写真の説明

　肝炎の原因ウイルスは，1970年代までにA型およびB型肝炎ウイルスが発見されたが，同時にこれらのウイルスでは引き起こされない血液媒介性の肝炎，いわゆる非A非B型肝炎の存在が明らかとなった．非A非B型肝炎の病原体の分離には困難を極めたが，唯一の動物実験系であるチンパンジーを用いた感染実験でフラビウイルスに近縁のRNAウイルスである事が予測され，1989年に米国カイロン社のホートン博士らが遺伝子クローニングに成功してC型肝炎ウイルス（HCV）ゲノムの構造と塩基配列が同定された．この遺伝子産物を抗原として世界中の患者血液が調べられた結果，現在全世界では約1億2千万人の抗体陽性者がいると推定される（図Ⅰ）．我が国でも抗体陽性者は200万人前後いると推定されているが，さらに問題となっているのはHCVが持続感染を成立しやすく，肝硬変，肝癌へと進展する点で，日本の肝癌患者さんの多くはHCV抗体陽性である．HCVはRNAウイルスであり細胞質で主に複製増殖すると同時に，典型的な癌遺伝子は持たず宿主細胞のDNAに組み込まれる事もないと考えられている（図Ⅱ）．このようなHCVがいかに細胞を癌化するかを解析するためには感染系が必要だが，現在までのところ肝癌細胞以外には確立されておらず腫瘍原性解析は困難な状況である．これに対して私達は，HCV全長遺伝子をCre/loxPシステムを用いてどんな細胞でも必要な時に発現できるユニットを構築し腫瘍原性解析を可能にしている（図Ⅲ-a）．本ユニットでHCV蛋白質をスイッチング発現すると持続的に発現が維持されるので慢性感染時の肝細胞におけるウイルスの作用のモデル系として容易に解析が可能となる（図Ⅲ-b）．継代44日以降には細胞の腫瘍原性が亢進し（図Ⅲ-c），標的経路の1つはcdk-Rb-E2F経路である事を明らかにした（図Ⅲ-d）．さらに，本システムを用いて抗体を樹立し，HCVの新たな標的経路を解明している．

Ⅰ．世界におけるC型肝炎ウイルス感染者の分布
　　HCV感染者は全世界で約1億2千万人とされ（*Lancet Infect. Dis.*, 5: 558-567, 2005），エジプトなど一部の地域では高い抗体陽性率を示す．

Ⅱ．C型肝炎ウイルスの生活環とウイルスゲノム構造
　　肝臓細胞におけるHCVの複製の場は細胞質であり，ウイルス遺伝子はプラス1本鎖RNA（約9.6kb）でウイルス蛋白質は1本のポリプロテインから切断されて機能を持つ．

Ⅲ．C型肝炎ウイルスによる腫瘍原性亢進
(a) Cre/loxPシステムを用いた全長HCV遺伝子発現システム（*J. Biol. Chem.*, 279 (15): 14531-14541, 2004）
　　HCV遺伝子は，Cre酵素で遺伝子発現後，リボザイム配列で正確に切断される．
(b) (a)のユニットを持つ細胞株でのHCVコア蛋白質発現（ELISAによる定量）
(c) HCV発現継代細胞はコロニー形成能やヌードマウス腫瘍形成能が亢進している．
(d) HCV発現継代細胞で活性化されるシグナル経路
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Original Article

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

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

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

INTRODUCTION

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

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

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

METHODS

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

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

RESULTS

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

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
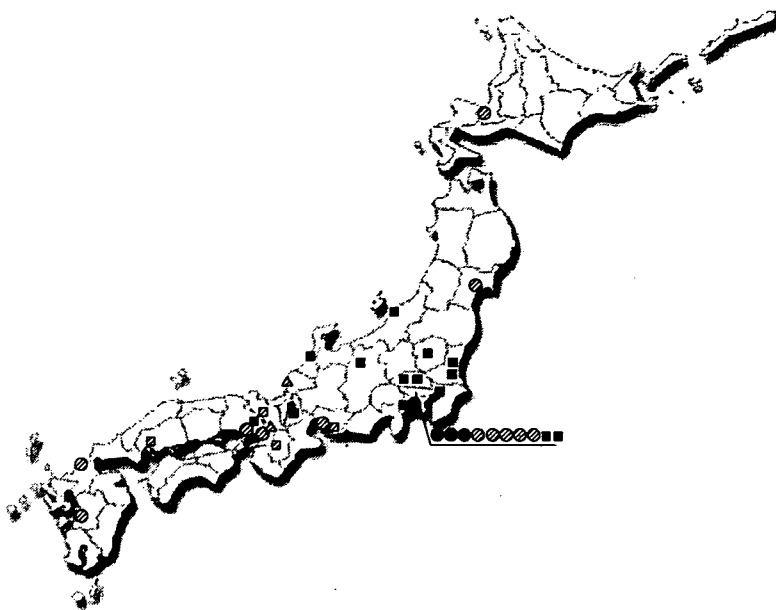
Figure 1 Nationwide distribution of hospitals in the HIV/AIDS Network of Japan that a number of HIV-positive or HIV/HCV coinfected patients are visiting regularly. Note that in the Kanto area, HIV/HCV coinfected patients were concentrated in the HIV/AIDS Network hospitals in the Tokyo city area. (△) 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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Pathogenesis of HCV-associated HCC: Dual-pass carcinogenesis through activation of oxidative stress and intracellular signaling

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Overwhelming lines of epidemiological evidence have indicated that persistent infection with hepatitis C virus (HCV) is a major risk toward development of hepatocellular carcinoma (HCC). It remains controversial, however, in the pathogenesis of HCC associated with HCV, whether the virus plays a direct role or merely an indirect one. The studies using transgenic mouse models by us and others, in which the core protein of HCV has oncogenic potential, indicate that HCV is directly involved in hepatocarcinogenesis, albeit other factors such as continued cell death and regeneration associated with inflammation would play a role, as well. The downstream events of the core protein are segregated into two components. One is the augmented production of oxidative stress along with the activation of scavenging system including catalase and glutathion (GSH) in the putative preneoplastic stage with steatosis in the liver. Thus, oxidative stress production in the absence of inflammation by the core protein would partly contribute to the development of HCC. The generation of oxidative stress is estimated to originate from mitochondrial dysfunction in hepatocytes by HCV infection. The other is the alteration of intracellular signaling cascade of MAPK (JNK), AP-1, cyclin D1, and CDK4. The combination of these pathways, collective with HCV-associated alterations in lipid and glucose metabolism, would lead to the frequent development of HCC in persistent HCV infection. Our results suggest that there would be a mechanism for hepatocarcinogenesis in persistent HCV infection that is distinct from those for other cancers. Similar to the pathogenesis of other cancers, the accumulation of a set of genetic aberrations may also be necessary for multistage development of HCC. However, HCV core protein, to which an oncogenic potential is ascribed, may allow some of the multiple steps to be bypassed in hepatocarcinogenesis. Therefore, unlike other cancers, HCV infection can elicit HCC in the absence of a complete set of genetic aberrations. Such a scenario, "non-Vogelstein-type" carcinogenesis, would explain the unusually high incidence and multicentric nature of HCC development in HCV infection.

Key words: hepatitis C virus, hepatocarcinogenesis, intracellular signaling transduction, oxidative stress, transgenic mouse

INTRODUCTION

WORLDWIDE, HEPATITIS C virus (HCV) infects hundreds of millions of people persistently, and induces a spectrum of chronic liver diseases.[1] Hence, it affects society in a number of domains including medical, sociological, and economic. Hepatocellular carcinoma (HCC) has become the most frequent cause of death in individuals persistently infected with HCV. In particular, HCV has been given increasing attention because of its wide and deep penetration in the community, coupled with a very high incidence of HCC in persistent HCV infection. Once liver cirrhosis is established in hosts infected with HCV, HCC develops at a yearly rate of 5–7%.[2] Knowledge of the mechanism of HCC development in chronic HCV infection, therefore, is imminently required for the prevention of HCC.

UNIQUENESS OF HCC DEVELOPMENT IN HCV INFECTION

HOW HCV INDUCES HCC is not yet clear, despite the finding that more than 70% of patients with HCC in Japan are infected with HCV.[1,3,4] HCV infection

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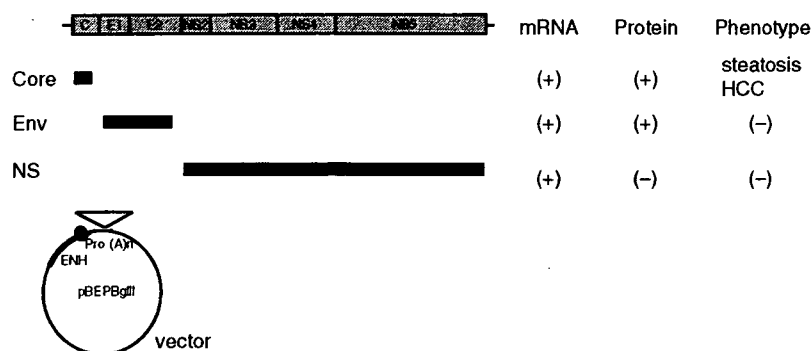
Figure 1 Hepatitis C virus (HCV) transgenic mouse lines. Among the three different transgenic mouse lines established, only the transgenic mice carrying the HCV core gene develop hepatocellular carcinoma (HCC) after an early phase with hepatic steatosis in two independent lineages. The mice transgenic for the envelope genes or non-structural genes do not develop HCC. core, core genes, env, envelope genes; NS, non-structural genes.

is also common in patients with HCC in other countries, albeit to a lesser extent. These lines of evidence obligate hepatologists to a considerable task of determining the role of HCV in hepatocarcinogenesis. Inflammation induced by HCV, manifesting in various forms of hepatitis, should be considered in a study on the carcinogenic capacity of hepatitis viruses. It has been proposed repeatedly that the necrosis of hepatocytes caused by chronic inflammation and ensuing regeneration enhances mutagenesis in host cells, the accumulation of which culminates in HCC. This theory presupposes an indirect involvement of hepatitis viruses in HCC *via* hepatic inflammation. However, this leaves specialists in hepatology with a serious question: can inflammation *per se* result in the development of HCC in such a high incidence or multicentric nature in HCV infection? The secondary role of HCV would have to be weighed against an extremely rare occurrence of HCC in patients with autoimmune hepatitis in whom severe inflammation in the liver persists indefinitely.

This background and reasoning lead to a possible activity of viral proteins for inducing HCC. This possibility has been evaluated by introducing genes of HCV into hepatocytes in culture with little success. A difficulty in using cultured cells is the carcinogenic capacity of HCV, if any, which would be weak and would take a long time to manifest. It takes 30–40 years for HCC to develop in individuals infected with HCV. Another constraint common to studies on carcinogenesis is the development of HCC by transformed cells that might have gone out of growth control and escaped surveillance of the host. Should this be the case, the analysis of transformed cells would not be sufficient for solving the mystery of carcinogenesis. On the basis of these viewpoints, we started tackling carcinogenesis in chronic viral hepatitis by transgenic mouse technology.

CORE PROTEIN OF HCV HAS ONCOGENIC ACTIVITY *IN VIVO*

AS ILLUSTRATED IN Figure 1, transgenic mouse lines with parts of the HCV genome were engineered by introducing the genes excised from the cDNA of the HCV genome of genotype 1b.[5,6] The background of the mouse lines is a C57BL/6 strain, which is known for a rare spontaneous occurrence of HCC.[7] Established are three different transgenic mouse lines, which carry the core gene, envelope genes, or non-structural genes, under the same transcriptional control element. Among these mouse lines, only the transgenic mice carrying the core gene develop HCC in two independent lineages (Fig. 1).[6] The envelope gene transgenic mice do not develop HCC, despite high expression levels of both E1 and E2 proteins.[8,9] The transgenic mice carrying the entire non-structural genes have not developed HCC.

The transgenic mice carrying the core gene express the core protein of an expected size, approximately 21 kDa, the level of which in the liver is similar to that in the liver of chronic hepatitis C patients. Early in life, these mice develop hepatic steatosis, which is a histologic characteristic of chronic hepatitis C, along with lymphoid follicle formation and bile duct damage.[10] Thus, the core gene transgenic mouse model well reproduces this feature of chronic hepatitis C. Of note, significant inflammation is not observed in the liver of this animal model. Late in life, these transgenic mice develop HCC. Most hepatic nodules disclose a pathology characterized by "nodule-in-nodule", and HCC with a low degree of differentiation develops within adenoma as well as within HCC with a higher degree of differentiation.[6] Although numerous lipid droplets are found in cells forming adenoma, as in non-tumorous cells, they are rarely observed in HCC cells. These histologic features

closely resemble those observed in HCC developing in chronic hepatitis C patients, in which prominent lipid droplets are found in small differentiated HCC and its precursors; poorly differentiated HCC without lipid droplets develops from within differentiated HCC.[6] Notably, the development of steatosis and HCC has been reproduced by other HCV transgenic mouse lines, which harbor the entire HCV genome or structural genes including the core gene.[11] These outcomes indicate that the core protein of HCV has an oncogenic potential when expressed *in vivo*.

MECHANISM OF HEPATOCARCINOGENESIS IN MOUSE MODEL FOR HCV-ASSOCIATED HCC

IT IS DIFFICULT to sort out the mechanism of carcinogenesis even for our simple model, in which only the core protein is expressed in otherwise normal liver tissue. There is a notable feature in the localization of the core protein in hepatocytes; while the core protein predominantly exists in the cytoplasm associated with lipid droplets, it is also present in the mitochondria and nuclei.[6,12] On the basis of this finding, the pathways related to these two organelles, the mitochondria and nuclei, were meticulously analyzed.

One activity of the core protein is an increased production of oxidative stress in the liver. The production of oxidative stress is increased in our transgenic mouse model in the absence of inflammation in the liver (hepatitis). This reflects a state of an overproduction of reactive oxygen species (ROS) in the liver, or predisposition to it, which is staged by the HCV core protein without any intervening inflammation.[13,14] The overproduction of oxidative stress results in the generation of deletions in the mitochondrial DNA, an indicator of genetic damage. Thus, the core protein induces oxidative stress overproduction in the absence of inflammation, and may, at least in part, contribute to hepatocarcinogenesis in HCV infection. If inflammation is induced in the liver with the HCV core protein, the production of oxidative stress is escalated to an extent that can no longer be scavenged by a physiologically antagonistic system. This indicates that the inflammation in chronic HCV infection would have a characteristic different in quality from those of other types of hepatitis, such as autoimmune hepatitis. The basis for the overproduction of oxidative stress may be ascribed to mitochondrial dysfunction.[13,15] The function of the electron transfer system of the mitochondrion is suggested in association

Table 1 Biomolecular alterations with core protein expression observed in the transgenic mouse model

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1.	Induction of cytokines including TNF-α and IL-1β[19]
2.	Activation of MAPK pathway and enhancement of AP-1 activation[19,20]
3.	Overproduction of oxidative stress or ROS in the absence of inflammation[13]
4.	Synergy of HCV core and alcohol in inducing oxidative stress and activating MAPK[13,20]
5.	Interaction of HCV core and RXR-α and PPAR-α[21]
6.	Induction of insulin resistance[17]
7.	Development of steatosis by inhibiting MTP activity[5,14,22]
8.	Interaction of HCV core and proteasome activator PA28γ[23]
9.	Inhibition of SOCS-1[24]

AP-1, activated protein-1; HCV, hepatitis C virus; IL-1β, interleukin-1β; MAPK, mitogen-activated protein kinase; MTP, microsomal triglyceride transfer protein; PPAR-α, peroxisome proliferator agonist receptor-α; ROS, reactive oxygen species; RXR-α, retinoid X receptor; SOCS-1, suppressor of cytokine signaling; TNF-α, tumor necrosis factor.

with the presence of the HCV core protein.[16] Hepatic steatosis in hepatitis C may work as fuel for oxidative stress overproduction.[14,17,18]

Other possible pathways are the alteration of the expression of cellular genes, interacting with cellular proteins, and modulation of intracellular signaling pathways (Table 1). For example, tumor necrosis factor (TNF)-α and interleukin-1β (IL-1β) have been found transcriptionally activated.[19] The core protein has also been found to interact with some cellular proteins, such as retinoid X receptor (RXR)-α, that play pivotal roles in cell proliferation and metabolism.[20] The mitogen-activated protein kinase (MAPK) cascade is also activated in the liver of the core gene transgenic mouse model. The MAPK pathway, which consists of three routes, c-Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK), is involved in numerous cellular events including cell proliferation. In the liver of the core gene transgenic mouse model prior to HCC development, only the JNK route is activated. Downstream in the JNK activation, transcription factor AP-1 activation is markedly enhanced.[19,21] Far downstream, both the mRNA and protein levels of cyclin D1 and CDK4 are increased. Thus, the HCV core protein modulates the intracellular signaling pathways and gives advantage for cell proliferation to hepatocytes.

Such an effect of the core protein on the MAPK pathway, combined with that on oxidative stress, may
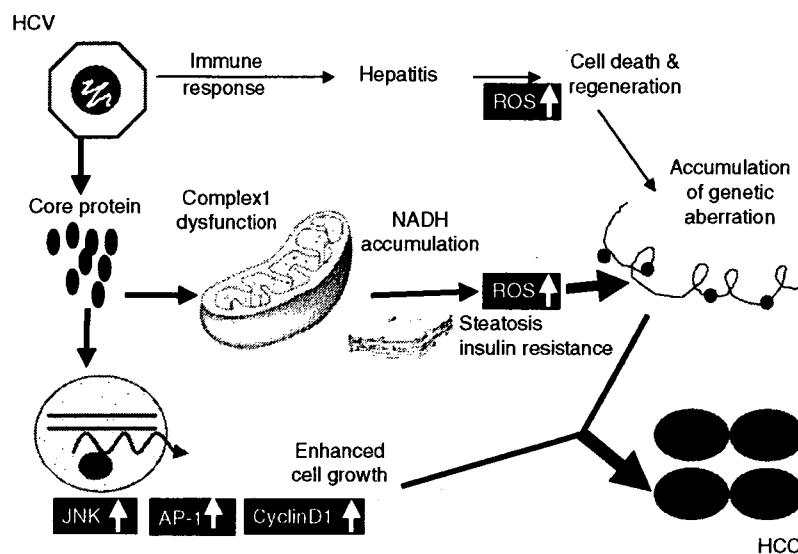
Figure 2   Mechanism of hepatitis C virus (HCV)-associated hepatocarcinogenesis. Inflammation should contribute to hepatocarcinogenesis by producing genetic aberrations via continual cell death and regeneration. In the case of HCV infection, the virus would contribute to hepatocarcinogenesis via two pathways: (i) the core protein acts on the function of mitochondrial electron transfer system, leading to the overproduction of oxidative stress. Inflammation may act synergistically with the core protein in inducing oxidative stress. The presence of steatosis and insulin resistance would enhance the production of oxidative stress; and (ii) modulation of cellular gene expression and signal transduction, which would give a growth advantage to hepatocytes. The combination of these alterations would escalate the development of hepatocellular carcinoma (HCC) in HCV infection. AP-1, activated protein-1; JNK, Jun N-terminal kinase; NADH, nicotinamide adenine dinucleotide; ROS, reactive oxygen species.

explain the extremely high incidence of HCC development in chronic hepatitis C.

HEPATOCARCINOGENESIS INDUCED BY HCV INFECTION: MECHANISM DISTINCT FROM OTHER CANCERS

THE RESULTS OF our studies on transgenic mice indicated a carcinogenic potential of the HCV core protein *in vivo*; thus, HCV may be directly involved in hepatocarcinogenesis.

In research studies of carcinogenesis, the theory by Kinzler and Vogelstein[25] has gained wide popularity. They proposed that the development of colorectal cancer is induced by the accumulation of a complete set of cellular gene mutations. They deduced that mutations in the *APC* gene for inactivation, those in *K-ras* for activation and those in the *p53* gene for inactivation accumulate, which cooperate toward the development of colorectal cancer.[25] The theory has been extended to the carcinogenesis of other cancers as well, called "Vogelstein-type" carcinogenesis (Fig. 2).

On the basis of results we obtained for the induction of HCC by the HCV core protein, we introduce a mechanism different from that of Kinzler and Vogelstein[25] for hepatocarcinogenesis in HCV infection. We allow multistages in the induction of all cancers; it would be mandatory for hepatocarcinogenesis that many mutations accumulate in hepatocytes. Some of these steps, however, may be skipped in the development of HCC in HCV infection to which the core protein would contribute (Fig. 3). The overall effects achieved by the expression of the viral protein would be the induction of HCC, even in the absence of a complete set of genetic aberrations, required for carcinogenesis.

By considering such a "non-Vogelstein-type" process for the induction of HCC, a plausible explanation may be given for many unusual events happening in HCV carriers.[26] Now it does not seem so difficult as before to determine why HCC develops in persistent HCV infection at an outstandingly high incidence. Our theory may also give an account of the non-metastatic and multicentric *de novo* occurrence characteristics of HCC, which would be the result of persistent HCV infection.
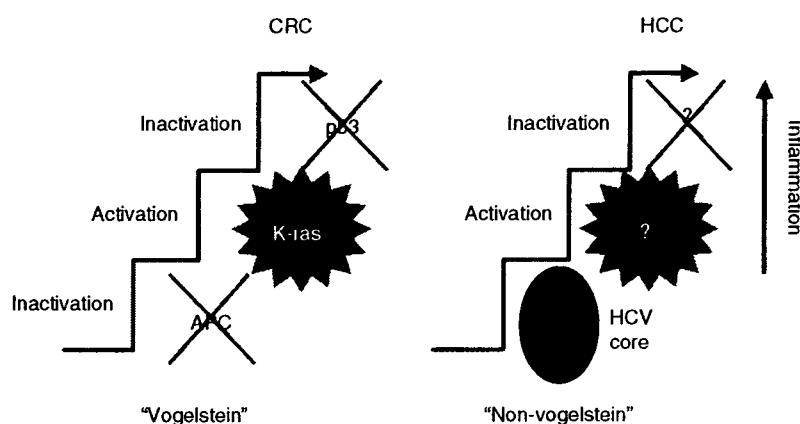
Figure 3 Hepatitis C virus (HCV)-associated hepatocarcinogenesis is a non-Vogelstein-type. Multiple steps are required in the induction of all cancers; it would be mandatory for hepatocarcinogenesis that genetic mutations accumulate in hepatocytes. However, in HCV infection, some of these steps may be skipped in the development of hepatocellular carcinoma (HCC) in the presence of core protein. Overall effects achieved by the expression of core protein would be the induction of HCC, even in the absence of a complete set of genetic aberrations, required for carcinogenesis. By considering such a "non-Vogelstein-type" process for the induction of HCC, a plausible explanation may be given for many unusual events in HCV carriers. CRC, colorectal cancer.

CONFLICT OF INTEREST

NO CONFLICT OF interest has been declared by the author.

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DYSFUNCTION OF ENERGY METABOLISM IN HEPATIC CARCINOGENESIS

Hepatitis C virus contributes to hepatocarcinogenesis by modulating metabolic and intracellular signaling pathways

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Abstract

Persistent infection with hepatitis C virus (HCV) is a major risk factor for development of hepatocellular carcinoma (HCC). However, it remains controversial in the pathogenesis of HCC associated with HCV as to whether the virus plays a direct or an indirect role. The studies using transgenic mouse models, in which the core protein of HCV has an oncogenic potential, indicate that HCV is directly involved in hepatocarcinogenesis, albeit other factors such as continued cell death and regeneration associated with inflammation would also play a role. The downstream events of the core protein are segregated into two components. One is the augmented production of oxidative stress along with the activation of scavenging system, including catalase and glutathione, in the putative pre-neoplastic stage with steatosis in the liver. Thus, oxidative stress production in the absence of inflammation by the core protein would partly contribute to the development of HCC. The generation of oxidative stress is estimated to originate from mitochondrial dysfunction in hepatocytes by HCV infection. The other component is the alteration of intracellular signaling cascade of mitogen-activated protein kinase and activating factor (AP)-1, leading to the activation of cell cycle control. The combination of these pathways, collective with HCV-associated alterations in lipid and glucose metabolism, would lead to the frequent development of HCC in persistent HCV infection. These results suggest that there would be a mechanism for hepatocarcinogenesis in persistent HCV infection that is distinct from those for the other cancers. Similar to the pathogenesis of other cancers, the accumulation of a set of genetic aberrations may also be necessary for a multistage development of HCC. However, HCV core protein, to which an oncogenic potential is ascribed, may allow some of the multiple steps to be bypassed in hepatocarcinogenesis. Therefore unlike for other cancers, HCV infection may be able to cause HCC in the absence of a complete set of genetic aberrations. Such a scenario, 'non-Vogelstein-type' carcinogenesis, would explain the rare feature of hepatocarcinogenesis in HCV infection, the extraordinarily high incidence and the multicentric nature of HCC development.

Introduction

Hepatitis C virus (HCV) infects hundreds of millions of people persistently, and induces a spectrum of chronic liver disease worldwide.[1] It impacts on society in a number of domains including the medical, sociological and economic. Hepatocellular carcinoma (HCC) has become the major cause of death in individuals persistently infected with HCV. In particular, HCV has been given increasing attention because of its wide and deep penetration in the community, coupled with a very high incidence of HCC in persistent HCV infection. Once liver cirrhosis is established in hosts infected with HCV, HCC develops at a yearly rate of 5-7%.[2] Knowledge on the mechanism of HCC development in chronic HCV infection therefore is urgently required for the prevention of HCC.

Hepatocellular carcinoma frequently develops in persistent HCV infection
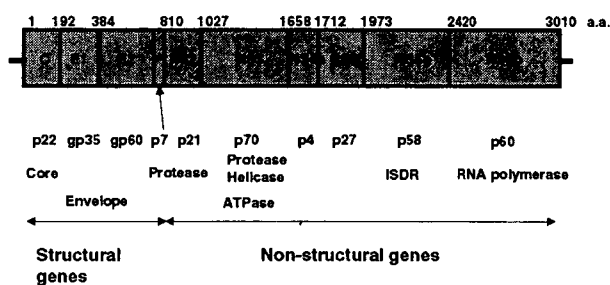
How HCV induces HCC is not clear yet, despite the fact that more than 70% of patients with HCC in Japan are infected with HCV.[1,3,4] Hepatitis C virus infection is also common in patients with HCC in other countries albeit to a lesser extent. These lines of evidence obligate hepatologists to the considerable task of determining the role of HCV in hepatocarcinogenesis. Inflammation induced by HCV, manifesting in various forms of hepatitis, should be considered in a study on the carcinogenic capacity of hepatitis viruses. It has been proposed repeatedly that the necrosis of hepatocytes due to chronic inflammation and ensuing regeneration enhances mutagenesis in host cells, the accumulation of which culminates in HCC. This theory presupposes an indirect involvement of hepatitis

viruses in HCC via hepatic inflammation. However, this leaves specialists in hepatology with a serious question: can inflammation per se result in the development of HCC in such a high incidence or multicentric nature in HCV infection? The secondary role of HCV would have to be weighed against an extremely rare occurrence of HCC in patients with autoimmune hepatitis in whom severe inflammation in the liver persists indefinitely.

This background and reasoning led to the suggestion that HCC may be induced, at least in part, by viral proteins. This possibility has been evaluated by introducing genes of HCV into hepatocytes in culture with little success. One of the difficulties in using cultured cells is the carcinogenic capacity of HCV, if any, which would be weak and would take a long time to manifest itself. It takes 30–40 years for HCC to develop in individuals infected with HCV. Another constraint common to studies on carcinogenesis is the development of HCC by transformed cells that might have gone out of growth control and escaped surveillance of the host. Should this be the case, the analysis of transformed cells would not be sufficient for solving the mystery of carcinogenesis. On the basis of these points, we chose to investigate carcinogenesis in chronic viral hepatitis using transgenic mouse technology.

HCV core protein has an oncogenic activity in transgenic mouse

Transgenic mouse lines with sections of the HCV genome were engineered by introducing genes excised from the cDNA of the HCV genome of genotype 1b.[5,6] The mouse lines were from a C57BL/6 strain, which is known for a rare spontaneous occurrence of HCC.[7] Three different transgenic mouse lines have been established, which carry the core gene, envelope genes or non-structural genes (Fig. 1), respectively, under the same transcriptional control element. Among these mouse lines, only the transgenic mice car-

1 192 384   810 1027   1658 1712 1973   2420   3010 a.a.

p22 gp35 gp60 p7 p21   p70   p4 p27   p58   p60
                          Protease
Core   Protease Helicase   ISDR   RNA polymerase

    Envelope   ATPase

Structural   Non-structural genes
genes

Figure 1 Structure of the hepatitis C virus (HCV) genome. The HCV genome consists of structural and non-structural regions. The structural region consists of the core, envelope and p7 genes. The non-structural region cones enzyme proteins of NS3 to NS5B. Among the three different transgenic mouse lines established, which carry the core, envelope and non-structural region, respectively, only the transgenic mice carrying the HCV core gene develop hepatocellular carcinoma (HCC) after an early phase with hepatic steatosis in two independent lineages. The mice transgenic for the envelope genes or non-structural genes do not develop HCC. ISDR, interferon-sensitivity determining region.

rying the core gene develop HCC in two independent lineages.[6] The envelope gene transgenic mice do not develop HCC, despite high expression levels of both E1 and E2 proteins.[8,9] The transgenic mice carrying the entire non-structural genes have developed no HCC.

The transgenic mice carry the core gene and express the core protein of an expected size, approximately 21 kDa, the level of which in the liver is similar to that in the liver of chronic hepatitis C patients. Early in life, these mice develop hepatic steatosis, which is one of the histologic characteristics of chronic hepatitis C, along with lymphoid follicle formation and bile duct damage.[10] Thus, the core gene transgenic mouse model well reproduces this feature of chronic hepatitis C. Of note, significant inflammation is not observed in the liver of this animal model. Late in life, these transgenic mice develop HCC. Most hepatic nodules have a pathology characterized by 'nodule in nodule', and HCC with a low degree of differentiation develops within adenoma as well as within HCC with a higher degree of differentiation.[6] Although numerous lipid droplets are found in cells forming adenoma, as in non-tumorous cells, they are rarely observed in HCC cells. These histological features closely resemble those observed in HCC developing in chronic hepatitis C patients, in whom prominent lipid droplets are found in small, well-differentiated HCC and its precursors; poorly differentiated HCC without lipid droplets develops from within differentiated HCC.[6] Notably, the development of steatosis and HCC has been reproduced in other HCV transgenic mouse lines, which harbor the entire HCV genome or structural genes including the core gene.[11] These outcomes indicate that the core protein per se of HCV has an oncogenic potential when expressed in vivo.

Sequence to the core protein expression in the liver

It is difficult to clarify the mechanism of carcinogenesis even for our simple model in which only the core protein is expressed in otherwise normal liver tissues. There is a notable feature of the localization of the core protein in hepatocytes: although the core protein predominantly exists in the cytoplasm associated with lipid droplets, it is also present in the mitochondria and nuclei.[6,12] On the basis of this finding, the pathways related to these two organelles, the mitochondria and nuclei, were analyzed.

One activity of the core protein is an increased production of oxidative stress in the liver. We note that the production of oxidative stress is increased in our transgenic mouse model in the absence of inflammation in the liver (hepatitis). This reflects overproduction of reactive oxygen species (ROS) in the liver, or predisposition to it, which is staged by the HCV core protein without any intervening inflammation.[13,14] The overproduction of oxidative stress results in the generation of deletions in the mitochondrial DNA, an indicator of genetic damage. Thus, the core protein induces oxidative stress overproduction in the absence of inflammation, and may, at least in part, contribute to hepatocarcinogenesis in HCV infection (Fig. 2). If inflammation is induced in the liver with the HCV core protein, the production of oxidative stress is escalated to an extent that cannot be scavenged by a physiological antagonistic system. This indicates that the inflammation in chronic HCV infection would be different to that produced in other
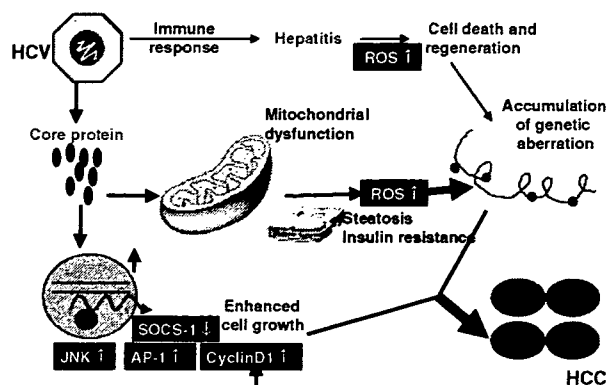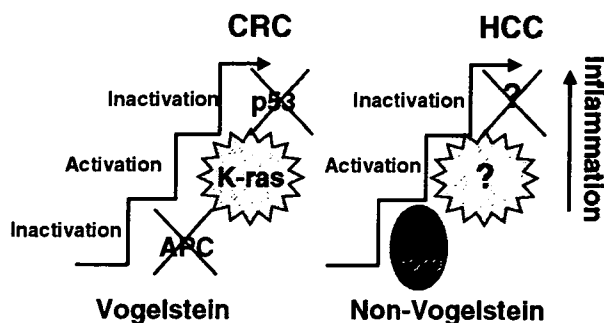
Figure 2 Molecular pathogenesis of liver disease in hepatitis C virus (HCV) infection. Induction of oxidative stress together with hepatic steatosis by the HCV core protein would play a pivotal role in the development of hepatocellular carcinoma (HCC). Alterations in cellular gene expressions, such as tumor necrosis factor-α (TNF-α) or suppressor of cytokine signaling-1 (SOCS-1), and those in the intracellular signaling pathways including c-Jun N-terminal kinase (JNK) would be coaccelerators to hepatocarcinogenesis in HCV infection. ROS, reactive oxygen species.

Figure 3 Mechanism of hepatitis C virus (HCV)-associated hepatocarcinogenesis. Multiple steps are required in the induction of all cancers; it would be mandatory for hepatocarcinogenesis that genetic mutations accumulate in hepatocytes. However, in HCV infection, some of these steps may be skipped in the development of hepatocellular carcinoma (HCC) in the presence of the core protein. The overall effects achieved by the expression of the core protein would be the induction of HCC, even in the absence of a complete set of genetic aberrations, required for carcinogenesis. By considering such a 'non-Vogelstein-type' process for the induction of HCC, a plausible explanation may be give for many unusual events occurring in HCV carriers. CRC, colorectal cancer; APC, adenomatous polyposis coli.

types of hepatitis, such as autoimmune hepatitis. The basis for the overproduction of oxidative stress may be ascribed to the mitochondrial dysfunction.[13,15] The function of the electron transfer system of the mitochondrion is suggested in association with the presence of the HCV core protein.[16] Hepatic steatosis in hepatitis C may work as fuel for oxidative stress overproduction.[14,17,18]

Other possible pathways would be the alteration of the expression of cellular genes, interacting with cellular proteins, and modulation of intracellular signaling pathways (Fig. 2). For an example, tumor necrosis factor (TNF)-α and interleukin-1β have been found to be transcriptionally activated.[19] The core protein has also been found to interact with some cellular proteins, such as retinoid X receptor (RXR)-α, which play pivotal roles in cell proliferation and metabolism.[20] The mitogen-activated protein kinase (MAPK) cascade is also activated in the liver of the core gene transgenic mouse model. The MAPK pathway, which consists of three routes, c-Jun N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK), is involved in numerous cellular events including cell proliferation. In the liver of the core gene transgenic mouse model prior to HCC development, only the JNK route is activated. Downstream of the JNK activation, transcription factor activating factor (AP)-1 activation is markedly enhanced.[19,21] Far downstream, both the mRNA and protein levels of cyclin D1 and CDK4 are increased (Fig. 2). The suppression by HCV core protein of the suppressor of cytokine signaling (SOCS)-1, a tumor suppressor gene, may also contribute to hepatocarcinogenesis. Thus, the HCV core protein modulates the intracellular signaling pathways and gives an advantage to hepatocytes for cell proliferation.

Such an effect of the core protein on the MAPK pathway, combined with that on oxidative stress, may explain the extremely high incidence of HCC development in chronic hepatitis C (Fig. 2).

Hepatocarcinogenesis in HCV infection: A mechanism distinct from those in other cancers

The results of our studies on transgenic mice have indicated a carcinogenic potential of the HCV core protein *in vivo*; thus, HCV may be directly involved in hepatocarcinogenesis. In research studies of carcinogenesis, the theory by Kinzler and Vogelstein has gained popularity.[22] They have proposed that the development of colorectal cancer is induced by the accumulation of a complete set of cellular gene mutations. They have deduced that mutations in the *adenomatous polyposis coli* gene for inactivation, those in *K-ras* for activation and those in the *p53* gene for inactivation accumulate, which cooperate toward the development of colorectal cancer.[22] Their theory has been extended to the carcinogenesis of other cancers as well, called 'Vogelstein-type' carcinogenesis (Fig. 3).

On the basis of the results for the induction of HCC by the HCV core protein, we would like to introduce a mechanism different from that of Kinzler and Vogelstein for hepatocarcinogenesis in HCV infection. We do allow a multistage process in the induction of all cancers; it would be mandatory for hepatocarcinogenesis that many mutations accumulate in hepatocytes. Some of these steps, however, may be skipped in the development of HCC in HCV infection to which the core protein would contribute (Fig. 3). The overall effects achieved by the expression of the viral protein would be the induction of HCC, even in the absence of a complete set of genetic aberrations, required for carcinogenesis.

By considering such a 'non-Vogelstein-type' process for the induction of HCC, a plausible explanation may be given for many unusual events happening in HCV carriers.[23] Now it does not seem so difficult as before to determine why HCC develops in persistent HCV infection at an outstandingly high incidence. Our theory may

also give an account of the non-metastatic and multicentric de novo occurrence characteristics of HCC, which would be the result of persistent HCV infection.

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

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

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

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

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

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

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

MATERIALS AND METHODS

Preparation of PA28γ-knockout HCV core gene-transgenic mice. C57BL/6 mice carrying the gene encoding HCV core protein genotype 1b (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
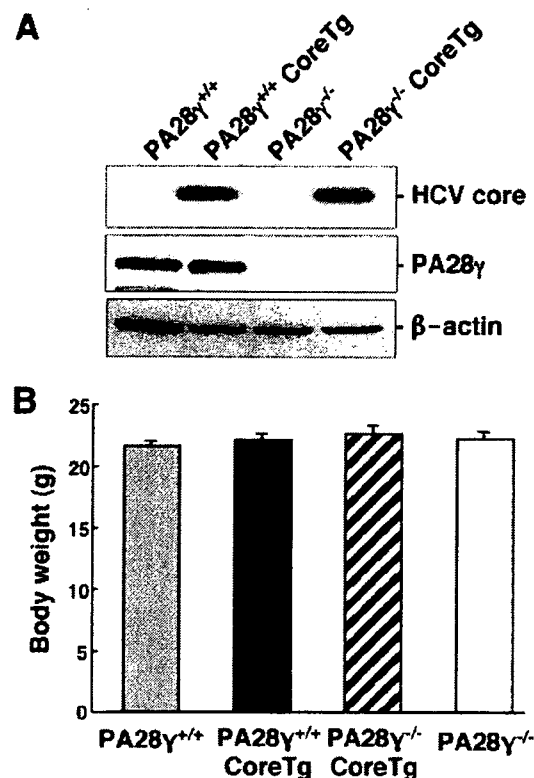

FIG. 1. Characterization of HCV core gene-transgenic mice deficient in the PA28γ gene. (A) Expression of the HCV core protein and PA28γ in the livers of PA28γ$^{+/+}$, PA28γ$^{+/+}$CoreTg, PA28γ$^{-/-}$, and PA28γ$^{-/-}$CoreTg mice. Lysates obtained from liver tissues of the mice (100 μg protein/lane)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

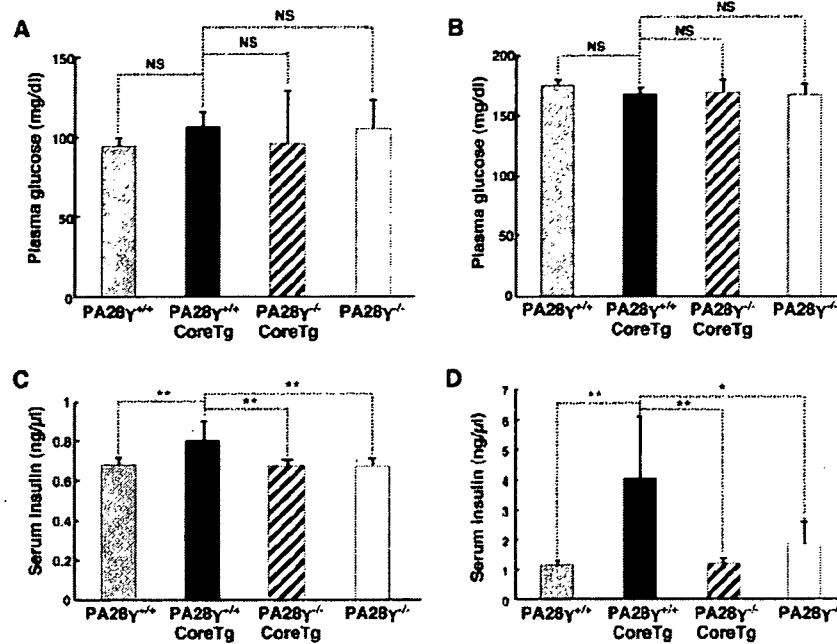FIG. 2. Knockout of the PA28γ gene inhibited the hyperinsulinemia induced by HCV core protein. Plasma glucose levels of PA28γ$^{+/+}$, PA28γ$^{+/+}$CoreTg, PA28γ$^{-/-}$CoreTg, and PA28γ$^{-/-}$ mice under fasting (A) or fed (B) conditions (n = 7 in each group) are shown. Serum insulin levels in fasting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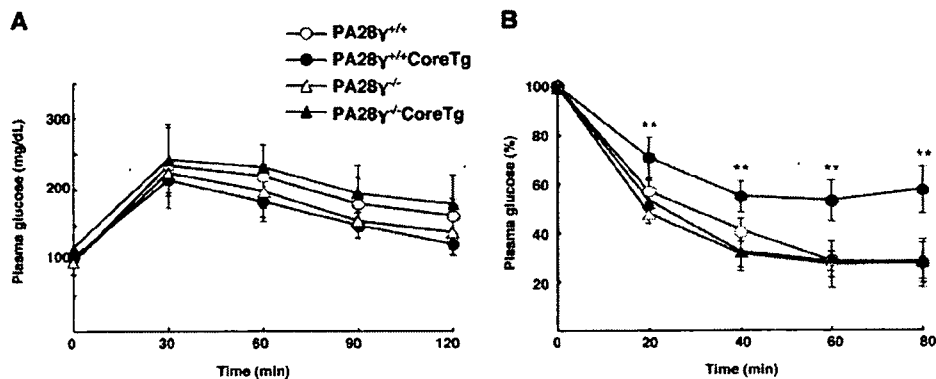

FIG. 3. Knockout of the PA28γ gene inhibits the insulin resistance induced by the HCV core protein. (A) Glucose tolerance test. D-Glucose was intraperitoneally administered to mice fasted for more than 16 h at 1 g/kg of body weight. Plasma glucose levels were estimated at the indicated times (n = 5 in 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

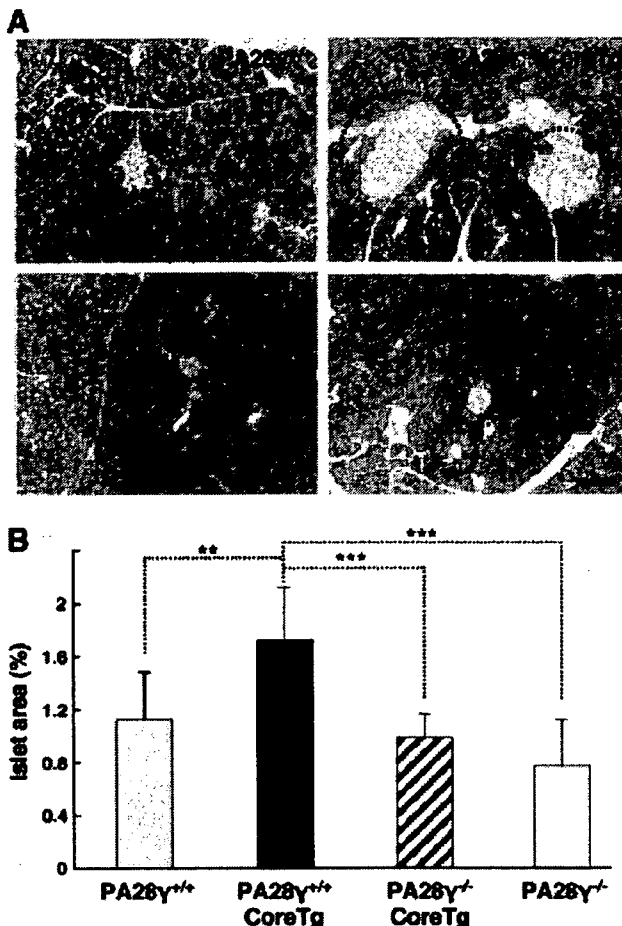
FIG. 4. PA28γ participated in the enlargement of pancreatic islets induced by the HCV core protein. (A) Histological sections prepared from pancreas tissues of PA28γ$^{+/+}$, PA28γ$^{+/+}$CoreTg, PA28γ$^{-/-}$, and PA28γ$^{-/-}$CoreTg mice were stained with hematoxylin and eosin. Dotted circles indicate 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
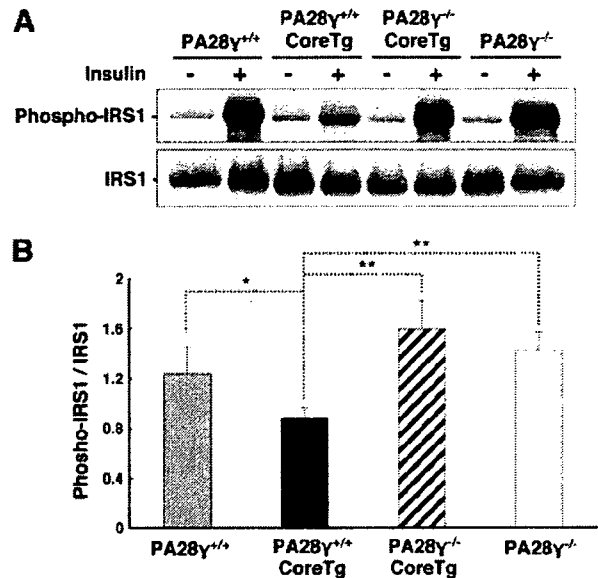
used in this study. Figure 1B shows the body weights of 2-month-old mice. There were no significant differences in body weight among PA28γ$^{+/+}$CoreTg, PA28γ$^{-/-}$CoreTg, PA28γ$^{-/-}$, and PA28γ$^{+/+}$ mice. Steatosis was not detected in the livers of the 2-month-old mice (data not shown).

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-
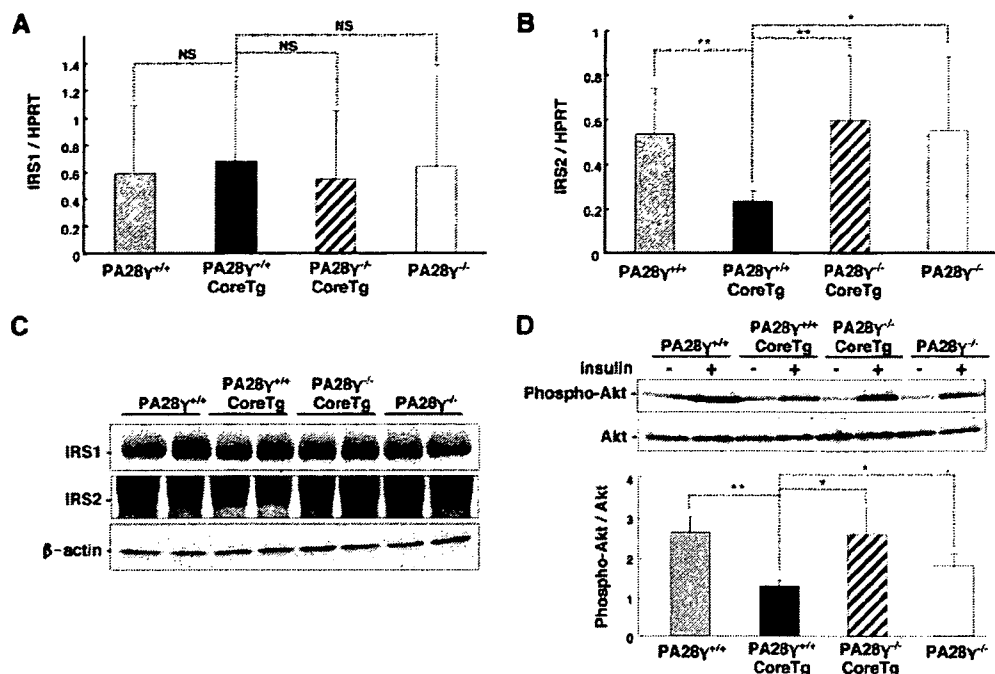
FIG. 6. PA28γ participated in the inhibition of the IRS2 expression and Akt phosphorylation induced by HCV core protein. The transcription of IRS1 (A) and IRS2 (B) was estimated by quantitative RT-PCR ($n = 5$ in each group). (C) The expression levels of IRS1 and IRS2 in the livers of the mice were determined 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