

互に干渉することがあり、例えば d4T+ZDV は禁忌であるとされている。

一方これらの薬剤自身も副作用も枚挙にいとまがない。NRTI のほとんどが乳酸血症と脂肪肝を生じ生命に危険を生ずる。また ZDV には骨髄抑制による貧血や好中球減少、下痢、頭痛、不眠、倦怠感が、ddI には肺炎と末梢神経炎が、ddC と d4T ではやはり末梢神経炎を生ずる。プロテアーゼ・インヒビターでは一般に、体脂肪の分布が変わり、脂質代謝異常を伴ったり、血友病患者では出血のエピソードが増加するなどの副作用が見られる。

治療がうまくいかない大きな理由は二つで、一つは薬に効果がなかった場合で、二つ目は薬の副作用である。後者の場合は副作用を起こしている薬物を同定し、同じような効果がある薬に変えることは比較的容易である。前者の場合は3種の薬剤の全てを変えることが望まれる。

確かにウイルスの塩基配列や培養によるウイルスの特異的な薬剤に対する抵抗性を検討することは極めて有意義なことではあるが、それらの情報がウイルスの生体内での病原性にどの程度反映しているかの確証はまだ得られていない。現在治療の有効性を最もよく反映できるのは、血漿中のウイルス量と CD4 数であり、これらのマーカーでの有効性がなかった時に治療の変更を考える。変更する際には決して単剤だけの変更ではなく、全ての薬剤を変更すべきである。

以前 ZDV を投与されていた患者に抵抗性のウイルスが出現して ZDV 投与を中止した場合は、患者の体内では ZDV 感受性のウイルスが置き換わる。しかし ZDV を再投与すると、ZDV 抵抗性ウイルスが容易に再出現してくることが知られている。また indinavir と ritonavir, delavirdine と nevirapine は似通った薬剤であり、交叉耐性が存在するので、変更のさいは注意を要する。

(服部俊夫)

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 dis-

tributing 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 coinfecting 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 coinfecting 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. jirovecii*) 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 coinfecting 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 coinfecting with HIV and HCV

No. of HIV(+)/HCV(+)	No. of HIV(+)				Total
	0	1-19	20-49	50+	
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 coinfecting with HIV and HCV in 75 (42.6%) of 176 hospitals, and there were 10 or more HIV/HCV coinfecting patients in 18 (10.2%) hospitals. HIV/HCV coinfecting patients were concentrated in specific hospitals in big cities around Japan. In particular, in the Kanto area, HIV/HCV coinfecting 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 coinfecting patients, 12 were the same hospitals (Table 1). Hospitals with 10 or more HIV/HCV coinfecting 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

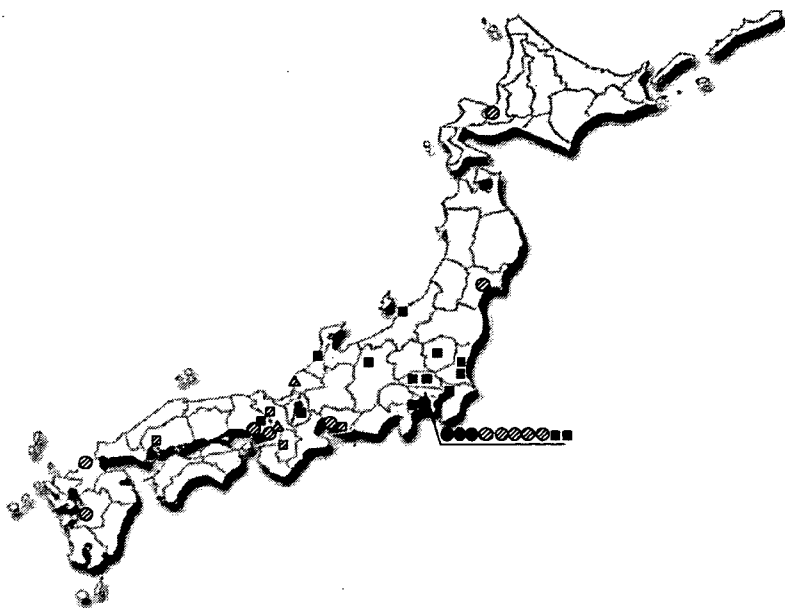


Figure 1 Nationwide distribution of hospitals in the HIV/AIDS Network of Japan that a number of HIV-positive or HIV/HCV coinfecting patients are visiting regularly. Note that in the Kanto area, HIV/HCV coinfecting patients were concentrated in the HIV/AIDS Network hospitals in the Tokyo city area. (Δ) hospitals with 1-19 HIV-positive patients; (\square) hospitals with 20-49 HIV-positive patients; (\circ) hospitals with 50+ HIV-positive patients. Hatched figures: hospitals with 10 or more HIV/HCV coinfecting patients. Closed figures: hospitals with less than 10 HIV/HCV coinfecting patients. For easier visual comprehension, hospitals with 19 or less HIV-positive patients and 9 or less HIV/HCV coinfecting 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 coinfecting patients, 786 (84.1%) of 935 patients were recipients of blood products. Thus, the majority of HIV/HCV coinfecting 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 coinfecting 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 coinfecting 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 coinfecting patients, it is essential to recognize the current status of the coinfection. In the present study, the number and transmission routes of HIV/HCV coinfecting patients in Japan were first described, although detailed information on the progression of HCV-associated liver diseases in HIV/HCV coinfecting patients has not yet been obtained. Undoubtedly, this will be the first step for improving the prognosis and quality of life of patients coinfecting 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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Short Communication

Reversal Periods and Patterns from Drug-Resistant to Wild-Type HIV Type 1 after Cessation of Anti-HIV Therapy

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ABSTRACT

Anti-HIV drug-resistant virus reverts to wild type following discontinuation of antiretroviral therapy (ART). This study aimed to determine the reversal period. ART was discontinued in 16 patients harboring drug-resistant viruses. Resistant mutations of reverse transcriptase (RT) and protease (PR) genes of plasma- and peripheral blood mononuclear cells (PBMC)-derived viruses were examined by direct sequencing monthly until the disappearance of mutants (median follow-up period: 8.9 months). Only wild-type virus was detected in 50% of patients at 6.3 months (quartiles, 3.2–20.7 months) and at 9.2 months (quartiles, 5.7–13.8 months) in plasma- and PBMC-derived viruses, respectively, after ART interruption. Among the 133 resistance-associated mutations identified at ART interruption, half the RT and PR mutations shifted to wild type in 3.2 months in plasma, 6.7 months of RT, and 5.7 months of PR in PBMC, respectively. In plasma- and PBMC-derived viruses, the PR mutations reverted earlier than the RT mutations. These results could be relevant as to when to perform drug-resistance testing.

THE EMERGENCE OF DRUG-RESISTANCE-ASSOCIATED MUTATIONS leads to treatment failure and may limit future treatment options. Therefore, inclusion of drug-resistance testing is recommended in anti-HIV-1 treatment guidelines, especially after failure of standard regimens.^{1,2} A number of studies showed that drug-resistance testing improved the benefits of antiretroviral therapy (ART).^{3–8} For drug resistance testing, plasma and peripheral blood mononuclear cell (PBMC) can be used as clinical specimens.⁹ Using direct sequencing, we reported previously the earlier detection of resistant mutations in plasma than in PBMC.¹⁰ Accordingly, we recommended the use of plasma for early detection of drug resistance during therapy in those patients who fail to respond to antiretroviral treatment. Clinically, even when patients develop virologic failure [rebound of plasma HIV-1 viral load (VL)], the CD4 count remains sufficiently high for treatment interruption, at least in some patients. In such cases, the timing of genotypic drug resistance testing is of practical importance. Discontinuation of treatment causes the reversion of resistance mutations to wild-type viruses.^{11–18}

Previous studies indicated that resistance mutations of plasma viruses could rapidly become undetectable either partially or entirely from 14 days to 4 months after ART cessation.^{12–18} The reversion of mutations to wild type is considered to be due to the low replication fitness of mutant variants and outgrowth of wild type viruses when the drug-selective pressure is withdrawn.^{17,21–22} However, the time course and pattern of this reversion have not been studied in detail in heavily treated patients. Clarification of this issue will help determine the most appropriate time and sample for performing genotypic-resistance testing after ART cessation.

The study subjects were 16 HIV-1-infected patients who had been known to have drug-resistance virus beforehand and discontinued antiretroviral therapy from August 1998 through December 2002 for a variety of reasons. All patients regularly consulted the AIDS Clinical Center at the International Medical Center of Japan, Tokyo, and gave written informed consent. Their demographic data and clinical characteristics at the time of quitting ART are listed in Table 1. Their blood samples were

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TABLE 1. DEMOGRAPHIC AND CLINICAL CHARACTERISTICS OF PATIENTS^a

Pt	Sex	Age (years)	Risk factor	CD4 cells/ μ l	log ₁₀ VL month 0	log ₁₀ VL month 1	Duration of ART (months)	Drugs ever used	Reasons of ART stop
1	M	40	Bisexual	404	5.2	5.3	70.4	AZT, 3TC, d4T, ddI, NFV, RTV, SQH	Virological failure
2	M	23	Hemophilia	103	5	5	46.6	ddC, d4T, 3TC, AZT, ddI, SQH, NFV, IDV	Virological failure
3	M	36	MSM	209	3.5	3.5	91.8	AZT, 3TC, d4T, ddI, NFV	Virological failure
4	M	22	Hemophilia	116	5.1	4.3	71.9	ddC, d4T, 3TC, ABC, ddI, AZT, EFV, SQH, NFV, APV	Virological failure
5	M	26	Hemophilia	30	5.7	5.2	50.2	d4T, 3TC, ddI, AZT, NFV, IDV, RTV	Virological failure
6	M	28	Hemophilia	93	3.6	3.7	129.7	ddI, AZT, 3TC, ABC, ddC, EFV, RTV, SQH, IDV, NFV, APV	Virological failure
7	M	29	Hemophilia	698	1.7	5	108.6	AZT, 3TC, d4T, ddI, ABC, EFV, RTV, SQH	Side effects
8	M	24	Hemophilia	35	5.1	5.1	67	AZT, 3TC, ddC, SQH, RTV	Virological failure
9	F	42	Heterosexual	690	1.8	4.4	90	AZT, ddI, d4T, 3TC, ABC, EFV, SQH, APV, NFV	Poor adherence
10	M	19	Hemophilia	586	1.8	4.2	107.6	AZT, ddC, d4T, 3TC, ddI, ABC, EFV, IDV, RTV, NFV, SQH, LPV/r	Poor adherence
11	M	24	Hemophilia	644	1.7	4.5	117.6	AZT, ddI, 3TC, IDV, RTV	Poor adherence
12	M	22	Hemophilia	138	4.5	4.5	53.4	AZT, ddC, d4T, 3TC, ddI, NVP, SQH, RTV, IDV	Virological failure
13	M	34	Bisexual	276	1.7	3.6	47.6	d4T, 3TC, ddI, ABC, AZT, NFV, IDV, RTV, LPV/r	Poor adherence
14	M	42	MSM	420	4.3	5.3	49.8	AZT, 3TC, d4T, ddI, IDV, RTV, SQH	Virological failure
15	M	39	Hemophilia	544	1.7	4.5	140.8	AZT, d4T, 3TC, NVP, NFV	Side effects
16	M	37	Bisexual	525	4.4	4.6	69.9	AZT, ddC	Virological failure
Mean		30.4		344	3.5	4.5	82		

^aM, male; F, female; MSM, men having sex with men; VL, HIV-1 viral load in plasma; month 0, time when ART was stopped; month 1, 1 month after ART was stopped. AZT, zidovudine; 3TC, lamivudine; d4T, stavudine; ddI, didanosine; ddC, zalcitabine; ABC, abacavir; EFV, efavirenz; NVP, nevirapine; NFV, nelfinavir; RTV, ritonavir; SQH, saquinavir hard gel capsule; DOV, indinavir; APV, amprenavir; LPV/r, lopinavir + ritonavir.

collected monthly. Measurements of VL (Amplicor HIV-Monitor, Roche Molecular Systems, Inc., NJ) and CD4 and CD8 lymphocyte counts (monoclonal antibodies and flow cytometry) were performed at each blood sampling.

PBMC were separated by centrifugation from 7 ml EDTA-treated blood. PBMC and plasma were stored at -80°C until sequence analysis. The method of sequence analysis was reported previously.¹⁰ Briefly, total RNA was extracted from 100 μl plasma and DNA was extracted from 1×10^6 PBMCs (SMITEST Ex R&D Kit, Japan). The RNA sample was subjected to reverse transcription (RT) followed by nested polymerase chain reaction (PCR) using primers targeting the RT gene and protease (PR) gene, respectively. A DNA sample was also subjected to nested PCR using the same primers for the same targets. The primers covered 1–100 base pairs of PR and 40–240 base pairs of RT. Sequences of primer sets were published elsewhere.¹⁰ Direct sequencing was performed on a 3730 DNA Analyzer (Applied Biosystems). A heterozygous base sequence was identified when the electrogram showed a minor peak at $>50\%$ of the major peak. The amino acid sequence was deduced with the GENETYX-WIN version 4.1 (Software Development, Tokyo) and the amino acid substitutions related to drug resistance were estimated from published data.² The clade of HIV-1 was determined by the sequences of RT and PR genes.

The reversal period was defined as the time interval between the date of ART interruption and the date of the disappearance of mutations confirmed by direct sequencing. When mutations (all minor mutations, in some patients) did not revert, the reversal period was defined as the date ART stopped to the date most mutations shifted to the wild-type amino acid sequence (for example, see Table 2; protease residues of plasma virus at month 5.9 of patient 2). As all HIV-1s amplified in this study were HIV-1 clade B, we regarded L63P as the polymorphism. The major mutant residues included M41L, A62V, D67N, K70R, L74V, M184V, G190S, L210W, T215F/Y, and K219E/Q of RT mutations and D30N, L33F, M46I, G48V, V82A/F, I84V, and L90M of PR mutations.² The follow-up period was the time interval from when ART was interrupted to when the resistance mutations disappeared.

A Kaplan–Meier survival curve was used to estimate the continuous periods of resistance mutations. The Mann–Whitney U test was used for group comparisons, the Wilcoxon signed rank test was used for paired comparison of the reversal period, the paired t -test was used for changes in CD4 count and HIV-1 viral load, and correlation analysis was used for the relationship between the reversal period and baseline CD4 count or baseline viral load, respectively. StatView version 5 was used for analysis and a p value less than 5% was considered statistically significant.

As shown in Table 1, most patients enrolled in this study had been treated over a long period of time [mean ART period: 82 months (SD, 31.6; range, 46.6–140.8 months)]. The reasons for discontinuation of ART were virologic failure in 10 cases, poor adherence in 4 cases, and side effects in 2 cases. The median follow-up period was 8.9 months (range: 2–25 months) and all patients provided blood samples for testing. None of the patients received any ART during the follow-up period. CD4 counts of 10 patients were more than $200/\mu\text{l}$ at the time of ART discontinuation. After withdrawal of ART, the CD4 count decreased a mean value of $66/\mu\text{l}$ 1 month later and continued to

decrease until the disappearance of resistant mutations. The VL of 6 patients (patients 7, 9, 10, 11, 13, and 15) who discontinued ART because of side effects or poor adherence ranged from <50 to 650 copies/ml at the time of ART cessation. The VL of these patients rebounded to a mean of $4.2 \log_{10}$ copies/ml 1 month later (designated as rebounded virus) but showed a plateau level thereafter. The VL of the other 10 patients who discontinued ART for virologic failure was stable after ART cessation.

In all 16 patients, a total of 133 resistance mutation residues with 59 RT and 74 PR were found in plasma and PBMC. The concordance of mutant residues between plasma and PBMC was 96.2% (RT mutations 93.2%, PR mutations 98.6%). All 16 patients possessed RT resistance mutations but 4 of them had no PR mutations (Table 2). In PR, both plasma and PBMC-derived viruses had 26 major resistance and 48 minor resistance residues. In contrast in RT, 52 and 50 major RT residues and 7 and 9 minor RT residues were detected in plasma and PBMC, respectively. The results showed that the resistance mutations could shift to wild type after 1 month or could persist for as long as 22 months after treatment stopped. Interestingly, in 6 patients with viral load rebound, the rebounded viruses in 5 patients (patients 7, 10, 11, 13, and 15) had the same resistant mutations as their predecessor viruses 1 month after ART cessation and then reverted to wild type thereafter. In patient 9, the rebounded virus was a wild-type virus.

As shown in Fig. 1A, after ART interruption, only wild-type virus was detected in 50% of patients at 6.3 months (quartiles, 3.2–20.7 months) and at 9.2 months (quartiles, 5.7–13.8 months) in plasma- and PBMC-derived viruses, respectively. In Fig. 1B, the reversion of 133 resistance mutations is shown by a Kaplan–Meier survival curve. Fifty percent of both PR and RT resistance mutations shifted to wild type in 3.2 months in plasma (quartiles, 1.5–3.7 months for PR, 2–10 months for RT). However, in PBMC, 50% of PR and RT mutations disappeared in 5.7 (quartile, 3.2–6.7 months) and 6.7 (quartile, 3.5–12 months) months, respectively. The reversal period of PR and RT mutations in plasma was 2.5 and 3.5 months, respectively, less than that in PBMC (both $p < 0.05$). Furthermore, the PR mutations shifted to wild type much more rapidly than RT mutations in both plasma and PBMC, although the half life of both mutation residues were the same in plasma (Wilcoxon test $p < 0.05$). In terms of the reversal period of major and minor mutations, there were no difference between them both in the PR and RT regions of plasma- or PBMC-derived viruses. There were no relationships found between the reversal periods of RT and PR mutations and the baseline CD4 cell count, baseline VL, and changes in these two surrogate markers 1 month later (data not shown).

Figure 2 shows how the mutation residues disappeared after ART cessation. We roughly divided the reversal process into two patterns. The first pattern was that resistant mutations persisted for some time and then disappeared abruptly (Fig. 2A). Most PR mutations of plasma viruses, 50% of PR mutations of provirus, and 50% of RT mutations in both types of specimens showed this pattern. The second pattern was that of a gradual decrease of mutations followed by their disappearance or persistence (Fig. 2B). One-third of RT mutations showed this pattern. Overall, all major mutations of RT and PR genes disappeared in all patients after withdrawal of ART. In contrast, the minor mutations did not disappear in some patients.

TABLE 2. RESISTANCE MUTATIONS AND REVERSAL PERIOD IN PLASMA AND PBMC AFTER ART CESSATION

Pt	Sample	Months after ART cessation	Reverse transcriptase residues	Months after ART cessation	Protease residues
1	Plasma	0	41L, 69D, 118I, 210W, 215Y	0	10I, 30N, 33F, 71T, 84I, 88D, 90M
		3.2	— ^a	3.2	—
2	PBMC	0	41L, 69D, 118I, 210W, 215Y	0	10I/L, 30N/D, 33F/L, 71T/A, 84I/V, 88D/N, 90M
		3.2	—	3.2	—
3	Plasma	0	41L, 67N, 69D, 118I, 210W, 215Y	0	10I, 20M, 36I, 48V, 54V, 82A
		15.2 ^b	41L, 210W/R	5.9	10F, 36I
		0	41L, 67N, 69D, 118I, 210W, 215Y	0	10I, 20M, 36I, 48V, 54V, 82A
		15.2 ^b	41L, 118I, 215Y	9	10F, 36I
4	PBMC	0	41L, 44D, 184V, 215Y	0	30N, 71V, 77I, 88D
		7	—	4.6	—
		0	41L, 44D, 184V, 215Y	0	30N, 71V, 77I, 88D
		8.6	—	4.6	—
5	PBMC	0	41L, 74V, 184V, 215Y	0	10I, 20I/M, 71V, 73S, 84V, 90M
		2.8	—	4.8	—
		0	41L, 184V, 215Y	0	10I/L, 20I, 71V/A, 73S/G, 84V/I, 90M
		4.8	—	4.8	—
6	PBMC	0	41L, 44D, 67N, 210W, 215Y	0	10I, 46I, 71T, 73S, 77I, 82F, 90M
		7.9	—	3.3	10I, 77I
		0	41L, 44D, 67N, 184V, 210W, 215Y	0	10I, 46I, 71V, 73S, 77I, 82F, 90M
		12.5	—	6.7	10I, 77I
7	Plasma	0	41L, 74V, 184V, 215Y	0	10I, 46I, 54L, 71V, 77I, 84V, 90M
		0	—	1.4	77I
		6.3	—	0	—
		0	41L, 74V/L, V118I, 184V/M, 190G/S, 210W, 215Y	0	10I, 20M/K, 46I, 54L/I, 71V, 77I, 84V, 90M
8	PBMC	11.3	—	3.2	77I
		0	67N, 70R, 184V, 219Q	0	N ^c
		1	67N/D, 70R, 184V/M, 219Q/K	0	—
		3.2	—	0	N
9	PBMC	0	67N, 70R, 184V, 219Q	0	—
		9.2	—	0	—
		0	41L, 184V, 215F	0	20R, 36I, 54V, 71V, 82A, 90M
		5.7	—	3.7	—
10	PBMC	0	41L, 184V, 215F	0	20R, 36I, 54V, 71V, 82A, 90M
		5.7	—	5.7	20R,
		0	41L, 67N, 70R, 215F, 219E	0	10L/I, 36I, 73S, 77I, 90M
		0	—	1	—
11	PBMC	1	67N, 184M/N, 210W, 219E	0	10L/I, 71T/I, 73S, 77I, 90M
		0	—	2	—

10	Plasma	0	41L, 67N, 215F, 219Q 41L, 67N, 215F, 219Q 219Q/K	0	101, 36I, 46I, 53L, 71V, 84V, 90M
	PBMC	1 8.5 ^d 0	41LM, 67N, 70R, 118V/I, 184V/M, 215F, 219Q	3.7 0	10I, 36I
11	Plasma	8.5 ^d 0	67N, 70R, 219Q	7.9 0	10I, 36I N
	PBMC	1.5 24	67N, 70R, 219Q 67N, 69N/D, 219Q	0	N
12	Plasma	0	184V, 62V	0	10I, 48V, 71T, 77I, 82A, 90M 71T, 77I
	PBMC	2	184V, 62V	2.2	10I, 48V, 71T, 77I, 82A, 90M 71T, 77I
	PBMC	0	—	0	10I, 46I, 71V, 77I, 88S 10I, 36M/L, 71V/T
13	Plasma	2.2	67N, 184V 67N, 184V	5	10I, 71V/T
	PBMC	0	67N/D, 184V/M	0	10I, 46M/L, 71V/T, 77I 10I, 36I/M, 71V/A
	PBMC	1	184V	1	10I, 20R, 24I, 36I, 53L, 54V, 71T, 82A
14	Plasma	3.5	—	2.3	—
	PBMC	0	—	0	10I/L, 20R/K, 24I/L, 36I, 53L/F, 54V, 71V/A, 82V/A
	PBMC	13.8	184V	7.4	—
15	Plasma	0	67N, 70R, 219Q 67N, 70R	0	N
	PBMC	1	67N, 70R, 219Q	6.2	—
	PBMC	20.7	67N, 219Q	0	N
16	Plasma	0	67N/D/G, 69A/D, 70R, 219Q 219Q	0	N
	PBMC	22.5	67N, 219Q	0	N
	PBMC	0	67N/D/G, 69A/D, 70R, 219Q 219Q	0	N
	PBMC	19.5	69A/D, 70R, 219Q	0	N
	PBMC	0	69A/D, 219Q	0	N
	PBMC	19.5	69A/D, 219Q	0	N

^a—, wild type.

^bThis patient died at this time point with RT mutations detected.

^cN, no resistance mutations.

^dNew ART was introduced at the time.

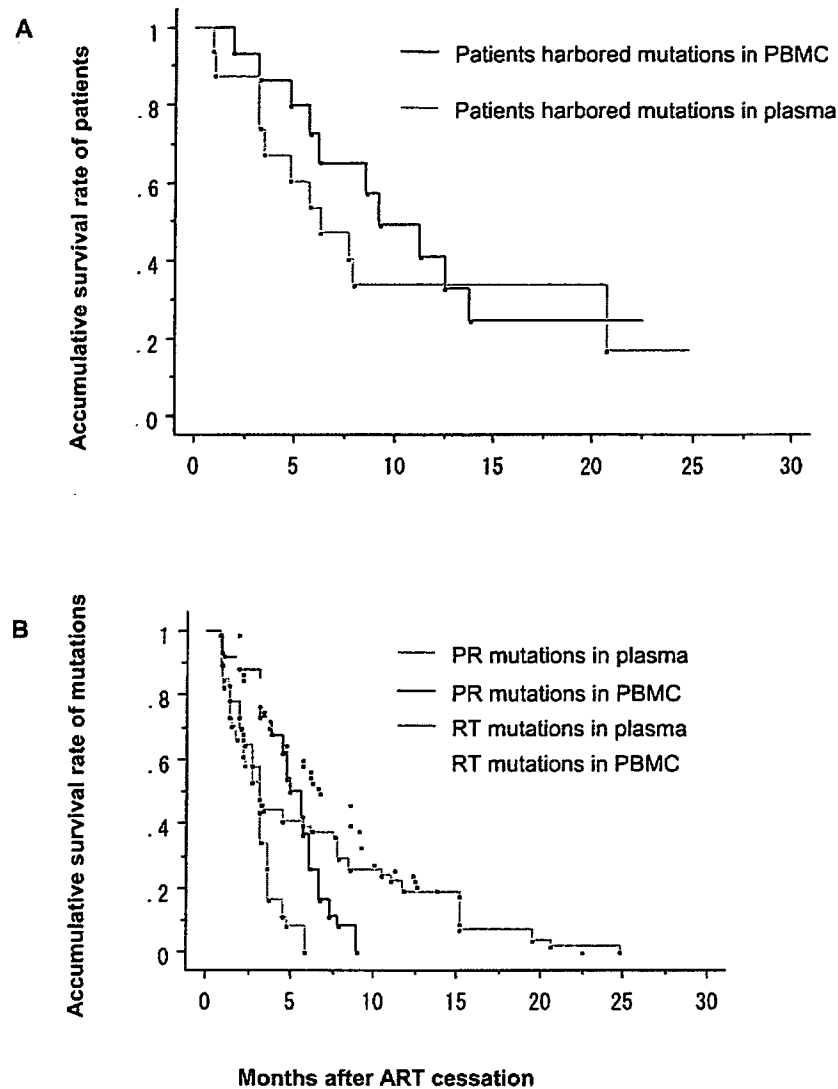


FIG. 1. (A) Kaplan-Meier curves showing percent of 16 patients with drug resistance mutations in plasma or PBMC. (B) Kaplan-Meier curves showing percent of 133 drug resistance mutations (59 RT and 74 PR) in plasma or PBMC. PR, in plasma vs. in PBMC ($p < 0.05$); RT, in plasma vs. in PBMC ($p < 0.05$); PR vs. RT ($p < 0.05$).

We designed the present study with the main objectives of determining the duration of the reversal period from the presence of resistant viruses to wild-type viruses and of elucidating the reversal patterns of plasma- and PBMC-derived viruses after discontinuation of ART. To determine the duration of the reversal period (i.e., from resistant mutations of RT and PR genes of plasma viruses and proviruses to wild type), sequential specimens of plasma and PBMC from patients with resistance mutations were sequenced after ART was interrupted. We found that the PR and RT resistance mutations shifted to wild type much more rapidly in plasma than in PBMC after ART cessation. In 3.2 months after ART stopped, 50% of the resistance mutations in plasma-derived viruses shifted to wild type and 50% of the major mutations of both RT and PR regions were undetected by direct sequencing. This period was similar to that reported by other investigators.^{13,14,16-20} However, 50% of the mutations of RT and PR were detected by 6.7 and 5.7

months, respectively, when PBMC samples were used. Accordingly, when the patient develops virologic failure and drug resistance testing is performed using plasma sample after 3.2 months of ART cessation, the results of the test should be interpreted with caution, especially when deciding subsequent ART regimens, because 50% of mutation residues were undetectable by testing. When a resistant virus is not detected by drug-resistant testing, therapy using the same antiretroviral drugs or the same class of agents that reveal cross resistance is usually associated with early drug failure by previously acquired resistant viruses.^{23,24} Therefore, like other recommendations,^{1,2} drug-resistance testing should be performed soon after ART cessation. However, according to our data, the testing period could be postponed for 2.5 months (from 3.2 to 5.7 months) after ART withdrawal if PBMCs are used instead for plasma. In this regard, PBMC is a suitable candidate specimen for drug-resistance testing during off therapy.

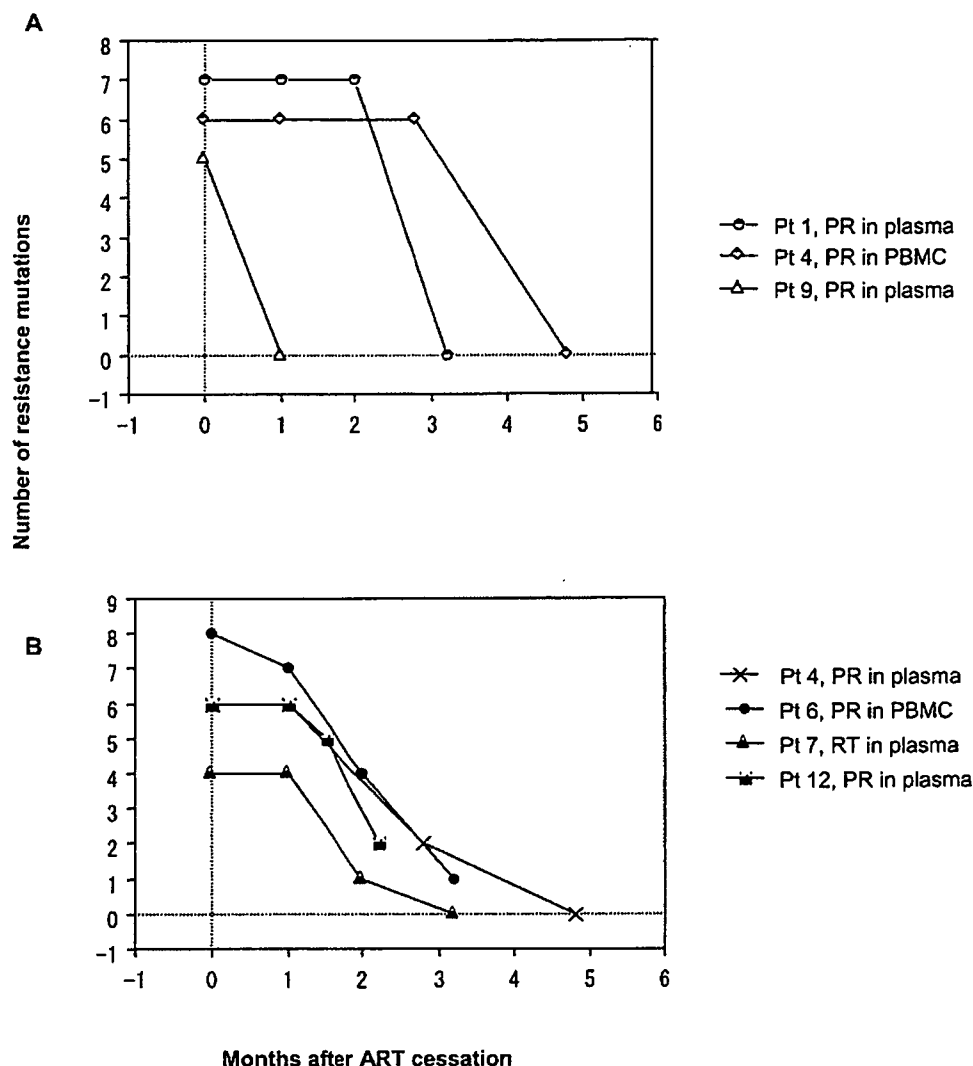


FIG. 2. Two Representative patterns of resistance mutations reverted to wild type after ART cessation. (A) Steep disappearance pattern of resistance mutations; (B) gradual reversal pattern of resistance mutations. RT, reverse transcriptase; PR, protease.

Drug-resistance testing is not advised for patients with VL <1000 copies/ml since amplification of the virus is unreliable.^{1,2} However, if ART has to be discontinued because of ART-related toxicities and VL was undetectable at the time of discontinuation, the timing of the test is a practical question. Others report¹⁷ a sharp reduction in the number of mutations at the time of viral load increase in patients during structured treatment interruption. Our results showed that at 1 month after ART cessation, VL dramatically increased from <1000 copies/ml to >4 log₁₀ copies/ml in 6 patients who stopped treatment due to causes other than ART failure. However, the rebounded viruses in 5 of these 6 patients were still resistance mutant but not the wild-type virus. We previously reported that drug resistance mutations emerged gradually when therapy failed.¹⁰ In contrast, the results here showed that nearly 50% of the mutations disappeared abruptly when ART completely stopped. Thus, waiting for several months after ART withdrawal until stabilization of the VL may potentially result in missing important information for selecting the subsequent ther-

apeutic regimen. Therefore, in such situations, drug-resistance testing should be performed after 1 month to obtain a reliable result after ART withdrawal.

We previously studied the emergence of drug resistance during therapy and reported that the appearance of drug resistance in plasma viruses precedes that in proviruses by more than 1 year and recommended the use of plasma samples for drug-resistance testing during therapy.¹⁰ Considering the high concordance of resistance mutations between plasma and PBMC, and the long persistence period of mutations in PBMC, we conclude that when ART stopped, if PBMC could be used as the sample for resistance assay, the test period may be postponed for 3 months.

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Involvement of the PA28 γ -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 (PA28 $\gamma^{+/+}$ CoreTg) mice develop marked insulin resistance and that the HCV core protein is degraded in the nucleus through a PA28 γ -dependent pathway. In this study, we examined whether PA28 γ is required for HCV core-induced insulin resistance in vivo. HCV core gene-transgenic mice lacking the PA28 γ gene (PA28 $\gamma^{-/-}$ CoreTg) were prepared by mating of PA28 $\gamma^{+/+}$ CoreTg with PA28 γ -knockout mice. Although there was no significant difference in the glucose tolerance test results among the mice, the insulin sensitivity in PA28 $\gamma^{-/-}$ 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 PA28 $\gamma^{+/+}$ CoreTg mice in response to insulin stimulation, whereas they were restored in the livers of PA28 $\gamma^{-/-}$ 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 PA28 γ gene. These results suggest that the HCV core protein suppresses insulin signaling through a PA28 γ -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 PA28 γ /REG γ (11S regulator)-dependent pathway in the nucleus (32). It is well known that PA28 γ enhances latent proteasome activity, although the biological significance of PA28 γ is largely unknown, with the exception that PA28 γ 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 PA28 γ 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 PA28 γ in the induction of insulin resistance by the HCV core protein in vivo.

MATERIALS AND METHODS

Preparation of PA28 γ -knockout HCV core gene-transgenic mice. C57BL/6 mice carrying the gene encoding HCV core protein genotype 1b (PA28 γ ^{+/+} CoreTg) line C49 and PA28 γ ^{-/-} mice have been described previously (35, 36). These two genotypes were crossed to create PA28 γ ^{+/-} CoreTg mice. PA28 γ ^{+/-} CoreTg mice were bred to generate PA28 γ ^{-/-} CoreTg mice (35, 36). The HCV core gene and the target sequence to knock out the PA28 γ 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, PA28 γ , 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 Na₂VO₄, 100 mM NaF, 50 mM Na₂P₂O₇, 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'-GGTACAACCCATCGCTGGCA-3' (forward) and 5'-GCCAGCTGGAAGTGCAGAAAG-3' (reverse) for TNF- α , 5'-ATAG

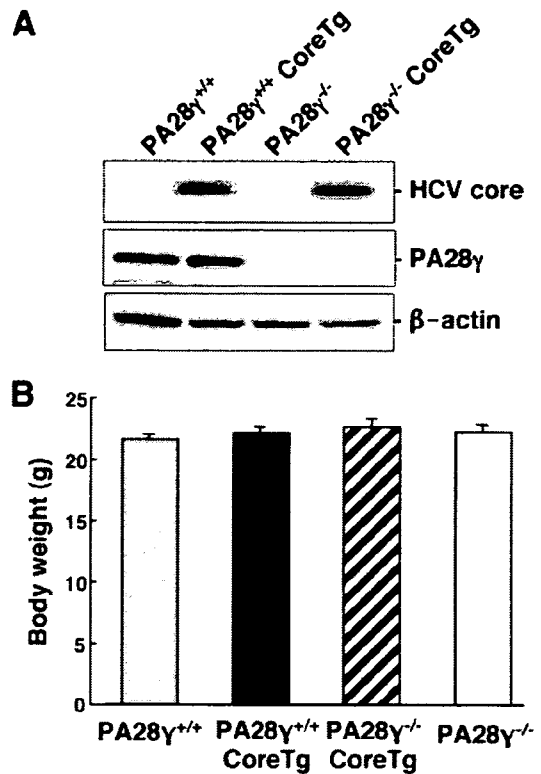


FIG. 1. Characterization of HCV core gene-transgenic mice deficient in the PA28 γ gene. (A) Expression of the HCV core protein and PA28 γ in the livers of PA28 γ ^{+/+}, PA28 γ ^{+/+} CoreTg, PA28 γ ^{-/-}, and PA28 γ ^{-/-} CoreTg mice. Lysates obtained from liver tissues of the mice (100 μ g protein/lane) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting using antibodies to the HCV core protein, PA28 γ , 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'-AGCCTGGGATAATGGTG 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. PA28 γ -knockdown cell lines were established by using pSilencer 2.1 U6 Hygro (Ambion) according to the manufacturer's protocol.

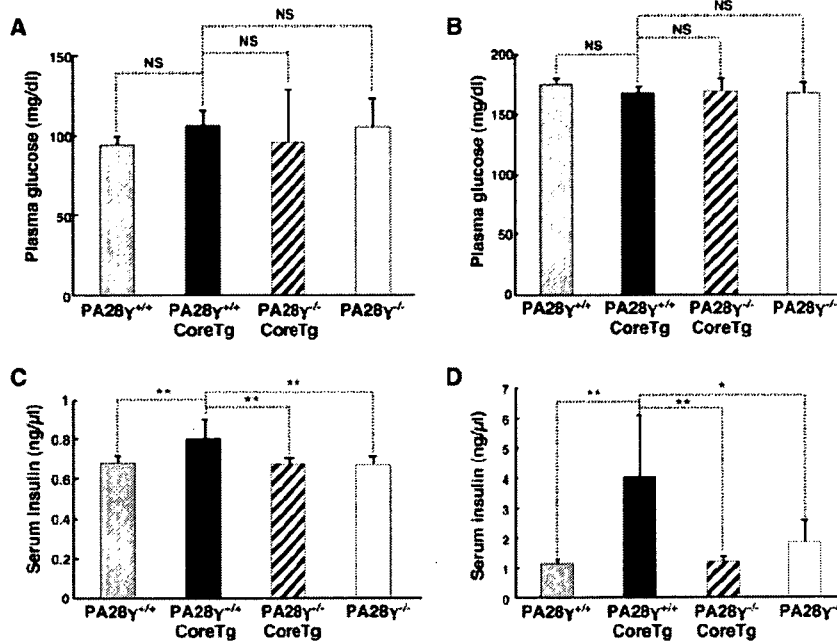


FIG. 2. Knockout of the PA28 γ gene inhibited the hyperinsulinemia induced by HCV core protein. Plasma glucose levels of PA28 $\gamma^{+/+}$, PA28 $\gamma^{+/+}$ CoreTg, PA28 $\gamma^{-/-}$ CoreTg, and PA28 $\gamma^{-/-}$ mice under fasting (A) or fed (B) conditions ($n = 7$ in each group) are shown.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

Statistical analysis. The results are presented as means \pm 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 PA28 γ gene.

To investigate the role of PA28 γ 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 PA28 γ gene (PA28 $\gamma^{-/-}$ CoreTg). A PA28 $\gamma^{+/+}$ CoreTg mouse expressing an amount of PA28 γ 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

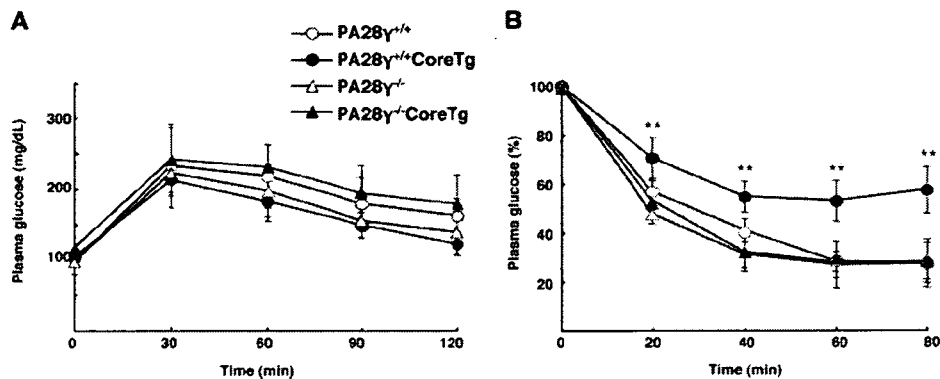


FIG. 3. Knockout of the PA28 γ gene inhibits the insulin resistance induced by the HCV core protein. (A) Glucose tolerance test. D-Glucose was intraperitoneally administered to mice fasted for more than 16 h at 1 g/kg of body weight. Plasma glucose levels were estimated at the indicated times ($n = 5$ 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 \pm 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 ($P > 0.05$).

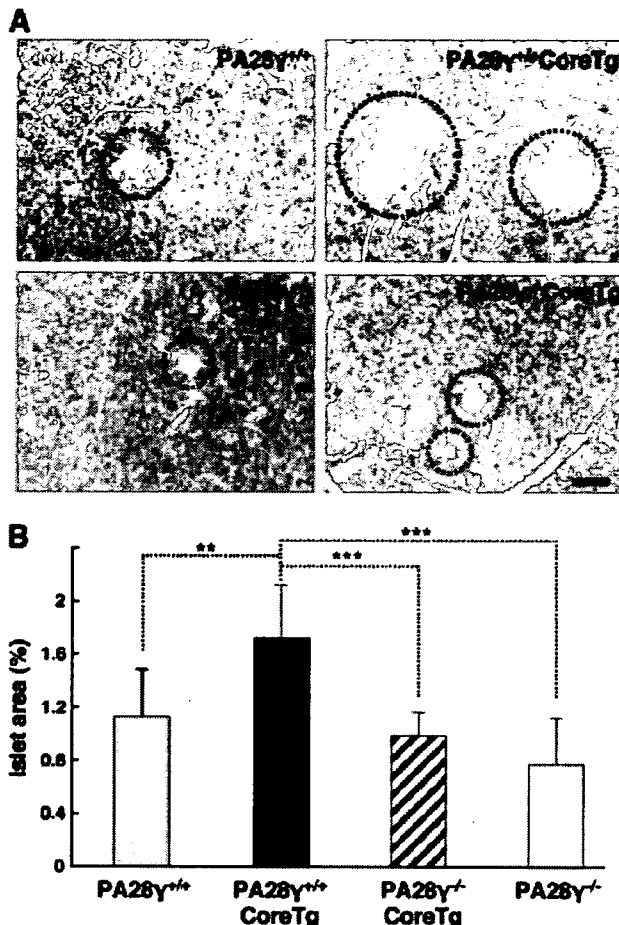


FIG. 4. PA28 γ participated in the enlargement of pancreatic islets induced by the HCV core protein. (A) Histological sections prepared from pancreas tissues of PA28 $\gamma^{+/+}$, PA28 $\gamma^{+/+}$ CoreTg, PA28 $\gamma^{-/-}$, and PA28 $\gamma^{-/-}$ CoreTg mice were stained with hematoxylin and 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 μ m.

mice but not in PA28 $\gamma^{+/+}$ (normal littermates) or PA28 $\gamma^{-/-}$ mice. PA28 γ 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

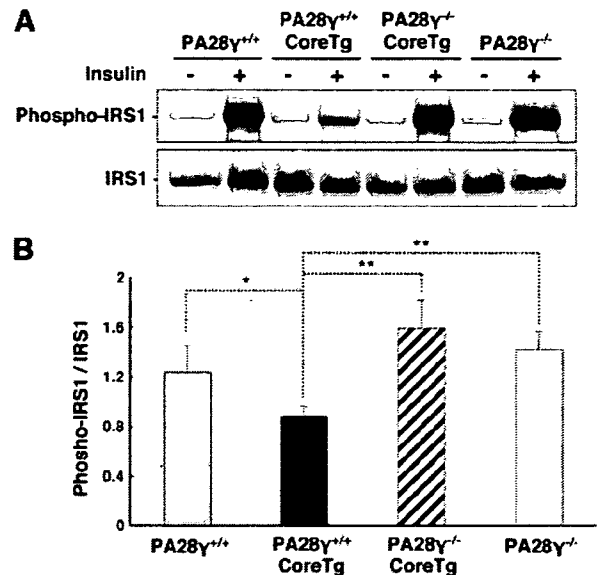


FIG. 5. PA28 γ participated in the inhibition of the tyrosine phosphorylation of IRS1 induced by the HCV core protein. Liver tissues from PA28 $\gamma^{+/+}$, PA28 $\gamma^{+/+}$ CoreTg, PA28 $\gamma^{-/-}$, and PA28 $\gamma^{-/-}$ CoreTg mice were prepared after administration of insulin (+) or phosphate-buffered saline (-). The samples (100 μ g 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. * $P < 0.05$; ** $P < 0.01$.

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).

PA28 γ 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 PA28 γ 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-

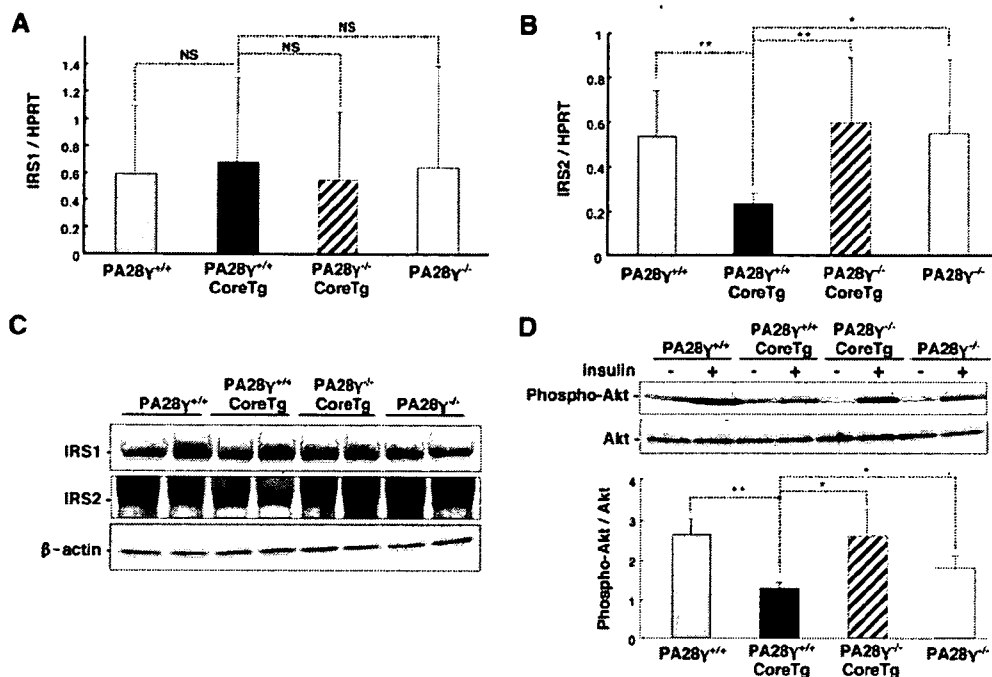


FIG. 6. PA28 γ participated in the inhibition of the IRS2 expression and Akt phosphorylation induced by HCV core protein. The transcription of IRS1 (A) and IRS2 (B) was estimated by quantitative RT-PCR ($n = 5$ in each group). (C) The expression levels of IRS1 and IRS2 in the livers of the mice were 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 PA28 $\gamma^{+/+}$ CoreTg mice (47). In this study, PA28 $\gamma^{-/-}$ CoreTg mice exhibited a normal insulin level comparable to those of PA28 $\gamma^{+/+}$ and PA28 $\gamma^{-/-}$ mice by an insulin tolerance test, in contrast to PA28 $\gamma^{+/+}$ 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 PA28 $\gamma^{+/+}$ CoreTg mice to compensate for insulin resistance and retain a physiological level of plasma glucose and that PA28 γ participates in the development of hyperinsulinemia and insulin resistance in PA28 $\gamma^{+/+}$ 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 PA28 $\gamma^{+/+}$ CoreTg mice. To clarify whether a knockout of the PA28 γ 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 PA28 $\gamma^{-/-}$ CoreTg mice was smaller than that of PA28 $\gamma^{+/+}$ CoreTg mice and comparable to that of PA28 $\gamma^{+/+}$ and PA28 $\gamma^{-/-}$ mice (Fig. 4B). Infiltration of inflammatory cells within or surrounding the islets was not found in all genotypes of mice. These results suggest that PA28 γ also participates in the enlargement of pancreatic islets induced in PA28 $\gamma^{+/+}$ CoreTg mice.

PA28 γ 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 PA28 $\gamma^{+/+}$ CoreTg mice and that the administration of anti-TNF- α antibody restores insulin sensitivity (47). We examined whether a knockout of the PA28 γ gene could restore the tyrosine phosphorylation of IRS1. Tyrosine phosphorylation of IRS1 was suppressed in the livers of PA28 $\gamma^{+/+}$ CoreTg mice in response to insulin stimulation, whereas it was recovered in PA28 $\gamma^{-/-}$ CoreTg mice to levels comparable to those in PA28 $\gamma^{+/+}$ and PA28 $\gamma^{-/-}$ 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 PA28 $\gamma^{+/+}$ 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

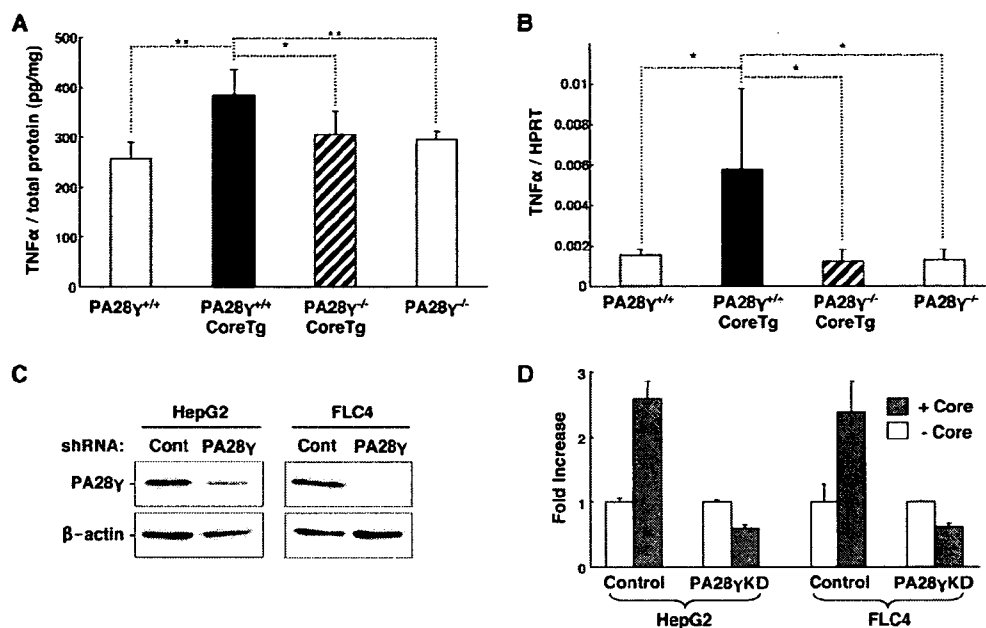


FIG. 7. PA28 γ was required for activation of the TNF- α promoter by the HCV core protein. (A) Expression of TNF- α in the livers of mice was determined by ELISA ($n = 5$ in each group). (B) TNF- α mRNA in the livers of mice was examined by quantitative RT-PCR ($n = 5$ in each group). (C) Knockdown of the expression of PA28 γ in the HepG2 and FLC-4 cell lines by the introduction of a plasmid encoding a short hairpin RNA (shRNA) targeted to the PA28 γ gene. The expression levels of PA28 γ 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 PA28 γ -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 PA28 $\gamma^{+/+}$ 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 PA28 $\gamma^{+/+}$ CoreTg mice but not in those of PA28 $\gamma^{+/+}$, PA28 $\gamma^{-/-}$, or PA28 $\gamma^{-/-}$ CoreTg mice (Fig. 6D). These results suggest that the expression of the HCV core protein in the livers of mice in the presence of PA28 γ impairs the insulin-signaling pathway through the suppression of both the tyrosine phosphorylation of IRS1 and the expression of IRS2.

PA28 γ 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 PA28 $\gamma^{+/+}$ CoreTg mice (47). To determine the involvement of PA28 γ 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 PA28 $\gamma^{+/+}$ CoreTg mice but were restored in the livers of PA28 $\gamma^{-/-}$ CoreTg mice to levels comparable to those of PA28 $\gamma^{+/+}$ and PA28 $\gamma^{-/-}$ mice. To determine the effect of PA28 γ expression on the promoter activity of TNF- α in human liver cells, PA28 γ -knockdown human hepatoma cell lines HepG2 and FLC4 were

established by the introduction of a plasmid encoding a short hairpin RNA targeting the PA28 γ gene in the cell lines. The expression of PA28 γ 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 PA28 γ -knockdown cell lines (Fig. 7D). These results suggest that PA28 γ 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 lipolysis (21). Activated PI3-kinase phosphorylates phosphatidylinositol 4,5-bisphosphate to produce phosphatidylinositol 3,4,5-trisphosphate, 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, PA28 $\gamma^{+/+}$ CoreTg mice may exhibit a milder phenotype than IRS1- and/or IRS2-knockout mice. In this study, knockout of the PA28 γ gene restored the insulin sensitivity and signaling of IRS1 and IRS2 in PA28 $\gamma^{+/+}$ CoreTg mice, suggesting that the expression of the HCV core protein leads to the dysfunction of both IRS1 and IRS2 through a PA28 γ -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 PA28 $\gamma^{+/+}$ CoreTg mice, resulting in the suppression of phosphorylation of IRS1, and insulin sensitivity in PA28 $\gamma^{+/+}$ CoreTg was improved by the administration of an anti-TNF- α antibody (47). In the present study, the expression level of TNF- α in PA28 $\gamma^{-/-}$ CoreTg mice was similar to that in PA28 $\gamma^{-/-}$ 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 PA28 γ gene by RNA interference (Fig. 7D). These data suggest that PA28 γ 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 PA28 γ 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 PA28 $\gamma^{+/+}$ CoreTg mice was similar to that in PA28 $\gamma^{-/-}$ 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 PA28 $\gamma^{+/+}$ 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 PA28 γ -dependent pathway (32). Recently, PA28 γ 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 PA28 γ -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/PA28 γ -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 PA28 γ -dependent pathway, thereby leading to insulin resistance. PA28 γ may be a novel target for the treatment of HCV-induced diabetes.

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