity and mortality of HIV/HCV coinfected patients, it is essential to recognize the current status of the coinfection. In the present study, the number and transmission routes of HIV/HCV coinfected patients in Japan were first described, although detailed information on the progression of HCV-associated liver diseases in HIV/HCV coinfected patients has not yet been obtained. Undoubtedly, this will be the first step for improving the prognosis and quality of life of patients coinfected with HIV and HCV in Japan. A detailed analysis of the progression and severity of HCV-associated liver diseases is expected.

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REFERENCES

1 Alter MJ. Hepatitis C virus infection in the United States. *J Hepatol* 1999; 31: 88–91.
2 Weinbaum CM, Sabin KM, Santibanez SS. Hepatitis B, hepatitis C, and HIV in correctional populations: a review of epidemiology and prevention. *AIDS* 2005; 19: S41–6.
3 Sulkowski MS. Hepatitis C virus infection in HIV-infected patients. *Curr HIV/AIDS Rep* 2004; 1: 128–35.
4 Bica I, McGovern B, Dhar R *et al.* Increasing mortality due to end-stage liver disease in patients with human immunodeficiency virus infection. *Clin Infect Dis* 2001; 32: 492–7.
5 HCV in HIV. Challenges and opportunities. *The PRN NOTEBOOK* 2001; 6: 14–18. [Cited 15 July 2004.] Available from URL: http//: www.prn.org.
6 Picchio GR, Nakatsuno M, Boggiano C *et al.* Hepatitis C (HCV) genotype and viral titer distribution among Argentinean hemophilic patients in the presence or absence of human immunodeficiency virus (HIV) co-infection. *J Med Virol* 1997; 52: 219–25.
7 Benhamou Y, Bochet M, Di Martino V *et al.* Liver fibrosis progression in human immunodeficiency virus and hepatitis C virus coinfected patients. *The Multivirc Group Hepatology* 1999; 30: 1054–8.
8 Marsen AH, Easterbrook PJ, Taylor C *et al.* Impact of human immunodeficiency virus (HIV) infection on the progression of liver fibrosis in hepatitis C virus infected patients. *Gut* 2003; 52: 1035–40.
9 Staples CT Jr, Rimland D, Dudas D. Hepatitis C in the HIV (human immunodeficiency virus) Atlanta V.A. (Veterans Affairs Medical Center) Cohort Study (HAVACS): the effect of coinfection on survival. *Clin Infect Dis* 1999; 29: 150–4.
10 Monga HK, Rodriguez-Barradas MC, Breaux K *et al.* Hepatitis C virus infection-related morbidity and mortality among patients with human immunodeficiency virus infection. *Clin Infect Dis* 2001; 33: 240–7.
11 Tatsunami S, Taki M, Shirahata A, Mimaya J, Yamada K. Increasing incidence of critical liver disease among causes of death in Japanese hemophiliacs with HIV-1. *Acta Haematol* 2004; 111: 181–4.
12 National AIDS Survey Report. *The Ministry of Health, Welfare and Labor of Japan.* [Cited 10 July 2006.] Available from URL: http://www.wam.go.jp/wamappl/bb14GS50.nsf/vAdmPBigcategory40/73F664B9EA8D63D4492570290023D454?OpenDocument.
13 Tanaka J, Kumagai J, Katayama K *et al.* Sex- and age-specific carriers of hepatitis B and C viruses in Japan estimated by the prevalence in the 3 485 648 first-time blood donors during 1995–2000. *Intervirology* 2004; 47: 32–40.

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Involvement of the PA28γ-Dependent Pathway in Insulin Resistance Induced by Hepatitis C Virus Core Protein▽

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The hepatitis C virus (HCV) core protein is a component of nucleocapsids and a pathogenic factor for hepatitis C. Several epidemiological and experimental studies have suggested that HCV infection is associated with insulin resistance, leading to type 2 diabetes. We have previously reported that HCV core gene-transgenic (PA28γ$^{+/+}$CoreTg) mice develop marked insulin resistance and that the HCV core protein is degraded in the nucleus through a PA28γ-dependent pathway. In this study, we examined whether PA28γ is required for HCV core-induced insulin resistance in vivo. HCV core gene-transgenic mice lacking the PA28γ gene (PA28γ$^{-/-}$CoreTg) were prepared by mating of PA28γ$^{+/+}$CoreTg with PA28γ-knockout mice. Although there was no significant difference in the glucose tolerance test results among the mice, the insulin sensitivity in PA28γ$^{-/-}$CoreTg mice was recovered to a normal level in the insulin tolerance test. Tyrosine phosphorylation of insulin receptor substrate 1 (IRS1), production of IRS2, and phosphorylation of Akt were suppressed in the livers of PA28γ$^{+/+}$CoreTg mice in response to insulin stimulation, whereas they were restored in the livers of PA28γ$^{-/-}$CoreTg mice. Furthermore, activation of the tumor necrosis factor alpha promoter in human liver cell lines or mice by the HCV core protein was suppressed by the knockdown or knockout of the PA28γ gene. These results suggest that the HCV core protein suppresses insulin signaling through a PA28γ-dependent pathway.

Hepatitis C virus (HCV) is the causative agent in most cases of acute and chronic non-A, non-B hepatitis (15). Over one-half of patients with the acute infection evolve into a persistent carrier state (24). Chronic infection with HCV frequently induces hepatic steatosis, cirrhosis, and eventually hepatocellular carcinoma (22) and is known to be associated with diseases of extrahepatic organs, including an essential mixed cryoglobulinemia, porphyria cutanea tarda, membranoproliferative glomerulonephritis, and type 2 diabetes (13).

HCV is classified into the genus *Hepacivirus* of the family *Flaviviridae* and possesses a viral genome consisting of a single positive-strand RNA with a nucleotide length of about 9.5 kb. This viral genome encodes a single polyprotein composed of approximately 3,000 amino acids (9). The polyprotein is post-translationally cleaved by host cellular peptidases and viral proteases, resulting in 10 viral proteins (6, 10, 12). The HCV core protein is known to interact with viral-sense RNA of HCV to form the viral nucleocapsid (44). The HCV core protein is cleaved off at residue 191 by the host signal peptidase to release it from the E1 envelope protein and then by the host signal peptide peptidase at around amino acid residues 177 to 179 within the C-terminal transmembrane region (30, 39, 40). The mature core protein is retained mainly on the endoplasmic reticulum, although a portion moves to the nucleus and mitochondria (11, 51).

Recent epidemiological studies have indicated that type 2 diabetes is an HCV-associated disease (7, 29). However, it remains unclear how insulin resistance is induced in patients chronically infected with HCV, since there is no suitable model for investigating HCV pathogenesis. Type 2 diabetes is a complex, multisystemic disease with pathophysiology that includes a high level of hepatic glucose production and insulin resistance, which contribute to the development of hyperglycemia (8, 18). Although the precise mechanism by which these factors contribute to the induction of insulin resistance is difficult to understand, a high level of insulin production by pancreatic β cells under a state of insulin resistance is common in the development of type 2 diabetes. The hyperinsulinemia in the fasting state that is observed relatively early in type 2 diabetes is considered to be a secondary response that compensates for the insulin resistance (8, 18).

The HCV core protein is also known as a pathogenic factor that induces steatosis and hepatocellular carcinoma in mice (33, 35). Previously, we reported that insulin resistance occurs in HCV core gene-transgenic mice due at least partly to an increase in tumor necrosis factor alpha (TNF-α) secretion (47) and that the HCV core protein is degraded through a PA28γ/REGγ (11S regulator)-dependent pathway in the nucleus (32). It is well known that PA28γ enhances latent proteasome activity, although the biological significance of PA28γ is largely unknown, with the exception that PA28γ is known to regulate steroid receptor coactivator 3 (28). Although several reports suggested that the degradation of insulin receptor substrate (IRS) proteins by a ubiquitin-dependent proteasome activity contributes to insulin resistance (43, 50), the involvement of the HCV core protein in cooperation with PA28γ in the stability of IRS proteins and in the development of insulin resis-

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tance is not known. In this study, we examined the involvement of PA28γ in the induction of insulin resistance by the HCV core protein in vivo.

MATERIALS AND METHODS

Preparation of PA28γ-knockout HCV core gene-transgenic mice. C57BL/6 mice carrying the gene encoding HCV core protein genotype 1b ($PA28\gamma^{+/-}$CoreTg) line C49 and $PA28\gamma^{-/-}$ mice have been described previously (35, 36). These two genotypes were crossbred to create $PA28\gamma^{+/-}$CoreTg mice. $PA28\gamma^{+/-}$CoreTg mice were bred to generate $PA28\gamma^{-/-}$CoreTg mice (35, 36). The HCV core gene and the target sequence to knock out the PA28γ gene were identified by PCR. The mice were given ordinary feed (CRF-1; Charles River Laboratories, Yokohama, Japan) and were maintained under specific-pathogen-free conditions.

Glucose tolerance test. The mice were fasted for more than 16 h before glucose administration. D-Glucose (1 g/kg body weight) was intraperitoneally administered to the mice. Blood samples were taken from the orbital sinus at the indicated time points. The plasma glucose concentration was measured by means of a MEDI-SAFE Mini blood glucose monitor (TERUMO, Tokyo, Japan). The serum insulin level was determined by a Mercodia (Uppsala, Sweden) ultrasensitive mouse insulin enzyme-linked immunosorbent assay (ELISA).

Insulin tolerance test. The mice were fed freely and then fasted during the study period. Human insulin (2 U/kg body weight) (Humulin; Eli Lilly, Indianapolis, IN) was intraperitoneally administered to the mice. The plasma glucose concentration was measured at the indicated time and was normalized based on the glucose concentration at the time just before insulin administration.

Histological analysis of pancreatic islets. Pancreas tissues were fixed with paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The relative islet area and islet number were determined with Image-Pro PLUS image analyzing software (NIPPON ROPER, Tokyo, Japan).

Estimation of tumor necrosis factor alpha and HCV core protein. Mouse TNF-α was measured by using a mouse TNF-α ELISA kit (Pierce, Rockford, IL) and normalized based on the amount of total protein in each sample. The protein concentration was estimated by using a BCA protein assay kit (Pierce). The amount of HCV core protein in the liver tissues was determined by using an ELISA system as described previously (4).

In vivo insulin stimulation and immunoblot analysis. Mice were fasted for more than 16 h before insulin stimulation and then anesthetized with ketamine and xylazine. Five units of insulin were injected into the mice via the interior vena cava. Livers of the mice were collected 5 min after the insulin injection and frozen in liquid nitrogen. Immunoblot analyses of the HCV core protein, PA28γ, and each of the insulin-signaling molecules were carried out with the liver tissue homogenates prepared in the homogenizing buffer containing 25 mM Tris-HCl (pH 7.4), 10 mM Na_3VO_4, 100 mM NaF, 50 mM $Na_4P_2O_7$, 10 mM EGTA, 10 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P40 supplemented with Complete Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany) (53). Tissue lysates were subjected to sodium dodecyl sulfate–2% to 15% gradient polyacrylamide gel electrophoresis (PAG Mini DAIICHI 2/15 13W; Daiichi Diagnostics, Tokyo, Japan) and electrotransferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA). The protein transferred onto the membrane was reacted with rabbit anti-HCV core (32), rabbit anti-Akt (Cell Signaling, Danvers, MA), rabbit anti-phospho-Ser473-Akt (Cell Signaling), rabbit anti-IRS1 (Upstate, Lake Placid, NY), rabbit anti-phospho-Tyr608 mouse insulin receptor substrate 1 (Sigma, St. Louis, MO), or rabbit anti-IRS2 (Upstate) polyclonal antibody and then incubated with horseradish peroxidase-conjugated anti-rabbit antibody. Blotted protein was visualized using Super Signal Femto (Pierce) and an LAS3000 imaging system (Fuji Photo Film, Tokyo, Japan).

Quantitative reverse transcription-PCR (RT-PCR). Total RNA was isolated from mouse liver using an RNeasy kit (QIAGEN, Valencia, CA). The RNA preparation was treated with a TURBO DNA-free kit (Ambion, Austin, TX) to remove DNA contamination in the samples. The first-strand cDNAs were synthesized by a first-strand cDNA synthesis kit (Amersham Biosciences, Franklin Lakes, NJ). The targeted cDNA was estimated by using Platinum SYBR Green qPCR Super Mix UDG (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The fluorescent signal was measured by using an ABI Prism 7000 (Applied Biosystems, Foster City, CA). The genes encoding mouse TNF-α, IRS1, IRS2, and hypoxanthine phosphoribosyl transferase were amplified with the following primer pairs: 5′-GGTACAACCCATCGGCTGGCA-3′ (forward) and 5′-GCGACGTGGAACTGGCAGAAG-3′ (reverse) for TNF-α, 5′-ATAG CTCTGAGACCTTCTCAGCACCTAC-3′ (forward) and 5′-GGAGTTGCCCT CATTGCTGCCTAA-3′ (reverse) for IRS1, 5′-AGCCTGGGGATAATGGTG ACTATACCGA-3′ (forward) and 5′-TTGTGGGCAAAGGATGGGGACAC T-3′ (reverse) for IRS2, and 5′-CCAGCAAGCTTGCAACCTTAACCA-3′ (forward) and 5′-GTAATGATCAGTCAACGGGGGAC-3′ (reverse) for hypoxanthine phosphoribosyl transferase. Each PCR product was found as a single band with the correct size by agarose gel electrophoresis (data not shown).

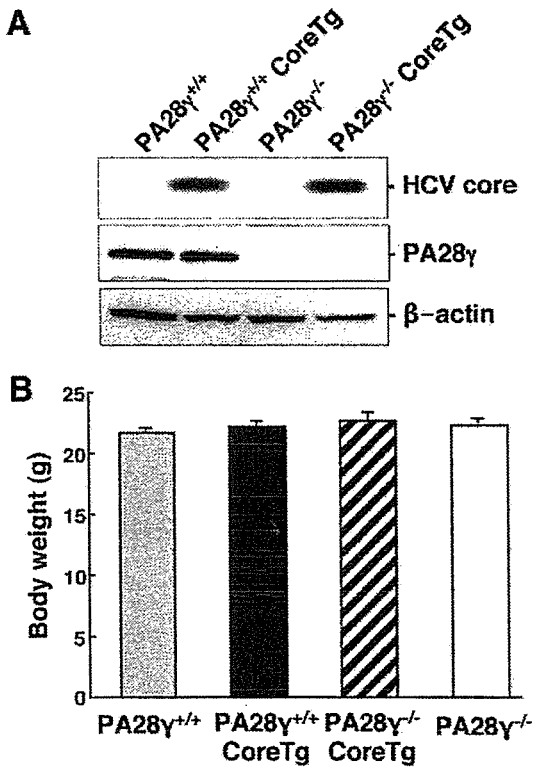

FIG. 1. Characterization of HCV core gene-transgenic mice deficient in the PA28γ gene. (A) Expression of the HCV core protein and PA28γ in the livers of $PA28\gamma^{+/+}$, $PA28\gamma^{+/+}$CoreTg, $PA28\gamma^{-/-}$, and $PA28\gamma^{-/-}$CoreTg mice. Lysates obtained from liver tissues of the mice (100 μg protein/lane) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting using antibodies to the HCV core protein, PA28γ, and β-actin. (B) Body weights of the mice. Body weights of 2-month-old mice were measured ($n = 7$ in each group). There were no statistically significant differences in body weights among the mice ($P > 0.05$).

Reporter assay for TNF-α promoter activity. The promoter region of the TNF-α gene (located from residues −1260 to +140) was amplified from mouse genomic DNA and was then introduced into the KpnI and BglII sites of pGL3-Basic (Promega, Madison, WI) (25). The resulting plasmid was designated as pGL3-tnf-aPro. The gene encoding the HCV core protein was amplified from HCV strain J1 (genotype 1b) and cloned into pCAG-GS (1, 38). To avoid contamination with endotoxin from *Escherichia coli*, the plasmid DNA was purified by using an EndoFree Plasmid Maxi kit (QIAGEN). The total amount of transfected DNA was normalized by the addition of empty plasmids. Plasmid vector was transfected into hepatoma cell lines by lipofection using Lipofectamine 2000 (Invitrogen). Cells were harvested at 24 h posttransfection. Luciferase activity was determined by using the Dual-Luciferase Reporter Assay system (Promega). Firefly luciferase activity was normalized to coexpressed *Renilla* luciferase activity. The amount of firefly luciferase activity was presented as the increase (*n*-fold) relative to the value for the sample lacking the HCV core protein, which was taken to be 1.0. PA28γ-knockdown cell lines were established by using pSilencer 2.1 U6 Hygro (Ambion) according to the manufacturer's protocol.

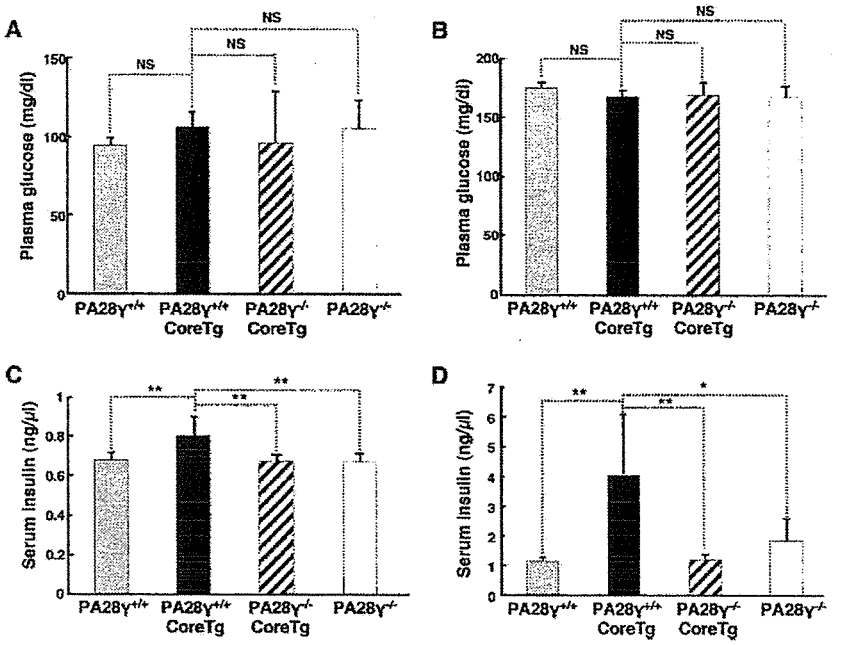

FIG. 2. Knockout of the PA28γ gene inhibited the hyperinsulinemia induced by HCV core protein. Plasma glucose levels of PA28$\gamma^{+/+}$, PA28$\gamma^{-/+}$CoreTg, PA28$\gamma^{-/-}$CoreTg, and PA28$\gamma^{-/-}$ mice under fasting (A) or fed (B) conditions (n = 7 in each group) are shown. Serum insulin levels in fasting (C) or fed (D) mice (n = 7 in each group) are also shown. Values are represented as means ± standard deviations. $*P < 0.05$; $**P < 0.01$. NS, not statistically significant.

Statistical analysis. The results are presented as means ± standard deviations. The significance of the differences was determined by Student's t test. P values of <0.05 were considered statistically significant.

RESULTS

HCV core gene-transgenic mice deficient in the PA28γ gene. To investigate the role of PA28γ in the development of insulin resistance in HCV core gene-transgenic (PA28$\gamma^{-/-}$CoreTg) mice, we generated HCV core gene-transgenic mice deficient in the PA28γ gene (PA28$\gamma^{-/-}$CoreTg). A PA28$\gamma^{+/+}$CoreTg mouse expressing an amount of PA28γ equal to that of its normal littermates (Fig. 1A) was crossbred with a PA28$\gamma^{-/-}$ mouse to generate a PA28$\gamma^{+/-}$ CoreTg mouse. PA28$\gamma^{+/-}$ CoreTg mice were bred with each other, and a PA28$\gamma^{-/-}$ CoreTg mouse was selected by PCR. The HCV core protein was expressed in PA28$\gamma^{+/+}$CoreTg and PA28$\gamma^{-/-}$CoreTg

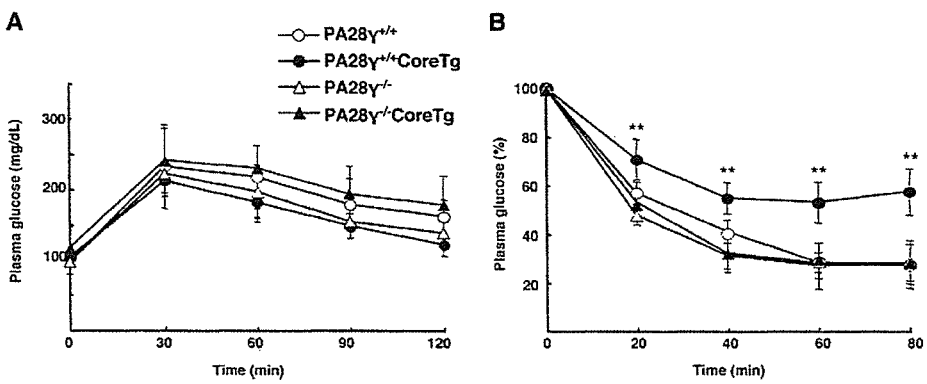

FIG. 3. Knockout of the PA28γ gene inhibits the insulin resistance induced by the HCV core protein. (A) Glucose tolerance test. D-Glucose was intraperitoneally administered to mice fasted for more than 16 h at 1 g/kg of body weight. Plasma glucose levels were estimated at the indicated times (n = 5 in each group). There were no significant differences in glucose levels among the mice ($P > 0.05$). (B) Insulin tolerance test. Human insulin (2 units/kg body weight) was intraperitoneally administered to the mice, and the plasma glucose levels were estimated at the indicated times. Values were normalized to the baseline glucose concentration at the time of insulin administration (n = 5 in each group). The values for the PA28$\gamma^{-/+}$ (open circles), PA28$\gamma^{+/+}$CoreTg (closed circles), PA28$\gamma^{-/-}$ (open triangles), and PA28$\gamma^{-/-}$CoreTg (closed triangles) mice are represented as means and ± standard deviations. Significant differences in insulin sensitivity ($P < 0.01$) in PA28$\gamma^{+/-}$CoreTg mice compared to that in PA28$\gamma^{-/+}$, PA28$\gamma^{-/-}$, or PA28$\gamma^{-/-}$CoreTg mice are indicated by double asterisks (**). There were no significant differences among PA28$\gamma^{-/+}$, PA28$\gamma^{-/-}$, and PA28$\gamma^{-/-}$CoreTg mice ($P > 0.05$).

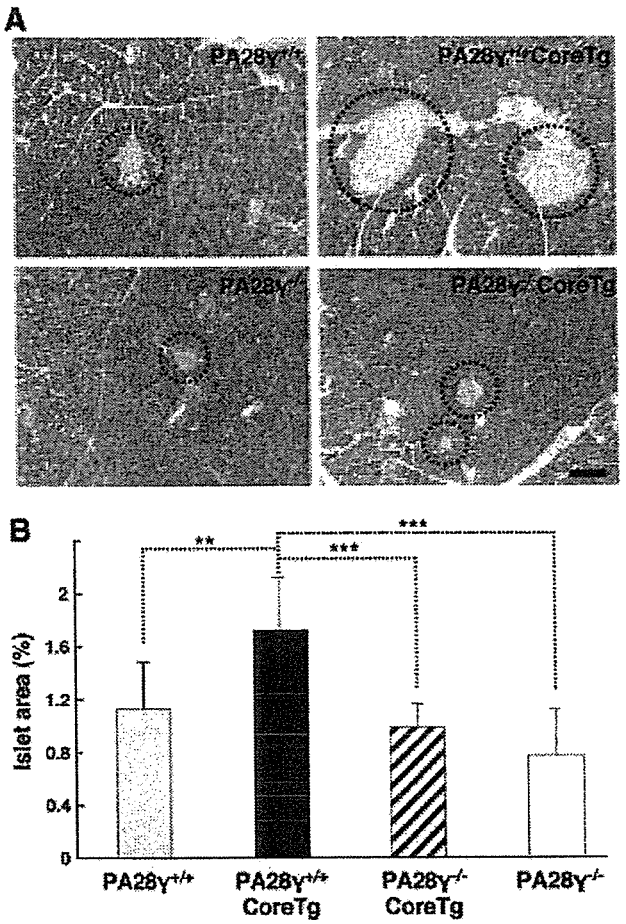

FIG. 4. PA28γ participated in the enlargement of pancreatic islets induced by the HCV core protein. (A) Histological sections prepared from pancreas tissues of PA28$\gamma^{+/+}$, PA28$\gamma^{+/+}$CoreTg, PA28$\gamma^{-/-}$, and PA28$\gamma^{-/-}$CoreTg mice were stained with hematoxylin and eosin. Dotted circles indicate pancreatic islets. (B) The area occupied by pancreatic islets was measured by computer software in three different fields of every six randomly selected sections of 10 mice per genotype and is represented as a percentage of the total pancreatic area. $**P < 0.01$; $***P < 0.001$. The scale bar indicates 100 μm.

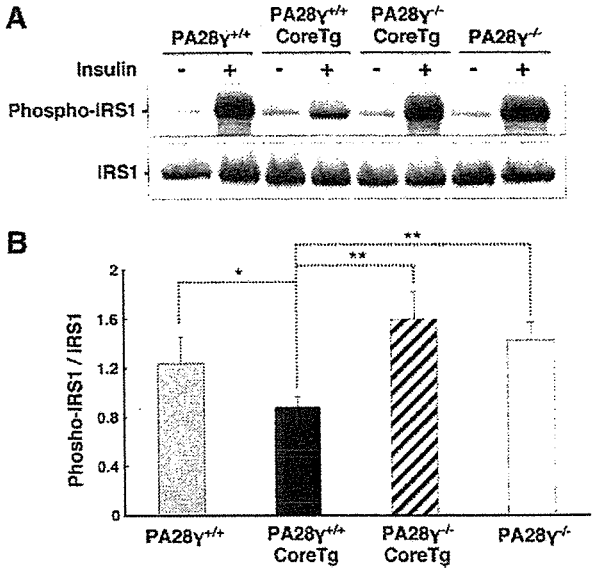

FIG. 5. PA28γ participated in the inhibition of the tyrosine phosphorylation of IRS1 induced by the HCV core protein. Liver tissues from PA28$\gamma^{+/+}$, PA28$\gamma^{+/+}$CoreTg, PA28$\gamma^{-/-}$, and PA28$\gamma^{-/-}$CoreTg mice were prepared after administration of insulin (+) or phosphate-buffered saline (−). The samples (100 μg of total protein) were examined by immunoblotting with antibodies against IRS1 and phospho-Tyr608 of mouse IRS1 (A). Phosphorylated IRS1 was estimated from the density on the immunoblotted membrane by using computer software (B) ($n = 5$ in each group). The data presented are representative of three independent experiments. $*P < 0.05$; $**P < 0.01$.

mice but not in PA28$\gamma^{+/+}$ (normal littermates) or PA28$\gamma^{-/-}$ mice. PA28γ was found at a similar level in PA28$\gamma^{+/+}$CoreTg and PA28$\gamma^{+/+}$ mice but was not present in either PA28$\gamma^{-/-}$ or PA28$\gamma^{-/-}$CoreTg mice (Fig. 1A). The expression of the HCV core protein in the livers of 2-month-old male mice was slightly higher in PA28$\gamma^{-/-}$CoreTg (1.36 ± 0.44 ng/mg of total protein; $n = 7$) than in PA28$\gamma^{+/+}$CoreTg (1.23 ± 0.22 ng/mg of total protein; $n = 7$) mice, but these values were not significantly different ($P > 0.05$). Insulin sensitivity is dependent on several conditions such as body weight, obesity, and liver steatosis (26). PA28$\gamma^{-/-}$ mice were slightly smaller than their normal littermates (PA28$\gamma^{+/+}$) at more than 3 months old, as described previously (36), but this was not significantly different in 2-month-old mice (Fig. 1B). PA28$\gamma^{+/+}$CoreTg mice exhibited severe hepatic steatosis from 4 months of age (35). To avoid the influence of hepatic steatosis and body weight on the examination of insulin resistance, 2-month-old mice were used in this study. Figure 1B shows the body weights of 2-month-old mice. There were no significant differences in body weight among PA28$\gamma^{+/+}$CoreTg, PA28$\gamma^{-/-}$CoreTg, PA28$\gamma^{-/-}$, and PA28$\gamma^{+/+}$ mice. Steatosis was not detected in the livers of the 2-month-old mice (data not shown).

PA28γ is involved in the development of hyperinsulinemia and insulin resistance in PA28$\gamma^{+/+}$CoreTg mice. In our previous study, we found a significant difference in serum insulin levels, but not in plasma glucose levels, between PA28$\gamma^{+/+}$ CoreTg mice and normal littermates (47). To determine the involvement of PA28γ in the development of insulin resistance in PA28$\gamma^{+/+}$CoreTg mice, we examined here the plasma glucose and insulin levels in the mice under fasting and fed conditions. Although no significant difference in plasma glucose levels was observed in the mice under either fasting (Fig. 2A) or fed (Fig. 2B) conditions, serum insulin levels were significantly higher in PA28$\gamma^{+/+}$CoreTg mice than in PA28$\gamma^{+/+}$ mice under both conditions (Fig. 2C and D), as described previously (47). In contrast, the serum insulin concentration in PA28$\gamma^{-/-}$CoreTg mice was recovered to a normal level similar to that of PA28$\gamma^{+/+}$ and PA28$\gamma^{-/-}$ mice under either fasting (Fig. 2C) or fed (Fig. 2D) conditions.

To determine the glucose intolerance among the mice, glucose was administered to the mice after fasting, and the plasma glucose level was then determined. There was no significant difference among the genotypes at any time point in the glucose tolerance test (Fig. 3A), suggesting that the volume of glucose was maintained at a normal level by the higher concentration of insulin in PA28$\gamma^{+/+}$CoreTg mice. In our previ-

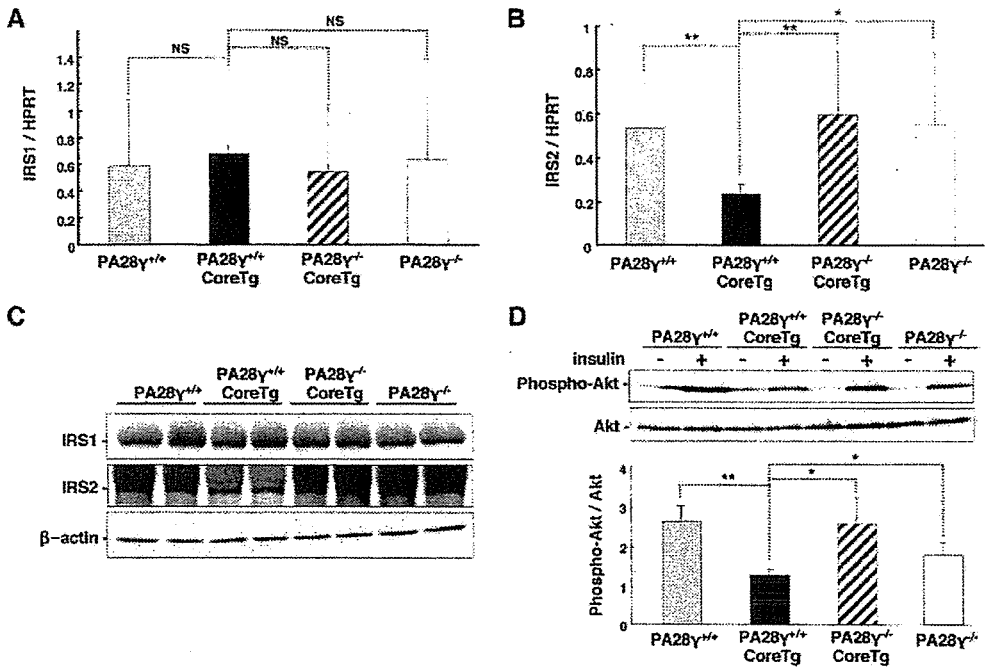

FIG. 6. PA28γ participated in the inhibition of the IRS2 expression and Akt phosphorylation induced by HCV core protein. The transcription of IRS1 (A) and IRS2 (B) was estimated by quantitative RT-PCR ($n = 5$ in each group). (C) The expression levels of IRS1 and IRS2 in the livers of the mice were determined by immunoblotting with specific antibodies. (D) Phosphorylation of Akt in the livers of the mice was examined by immunoblotting with antibodies against Akt and phosphorylated Akt. The ratio of Akt phosphorylation was determined by computer software based on the densities of phosphorylated Akt and a total amount of Akt ($n = 3$ in each group). The data presented are representative of three independent experiments. $*P < 0.05$; $**P < 0.01$. NS, not statistically significant; HPRT, hypoxanthine phosphoribosyl transferase.

ous study, the reduction in the plasma glucose concentration after insulin administration was impaired in PA28γ$^{+/+}$CoreTg mice (47). In this study, PA28γ$^{-/-}$CoreTg mice exhibited a normal insulin level comparable to those of PA28γ$^{+/+}$ and PA28γ$^{-/-}$ mice by an insulin tolerance test, in contrast to PA28γ$^{+/+}$CoreTg mice, in which a high concentration of plasma glucose was detected at all time points, as previously reported (Fig. 3B). These data suggest that hyperinsulinemia was induced in PA28γ$^{+/+}$CoreTg mice to compensate for insulin resistance and retain a physiological level of plasma glucose and that PA28γ participates in the development of hyperinsulinemia and insulin resistance in PA28γ$^{+/+}$CoreTg mice.

Morphology of pancreatic islets. Hyperinsulinemia and insulin resistance are expected to enlarge the pancreatic islet mass due to the overexpression of insulin. Our previous report showed the enlargement of the pancreatic islets in PA28γ$^{+/+}$ CoreTg mice. To clarify whether a knockout of the PA28γ gene restores the enlarged pancreatic islets to their normal size, the morphology of the pancreatic islets of the mice was evaluated by histologic examination (Fig. 4A). The relative islet area in the pancreatic cells of the PA28γ$^{-/-}$CoreTg mice was smaller than that of PA28γ$^{+/+}$CoreTg mice and comparable to that of PA28γ$^{+/+}$ and PA28γ$^{-/-}$ mice (Fig. 4B). Infiltration of inflammatory cells within or surrounding the islets was not found in all genotypes of mice. These results suggest that PA28γ also participates in the enlargement of pancreatic islets induced in PA28γ$^{+/+}$CoreTg mice.

PA28γ impairs the insulin-signaling pathway through the suppression of both tyrosine phosphorylation of IRS1 and expression of IRS2. Insulin binds to insulin receptors, resulting in the activation of downstream signaling (26). The activated insulin receptors phosphorylate themselves, IRS1, and IRS2. Phosphorylated IRS1 and IRS2 can activate phosphatidylinositol 3 (PI3)-kinase signaling, leading to the activation of glucose metabolism and cell growth. Our previous report showed that tyrosine phosphorylation of IRS1 is suppressed in the livers of PA28γ$^{+/+}$CoreTg mice and that the administration of anti-TNF-α antibody restores insulin sensitivity (47). We examined whether a knockout of the PA28γ gene could restore the tyrosine phosphorylation of IRS1. Tyrosine phosphorylation of IRS1 was suppressed in the livers of PA28γ$^{+/+}$ CoreTg mice in response to insulin stimulation, whereas it was recovered in PA28γ$^{-/-}$CoreTg mice to levels comparable to those in PA28γ$^{+/+}$and PA28γ$^{-/-}$ mice (Fig. 5).

Chronic hyperinsulinemia downregulates the expression of IRS2, which is one of the essential components of the insulin-signaling pathway in the liver (46). However, in our previous study, we showed that there was no significant difference in the phosphorylation of IRS2 between PA28γ$^{+/+}$CoreTg mice and their normal littermates (47). To gain more insight into the mechanisms of regulation of IRS expression, we determined the transcription and translation of IRS1 and IRS2 in the livers of the mice by real-time PCR and Western blotting, respectively. Although there was no significant difference in IRS1 expression at either the transcriptional or translational level among the mice

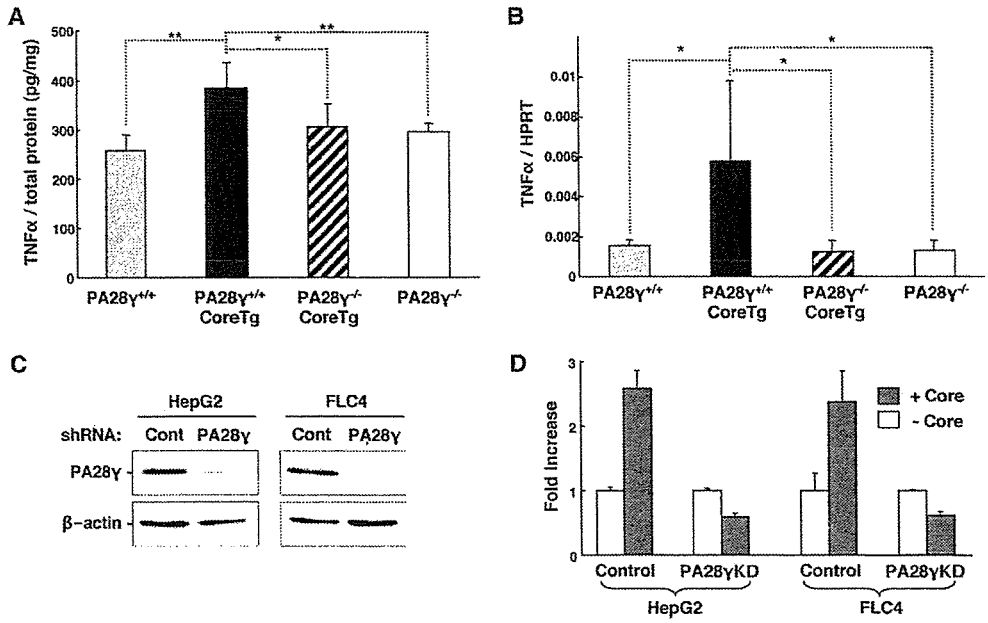

FIG. 7. PA28γ was required for activation of the TNF-α promoter by the HCV core protein. (A) Expression of TNF-α in the livers of mice was determined by ELISA (n = 5 in each group). (B) TNF-α mRNA in the livers of mice was examined by quantitative RT-PCR (n = 5 in each group). (C) Knockdown of the expression of PA28γ in the HepG2 and FLC-4 cell lines by the introduction of a plasmid encoding a short hairpin RNA (shRNA) targeted to the PA28γ gene. The expression levels of PA28γ and β-actin were determined by immunoblotting with specific antibodies. (D) Promoter activity of TNF-α in the presence or absence of the HCV core protein was determined by luciferase assay in the PA28γ-knockdown and control cell lines. The data presented are representative of three independent experiments. HPRT, hypoxanthine phosphoribosyl transferase.

(Fig. 6A and C), the expression of IRS2 was clearly impaired in PA28γ$^{+/+}$CoreTg mice at both the transcriptional and translational levels compared with that in other mice (Fig. 6B and C). The serine/threonine protein kinase Akt is phosphorylated by phosphoinositide-dependent kinase 1 (PDK1) under the activated condition of IRS family proteins (26). The insulin-induced phosphorylation of Akt was suppressed in the livers of PA28γ$^{+/+}$ CoreTg mice but not in those of PA28γ$^{+/+}$, PA28γ$^{-/-}$, or PA28γ$^{-/-}$CoreTg mice (Fig. 6D). These results suggest that the expression of the HCV core protein in the livers of mice in the presence of PA28γ impairs the insulin-signaling pathway through the suppression of both the tyrosine phosphorylation of IRS1 and the expression of IRS2.

PA28γ is required for activation of the TNF-α promoter by HCV core protein. TNF-α is an adipokine (54) and suppresses the signaling pathway of IRS1 and IRS2 (14, 42). Several reports suggested that the serum TNF-α level is higher in HCV patients than in healthy individuals (19, 37). Elevations of TNF-α levels have also been demonstrated in the livers of PA28γ$^{+/+}$CoreTg mice (47). To determine the involvement of PA28γ in the enhancement of TNF-α expression, the expression of TNF-α in the livers of each genotype was determined by ELISA and real-time PCR (Fig. 7A and B). Transcription and translation of TNF-α were increased in the livers of PA28γ$^{+/+}$ CoreTg mice but were restored in the livers of PA28γ$^{-/-}$ CoreTg mice to levels comparable to those of PA28γ$^{+/+}$ and PA28γ$^{-/-}$ mice. To determine the effect of PA28γ expression on the promoter activity of TNF-α in human liver cells, PA28γ-knockdown human hepatoma cell lines HepG2 and FLC4 were established by the introduction of a plasmid encoding a short hairpin RNA targeting the PA28γ gene in the cell lines. The expression of PA28γ was clearly suppressed in the cell lines (Fig. 7C). The expression of HCV core protein in the hepatoma cell lines potentiated TNF-α promoter activity, whereas the promoter activation by the HCV core protein was suppressed in the PA28γ-knockdown cell lines (Fig. 7D). These results suggest that PA28γ is required for the activation of the TNF-α promoter induced by the expression of the HCV core protein in human hepatoma cell lines.

DISCUSSION

HCV infection has a close association with type 2 diabetes, which is a polygenic disease with a pathophysiology that includes a defect in insulin secretion, increased hepatic glucose production, and resistance to the action of insulin (2, 8, 18). Insulin binds to insulin receptors, which exhibit tyrosine kinase activity, leading to the autophosphorylation and phosphorylation of IRS (56). Tyrosine phosphorylation in IRS proteins leads to the interaction between IRS proteins and the regulatory subunit p85 of PI3-kinase, which enhances glucose uptake and inhibits lypolysis (21). Activated PI3-kinase phosphorylates phosphatidylinositol 4,5-biphosphate to produce phosphatidylinositol 3,4,5-triphosphate, which contributes to the activation of PDK1 (55). Activated PDK1 phosphorylates downstream substrates including Akt and other kinases (55). A diabetic phenotype that included insulin resistance was found in IRS2-knockout mice with normal growth (57), although a

knockout of the IRS1 gene has been shown to lead to growth retardation and insulin resistance but not overt diabetes (5, 52). The double knockdown of IRS1 and IRS2 genes in the liver induces hyperinsulinemia and insulin resistance in mice (53). The reduction of both IRS1 and IRS2 under conditions of insulin resistance and hyperinsulinemia (3) and in the livers of *ob/ob* mice, an obese diabetic mouse model (20), has been reported previously. In the present study, the expression of the HCV core protein reduced the phosphorylation of tyrosine on IRS1 and the production of IRS2 in the livers of mice but did not completely abolish the activities of these genes, suggesting that residual activities of IRS transfer a faint signal to the downstream region of IRS. Therefore, PA28γ$^{+/+}$CoreTg mice may exhibit a milder phenotype than IRS1- and/or IRS2-knockout mice. In this study, knockout of the PA28γ gene restored the insulin sensitivity and signaling of IRS1 and IRS2 in PA28γ$^{+/+}$CoreTg mice, suggesting that the expression of the HCV core protein leads to the dysfunction of both IRS1 and IRS2 through a PA28γ-dependent pathway.

Our previous study suggested that the induction of TNF-α by the HCV core protein plays a role in insulin resistance (47). An increase in TNF-α levels has been correlated with obesity and insulin resistance in animal models and humans (14, 42). However, the mechanism by which TNF-α induces insulin resistance is not completely known. The expression of TNF-α has been shown to be increased in PA28γ$^{-/-}$CoreTg mice, resulting in the suppression of phosphorylation of IRS1, and insulin sensitivity in PA28γ$^{+/+}$CoreTg was improved by the administration of an anti-TNF-α antibody (47). In the present study, the expression level of TNF-α in PA28γ$^{-/-}$CoreTg mice was similar to that in PA28γ$^{-/-}$ mice or their normal littermates. The expression of the HCV core protein enhanced the promoter activity of the TNF-α gene in human liver cell lines but not in those with a knockdown of the PA28γ gene by RNA interference (Fig. 7D). These data suggest that PA28γ plays a crucial role in HCV core-induced expression of TNF-α. Sterol regulatory element-binding proteins (SREBPs) were shown to be increased at the stage of viremia in HCV-infected chimpanzees (49). SREBPs are known to regulate not only the biosynthesis of lipid but also the transcription of IRS2 and TNF-α (17, 45). Therefore, it might be feasible to speculate that the HCV core protein may cooperate with PA28γ to regulate the expression of SREBPs.

Houstis et al. previously reported that reactive oxygen species (ROS) are increased in both cellular and mouse models of insulin resistance induced by treatment with TNF-α or dexamethasone and that insulin sensitivity was restored by treatment with small antioxidant molecules (16). The HCV core protein potentiates ROS production in hepatoma cells and HCV core gene-transgenic mice (23, 34, 41). Accelerated production of ROS results in mitochondrion dysfunction, which contributes to a decrease in fatty acid oxidation. Defects in mitochondrial fatty acid oxidation enhance the production of intracellular fatty acyl coenzyme A (CoA) and diacylglycerol (48, 58). Mitochondrion dysfunction and accumulation of lipid droplets in mice expressing the HCV core or the full-length HCV polyprotein have been reported (27, 34). An increase in lipid droplets also leads to the accumulation of fatty acid CoA and diacylglycerol (48, 58). Fatty acyl CoA and diacylglycerol nonspecifically activate the Ser/Thr kinase cascade, leading to the enhancement of the serine phosphorylation of IRS1 (26). Serine phosphorylation on IRS1 blocks the tyrosine phosphorylation of IRS1 by insulin receptors (26). In the present study, however, serine phosphorylation of IRS1 in PA28γ$^{+/+}$ CoreTg mice was similar to that in PA28γ$^{-/-}$CoreTg mice (data not shown). TNF-α signaling pathways other than the accumulation of ROS and fatty acid intermediates may also participate in the inhibition of tyrosine phosphorylation on IRS1 in PA28γ$^{+/+}$ CoreTg mice.

How does the HCV core protein induce TNF-α production? Our previous report suggests that the HCV core protein is degraded through a PA28γ-dependent pathway (32). Recently, PA28γ has been shown to participate in the proteasome-dependent degradation of steroid receptor coactivator 3 (28). Degradation products of the HCV core protein via the PA28γ-dependent pathway may regulate the promoter activity of the TNF-α gene. PA28 proteins are necessary and sufficient to fully reconstitute Hsp90-initiated refolding together with Hsc70 and Hsp40 (31). Therefore, it might also be feasible to speculate that the HCV core protein refolded by an Hsp90/PA28γ-dependent pathway activates the promoter of the TNF-α gene together with an unknown transcription factor(s) or regulator(s).

In conclusion, the data obtained in this study suggest that the expression of the HCV core protein enhances the production of TNF-α and suppresses the phosphorylation of tyrosine on IRS1 and the production of IRS2 through a PA28γ-dependent pathway, thereby leading to insulin resistance. PA28γ may be a novel target for the treatment of HCV-induced diabetes.

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REFERENCES

1. **Aizaki, H., Y. Aoki, T. Harada, K. Ishii, T. Suzuki, S. Nagamori, G. Toda, Y. Matsuura, and T. Miyamura.** 1998. Full-length complementary DNA of hepatitis C virus genome from an infectious blood sample. Hepatology **27:** 621–627.
2. **Allison, M. E., T. Wreghitt, C. R. Palmer, and G. J. Alexander.** 1994. Evidence for a link between hepatitis C virus infection and diabetes mellitus in a cirrhotic population. J. Hepatol. **21:**1135–1139.
3. **Anai, M., M. Funaki, T. Ogihara, J. Terasaki, K. Inukai, H. Katagiri, Y. Fukushima, Y. Yazaki, M. Kikuchi, Y. Oka, and T. Asano.** 1998. Altered expression levels and impaired steps in the pathway to phosphatidylinositol 3-kinase activation via insulin receptor substrates 1 and 2 in Zucker fatty rats. Diabetes **47:**13–23.
4. **Aoyagi, K., C. Ohue, K. Iida, T. Kimura, E. Tanaka, K. Kiyosawa, and S. Yagi.** 1999. Development of a simple and highly sensitive enzyme immunoassay for hepatitis C virus core antigen. J. Clin. Microbiol. **37:**1802–1808.
5. **Araki, E., M. A. Lipes, M. E. Patti, J. C. Bruning, B. Haag III, R. S. Johnson, and C. R. Kahn.** 1994. Alternative pathway of insulin signalling in mice with targeted disruption of the IRS-1 gene. Nature **372:**186–190.
6. **Bukh, J., R. H. Purcell, and R. H. Miller.** 1994. Sequence analysis of the core gene of 14 hepatitis C virus genotypes. Proc. Natl. Acad. Sci. USA **91:**8239–8243.
7. **Caronia, S., K. Taylor, L. Pagliaro, C. Carr, U. Palazzo, J. Petrik, S. O'Rahilly, S. Shore, B. D. Tom, and G. J. Alexander.** 1999. Further evidence for an association between non-insulin-dependent diabetes mellitus and chronic hepatitis C virus infection. Hepatology **30:**1059–1063.
8. **Cavaghan, M. K., D. A. Ehrmann, and K. S. Polonsky.** 2000. Interactions between insulin resistance and insulin secretion in the development of glucose intolerance. J. Clin. Investig. **106:**329–333.
9. **Choo, Q. L., G. Kuo, A. J. Weiner, L. R. Overby, D. W. Bradley, and M. Houghton.** 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science **244:**359–362.

10. **Choo, Q. L., K. H. Richman, J. H. Han, K. Berger, C. Lee, C. Dong, C. Gallegos, D. Coit, R. Medina-Selby, P. J. Barr, et al.** 1991. Genetic organization and diversity of the hepatitis C virus. Proc. Natl. Acad. Sci. USA **88**:2451–2455.
11. **Falcon, V., N. Acosta-Rivero, G. Chinea, J. Gavilondo, M. C. de la Rosa, I. Menendez, S. Duenas-Carrera, A. Vina, W. Garcia, B. Gra, M. Noa, E. Reytor, M. T. Barcelo, F. Alvarez, and J. Morales-Grillo.** 2003. Ultrastructural evidences of HCV infection in hepatocytes of chronically HCV-infected patients. Biochem. Biophys. Res. Commun. **305**:1085–1090.
12. **Grakoui, A., D. W. McCourt, C. Wychowski, S. M. Feinstone, and C. M. Rice.** 1993. Characterization of the hepatitis C virus-encoded serine proteinase: determination of proteinase-dependent polyprotein cleavage sites. J. Virol. **67**:2832–2843.
13. **Gumber, S. C., and S. Chopra.** 1995. Hepatitis C: a multifaceted disease. Review of extrahepatic manifestations. Ann. Intern. Med. **123**:615–620.
14. **Hotamisligil, G. S.** 1999. The role of TNFalpha and TNF receptors in obesity and insulin resistance. J. Intern. Med. **245**:621–625.
15. **Houghton, M., A. Weiner, J. Han, G. Kuo, and Q. L. Choo.** 1991. Molecular biology of the hepatitis C viruses: implications for diagnosis, development and control of viral disease. Hepatology **14**:381–388.
16. **Houstis, N., E. D. Rosen, and E. S. Lander.** 2006. Reactive oxygen species have a causal role in multiple forms of insulin resistance. Nature **440**:944–948.
17. **Ide, T., H. Shimano, N. Yahagi, T. Matsuzaka, M. Nakakuki, T. Yamamoto, Y. Nakagawa, A. Takahashi, H. Suzuki, H. Sone, H. Toyoshima, A. Fukamizu, and N. Yamada.** 2004. SREBPs suppress IRS-2-mediated insulin signalling in the liver. Nat. Cell Biol. **6**:351–357.
18. **Kahn, B. B.** 1998. Type 2 diabetes: when insulin secretion fails to compensate for insulin resistance. Cell **92**:593–596.
19. **Kallinowski, B., K. Haseroth, G. Marinos, C. Hanck, W. Stremmel, L. Theilmann, M. V. Singer, and S. Rossol.** 1998. Induction of tumour necrosis factor (TNF) receptor type p55 and p75 in patients with chronic hepatitis C virus (HCV) infection. Clin. Exp. Immunol. **111**:269–277.
20. **Kerouz, N. J., D. Horsch, S. Pons, and C. R. Kahn.** 1997. Differential regulation of insulin receptor substrates-1 and -2 (IRS-1 and IRS-2) and phosphatidylinositol 3-kinase isoforms in liver and muscle of the obese diabetic (ob/ob) mouse. J. Clin. Investig. **100**:3164–3172.
21. **Kido, Y., J. Nakae, and D. Accili.** 2001. Clinical review 125: the insulin receptor and its cellular targets. J. Clin. Endocrinol. Metab. **86**:972–979.
22. **Kiyosawa, K., T. Sodeyama, E. Tanaka, Y. Gibo, K. Yoshizawa, Y. Nakano, S. Furuta, Y. Akahane, K. Nishioka, R. H. Purcell, et al.** 1990. Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. Hepatology **12**:671–675.
23. **Korenaga, M., T. Wang, Y. Li, L. A. Showalter, T. Chan, J. Sun, and S. A. Weinman.** 2005. Hepatitis C virus core protein inhibits mitochondrial electron transport and increases reactive oxygen species (ROS) production. J. Biol. Chem. **280**:37481–37488.
24. **Kuo, G., Q. L. Choo, H. J. Alter, G. L. Gitnick, A. G. Redeker, R. H. Purcell, T. Miyamura, J. L. Dienstag, M. J. Alter, C. E. Stevens, et al.** 1989. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. Science **244**:362–364.
25. **Kuprash, D. V., I. A. Udalova, R. L. Turetskaya, D. Kwiatkowski, N. R. Rice, and S. A. Nedospasov.** 1999. Similarities and differences between human and murine TNF promoters in their response to lipopolysaccharide. J. Immunol. **162**:4045–4052.
26. **Lazar, D. F., and A. R. Saltiel.** 2006. Lipid phosphatases as drug discovery targets for type 2 diabetes. Nat. Rev. Drug Discov. **5**:333–342.
27. **Lerat, H., M. Honda, M. R. Beard, K. Loesch, J. Sun, Y. Yang, M. Okuda, R. Gosert, S. Y. Xiao, S. A. Weinman, and S. M. Lemon.** 2002. Steatosis and liver cancer in transgenic mice expressing the structural and nonstructural proteins of hepatitis C virus. Gastroenterology **122**:352–365.
28. **Li, X., D. M. Lonard, S. Y. Jung, A. Malovannaya, Q. Feng, J. Qin, S. Y. Tsai, M. J. Tsai, and B. W. O'Malley.** 2006. The SRC-3/AIB1 coactivator is degraded in a ubiquitin- and ATP-independent manner by the REGgamma proteasome. Cell **124**:381–392.
29. **Mason, A. L., J. Y. Lau, N. Hoang, K. Qian, G. J. Alexander, L. Xu, L. Guo, S. Jacob, F. G. Regenstein, R. Zimmerman, J. E. Everhart, C. Wasserfall, N. K. Maclaren, and R. P. Perrillo.** 1999. Association of diabetes mellitus and chronic hepatitis C virus infection. Hepatology **29**:328–333.
30. **McLauchlan, J., M. K. Lemberg, G. Hope, and B. Martoglio.** 2002. Intramembrane proteolysis promotes trafficking of hepatitis C virus core protein to lipid droplets. EMBO J. **21**:3980–3988.
31. **Minami, Y., H. Kawasaki, M. Minami, N. Tanahashi, K. Tanaka, and I. Yahara.** 2000. A critical role for the proteasome activator PA28 in the Hsp90-dependent protein refolding. J. Biol. Chem. **275**:9055–9061.
32. **Moriishi, K., T. Okabayashi, K. Nakai, K. Moriya, K. Koike, S. Murata, T. Chiba, K. Tanaka, R. Suzuki, T. Suzuki, T. Miyamura, and Y. Matsuura.** 2003. Proteasome activator PA28γ-dependent nuclear retention and degradation of hepatitis C virus core protein. J. Virol. **77**:10237–10249.
33. **Moriya, K., H. Fujie, Y. Shintani, H. Yotsuyanagi, T. Tsutsumi, K. Ishibashi, Y. Matsuura, S. Kimura, T. Miyamura, and K. Koike.** 1998. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nat. Med. **4**:1065–1067.
34. **Moriya, K., K. Nakagawa, T. Santa, Y. Shintani, H. Fujie, H. Miyoshi, T. Tsutsumi, T. Miyazawa, K. Ishibashi, T. Horie, K. Imai, T. Todoroki, S. Kimura, and K. Koike.** 2001. Oxidative stress in the absence of inflammation in a mouse model for hepatitis C virus-associated hepatocarcinogenesis. Cancer Res. **61**:4365–4370.
35. **Moriya, K., H. Yotsuyanagi, Y. Shintani, H. Fujie, K. Ishibashi, Y. Matsuura, T. Miyamura, and K. Koike.** 1997. Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. J Gen. Virol. **78**:1527–1531.
36. **Murata, S., H. Kawahara, S. Tohma, K. Yamamoto, M. Kasahara, Y. Nabeshima, K. Tanaka, and T. Chiba.** 1999. Growth retardation in mice lacking the proteasome activator PA28gamma. J. Biol. Chem. **274**:38211–38215.
37. **Nelson, D. R., H. L. Lim, C. G. Marousis, J. W. Fang, G. L. Davis, L. Shen, M. S. Urdea, J. A. Kolberg, and J. Y. Lau.** 1997. Activation of tumor necrosis factor-alpha system in chronic hepatitis C virus infection. Dig. Dis. Sci. **42**:2487–2494.
38. **Niwa, H., K. Yamamura, and J. Miyazaki.** 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene **108**:193–199.
39. **Ogino, T., H. Fukuda, S. Imajoh-Ohmi, M. Kohara, and A. Nomoto.** 2004. Membrane binding properties and terminal residues of the mature hepatitis C virus capsid protein in insect cells. J. Virol. **78**:11766–11777.
40. **Okamoto, K., K. Moriishi, T. Miyamura, and Y. Matsuura.** 2004. Intramembrane proteolysis and endoplasmic reticulum retention of hepatitis C virus core protein. J. Virol. **78**:6370–6380.
41. **Okuda, M., K. Li, M. R. Beard, L. A. Showalter, F. Scholle, S. M. Lemon, and S. A. Weinman.** 2002. Mitochondrial injury, oxidative stress, and antioxidant gene expression are induced by hepatitis C virus core protein. Gastroenterology **122**:366–375.
42. **Ozes, O. N., H. Akca, L. D. Mayo, J. A. Gustin, T. Maehama, J. E. Dixon, and D. B. Donner.** 2001. A phosphatidylinositol 3-kinase/Akt/mTOR pathway mediates and PTEN antagonizes tumor necrosis factor inhibition of insulin signaling through insulin receptor substrate-1. Proc. Natl. Acad. Sci. USA **98**:4640–4645.
43. **Rui, L., T. L. Fisher, J. Thomas, and M. F. White.** 2001. Regulation of insulin/insulin-like growth factor-1 signaling by proteasome-mediated degradation of insulin receptor substrate-2. J. Biol. Chem. **276**:40362–40367.
44. **Shimoike, T., S. Mimori, H. Tani, Y. Matsuura, and T. Miyamura.** 1999. Interaction of hepatitis C virus core protein with viral sense RNA and suppression of its translation. J. Virol. **73**:9718–9725.
45. **Shimomura, I., R. E. Hammer, J. A. Richardson, S. Ikemoto, Y. Bashmakov, J. L. Goldstein, and M. S. Brown.** 1998. Insulin resistance and diabetes mellitus in transgenic mice expressing nuclear SREBP-1c in adipose tissue: model for congenital generalized lipodystrophy. Genes Dev. **12**:3182–3194.
46. **Shimomura, I., M. Matsuda, R. E. Hammer, Y. Bashmakov, M. S. Brown, and J. L. Goldstein.** 2000. Decreased IRS-2 and increased SREBP-1c lead to mixed insulin resistance and sensitivity in livers of lipodystrophic and ob/ob mice. Mol. Cell **6**:77–86.
47. **Shintani, Y., H. Fujie, H. Miyoshi, T. Tsutsumi, K. Tsukamoto, S. Kimura, K. Moriya, and K. Koike.** 2004. Hepatitis C virus infection and diabetes: direct involvement of the virus in the development of insulin resistance. Gastroenterology **126**:840–848.
48. **Shulman, G. I.** 2000. Cellular mechanisms of insulin resistance. J. Clin. Investig. **106**:171–176.
49. **Su, A. I., J. P. Pezacki, L. Wodicka, A. D. Brideau, L. Supekova, R. Thimme, S. Wieland, J. Bukh, R. H. Purcell, P. G. Schultz, and F. V. Chisari.** 2002. Genomic analysis of the host response to hepatitis C virus infection. Proc. Natl. Acad. Sci. USA **99**:15669–15674.
50. **Sun, X. J., J. L. Goldberg, L. Y. Qiao, and J. J. Mitchell.** 1999. Insulin-induced insulin receptor substrate-1 degradation is mediated by the proteasome degradation pathway. Diabetes **48**:1359–1364.
51. **Suzuki, R., S. Sakamoto, T. Tsutsumi, A. Rikimaru, K. Tanaka, T. Shimoike, K. Moriishi, T. Iwasaki, K. Mizumoto, Y. Matsuura, T. Miyamura, and T. Suzuki.** 2005. Molecular determinants for subcellular localization of hepatitis C virus core protein. J. Virol. **79**:1271–1281.
52. **Tamemoto, H., T. Kadowaki, K. Tobe, T. Yagi, H. Sakura, T. Hayakawa, Y. Terauchi, K. Ueki, Y. Kaburagi, S. Satoh, et al.** 1994. Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1. Nature **372**:182–186.
53. **Taniguchi, C. M., K. Ueki, and R. Kahn.** 2005. Complementary roles of IRS-1 and IRS-2 in the hepatic regulation of metabolism. J. Clin. Investig. **115**:718–727.
54. **Uysal, K. T., S. M. Wiesbrock, M. W. Marino, and G. S. Hotamisligil.** 1997. Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. Nature **389**:610–614.
55. **Vanhaesebroeck, B., and D. R. Alessi.** 2000. The PI3K-PDK1 connection: more than just a road to PKB. Biochem. J. **346**:561–576.

56. **White, M. F.** 1998. The IRS-signalling system: a network of docking proteins that mediate insulin action. Mol. Cell. Biochem. **182**:3–11.
57. **Withers, D. J., J. S. Gutierrez, H. Towery, D. J. Burks, J. M. Ren, S. Previs, Y. Zhang, D. Bernal, S. Pons, G. I. Shulman, S. Bonner-Weir, and M. F. White.** 1998. Disruption of IRS-2 causes type 2 diabetes in mice. Nature **391**:900–904.
58. **Yu, C., Y. Chen, G. W. Cline, D. Zhang, H. Zong, Y. Wang, R. Bergeron, J. K. Kim, S. W. Cushman, G. J. Cooney, B. Atcheson, M. F. White, E. W. Kraegen, and G. I. Shulman.** 2002. Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. J. Biol. Chem. **277**: 50230–50236.

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Critical role of PA28{gamma} in hepatitis C virus-associated steatogenesis and hepatocarcinogenesis

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