Case report

N. Shin
Y. Sugawara
K. Tsukada
S. Tamura
N. Akamatsu
S. Okugawa
K. Koike
K. Kikuchi
M. Makuuchi

Successful treatment of disseminated *Nocardia farcinica* infection in a living-donor liver transplantation recipient

Abstract: Nocardiosis is a serious infection with high mortality. We report a case of subcutaneous and neural lesions due to *Nocardia farcinica* infection after living-donor liver transplantation. The neural lesion was cured with antibiotics without drainage.

Authors' affiliations:

N. Shin[1],
Y. Sugawara[1],
K. Tsukada[2],
S. Tamura[1],
N. Akamatsu[1],
S. Okugawa[2],
K. Koike[2],
K. Kikuchi[3],
M. Makuuchi[1]

[1]Artificial Organ and Transplantation Division, Department of Surgery.
[2]Department of Infectious Diseases, University of Tokyo, Tokyo, Japan
[3]Department of Infectious Diseases, Tokyo Women's Medical University, Tokyo, Japan

Corespondence to:
Yasuhiko Sugawara, MD
Artificial Organ and
Transplantation Division
Department of Surgery
Graduate School of Medicine
University of Tokyo
7-3-1 Hongo, Bunkyo-ku
Tokyo 113-8655
Japan
Tel: + 81-3-3815-5411
Fax: + 81-3-5684-3989
e-mail:
yasusuga-tky@umin.ac.jp

Nocardia species are gram-positive, variably acid-fast, filamentous aerobic actinomycetes with branching rods (1–3). *Nocardia* is found widely in nature, such as in soil. Common primary sites of infection in man are the respiratory tract and injured skin (1, 4). *Nocardia* disseminates hematogenously, which can lead to brain abscesses. *Nocardia* species are a well-recognized cause of infection in humans and animals (3). T-cell-mediated immune response is important for preventing nocardial infection (5, 6), and those with impaired cell-mediated immunity, such as post-transplant recipients and patients with human immunodeficiency virus, are at risk for nocardiosis (6, 7).

In a recent review of the published literature, 22% of nocardiosis occurred in organ transplant recipients, among which approximately 60% were renal transplant recipients (6). Wilson et al. (8) reviewed 76 renal transplant recipients with nocardiosis. The overall mortality was 25%, and in cases with brain abscesses the rate increased to 42%. More recently, Kontoyiannis et al. (4) examined 36 cases of *Nocardia* infection. Two of the cases had brain lesions and both patients died of infection. Fourteen liver transplant patients (4, 9–11) had complications of nocardial infection: 11 had lesions in the lung and the other 3 (21%) (9) had sub-

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cutaneous lesions. To our knowledge, there are no reports of nocardiosis disseminating to the brain after liver transplantation.

Case report

A 63-year-old male underwent living-donor liver transplantation for hepatitis C-related liver cirrhosis. Immunosuppressive drugs consisted of tacrolimus and prednisolone; detailed protocols were described previously (12). The tacrolimus trough level was monitored twice a day during hospitalization. The dose was adjusted to maintain the target level around 5 ng/mL because he was thought to have active infection (13). The postoperative course was uneventful other than positive cytomegalovirus antigenemia 3 months after the transplantation, which was successfully treated with intravenous ganciclovir.

The patient was admitted to our hospital on postoperative day 120 with fever up to 38°C and pain in the right lower leg. Chest x-ray was normal. An ultrasound test revealed abscess formation in the soleus muscle on the right side, and 20 mL of odor-free pale yellow pus was aspirated. A direct smear of the aspirate revealed gram-positive bacilli. A drainage tube was inserted and imipenem was administered intravenously (500 mg/12 h). The creatinine clearance was 40 mL/min and the body weight was 50 kg. After 3 days of incubation, Nocardia species was cultured from aerobic culture of the aspirate. Administration of imipenem was continued and the clinical manifestations, such as fever and local pain, improved.

Ten days after the episode, the patient developed systemic convulsions. His left upper and lower extremities become paretic, and neurologic examination demonstrated dense left-sided neglect. Phenytoin (200 mg/day) was administered orally to prevent further convulsions. Computed tomography (CT) and magnetic resonance imaging (MRI) (Fig. 1) revealed 3 lesions in the brain. Neurosurgeons advised against biopsy of the brain lesions. Lumbar puncture was also not performed because the patient could not maintain the appropriate position. Imipenem was switched to a combination of ceftriaxone (2 g) and amikacin (200 mg) every 12 h. Gallium scintigraphy performed for screening on the 19th day of hospitalization revealed an additional asymptomatic lesion, approximately 2 cm in diameter, in his left inguinal area, which was also drained percutaneously. Nocardia species were again cultured from the aspirate.

During treatment with ceftriaxone and amikacin, the neurologic symptoms gradually improved, and brain MRI scan revealed a size reduction of the brain abscesses. The causative organism in the percutaneous lesion was identified as Nocardia farcinica using a 16s ribosomal RNA gene sequencing method (14, 15). Susceptibility testing (16) revealed good susceptibility to amikacin and sulfamethoxazole, but moderate resistance to ceftriaxone (Table 1). On the 54th day after admission (postoperative day 174), antibiotics were switched to oral trimethoprim–sulfamethoxazole (single-strength tablet) 2 tablets 3 times a day. The creatinine clearance at that time was 42 mL/min. The patient was discharged from the hospital the next day. There were no signs of relapse, and the brain lesions disappeared on the CT 6 months after onset.
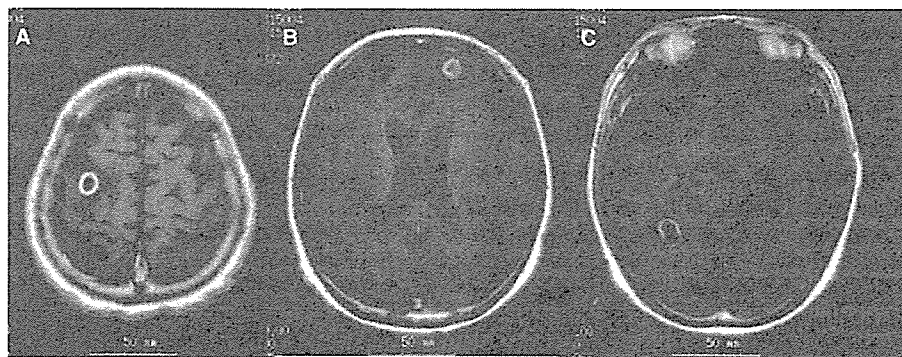

Fig. 1. Gadolinium-enhanced T1-weighted magnetic resonance scanning images demonstrate a lesion in the superior part of right precentral gyrus (A), left frontal pole (B), and medial occipitotemporal gyrus (C). Note the enhanced rings surrounded by the low-signal lesions, suggestive of edematous parenchyma.

Susceptibility of *Nocardia farcinica* to various antibiotics

Antimicrobial agent	MIC (mg/L)	Susceptibility
Amikacin*	1	Susceptible
Amoxicillin/clavulanic acid*	3	Susceptible
Ceftriaxone	32	Intermediate
Cefotaxime	32	Intermediate
Ciprofloxacin*	6	Resistant
Imipenem	4	Susceptible
Linezolid*	4	Susceptible
Minocycline*	3	Intermediate
Trimethoprim–sulfamethoxazole	2	Susceptible

*Susceptibility testing was performed with Etest™ (Med-Ox Diagnostics Inc., Ottawa, ON, Canada) and a broth microdilution method.
MIC, minimum inhibitory concentration.

Table 1

Discussion

The most common CT findings of nocardial brain abscesses are single or multiple ring-enhanced lesions with surrounding edema (17), which were compatible with the findings of the present case. Unfortunately, the findings were not specific for nocardial infection. The abscess capsule is generally smooth and of uniform thickness, distinct from the more irregular margin of a neoplasm. Multiple concentric rims on T2-weighted MRI are pathognomonic for the infection, which might be due to organization of the necrotic debris and phagocytosis of the capsule by macrophages (18).

It is a matter of debate whether resection or aspiration of the neural lesion is necessary for diagnosis or therapy. Fleetwood et al. (19) reported 3 cases of successfully treated nocardial brain abscesses. They emphasized that the etiology of a cerebral lesion is not always the same as that of an extra-neural lesion and recommended open or stereotactic biopsy of the brain lesion early in the therapeutic course. In contrast, Peters et al. (20) reported a case with disseminated and cerebral nocardial infection, who was cured with antibiotics alone. The present case suggests that invasive resection or aspiration of the neural lesion is not always necessary.

In our patient we initially preferred imipenem, but there was no clinical improvement and therefore a combination of ceftriaxone and amikacin was ordered as a second-line an-

tibiotic treatment. Because of its excellent central nervous system penetration, trimethoprim–sulfamethoxazole is widely used for cases with brain abscess (2). It was not used at first in our case because it can increase the plasma concentration of phenytoin. A wide range of antibiotics is suggested to be effective for nocardiosis, including sulfonamide, trimethoprim–sulfamethoxazole, imipenem, amikacin (1–3), minocycline, third-generation cephalosporins, and fluoroquinolones (4, 21, 22). Resistance to antimicrobial agents occurs more frequently with *N. farcinica* than with other *Nocardia* species (23). Therefore, the results of antimicrobial susceptibility testing must be considered carefully.

We present a case of *N. farcinica* infection after liver transplantation. Nocardiosis is related to a high mortality in immunocompromised hosts, especially in those with brain lesions. To our knowledge, the present case is the first reported case with brain nocardiosis after liver transplantation. The findings indicate that the brain lesion can be cured non-invasively if treated with prompt antibiotic therapy based on susceptibility testing of a specimen obtained from an extra-neural lesion.

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Original Article

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

Kazuhiko Koike,[1] Kunihisa Tsukada,[1] Hiroshi Yotsuyanagi,[1] Kyoji Moriya,[1] Yoshimi Kikuchi,[2] Shinichi Oka[1] and Satoshi Kimura[2]

[1]Department of Internal Medicine, Graduate School of Medicine, University of Tokyo, Tokyo and [2]AIDS Clinical Center, International Medical Center of Japan, Tokyo, Japan

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

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

a majority of HIV-positive patients in Japan were enrolled in the study.

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
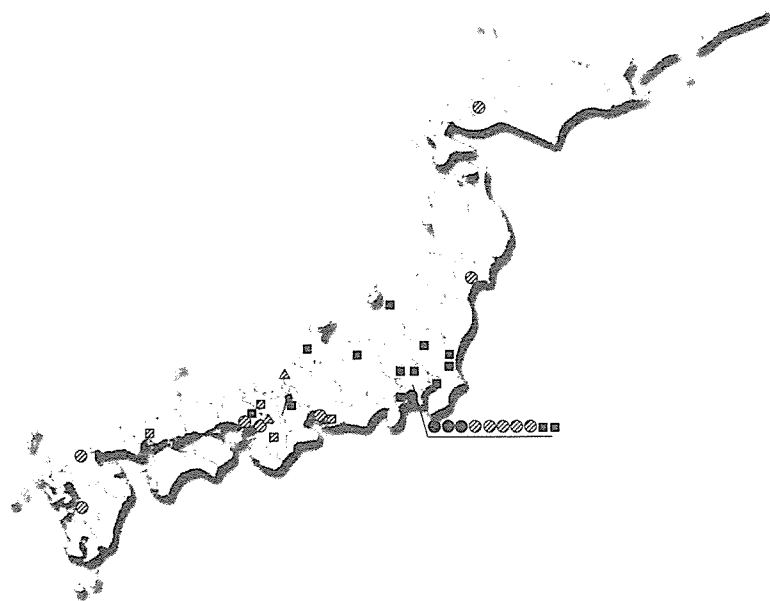
Figure 1 Nationwide distribution of hospitals in the HIV/AIDS Network of Japan that a number of HIV-positive or HIV/HCV coinfected patients are visiting regularly. Note that in the Kanto area, HIV/HCV coinfected patients were concentrated in the HIV/AIDS Network hospitals in the Tokyo city area. (△) 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.

ACKNOWLEDGMENTS

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

Hironobu Miyamoto,[1] Kohji Moriishi,[1] Kyoji Moriya,[2] Shigeo Murata,[3] Keiji Tanaka,[3] Tetsuro Suzuki,[4] Tatsuo Miyamura,[4] Kazuhiko Koike,[2] and Yoshiharu Matsuura[1]*

Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Osaka,[1] Department of Internal Medicine, Graduate School of Medicine, University of Tokyo, Tokyo,[2] Department of Molecular Oncology, Tokyo Metropolitan Institute of Medical Science, Tokyo,[3] and Department of Virology II, National Institute of Infectious Diseases, Tokyo,[4] Japan

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-

* Corresponding author. Mailing address: Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan. Phone: 81-6-6879-8340. Fax: 81-6-6879-8269. E-mail: matsuura@biken.osaka-u.ac.jp.
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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 (PA28γ$^{+/+}$ CoreTg) line C49 and PA28γ$^{-/-}$ mice have been described previously (35, 36). These two genotypes were crossbred to create PA28γ$^{+/-}$CoreTg mice. PA28γ$^{+/-}$CoreTg mice were bred to generate PA28γ$^{-/-}$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$_3$VO$_4$, 100 mM NaF, 50 mM Na$_4$P$_2$O$_7$, 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 UDC (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
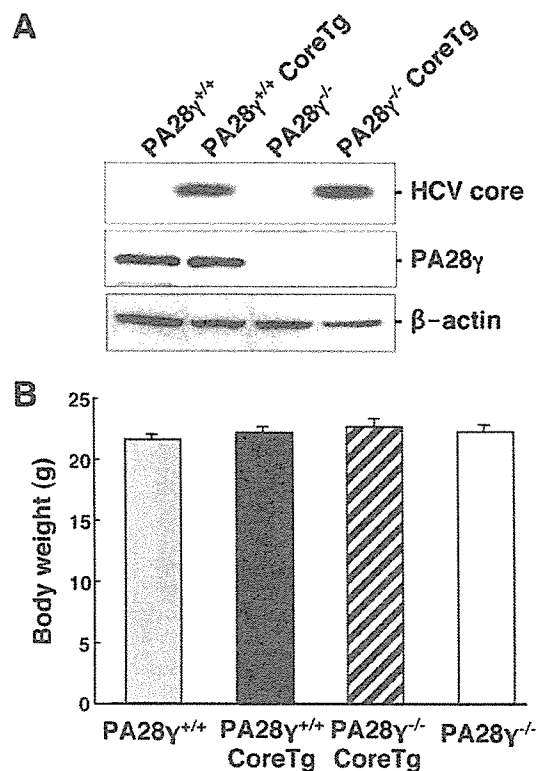

FIG. 1. Characterization of HCV core gene-transgenic mice deficient in the PA28γ gene. (A) Expression of the HCV core protein and PA28γ in the livers of PA28γ$^{+/+}$, PA28γ$^{+/+}$CoreTg, PA28γ$^{-/-}$, and PA28γ$^{-/-}$CoreTg mice. Lysates obtained from liver tissues of the mice (100 μg protein/lane) 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 ($P > 0.05$).

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).

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.
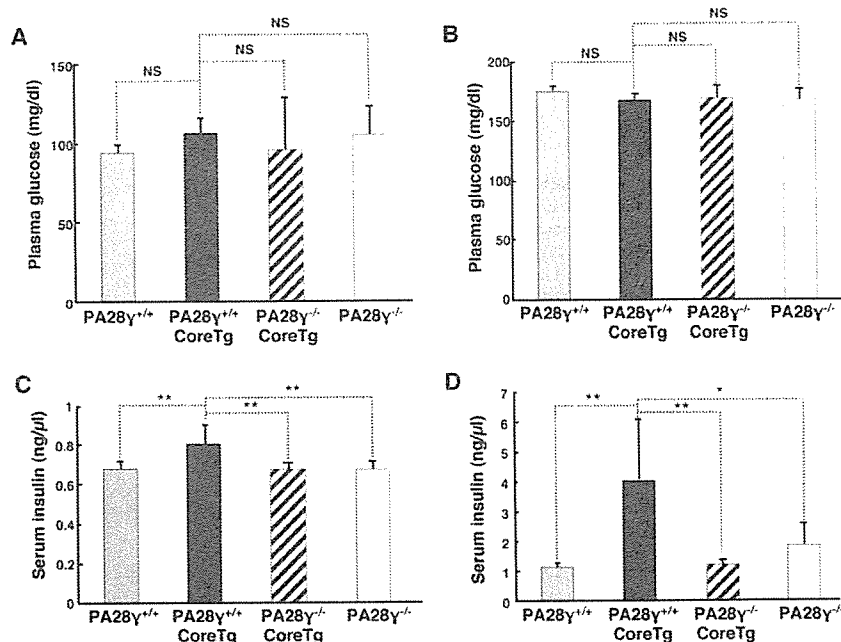
FIG. 2. Knockout of the PA28γ gene inhibited the hyperinsulinemia induced by HCV core protein. Plasma glucose levels of PA28γ$^{+/+}$, PA28γ$^{+/+}$CoreTg, PA28γ$^{-/-}$CoreTg, and PA28γ$^{-/-}$ mice under fasting (A) or fed (B) conditions ($n = 7$ in each group) are shown. Serum insulin levels in fasting (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 (PA28γ$^{+/+}$CoreTg)

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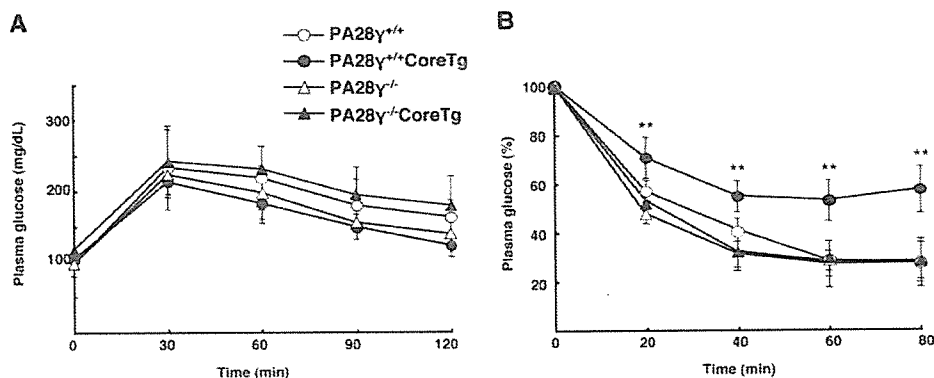

FIG. 3. Knockout of the PA28γ gene inhibits the insulin resistance induced by the HCV core protein. (A) Glucose tolerance test. D-Glucose was intraperitoneally administered to mice fasted for more than 16 h at 1 g/kg of body weight. Plasma glucose levels were estimated at the indicated times ($n = 5$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

A

B
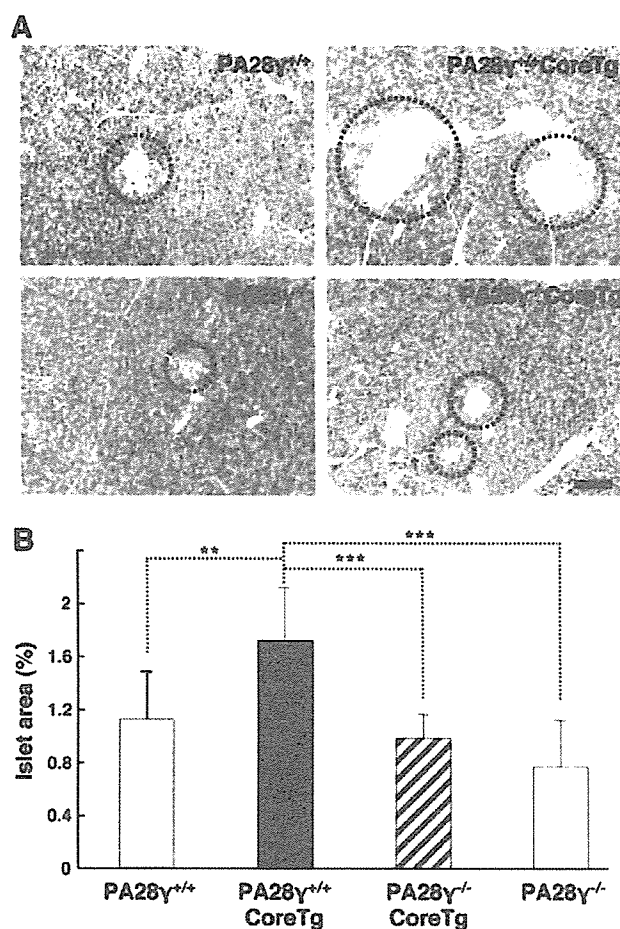

FIG. 4. PA28γ participated in the enlargement of pancreatic islets induced by the HCV core protein. (A) Histological sections prepared from pancreas tissues of PA28γ$^{+/+}$, PA28γ$^{+/+}$CoreTg, PA28γ$^{-/-}$, and PA28γ$^{-/-}$CoreTg mice were stained with hematoxylin and eosin. Dotted circles indicate 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.

A

B

FIG. 5. PA28γ participated in the inhibition of the tyrosine phosphorylation of IRS1 induced by the HCV core protein. Liver tissues from PA28γ$^{+/+}$, PA28γ$^{+/+}$CoreTg, PA28γ$^{-/-}$, and PA28γ$^{-/-}$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 PA28γ$^{+/+}$ (normal littermates) or PA28γ$^{-/-}$ mice. PA28γ was found at a similar level in PA28γ$^{+/+}$CoreTg and PA28γ$^{+/+}$ mice but was not present in either PA28γ$^{-/-}$ or PA28γ$^{-/-}$CoreTg mice (Fig. 1A). The expression of the HCV core protein in the livers of 2-month-old male mice was slightly higher in PA28γ$^{-/-}$CoreTg (1.36 ± 0.44 ng/mg of total protein; n = 7) than in PA28γ$^{+/+}$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). PA28γ$^{-/-}$ mice were slightly smaller than their normal littermates (PA28γ$^{+/+}$) at more than 3 months old, as described previously (36), but this was not significantly different in 2-month-old mice (Fig. 1B). PA28γ$^{+/+}$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
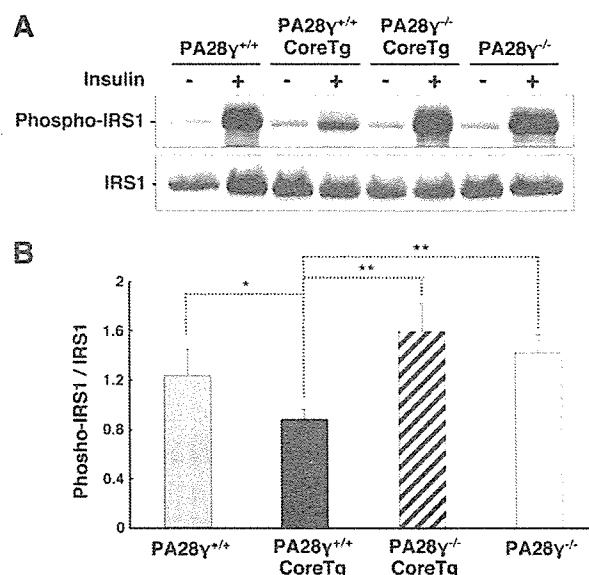
used in this study. Figure 1B shows the body weights of 2-month-old mice. There were no significant differences in body weight among PA28γ$^{+/+}$CoreTg, PA28γ$^{-/-}$CoreTg, PA28γ$^{-/-}$, and PA28γ$^{+/+}$ mice. Steatosis was not detected in the livers of the 2-month-old mice (data not shown).

PA28γ is involved in the development of hyperinsulinemia and insulin resistance in PA28γ$^{+/+}$CoreTg mice. In our previous study, we found a significant difference in serum insulin levels, but not in plasma glucose levels, between PA28γ$^{+/+}$ CoreTg mice and normal littermates (47). To determine the involvement of PA28γ in the development of insulin resistance in PA28γ$^{+/+}$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 PA28γ$^{+/+}$CoreTg mice than in PA28γ$^{+/+}$ mice under both conditions (Fig. 2C and D), as described previously (47). In contrast, the serum insulin concentration in PA28γ$^{-/-}$CoreTg mice was recovered to a normal level similar to that of PA28γ$^{+/+}$ and PA28γ$^{-/-}$ 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 PA28γ$^{+/+}$CoreTg mice. In our previ-
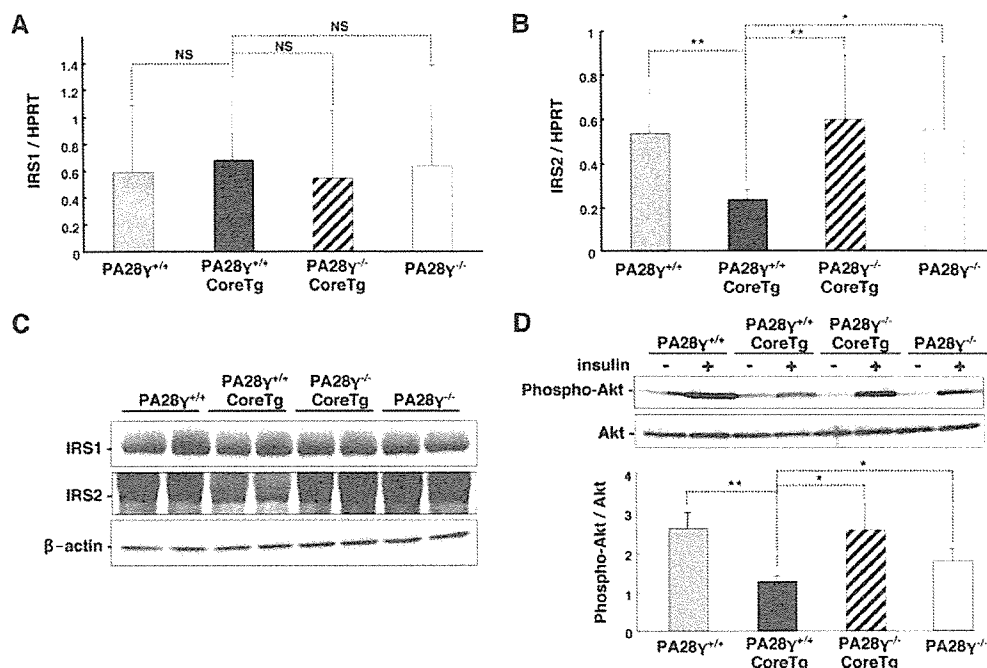
FIG. 6. PA28γ participated in the inhibition of the IRS2 expression and Akt phosphorylation induced by HCV core protein. The transcription of IRS1 (A) and IRS2 (B) was estimated by quantitative RT-PCR ($n = 5$ in each group). (C) The expression levels of IRS1 and IRS2 in the livers of the mice were determined 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
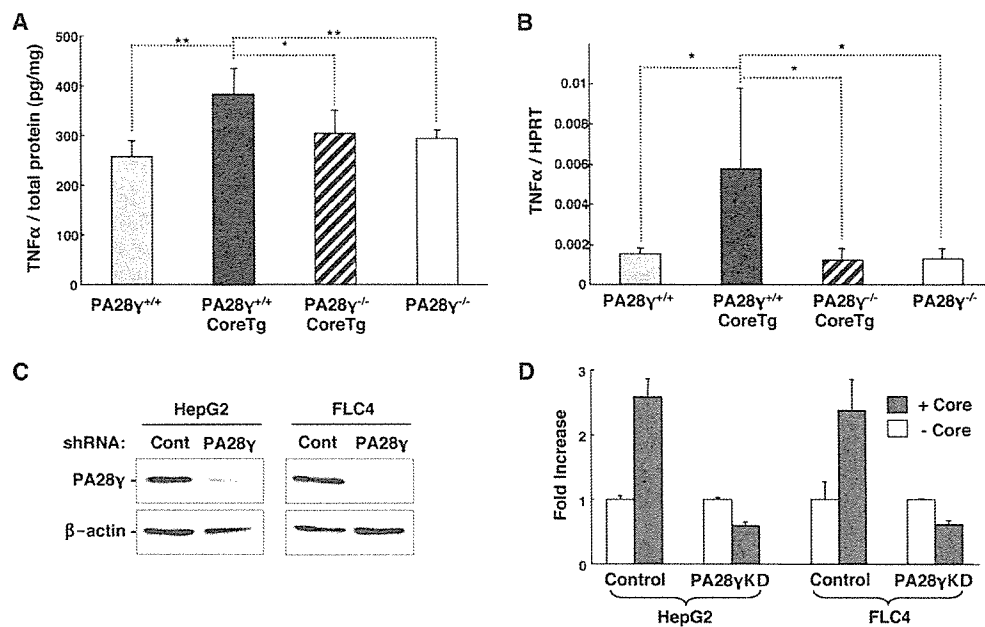
FIG. 7. PA28γ was required for activation of the TNF-α promoter by the HCV core protein. (A) Expression of TNF-α in the livers of mice was determined by ELISA ($n = 5$ in each group). (B) TNF-α mRNA in the livers of mice was examined by quantitative RT-PCR ($n = 5$ in each group). (C) Knockdown of the 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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Critical role of PA28γ in hepatitis C virus-associated steatogenesis and hepatocarcinogenesis

Kohji Moriishi*, Rika Mochizuki*, Kyoji Moriya[†], Hironobu Miyamoto*, Yoshio Mori*, Takayuki Abe*, Shigeo Murata[‡], Keiji Tanaka[‡], Tatsuo Miyamura[§], Tetsuro Suzuki[§], Kazuhiko Koike[†], and Yoshiharu Matsuura*[¶]

*Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan; [†]Department of Internal Medicine, Graduate School of Medicine, University of Tokyo, Tokyo 113-8655, Japan; [‡]Department of Molecular Oncology, Tokyo Metropolitan Institute of Medical Science, Tokyo 113-8613, Japan; and [§]Department of Virology II, National Institute of Infectious Diseases, Tokyo 162-8640, Japan

Hepatitis C virus (HCV) is a major cause of chronic liver disease that frequently leads to steatosis, cirrhosis, and eventually hepatocellular carcinoma (HCC). HCV core protein is not only a component of viral particles but also a multifunctional protein because liver steatosis and HCC are developed in HCV core gene-transgenic (CoreTg) mice. Proteasome activator PA28γ/REGγ regulates host and viral proteins such as nuclear hormone receptors and HCV core protein. Here we show that a knockout of the PA28γ gene induces the accumulation of HCV core protein in the nucleus of hepatocytes of CoreTg mice and disrupts development of both hepatic steatosis and HCC. Furthermore, the genes related to fatty acid biosynthesis and srebp-1c promoter activity were up-regulated by HCV core protein in the cell line and the mouse liver in a PA28γ-dependent manner. Heterodimer composed of liver X receptor α (LXRα) and retinoid X receptor α (RXRα) is known to up-regulate srebp-1c promoter activity. Our data also show that HCV core protein enhances the binding of LXRα/RXRα to LXR-response element in the presence but not the absence of PA28γ. These findings suggest that PA28γ plays a crucial role in the development of liver pathology induced by HCV infection.

fatty acid | proteasome | sterol regulatory element-binding protein (SREBP) | RXRα | LXRα

Hepatitis C virus (HCV) belongs to the Flaviviridae family, and it possesses a positive, single-stranded RNA genome that encodes a single polyprotein composed of ≈3,000 aa. The HCV polyprotein is processed by host and viral proteases, resulting in 10 viral proteins. Viral structural proteins, including the capsid (core) protein and two envelope proteins, are located in the N-terminal one-third of the polyprotein, followed by nonstructural proteins.

HCV infects >170 million individuals worldwide, and then it causes liver disease, including hepatic steatosis, cirrhosis, and eventually hepatocellular carcinoma (HCC) (1). The prevalence of fatty infiltration in the livers of chronic hepatitis C patients has been reported to average ≈50% (2, 3), which is higher than the percentage in patients infected with hepatitis B virus and other liver diseases. However, the precise functions of HCV proteins in the development of fatty liver remain unknown because of the lack of a system sufficient to investigate the pathogenesis of HCV. HCV core protein expression has been shown to induce lipid droplets in cell lines and hepatic steatosis and HCC in transgenic mice (4–6). These reports suggest that HCV core protein plays an important role in the development of various types of liver failure, including steatosis and HCC.

Recent reports suggest that lipid biosynthesis affects HCV replication (7–9). Involvement of a geranylgeranylated host protein, FBL2, in HCV replication through the interaction with NS5A suggests that the cholesterol biosynthesis pathway is also important for HCV replication (9). Increases in saturated and monounsaturated fatty acids enhance HCV RNA replication, whereas increases in polyunsaturated fatty acids suppress it (7). Lipid homeostasis is regulated by a family of steroid regulatory element-binding proteins (SREBPs), which activate the expression of >30 genes involved in

the synthesis and uptake of cholesterol, fatty acids, triglycerides, and phospholipids. Biosynthesis of cholesterol is regulated by SREBP-2, whereas that of fatty acids, triglycerides, and phospholipids is regulated by SREBP-1c (10–14). In chimpanzees, host genes involved in SREBP signaling are induced during the early stages of HCV infection (8). SREBP-1c regulates the transcription of acetyl-CoA carboxylase, fatty acid synthase, and stearoyl-CoA desaturase, leading to the production of saturated and monounsaturated fatty acids and triglycerides (15). SREBP-1c is transcriptionally regulated by liver X receptor (LXR) α and retinoid X receptor (RXR) α, which belong to a family of nuclear hormone receptors (15, 16). Accumulation of cellular fatty acids by HCV core protein is expected to be modulated by the SREBP-1c pathway because RXRα is activated by HCV core protein (17). However, it remains unknown whether HCV core protein regulates the srebp-1c promoter.

We previously reported (18) that HCV core protein specifically binds to the proteasome activator PA28γ/REGγ in the nucleus and is degraded through a PA28γ-dependent pathway. PA28γ is well conserved from invertebrates to vertebrates, and amino acid sequences of human and murine PA28γs are identical (19). The homologous proteins, PA28α and PA28β, form a heteroheptamer in the cytoplasm, and they activate chymotrypsin-like peptidase activity of the 20S proteasome, whereas PA28γ forms a homoheptamer in the nucleus, and it enhances trypsin-like peptidase activity of 20S proteasome (20). Recently, Li and colleagues (21) reported that PA28γ binds to steroid receptor coactivator-3 (SRC-3) and enhances the degradation of SRC-3 in a ubiquitin- and ATP-independent manner. However, the precise physiological functions of PA28γ are largely unknown *in vivo*. In this work, we examine whether PA28γ is required for liver pathology induced by HCV core protein *in vivo*.

Results

PA28γ-Knockout HCV Core Gene Transgenic Mice. To determine the role of PA28γ in HCV core-induced steatosis and the development of HCC *in vivo*, we prepared PA28γ-knockout core gene transgenic mice. The PA28γ-deficient, PA28γ^{−/−} mice were born without

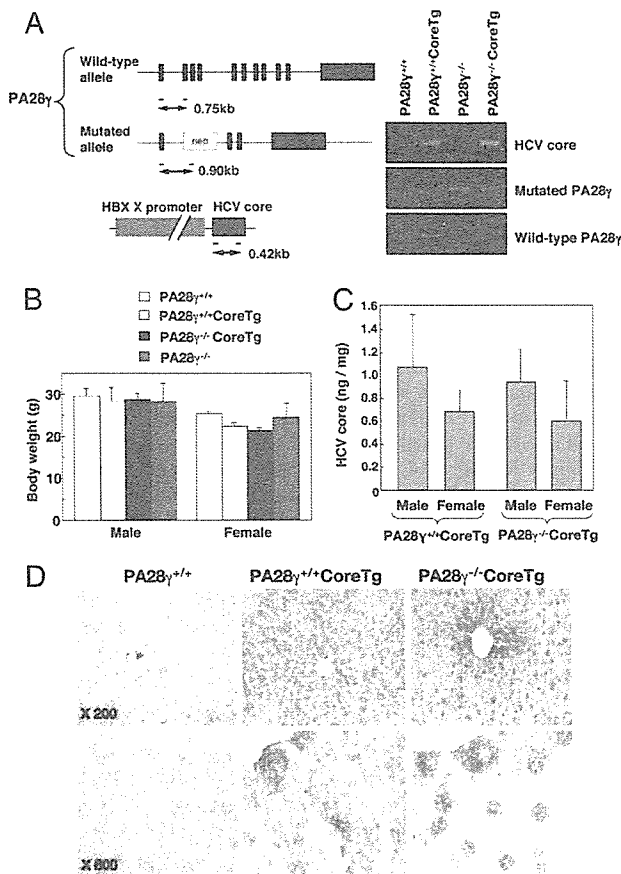Fig. 1. Preparation and characterization of PA28γ-knockout HCV core-transgenic mice. (A) The structures of the wild-type and mutated PA28γ genes and the transgene encoding the HCV core protein under the control of the HBV X promoter were investigated. Positions corresponding to the screening primers and sizes of PCR products are shown. PCR products of the HCV core gene as well as wild-type and mutated PA28γ alleles were amplified from the genomic DNAs of PA28γ$^{+/+}$, PA28γ$^{+/+}$CoreTg, PA28γ$^{-/-}$, and PA28γ$^{-/-}$CoreTg mice. (B) Body weights of PA28γ$^{+/+}$, PA28γ$^{+/+}$CoreTg, PA28γ$^{-/-}$CoreTg, and PA28γ$^{-/-}$ mice at the age of 6 months. (C) HCV core protein levels in the livers of PA28γ$^{+/+}$CoreTg and PA28γ$^{-/-}$CoreTg mice were determined by ELISA (mean ± SD, n = 10). (D) Localization of HCV core protein in the liver. Liver sections of PA28γ$^{+/+}$, PA28γ$^{+/+}$CoreTg, and PA28γ$^{-/-}$CoreTg mice at the age of 2 months were stained with anti-HCV core antibody.

appreciable abnormalities in all tissues examined, with the exception of a slight retardation of growth (22). HCV core gene-transgenic (PA28γ$^{+/+}$CoreTg) mice were bred with PA28γ$^{-/-}$ mice to create PA28γ$^{+/-}$CoreTg mice. The PA28γ$^{+/-}$CoreTg offspring were bred with each other, and PA28γ$^{-/-}$CoreTg mice were selected by PCR using primers specific to the target sequences (Fig. 1A). No significant differences in body weight were observed among the 6-month-old mice, although PA28γ$^{-/-}$ mice exhibited a slight retardation of growth (Fig. 1B). A similar level of PA28γ expression was detected in PA28γ$^{+/+}$CoreTg and PA28γ$^{+/+}$ mice (see Fig. 5B). The expression levels and molecular size of HCV core protein were similar in the livers of PA28γ$^{+/+}$CoreTg and PA28γ$^{-/-}$CoreTg mice (Fig. 1C; see also Fig. 5B).

PA28γ Is Required for Degradation of HCV Core Protein in the Nucleus and Induction of Liver Steatosis. HCV core protein has been detected at various sites, such as the endoplasmic reticulum, mitochondria, lipid droplets, and nucleus of cultured cell lines, as well as in hepatocytes of PA28γ$^{+/+}$CoreTg mice and hepatitis C patients
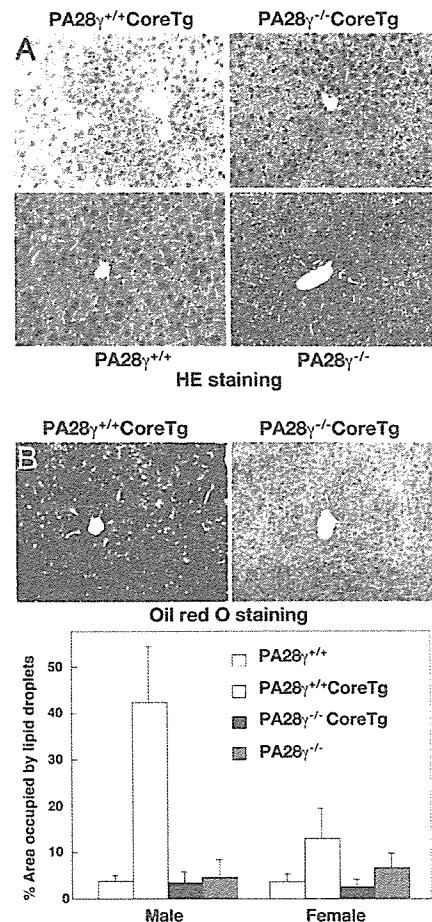

Fig. 2. Accumulation of lipid droplets by expression of HCV core protein. (A) Liver sections of the mice at the age of 6 months were stained with hematoxylin/eosin (HE). (B) (Upper) Liver sections of PA28γ$^{+/+}$CoreTg and PA28γ$^{-/-}$CoreTg mice at the age of 6 months were stained with oil red O. (Lower) The area occupied by lipid droplets of PA28γ$^{+/+}$ (white), PA28γ$^{+/+}$CoreTg (gray), PA28γ$^{-/-}$CoreTg (black), and PA28γ$^{-/-}$ (dark gray) mice was calculated by Image-Pro software (MediaCybernetics, Silver Spring, MD) (mean ± SD, n = 10).

(6, 23, 24). Although HCV core protein is predominantly detected in the cytoplasm of the liver cells of PA28γ$^{+/+}$CoreTg mice, as reported in ref. 6, in the present study a clear accumulation of HCV core protein was observed in the liver cell nuclei of PA28γ$^{-/-}$CoreTg mice (Fig. 1D). These findings clearly indicate that at least some fraction of the HCV core protein is translocated into the nucleus and is degraded through a PA28γ-dependent pathway. Mild vacuolation was observed in the cytoplasm of the liver cells of 4-month-old PA28γ$^{+/+}$CoreTg mice, and it became more severe at 6 months, as reported in ref. 25. Hematoxylin/eosin-stained liver sections of 6-month-old PA28γ$^{+/+}$CoreTg mice exhibited severe vacuolating lesions (Fig. 2A), which were clearly stained with oil red O (Fig. 2B Upper), whereas no such lesions were detected in the livers of PA28γ$^{-/-}$CoreTg, PA28γ$^{+/+}$, or PA28γ$^{-/-}$ mice at the same age. The areas occupied by the lipid droplets in the PA28γ$^{+/+}$CoreTg mouse livers were ≈10 and 2–4 times larger than those of male and female of PA28γ$^{+/+}$, PA28γ$^{-/-}$, and PA28γ$^{-/-}$CoreTg mice, respectively (Fig. 2B Lower). These results suggest that PA28γ is required for the induction of liver steatosis by HCV core protein in mice.

PA28γ Is Required for the Up-Regulation of SREBP-1c Transcription by HCV Core Protein in the Mouse Liver. To clarify the effects of a knockout of the PA28γ gene in PA28γ$^{+/+}$CoreTg mice on lipid
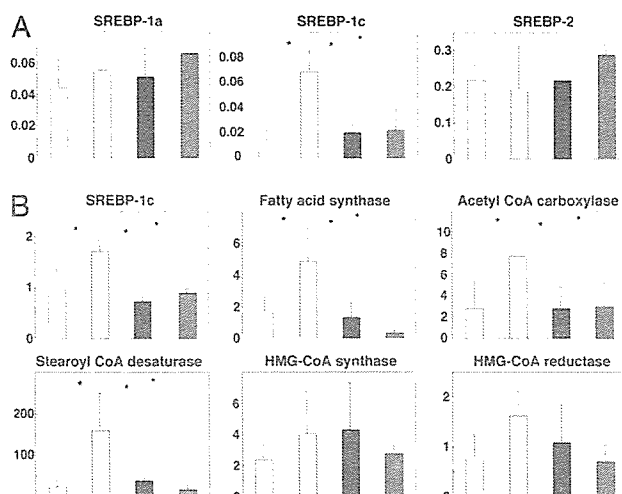
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Fig. 3. Transcription of genes regulating lipid biosynthesis in the mouse liver. (A) Total RNA was prepared from the livers of 2-month-old mice; and the transcription of genes encoding SREBP-1a, SREBP-1c, and SREBP-2 was determined by real-time PCR. (B) The transcription of genes encoding SREBP-1c, fatty acid synthase, acetyl-CoA carboxylase, stearoyl-CoA desaturase, HMG-CoA synthase, and HMG-CoA reductase of 6-month-old mice was measured by real-time PCR. The transcription of the genes was normalized with that of hypoxanthine phosphoribosyltransferase, and the values are expressed as relative activity ($n = 5$; *, $P < 0.05$; **, $P < 0.01$). The transcription of each gene in PA28$\gamma^{+/+}$, PA28$\gamma^{+/+}$CoreTg, PA28$\gamma^{-/-}$CoreTg, and PA28$\gamma^{-/-}$ mice is indicated by white, gray, black, and dark gray bars, respectively.

metabolism, genes related to the lipid biosyntheses were examined by real-time quantitative PCR. Transcription of SREBP-1c was higher in the livers of PA28$\gamma^{+/+}$CoreTg mice than in those of PA28$\gamma^{+/+}$, PA28$\gamma^{-/-}$, and PA28$\gamma^{-/-}$CoreTg mice at 2 months of age, but no such increases in SREBP-2 and SREBP-1a were observed (Fig. 3A). Although transcription of SREBP-1c and its regulating enzymes, such as acetyl-CoA carboxylase, fatty acid synthase, and stearoyl-CoA desaturase, was also enhanced in the livers of 6-month-old PA28$\gamma^{+/+}$CoreTg mice compared with the levels in the livers of PA28$\gamma^{+/+}$, PA28$\gamma^{-/-}$, and PA28$\gamma^{-/-}$CoreTg mice, no statistically significant differences were observed with respect to the transcription levels of cholesterol biosynthesis-related genes that are regulated by SREBP-2 (e.g., HMG-CoA synthase and HMG-CoA reductase) (Fig. 3B). These results suggest the

following: (*i*) the up-regulation of SREBP-1c transcription in the livers of mice requires both HCV core protein and PA28γ; and (*ii*) the nuclear accumulation of HCV core protein alone, which occurs because of the lack of degradation along a PA28γ-dependent proteasome pathway, does not activate the *srebp-1c* promoter.

HCV Core Protein Indirectly Potentiates *srebp-1c* Promoter Activity in an LXRα/RXRα-Dependent Manner. LXRα, which is primarily expressed in the liver, forms a complex with RXRα and synergistically potentiates *srebp-1c* promoter activity (16). Activation of RXRα by HCV core protein suggests that cellular fatty acid synthesis is modulated by the SREBP-1c pathway, although HCV core protein was not included in the transcription factor complex in the electrophoresis mobility shift assay (EMSA) (17). To analyze the effect of HCV core protein and PA28γ on the activation of the *srebp-1c* promoter, we first examined the effect of HCV core protein on the binding of the LXRα/RXRα complex to the LXR-response element (LXRE) located upstream of the SREBP-1c gene (Fig. 4A). Although a weak shift of the labeled LXRE probe was observed by incubation with nuclear extracts prepared from 293T cells expressing FLAG-tagged LXRα and HA-tagged RXRα, a clear shift was obtained by the treatment of cells with 9-*cis*-retinoic acid and 22(*R*)-hydroxylcholesterol, ligands for LXRα and RXRα, respectively. In contrast, coexpression of HCV core protein with LXRα and RXRα potentiated the shift of the probe irrespective of the treatment with the ligands. Addition of 500 times the amount of nonlabeled LXRE probe (competitor) diminished the shift of the labeled probe induced by the ligands and/or HCV core protein. Furthermore, coincubation of the nuclear fraction with antibody to FLAG or HA tag but not with antibody to either HCV core or PA28γ caused a supershift of the labeled probe. These results indicate that HCV core protein does not participate in the LXRα/RXRα–LXRE complex but indirectly enhances the binding of LXRα/RXRα to the LXRE.

The activity of the *srebp-1c* promoter was enhanced by the expression of HCV core protein in 293T cells, and it was further enhanced by coexpression of LXRα/RXRα (Fig. 4B). Enhancement of the *srebp-1c* promoter by coexpression of HCV core protein and LXRα/RXRα was further potentiated by treatment with the ligands for LXRα and RXRα. The cells treated with 9-*cis*-retinoic acid exhibited more potent enhancement of the *srebp-1c* promoter than those treated with 22(*R*)-hydroxylcholesterol. HCV core protein exhibited more potent enhancement of the *srebp-1c* promoter in cells treated with both ligands than in those treated with either ligand alone. These results suggest that HCV core protein poten-
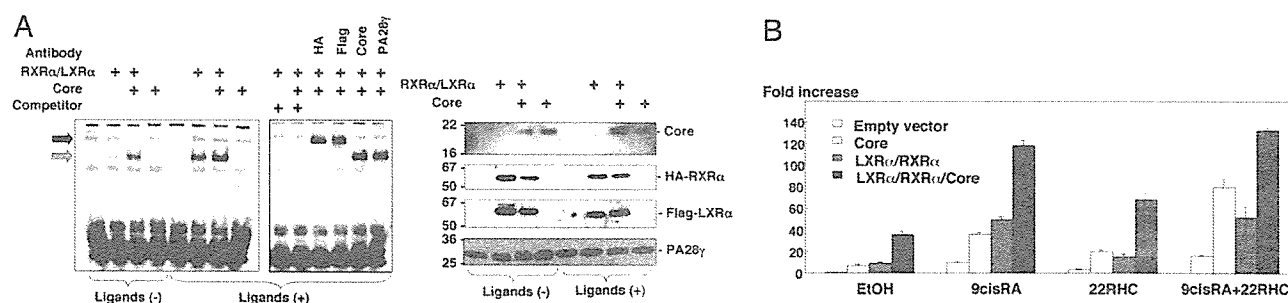

Fig. 4. Activation of the *srebp-1c* promoter by HCV core protein. (A) FLAG-LXRα and HA-RXRα were expressed in 293T cells together with or without HCV core protein. Ligands for LXRα and RXRα dissolved in ethanol [Ligands (+)] or ethanol alone [Ligands (−)] were added to the culture supernatant at 24 h posttransfection. Cells were harvested at 48 h posttransfection, and nuclear extracts were mixed with the reaction buffer for EMSA in the presence or absence of antibody (100 ng) against HA, FLAG, HCV core or PA28γ, or nonlabeled LXRE probe (Competitor). (Left) The resulting mixtures were subjected to PAGE and blotted with horseradish peroxidase/streptavidin. The mobility shift of the LXRE probe and its supershift are indicated by a gray and black arrow, respectively. (Right) Expression of HCV core, HA-RXRα, FLAG-LXRα, and PA28γ in cells was detected by immunoblotting. (B) Effects of ligands for RXRα, 9-*cis*-retinoic acid (9cisRA), and for LXRα, 22(*R*)-hydroxylcholesterol (22RHC), on the activation of the *srebp-1c* promoter in 293T cells expressing RXRα, LXRα, and/or HCV core protein. Ligands were added into the medium at 24 h posttransfection at a concentration of 5 μM, and the cells were harvested after 24 h of incubation.