alpha10, and 0.557 IU/ml (0.271 pg/ml) for alpha con1, respectively. Similarly to the Huh7 cells, IFN-alpha8 was the strongest to suppress expression of HCV replicon. On the other hand, IFN-alpha5 and alpha con1 showed weaker antiviral effects on HCV replicon in HeLa cells than in Huh7 cells. Western blotting of HeLa/Rep-Reo cells treated with IFNs-alpha1 and alpha8 showed dose-dependent suppression of HCV replication, and differential activities of IFN subtypes, which were comparable to that of luciferase activities (Fig. 4B).

Reporter assays were performed by transfecting plasmids expressing ISRE-, GAS-, AP1-, NF-kappa B-, CRE-, and SRE-luciferase reporters into HeLa cells. The ISRE- and GAS-luciferase constructs responded to treatment with IFN subtypes similarly to that in Huh7 cells (Fig. 5). There was no significant difference in induction velocity of ISRE and GAS reporter activities between different IFN subtypes as were seen in Huh7 cells. IFN treatment showed no significant effects on AP1, NF-kappa B, CRE, and SRE activities on HeLa cells.

3.5. Analyses of IFN receptors expression

It is possible that the differences in expression levels of the cell-surface IFN receptors may associate with the response to IFN. We then analyzed expression of the respective subunits of type I IFN receptor mRNAs of Huh7 and HeLa cells by RT-PCR. Type I IFN receptor, IFNAR, is constituted by two subunits; 110 kilo-dalton (kDa) alpha subunit (IFNAR-1), and a 102 kDa beta subunit (IFNAR-2). IFNAR-2 has three isoforms that are translated from alternatively spliced mRNA transcripts; a 40 kDa soluble form of IFNAR-2a, a 55 kDa short form of IFNAR-2b and a 102 KDa long form of IFNAR-2c [34–36]; IFNAR-2c is the authentic beta subunit that is functionally active and coexpressed with IFNAR-1 (Fig. 6A). An RT-PCR analysis of IFN receptors showed that both cell lines expressed IFNAR-1 and IFNAR-2 (Fig. 6B). Although the relative expression levels of IFNAR-2a was slightly higher in Huh7 cells that in HeLa cells, There were no apparent differences in the expression level of the major subunits, IFNAR-1 and IFNAR-2c between Huh7 and HeLa cells.

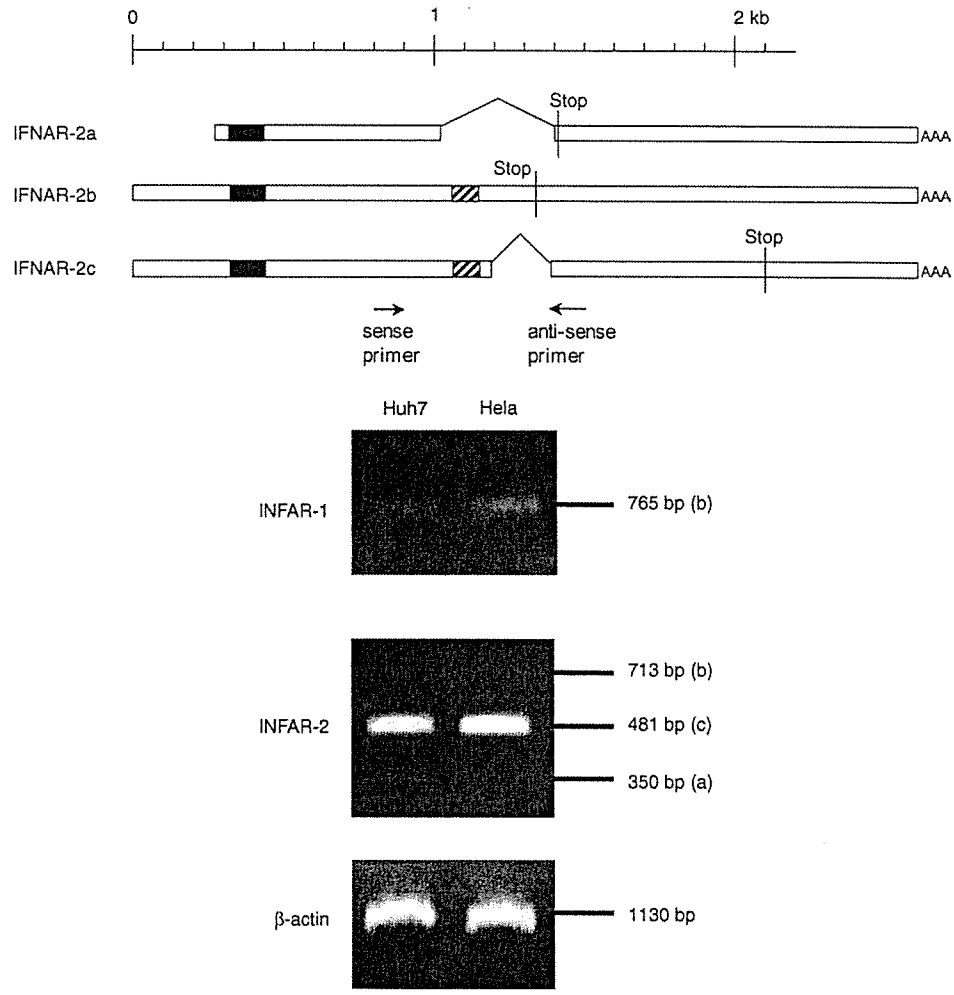

Fig. 6. IFN receptor expression: (A) structures of 3 IFNAR2 mRNA isoforms. Closed boxes indicate the leader peptide domains. The striped boxes indicate the transmembrane domain. Stop codons are indicated by vertical bars. Position of the sense and antisense primers are shown at the bottom. (B). Expression of IFN-alpha receptors in Huh7 and HeLa cells were evaluated by RT-PCR. The top panel: IFNAR-1 PCR-amplified DNA of 765 bp. The middle panel: IFNAR-2 PCR products, (a) 350 bp as IFNAR-2a, (b) 713 bp as IFNAR-2b, and (c) 481 bp as IFNAR-2c in size, respectively. The bottom panel: beta actin DNA.

4. Discussion

In this study, we used two cell lines that support expression of HCV replicon, in which the level of the viral genomic replication can by readily monitored by luciferase reporter assay. We showed that the five IFN-alpha subtypes have different activities to suppress expression of HCV replicon (Figs. 1 and 3). Using two IFN titers standardized in IU/ml and in pg/ml, IFN-alpha8 had the strongest antiviral effect on replicon, while IFN-alpha1 had the weakest effect in both titers. These finding are consistent with those reported by Foster et al. that IFN-alpha8 had the greatest antiviral activity in cells of three human tumor cell lines challenged with murine encephalomyelitis virus [17]. On the other hand, the reporter assay showed that activation of ISRE-dependent promoter, which is the primary signal transduction pathway, showed very similar results between Huh7 and HeLa cells, while the ISRE activities in neither of the cell lines correlated with the anti-HCV activities of the IFN subtypes (Figs. 2 and 4). GAS reporter activity, which bound by phosphorylated STAT1 homodimer, showed similar activation between each IFN subtypes. Other reporter assays, NF-kappa B, CRE, and SRE, showed no activation by the IFNs. These findings suggest that the divergent action of IFN subtypes may be independent of the classical JAK-STAT pathway.

Beside the classical JAK1-STAT1 and -2 pathway, type I IFN activates alternative signaling pathways. JAK2 mediates activity of IFNs as well as JAK1. As for STAT family, dimmers of STAT1:1, STAT3:3, STAT1:3, STAT5:5, and a heterodimer CrkL:STAT5 have been reported to be formed during the IFN-alpha signaling [18,19]. Furthermore, IFN-alpha treatment of cells activates expression of various genes that modulate virus infection and replication in JAK-STAT-independent manner; those include the insulin receptor substrate family, CrkL adaptor, protooncogene Vav, PKC-delta, p38 kinase, ERK 1/2, and PI-3 kinase, although the targets for these signaling pathways have not been well understood (reviewed in [18,19]).

Actions of IFN-alpha is initiated by binding the type I IFN receptors. It has been suggested that biologic activities of different IFN-alpha subtypes correlate with their respective binding affinities to the cells used [37]. Although we have not tested the cell-binding affinity of the IFN subtypes onto their receptor, activation of ISRE promoter, which is triggered by the receptor binding of IFN, did not correlate with their antiviral activities. Furthermore, analyses of IFN receptors by RT-PCR did not find differences in expression profiles the type I IFN receptor subunits, IFNAR1 and three isoforms of IFNAR2 [34–36] in Huh7 and HeLa cells that support expression of HCV replicon (Fig. 6B), suggesting that the differential effects of IFN subtypes may not be due to different expression profiles of their receptor subunits. Alternatively, binding of IFNs onto their receptor might recruit unidentified subunits or adaptor molecules that may activate aberrant signal transduction pathways.

Most studies on actions of the IFN subtypes focused only on the effects of the individual subtypes, and very little is known about their effects in combination [38–40]. On the other hand, because of the existence of multiple IFN subtypes, mutual interactions between the subtypes may be involved in the cellular responses, although these interaction between IFN subtypes are not well understood. Greiner et al. had reported IFN-alpha1 competes with IFN-alpha2 for binding to its receptor [16]. Our previous study also demonstrated additive and antagonistic effect of IFN-alpha subtypes, for instance, IFN-alpha2 and alpha8 had synergistic antiviral effect against VSV virus in HepG2 cells. [29] In our present study, we could not find such synergistic effects of IFN-alpha2 and alpha8 subtypes on cellular HCV replication (Fig. 3B). The result suggests that effects of IFN subtypes and their combination may show different effects depending on the target pathogens.

IFN-alpha con1 is a recombinant IFN that has consensus amino acid sequence of multiple IFN-alpha subtypes on its receptor-binding domain. The IFN-alpha con1 shows greater antiviral activity against HCV replication than the individual IFN-alpha subtypes in vitro [41] as well as in vivo [42]. In our present study, IFN con1 was moderately effective to suppress HCV replication with the IC50 of close to that of IFN-alpha5. However, activation of ISRE and GAS by IFN-alpha con1 seemed to be slight weaker, compared to the other IFN subtypes. Although it might be due to the different definition of units from that of the other IFN-alpha subtypes [30,41], the reportedly strong biological activity of IFN-alpha con1 might also involve pathway other than the Jak-STAT pathway.

Our present results using HCV replicon system have shown that IFN-alpha8 was the strongest to suppress HCV replication among 5 IFN-alpha subtypes 1, 2, 5, 8, and 10. Among clinically used IFN-alpha preparations, natural IFN-alpha preparations contain substantial amounts of IFN-alpha8 [29]. The differential activity shown in this study might direct a spotlight to the drugs, and might propose a hint for more effective IFN drugs used alone or in combination with ribavirin. Taken togather, IFN-alpha8 showed the strongest suppressive effect on in vitro HCV replication. The discrepancy between cellular ISRE responses and the anti-HCV effect implies other pathways other than IFN-activated JAK-STAT pathway. Further investigation of their differential antiviral actions may help elucidating the IFN-mediated cellular defense mechanisms against virus infection.

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Analysis of prognostic factors in therapeutic responses to interferon in patients with chronic hepatitis C

SHIGEMI HOSOGAYA, YUKIO OZAKI, NOBUYUKI ENOMOTO, and YOSHIHIRO AKAHANE

YAMANASHI, JAPAN

The genotype of hepatitis C virus (HCV) and the amount of HCV RNA are often used to predict the efficacy of interferon (IFN) therapy on chronic hepatitis C. In addition to these factors, there may be several factors related to the host. Therefore, the authors undertook a retrospective study in which physical findings and laboratory data before therapy were evaluated by multiple logistic analysis. Two-hundred and five cases with chronic hepatitis C treated with interferon were analyzed in this study. Sustained virological response was observed with 68 cases. Multiple logistic analysis was performed with 29 explanatory variables including HCV genotype, HCV RNA, IFN types, and total dose, along with gender, age, alcohol consumption, body mass index (BMI), histological findings of liver biopsy, platelet counts, and laboratory data of serum enzymes. Analysis on the factors that correlated well with therapeutic efficacy revealed that genotype 2a, 2b showed higher therapeutic responses than genotype 1b with reference to HCV genotypes, and higher IFN dose or lower HCV RNA levels gave better results. With reference to host factors, higher total protein level, lower levels of BMI, total bilirubin, and aspartate aminotransferase were highly correlated with therapeutic efficacy. HCV genotypes and HCV RNA levels have been already identified as prognostic factors. However, the high correlation values of BMI and the total protein level are new findings. It is suggested that probability estimation of therapeutic effects using the logistic regression equation may be a good tool for predicting therapeutic efficacy of IFN therapy on individual cases. (Translational Research 2006;148:79-86)

Abbreviations: AFP alpha-fetoprotein; ALB albumin; ALP orthophosphoric acid monoester phosphohydrolase; AST aspartate aminotransferase; bDNAp branched DNA probe method; BMI body mass index; BUN blood urea nitrogen; CAH2A chronic active hepatitis, mild; CAH2B chronic active hepatitis, severe; CHE acylcholine acylhydrolase; Cl chloride; CPH chronic persistent hepatitis; CRE creatinine; DB direct bilirubin; GGT gamma glutamyltransferase; HCV hepatitis C virus; HLBI human lymphoblastic interferon; ICG indocyanine green; IFN interferon; K potassium; LAP leucine aminopeptidase; LC liver cirrhosis; LD lactate dehydrogenase; MU mega-unit; Na sodium ions; NR nonresponder; PEG polyethylene glycol; PLT platelet count; SD standard deviation; SE standard error; SVR sustained viral responder; TB total bilirubin; TC total cholesterol; TG triacylglycerol; TP total protein; UA uric acid

From the Department of Clinical and Laboratory Medicine, Faculty of Medicine, University of Yamanashi, Yamanashi, Japan; First Department of Internal Medicine, Faculty of Medicine, University of Yamanashi, Yamanashi, Japan; and Kofu Municipal Hospital, Yamanashi, Japan.

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Reprint requests: Shigemi Hosogaya, Department of Clinical and Laboratory Medicine, Faculty of Medicine, University of Yamanashi, 1110 Tamaho, Nakakoma, Yamanashi 409-3898, Japan; e-mail: hosogaya@yamanashi.ac.jp.

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If chronic hepatitis induced by HCV is persistent for a long period of time, there is a high occurrence rate of liver cirrhosis and eventually of hepatocellular carcinoma. There are 1.5 to 2 million carriers of HCV in Japan, and approximately 80% of hepatocellular carcinoma originate from HCV infection.[1] Therefore, therapeutic approaches to chronic hepatitis C are an important issue with respect to prevention of hepatocellular carcinoma.[2] IFNs are one of the mainstream anti-viral therapies to treat chronic hepatitis C, and the factors that determine their therapeutic efficacy include the HCV genotype and the amount of HCV RNA, many of which are attributed to the HCV infection itself.[2–6] As for the therapeutic side of the story, major determinants include the total amount of IFN, therapeutic periods,[7,8] combination therapy with ribavirin,[9,10] and IFN products with PEG modification. [11]

Although factors belonging to the viral and therapeutic aspects can certainly predict therapeutic efficacy in a large portion of chronic hepatitis C, some cases show remarkable SVR despite HCV genotype 1b and high RNA values that ordinarily preclude therapeutic efficacy of IFN. Also, some cases show a poor response despite the HCV genotype 2a and low RNA values that are supposed to be good indicators of therapeutic efficacy. The presence of these cases implies that other factors are related to the host that determine the therapeutic efficacy of IFN against HCV infection. The candidates for host-related factors that may affect the therapeutic efficacy of IFN include gender, age, alcohol uptake, obesity, serum biochemical values, and platelet counts before IFN therapy, which may outline liver function. Therefore, the authors conducted a retrospective study to determine the host-related factors that may influence the therapeutic efficacy of IFN with chronic hepatitis C cases. To predict major prognostic factors out of several factors related to therapeutic efficacy, multiple logistic analysis was used that can analyze contributing factors independent of their mutual interactions. In this study, only the cases treated with IFN alone were analyzed so that the variance in the therapeutic approaches would be minimized, and that prognostic factors related to the host would be more clearly outlined. If the results of this analysis, based on physical findings and laboratory data before therapy, can predict therapeutic efficacy with high probability, it would lead to more efficient IFN therapy and prove the usefulness of clinical laboratory tests.

METHODS

Patients: Two hundred and five cases with chronic hepatitis C treated with IFN at the First Department of Internal Medicine (Digestive Organs), University of Yamanashi, from 1992 to 1996 were analyzed in this study. Of these, there were 155 men with the average age of 43.7 years (SD 9.8 years) and 50 women with the average age of 49.7 years (SD 8.6 years). The total demographic profile is 45.2 9.8 years. All diagnostic and therapeutic procedures were carried out according the principles of the Declaration of Helsinki, and informed consent was obtained from all participants. Furthermore, the study was approved by the review board of the University of Yamanashi. The experimental design of this retrospective study is, in brief, a case control study that enrolls 205 cases of chronic hepatitis C who underwent 24–26 weeks of IFN therapy and was evaluated for its efficacy 6 months after the therapy. Among the prognostic factors including physical findings and laboratory data before therapy, the major factors related to the efficacy of IFN therapy were analyzed with the therapeutic achievement of these 205 patients.

Therapeutic efficacy: The object variable of this study to be analyzed is the therapeutic efficacy of IFN, namely, probability of SVR. The evaluation standard for therapeutic efficacy is negative conversion of HCV-RNA assessed by COBAS AMPLICOR HCV assay (Roche Diagnostics, Switzerland) at some point elapsing more than 6 months after cessation of IFN therapy. Based on this evaluation standard, 68 cases were found to have SVR. The cases without negative conversion of HCV-RNA and with persistent ALT elevation were designated as NRs, and 137 cases in this study fell into this category.

Interferon therapy: With reference to IFN regimens, 166 cases received IFN alpha monotherapy (HLBI, human lymphoblastic interferon; Sumiferon, Sumitomo Pharmaceuticals, Japan), whereas 39 cases underwent IFN alpha-2b monotherapy (Intron A; Schering-Plough, Kenilworth, NJ). With IFN alpha, 6 MU were given daily for the first 2 to 4 weeks, and during the following 22 weeks, 6 MU were given 3 days a week, amounting to the total dose of 480–560 MU. With IFN alpha-2b, 9 MU instead of 6 MU were given with the protocol similar to IFN alpha, amounting to the total dose of 720 to 840 MU.

Explanatory variables: The explanatory variables in this study include HCV genotype and HCV RNA, which have been alleged to be major determinants, and IFN types and total dose. As for those related to the host, 29 factors were analyzed, including gender, age, alcohol consumption, BMI, histological findings of liver biopsy, laboratory data of 19 serum enzymes, and platelet counts. Laboratory tests were measured with fasting blood in the early morning soon after admission into hospital and before the beginning of IFN therapy.

The details of the explanatory variables in this study are as follows: HCV genotypes are classified into genotypes 1 to 6, and subclassified as a, b, c, and so on. The genetic distribution of HCV among Japanese patients are 1b approximately 70%, 2a 20%, and 2b 10%, and there are virtually no other types among Japanese patients.[12] HCV RNA was determined by the branched DNA probe method (bDNAp, Ver. 1.0; Chiron, UAS/ Daiichi Pure Chemicals, Japan) and presented as quantitative values (kMeq/L). Alcoholic consumption was expressed as average daily alcohol intake (g/day, questionnaire-

Table I. Comparison of explanatory variables between SVR and NR groups

		SVR			NR			
Variable	Unit	25%	50%	75%	25%	50%	75%	*P* value
HCV genotype		1b	2b	2a	1b	1b	1b	0.000
bDNAp	kMeq/L	0.20	0.29	0.83	0.58	1.80	3.81	0.000
IFN type		HLBI	HLBI	HLBI	HLBI	HLBI	HLBI	0.981
IFN dose	MU	516	522	774	516	516	774	0.709
Gender		Male	Male	Male	Male	Male	Female	0.114
Age	years	37	43	53	39	45	52	0.417
Alcohol	g/day	0	25	63	0	25	50	0.544
BMI	Kg/m^2	20.7	22.9	24.7	22.1	24.0	25.8	0.010
Histological type		CAH2A	CAH2A	CAH2A	CPH	CAH2A	CAH2B	0.533
PLT	$10^9/L$	150	188	218	150	182	220	0.856
T P	g/L	73	75	79	70	73	77	0.001
ALB	g/L	41	43	45	40	42	44	0.066
T B	mol/L	10	14	17	12	14	19	0.290
D B	mol/L	3	3	5	3	3	5	0.916
CHE	U/L	262	312	372	261	315	358	0.897
ALP	U/L	133	169	216	134	170	219	0.985
LAP	U/L	48	61	72	49	58	71	0.863
GGT	U/L	33	46	103	30	54	101	0.738
LD	U/L	172	249	305	175	244	316	0.756
AST	U/L	38	52	86	35	52	78	0.673
ALT	U/L	63	100	145	51	82	132	0.090
T G	mmol/L	0.84	1.11	1.52	0.88	1.11	1.41	0.829
T C	mmol/L	3.62	4.09	4.53	3.72	4.19	4.65	0.310
BUN	mmol/L	3.6	4.6	5.4	3.9	5.0	5.7	0.030
CRE	mol/L	53	62	71	53	62	71	0.901
U A	mol/L	280	333	404	274	327	369	0.301
Na	mmol/L	140	141	142	139	140	142	0.036
K	mmol/L	3.9	4.1	4.4	3.9	4.0	4.3	0.517
Cl	mmol/L	101	103	105	101	103	105	0.625

based). Quantitated alcohol intake was checked as those previous to the study period. The histological findings of the liver were classified as CPH, CAH2A, CAH2B, and LC, and these classifications practically corresponded with liver fibrosis stage classifications of F1 to F4, respectively. Serum chemical constituents were analyzed by a 7250 Clinical Analyzer (Hitachi High-Technologies, Japan), and the total protein, serum bilirubin, and enzyme activities were determined by the biuret method and bilirubin-oxidase method,[13,14] and the standardized methods proposed by Japanese Society of Clinical Chemistry,[15] respectively. Platelet counts were measured with a SE9000 Automated Hematology Analyzer (Sysmex, Japan).

Statistics: Statistical analysis was performed using multiple logistic analysis to estimate odds ratios of prognostic factors and to make a prognostic equation of therapeutic effects.[16,17] Briefly, logistic regression equation was estimated with a probability of SVR as object variable and related predictive variables as explanatory variables. All factors were first analyzed in one step analysis, and then a stepwise model was used to select appropriate explanatory variables. A test of the significance of the regression coefficients was performed, and odds ratios of each predictive variables were also estimated. A SPSS Professional Statistics software (SPSS Inc., Japan) was used for computer analysis of all data.

RESULTS

The authors first performed monovariate analysis on all cases. The patients were divided into 2 groups, SVR and NR, and with each group, the values for 25th, 50th (median), and 75th percentile of the explanatory variable are presented in Table I. The data are presented this way, because simple presentation of the data as the means SD could not give an idea as to whether the data assume normal distribution or skewed distribution of continuous variables, and it was not applicable to the categorical variables. The *P*-value for each explanatory variable is listed in the right-most lane, which represents the probability of type I error of the Mann–Whitney *U*-test (the probability of discarding the null hypothesis that there are no differences between 2 groups). The explanatory variables with significant differences between the 2 groups are HCV genotype, HCV-RNA, BMI, TP, BUN, and Na. Most measurement values were the results of standardized methods of

Table II. Multiple logostic analysis of all 29 factors for estimate of the therapeutic efficacy of IFN

Variable	Beta	SE(Beta)	z value	*P* value
HCV genotype	2.288	0.500	4.578	0.0000
bDNAp	0.631	0.198	3.190	0.0014
IFN type	0.053	0.995	0.054	0.9571
IFN dose	0.006	0.003	1.928	0.0539
Gender	0.833	1.156	0.721	0.4712
Age	0.036	0.041	0.875	0.3816
Alcohol	0.112	0.177	0.633	0.5270
BMI	0.238	0.127	1.875	0.0608
Histological type	0.698	0.493	1.417	0.1566
PLT	0.008	0.008	1.087	0.2770
TP	0.305	0.093	3.282	0.0010
ALB	0.051	0.180	0.286	0.7752
TB	0.352	0.132	2.662	0.0078
DB	0.775	0.352	2.203	0.0276
CHE	0.003	0.004	0.793	0.4276
ALP	0.007	0.005	1.449	0.1475
LAP	0.046	0.034	1.353	0.1761
GGT	0.007	0.007	1.060	0.2892
LD	0.003	0.007	0.391	0.6962
AST	0.025	0.017	1.442	0.1494
ALT	0.000	0.009	0.033	0.9737
TG	1.325	0.791	1.676	0.0938
TC	0.697	0.460	1.514	0.1300
BUN	0.360	0.322	1.119	0.2631
CRE	0.013	0.032	0.388	0.6978
UA	0.007	0.007	1.116	0.2645
Na	0.050	0.174	0.289	0.7724
K	0.144	0.937	0.153	0.8782
Cl	0.145	0.127	1.142	0.2533

measurement, and the sample data are considered to represent the range of distribution generally observed in patients with chronic hepatitis C. In addition to the measurement items listed here, AFP, ICG, and type IV collagen 7S, which is a marker of liver fibrosis, were analyzed in a preliminary study. However, as these factors were not measured in all cases analyzed in this study, and furthermore they were found to have no significant correlation with therapeutic efficacy, possibly due to a small number of samples, these data were omitted from this report.

For multivariate analysis of these data, logistic regression analysis was first performed with all 29 factors for prediction of therapeutic efficacy in all patients receiving IFN therapy (Table II). The contribution of each variable to therapeutic efficacy can be estimated by the z value, which is the regression coefficient of each variable (Beta) divided by its standard error [SE-(Beta)], and by the *P* value, which represents the probability of *z* value occurrence. The factors with a high probability of contributing to the therapeutic efficacy thus identified are HCV genotype, HCV RNA, TP, TB, and DB. However, in contrast to expectation, alcohol consumption, IFN type, histological findings of the liver, and platelet counts were not closely correlated with the therapeutic efficacy.

Taking into consideration the preliminary results of the aforementioned analysis with all 29 factors, variable selection in stepwise trials of increment/decrement in the number of variables was repeatedly performed so that reliable and reproducible regression could be attained with the minimal number of variables. This process allowed us to select appropriate explanatory variables and best-fit regression, as shown in Table III. The factors that can best estimate the therapeutic efficacy of IFN are HCV genotype (x_1), HCV RNA (x_2), IFN dose (x_3), BMI (x_4), TP (x_5), TB (x_6), DB (x_7), and AST (x_8). Assessed with *z* and *P* values, HCV genotype, HCV RNA and TP are shown to be most closely correlated with the therapeutic efficacy (*P* 0.1%), followed by IFN dose, BMI, bilirubin, and AST with the *P* values approximately 1%, which is within the level of significance. The logistic regression equation based on these regression coefficients (Beta) to estimate the probability of SVR (p(x)) of IFN therapy is as follows:

$$1 \quad p\,x \quad 1/[1 \quad \exp \quad (\quad 19.006 \quad 2.188\,x_1 \quad 0.554\,x_2 \quad 0.004\,x_3 \quad 0.232\,x_4 \quad 0.246\,x_5 \quad 0.224\,x_6 \quad 0.543\,x_7 \quad 0.014\,x_8) \ ]$$

It should be added that, because the distribution of each variable more closely resembles skew logarithmic normal distribution than normal distribution, the data with each variable in logarithmic transformation were also evaluated with logistic analysis, which produced the results virtually similar to those without variable transformation.

These analytical processes extracted several prognostic factors closely related with the therapeutic efficacy of IFN, as shown in Table III. The authors then evaluated how each of these prognostic factors as a single variable works in the SVR group as compared with the NR group, as shown in Table I. With SVR, HCV genotype with 2a, 2b, 1b in order of power works in favor of therapeutic efficacy, whereas with the NR group, HCV genotype of 1b predominates. Similarly, with the SVR group, there are smaller amounts of HCV RNA, less BMI, and with higher TP values than the NR group (*P* 0.01). However, there are no significant differences between the SVR and NR groups with IFN dose, TB, DB, and AST. On the other hand, with multivariate analysis, the results of which are presented in Table III, all of these factors including IFN dose, TB, DB, and AST are closely related to the therapeutic efficacy of IFN. Thus, the results of factor determina-

Table III. Multiple logistic regression equation for estimate of the therapeutic efficacy of IFN

Degree	Variable	Beta	SE(Beta)	z value	*P* value
x_0		19.006	4.810	3.951	0.00008
x_1	Genotype	2.188	0.397	5.513	0.00000
x_2	bDNAp	0.554	0.164	3.385	0.00071
x_3	IFN dose	0.004	0.002	2.563	0.01037
x_4	BMI	0.232	0.090	2.574	0.01007
x_5	TP	0.246	0.055	4.472	0.00001
x_6	TB	0.224	0.081	2.763	0.00573
x_7	DB	0.543	0.222	2.449	0.01431
x_8	AST	0.014	0.006	2.485	0.01295

Table IV. Odds ratios of prognostic factors for the therapeutic efficacy of IFN

Variable	Change	Odds Ratio	95% Confidence Interval
Genotype	1b to 2b	8.91	4.10–19.40
bDNAp	1.00 kMeq/L	1.74	1.26–2.40
IFN dose	100 MU	1.55	1.11–2.18
BMI	5.0 Kg/m^2	3.19	1.32–7.73
TP	5.0 g/L	3.42	2.00–5.87
TB	9 mol/L	7.51	1.80–31.34
DB	2 mol/L	2.96	1.24–7.07
AST	50 U/L	2.01	1.16–3.48

Example: A case with BMI 2.0 Kg/m^2, TP 5.0 g/L, and TB 9 mol/L would result in 40.85-fold increase in odds ratio.

tion related to IFN therapeutic efficacy differ between single-variate analysis and multivariate analysis.

How each prognostic factor affects the therapeutic efficacy of IFN can be learned from Table IV. The "change" column in Table IV refers to a certain level of increase/decrease in each variable or transition in the categorical variable. These changes can affect the therapeutic efficacy of IFN, irrespective of their reference values. The effect of a particular rate of changes in each variable on IFN therapeutic efficacy is expressed by odds ratios in Table IV, and an 8.91-fold therapeutic efficacy can be expected with HCV genotype of 2b over genotype 1b, 1.74-fold with bDNAp 100 kMeq/L lower, and with IFN dose 100 MU higher, 1.55-fold efficacy can be expected. Similarly, with the host-related prognostic factors, if BMI is 5.0 Kg/m^2 lower, 3.19-fold higher IFN therapeutic efficacy, 3.42-fold with TP 5.0 g/L higher, 7.51-fold with TB 9 mol/L lower, 2.96-fold with DB 2 mol/L higher, and 2.01-fold with AST 50 U/L lower can be expected. These factors are independently related to the therapeutic efficacy of IFN with statistically significant contribution, as shown by the 95% confidential intervals of odds ratios for each variable. Furthermore, the combined effects of several prognostic factors can be expressed as odds ratios. For example, as shown in the lower panel of Table IV, given that HCV genotype, HCV RNA, and IFN dose are the same, if a patient has a profile of BMI 2.0 Kg/m^2, TP 5.0 g/L, TB –9 mol/L, the odds ratio for therapeutic efficacy is 40.85, even though the changes in BMI, TP, and TB are minimal.

$$2 \quad \text{Odds ratio} \quad \exp \quad 0.232 \quad (\ 2.0)$$
$$0.246 \quad 5.0 - 0.224 \quad (\ 9.0) \quad 40.85$$

Thus, logistic analysis employed in this study can be used not only to estimate the prognostic factors, but also to estimate the odds ratios of each factor or multiple factors combined on object variables.

DISCUSSION

Virus-related factors such as HCV genotype, HCV RNA levels, and genetic mutations in NS5A region are alleged to be good markers to predict the effect of anti-viral therapy on chronic hepatitis C.[2–6] The factors related to therapeutic agents include IFN dose, duration of therapy,[7,8] origin of IFN such as those of recombinant IFN versus cell-derived,[18] combined therapy with ribavirin,[9,10] and IFN modified with PEG. [19] Most recently, it is being recognized that the combined therapy of PEG-IFN and ribavirin is the first choice as anti-viral therapy for chronic hepatitis C.[20]

On the other hand, to further augment the efficacy of IFN therapy, it is imperative to investigate the prog-

nostic factors related to recipients with which the therapeutic efficacy of IFN can be predicted. Gender, age, alcohol intake, liver function before therapy, platelet counts, and liver fibrosis have been considered as the host-related factors that determine the IFN therapeutic efficacy. Retrospective studies have disclosed that BMI higher than 30 kg/m^2 negatively affects anti-viral therapy and that it works independently of HCV genotype or liver fibrosis.[21,22]

As described, the factors that modify the efficacy of IFN therapy are multiple, and there seem to be large individual differences in the factors involved and the degrees of contributing factors. To make the issue more complicated, it would take a long time to evaluate the effects of these prognostic factors. With these cases in which the relationship between prognostic factors and therapeutic efficacy is difficult to prove directly, an analytic method is useful that can evaluate in terms of probabilities the contribution of explanatory variables to predict object variables. Thus, in this study, multiple logistic regression analysis is employed that proved extremely useful in analyzing risks of coronary heart diseases in the Framingham study. This analytical method has several advantages over the other methods; it can analyze the data in which explanatory variables include binary variables as well as quantitative data. It can express as odds ratio the contribution of a particular explanatory variable to object variables independently of other interacting explanatory variables. It can calculate the probability of the object occurrence for each case. Thus, multiple logistic regression analysis is a useful and practical tool in analyzing prognostic factors for clinical outcome.

The results of this study in analyzing prognostic factors for the therapeutic efficacy of IFN extracted out of 29 factors several major contributing factors. Those related to HCV include HCV genotype and HCV RNA, and IFN dose is also one major contributing factor. Their correlations with IFN therapeutic efficacy are in good agreement with previous reports. With regard to the host-related factors, the authors found that in addition to BMI, TP, TB, DB, and AST are contributing factors. Particularly, the magnitude of contribution of TP to the overall prediction of IFN therapeutic effect is higher than that of HCV RNA or BMI, and it is also to be noted that this report is the first to propose TP as a major prognostic factor in IFN therapeutic efficacy, to the best of the authors' knowledge. A previous paper suggested that GGT is an independent predictive factor of SVR,[23] although in this study, GGT was not a significant indicator of SVR. Their study dealt with a group of patients treated with IFN and ribavirin, and it is conceivable that the differences in the composition of patients and the modes of therapy are responsible for the discrepancy.

The authors then sought to elucidate why TP among other host-related prognostic factors was most closely correlated with the therapeutic efficacy of IFN. It is widely accepted that the more intact hepatic cells remain, the more effective is IFN therapy. In this line of reasoning, TP and ALB represents the protein producing activity of the liver, and they are expected to positively correlate with the efficacy of IFN therapy. There is no clear explanation for the superior predictability of TP to ALB in IFN therapeutic efficacy, as shown in this study. It would be of interest to explore contributing factors (proteins) that make TP more superior to ALB in predicting the therapeutic efficacy of IFN in further studies. AST and bilirubin levels represent the level of hepatic damage or impairment of its function, and they should be negatively correlated with the IFN therapeutic efficacy. With higher BMI index, there are more lipids in hepatocytes, and this is assumed to absorb IFN and to interfere with the signal transduction pathways related to IFN receptors. BMI may be also an index of underlying liver fibrosis that antagonizes the action of IFN.[21,22]

To further explore the cause for TP superiority over other prognostic factors, the data were divided into 3 groups based on HCV genotype (Table V). With HCV genotypes of 2a and 2b, which are comparatively good responders to IFN therapy, TP is positively, and BMI, bilirubin, and AST are negatively, correlated with IFN therapy. As the number of cases with genotype 2a and 2b are small, the authors did not perform multivariate analysis with these groups alone. With HCV genotype 1b, NRs predominated over SVR (106 vs 25). In contrast to HCV genotype 2a and 2b, there were only small differences between SVR and NR with regard to BMI, bilirubin in this group, and with AST, SVR had a tendency to have higher AST values than NR in the HCV genotype 1b group. On the other hand, TP consistently gave higher values with SVR, as in the case of HCV genotype 2a and 2b. Although the reasons BMI, bilirubin, or AST are not related to the IFN therapeutic efficacy with HCV genotype 1b remain elusive, it is of interest that TP can serve as a good predictive factor even with this group of patients. Based on these findings, the authors suggest that TP is judged to be the prognostic factor most closely correlated with the therapeutic efficacy of IFN in logistic regression analysis of this study, because it can consistently serve as a good marker for IFN efficacy, regardless of HCV genotype.

The logistic regression equation determined in this study can be used to estimate the therapeutic efficacy of IFN for each case. The authors postulated 4 cases in which the virus- and IFN-related factors are in various

Table V. Comparison of prognostic factors between SVR and NR groups, subdivided into different HCV genotypes

Genotype	1 b		2 b		2 a	
Response	SVR	NR	SVR	NR	SVR	NR
Sample size	n 25	n 106	n 12	n 19	n 28	n 10
Variable	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
BMI	23.19 (2.32)	23.90 (2.46)	22.83 (3.56)	25.34 (4.49)	22.44 (2.67)	23.27 (2.08)
TP	76.9 (4.5)	73.6 (4.7)	75.3 (4.3)	72.2 (4.4)	75.1 (5.4)	73.0 (4.5)
TB	14.4 (4.4)	14.9 (5.3)	15.6 (8.2)	17.6 (7.5)	14.4 (4.8)	18.5 (10.1)
DB	4.4 (2.6)	4.1 (1.7)	4.4 (2.1)	5.3 (2.9)	3.9 (1.5)	5.0 (2.7)
AST	70.84 (44.54)	61.23 (36.71)	60.50 (49.54)	77.32 (53.63)	68.93 (47.67)	74.67 (62.86)

Table VI. Estimate of the therapeutic efficacy of IFN in 4 typical hypothetical cases with logistic regression equations

Degree	Variable	Unit	Case 1	Case 2	Case 3	Case 4
x_1	HCV genotype		2 b	2 b	1 b	1 b
x_2	bDNAp	kMeq/L	0.5	0.5	1.5	1.5
x_3	IFN dose	MU	800	800	600	600
x_4	BMI	Kg/m^2	20.0	25.0	20.0	25.0
x_5	TP	g/L	80	70	80	70
x_6	TB	mol/L	17	26	17	26
x_7	DB	mol/L	5	5	5	5
x_8	AST	U/L	50	100	50	100
Probability			0.977	0.042	0.546	0.002

relationships with the host-related factors, respectively (Table VI). With case 1, virus/IFN-related factors and the host-related factors are both in favor of the therapeutic efficacy of IFN, and the estimated probability of SVR is 0.977. IFN therapy is strongly recommended for cases that fulfill these factors. On the other hand, case 4 represents a condition in which virus/IFN-related factors and the host-related factors are at odds with the therapeutic efficacy of IFN. The probability of SVR is thus estimated to be 0.002. With case 2, there are favorable virus/IFN-related factors despite undesirable host-related factors, and case 3 represents the combinations of the factors opposite to case 2. In each case, it is learned that the therapeutic effect of IFN is not only determined by the virus/IFN-related factors and that, in some cases, the host-related factors more strongly affect the final outcome. Although the results of this study only provide retrospective analysis, if they are applied prospectively, they would provide important information in estimating the therapeutic efficacy of IFN and possibly in choosing cases to be treated with particular regimen.

This study on the prognostic factor analysis of IFN therapy has been performed only on cases with IFN monotherapy. It is desirable that this mode of analysis should be extended to comparative studies of other types of IFNs including PEG-IFN, combination therapy with ribavirin, which is now widely applied clinically, and the therapeutic outcome of IFN including side effects. It is also important to apply the results of this logistic regression analysis to prospective studies, and evaluate whether these prognostic factors can really estimate the therapeutic efficacy of IFN in a wide variety of clinical settings, particularly with patients more difficult to treat with IFN. For the results of this type of research to provide universal evidence, it is imperative to facilitate standardization of analytical methods and establish the long-standing system of quality assurance.

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Involvement of the PA28 -Dependent Pathway in Insulin Resistance Induced by Hepatitis C Virus Core Protein

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

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

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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 / CoreTg) mice develop marked insulin resistance and that the HCV core protein is degraded in the nucleus through a PA28 -dependent pathway. In this study, we examined whether PA28 is required for HCV core-induced insulin resistance in vivo. HCV core gene-transgenic mice lacking the PA28 gene (PA28 / CoreTg) were prepared by mating of PA28 / CoreTg with PA28 -knockout mice. Although there was no significant difference in the glucose tolerance test results among the mice, the insulin sensitivity in PA28 / 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 / CoreTg mice in response to insulin stimulation, whereas they were restored in the livers of PA28 / 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 gene. These results suggest that the HCV core protein suppresses insulin signaling through a PA28 -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 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-) secretion (47) and that the HCV core protein is degraded through a PA28 / REG (11S regulator)-dependent pathway in the nucleus (32). It is well known that PA28 enhances latent proteasome activity, although the biological significance of PA28 is largely unknown, with the exception that PA28 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 in the stability of IRS proteins and in the development of insulin resis-

* Corresponding author. Mailing address: Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan. Phone: 81-6-6879-8340. Fax: 81-6-6879-8269. E-mail: matsuura@biken.osaka-u.ac.jp.
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tance is not known. In this study, we examined the involvement of PA28 in the induction of insulin resistance by the HCV core protein in vivo.

MATERIALS AND METHODS

Preparation of PA28 -knockout HCV core gene-transgenic mice. C57BL/6 mice carrying the gene encoding HCV core protein genotype 1b (PA28 / CoreTg) line C49 and PA28 / mice have been described previously (35, 36). These two genotypes were crossbred to create PA28 / CoreTg mice. PA28 / CoreTg mice were bred to generate PA28 / CoreTg mice (35, 36). The HCV core gene and the target sequence to knock out the PA28 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- was measured by using a mouse TNF- 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 , and each of the insulin-signaling molecules were carried out with the liver tissue homogenates prepared in the homogenizing buffer containing 25 mM Tris-HCl (pH 7.4), 10 mM Na_3VO_4, 100 mM NaF, 50 mM $Na_4P_2O_7$, 10 mM EGTA, 10 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P40 supplemented with Complete Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany) (53). Tissue lysates were subjected to sodium dodecyl sulfate–2% to 15% gradient polyacrylamide gel electrophoresis (PAG Mini DAIICHI 2/15 13W; Daiichi Diagnostics, Tokyo, Japan) and electrotransferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA). The protein transferred onto the membrane was reacted with rabbit anti-HCV core (32), rabbit anti-Akt (Cell Signaling, Danvers, MA), rabbit anti-phospho-Ser473-Akt (Cell Signaling), rabbit anti-IRS1 (Upstate, Lake Placid, NY), rabbit anti-phospho-Tyr608 mouse insulin receptor substrate 1 (Sigma, St. Louis, MO), or rabbit anti-IRS2 (Upstate) polyclonal antibody and then incubated with horseradish peroxidase-conjugated anti-rabbit antibody. Blotted protein was visualized using Super Signal Femto (Pierce) and an LAS3000 imaging system (Fuji Photo Film, Tokyo, Japan).

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

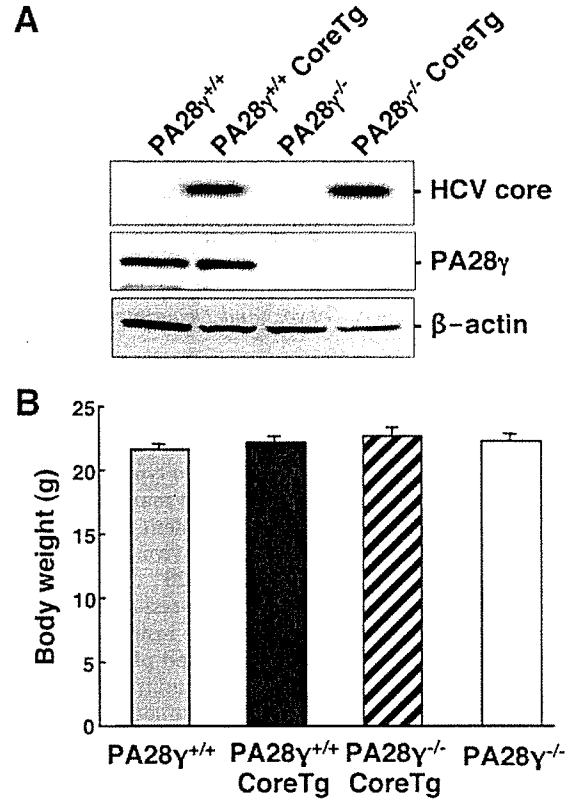

FIG. 1. Characterization of HCV core gene-transgenic mice deficient in the PA28 gene. (A) Expression of the HCV core protein and PA28 in the livers of PA28 / , PA28 / CoreTg, PA28 / , and PA28 / CoreTg mice. Lysates obtained from liver tissues of the mice (100 g protein/lane) were subjected to sodium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

Reporter assay for TNF- promoter activity. The promoter region of the TNF- 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 -knockdown cell lines were established by using pSilencer 2.1 U6 Hygro (Ambion) according to the manufacturer's protocol.

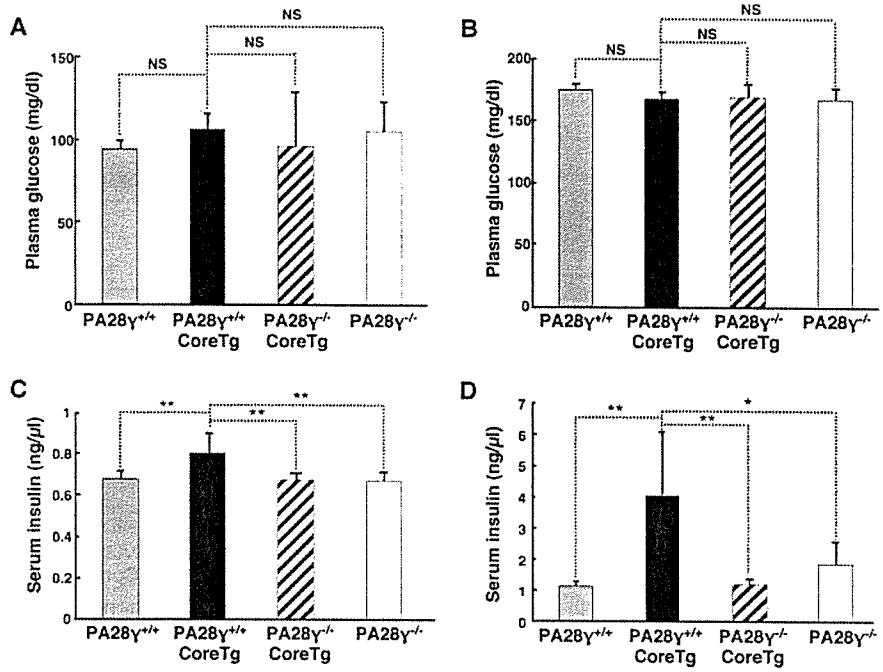

FIG. 2. Knockout of the PA28 gene inhibited the hyperinsulinemia induced by HCV core protein. Plasma glucose levels of PA28 / , PA28 / CoreTg, PA28 / CoreTg, and PA28 / mice under fasting (A) or fed (B) conditions (n 7 in each group) are shown. Serum insulin levels in fasting (C) or fed (D) mice (n 7 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 PA28 gene. To investigate the role of PA28 in the development of insulin resistance in HCV core gene-transgenic (PA28 / CoreTg) mice, we generated HCV core gene-transgenic mice deficient in the PA28 gene (PA28 / CoreTg). A PA28 / CoreTg mouse expressing an amount of PA28 equal to that of its normal littermates (Fig. 1A) was crossbred with a PA28 / mouse to generate a PA28 / CoreTg mouse. PA28 / CoreTg mice were bred with each other, and a PA28 / CoreTg mouse was selected by PCR. The HCV core protein was expressed in PA28 / CoreTg and PA28 / CoreTg

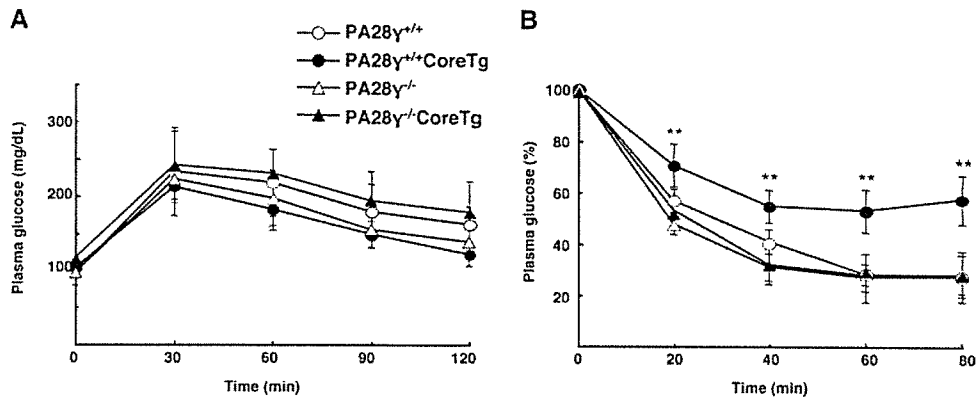

FIG. 3. Knockout of the PA28 gene inhibits the insulin resistance induced by the HCV core protein. (A) Glucose tolerance test. D-Glucose was intraperitoneally administered to mice fasted for more than 16 h at 1 g/kg of body weight. Plasma glucose levels were estimated at the indicated times (n 5 in each 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 / (open circles), PA28 / CoreTg (closed circles), PA28 / (open triangles), and PA28 / CoreTg (closed triangles) mice are represented as means and standard deviations. Significant differences in insulin sensitivity (P 0.01) in PA28 / CoreTg mice compared to that in PA28 / , PA28 / , or PA28 / CoreTg mice are indicated by double asterisks (**). There were no significant differences among PA28 / , PA28 / , and PA28 / CoreTg mice (P 0.05).

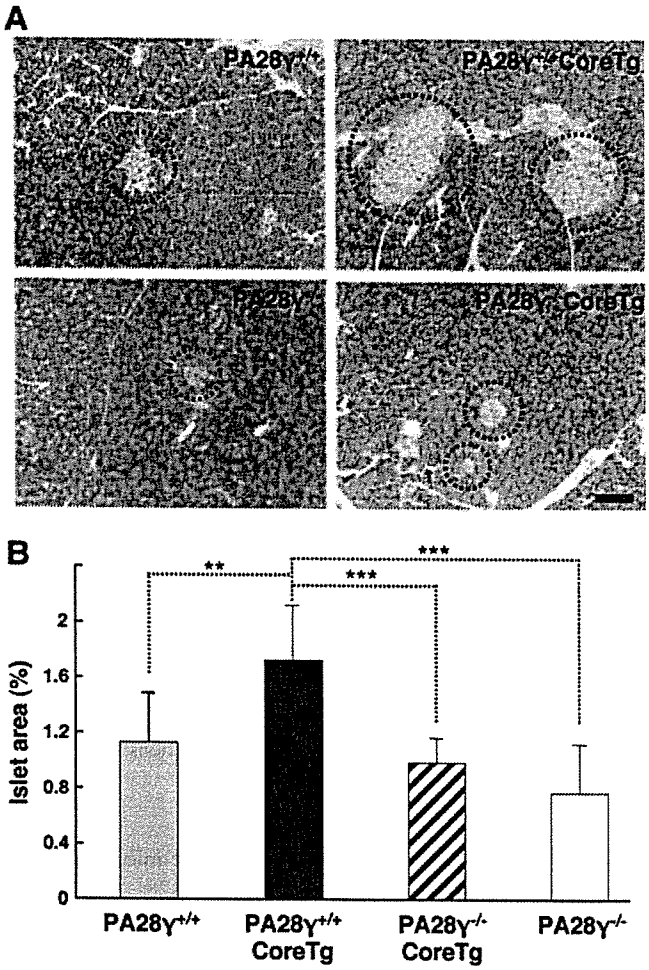

FIG. 4. PA28 participated in the enlargement of pancreatic islets induced by the HCV core protein. (A) Histological sections prepared from pancreas tissues of PA28 / , PA28 / CoreTg, PA28 / , and PA28 / CoreTg mice were stained with hematoxylin and eosin. Dotted circles indicate pancreatic islets. (B) 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 m.

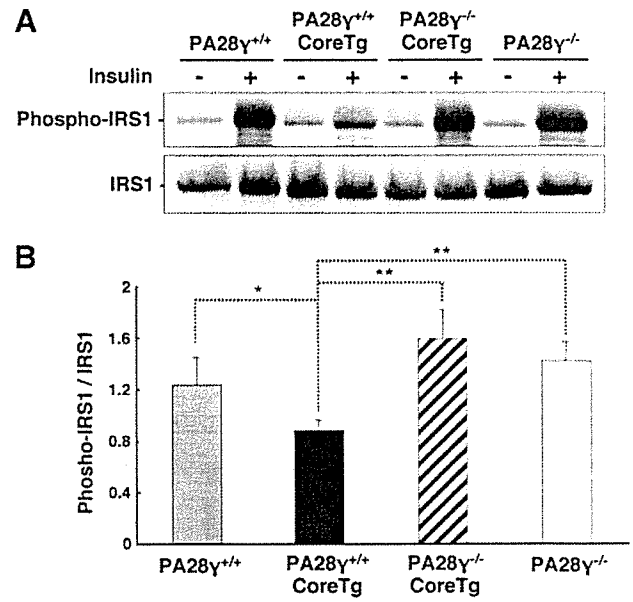

FIG. 5. PA28 participated in the inhibition of the tyrosine phosphorylation of IRS1 induced by the HCV core protein. Liver tissues from PA28 / , PA28 / CoreTg, PA28 / , and PA28 / CoreTg mice were prepared after administration of insulin ( ) or phosphate-buffered saline ( ). The samples (100 g of total 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.

mice but not in PA28 / (normal littermates) or PA28 / mice. PA28 was found at a similar level in PA28 / CoreTg and PA28 / mice but was not present in either PA28 / or PA28 / 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 / CoreTg (1.36 0.44 ng/mg of total protein; n 7) than in PA28 / 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 / mice were slightly smaller than their normal littermates (PA28 /) at more than 3 months old, as described previously (36), but this was not significantly different in 2-month-old mice (Fig. 1B). PA28 / CoreTg mice exhibited severe hepatic steatosis from 4 months of age (35). To avoid the influence of hepatic steatosis and body weight on the examination of insulin resistance, 2-month-old mice were used in this study. Figure 1B shows the body weights of 2-month-old mice. There were no significant differences in body weight among PA28 / CoreTg, PA28 / CoreTg, PA28 / , and PA28 / mice. Steatosis was not detected in the livers of the 2-month-old mice (data not shown).

PA28 is involved in the development of hyperinsulinemia and insulin resistance in PA28 / CoreTg mice. In our previous study, we found a significant difference in serum insulin levels, but not in plasma glucose levels, between PA28 / CoreTg mice and normal littermates (47). To determine the involvement of PA28 in the development of insulin resistance in PA28 / 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 / CoreTg mice than in PA28 / mice under both conditions (Fig. 2C and D), as described previously (47). In contrast, the serum insulin concentration in PA28 / CoreTg mice was recovered to a normal level similar to that of PA28 / and PA28 / 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 / CoreTg mice. In our previ-

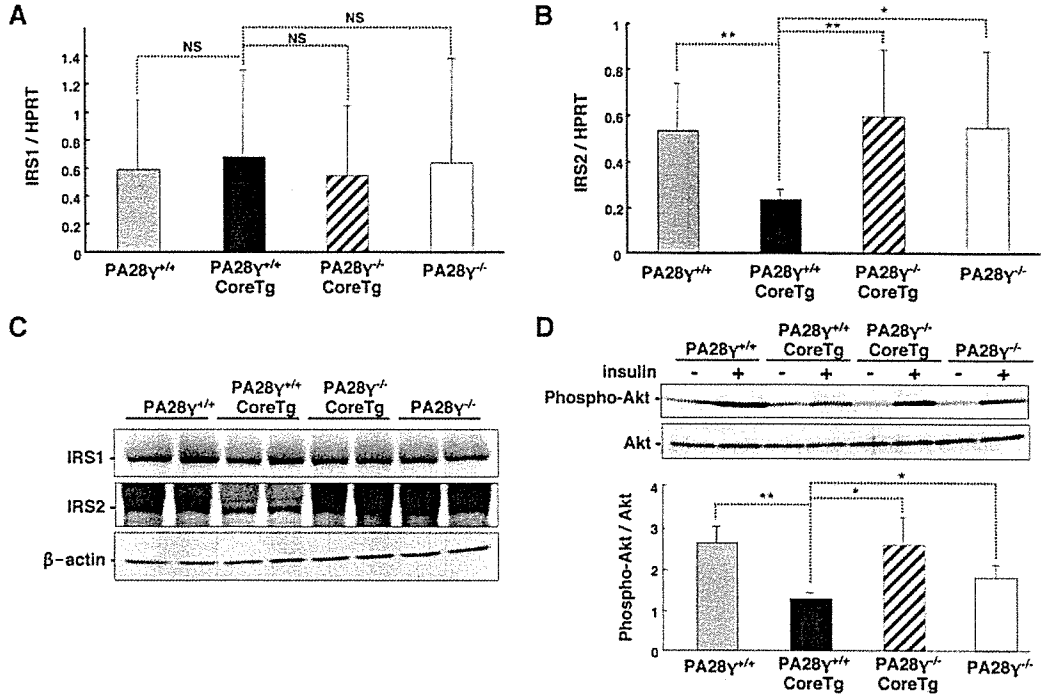

FIG. 6. PA28 participated in the inhibition of the IRS2 expression and Akt phosphorylation induced by HCV core protein. The transcription of IRS1 (A) and IRS2 (B) was estimated by quantitative RT-PCR (n 5 in each group). (C) The expression levels of IRS1 and IRS2 in the livers of the mice were determined 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 / CoreTg mice (47). In this study, PA28 / CoreTg mice exhibited a normal insulin level comparable to those of PA28 / and PA28 / mice by an insulin tolerance test, in contrast to PA28 / 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 / CoreTg mice to compensate for insulin resistance and retain a physiological level of plasma glucose and that PA28 participates in the development of hyperinsulinemia and insulin resistance in PA28 / 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 / CoreTg mice. To clarify whether a knockout of the PA28 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 / CoreTg mice was smaller than that of PA28 / CoreTg mice and comparable to that of PA28 / and PA28 / 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 also participates in the enlargement of pancreatic islets induced in PA28 / CoreTg mice.

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

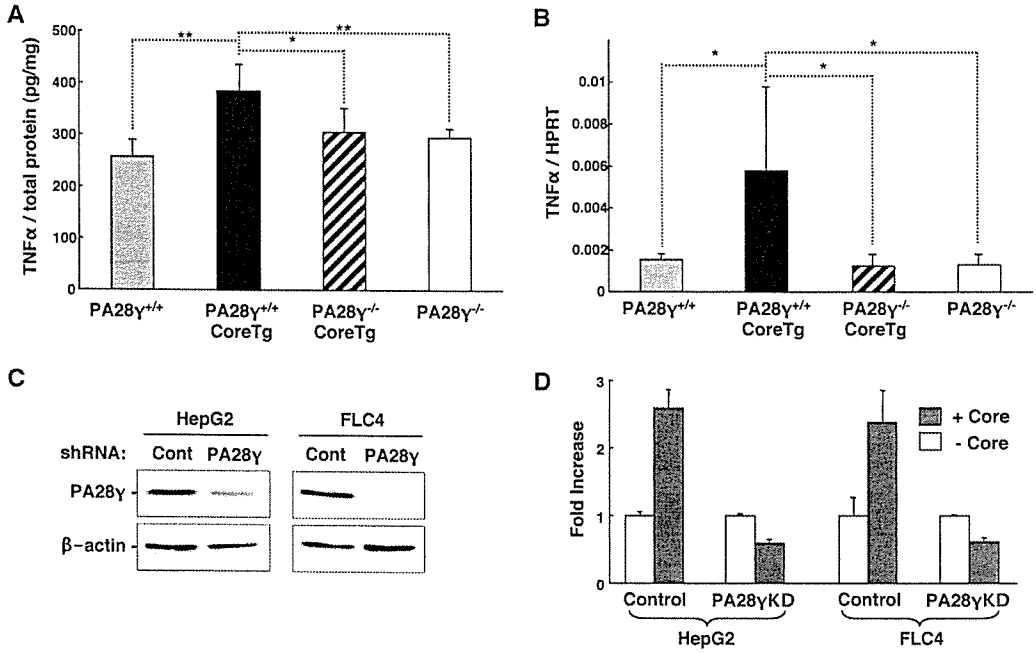

FIG. 7. PA28 was required for activation of the TNF- promoter by the HCV core protein. (A) Expression of TNF- in the livers of mice was determined by ELISA (*n* 5 in each group). (B) TNF- mRNA in the livers of mice was examined by quantitative RT-PCR (*n* 5 in each group). (C) Knockdown of the expression of PA28 in the HepG2 and FLC-4 cell lines by the introduction of a plasmid encoding a short hairpin RNA (shRNA) targeted to the PA28 gene. The expression levels of PA28 and -actin were determined by immunoblotting with specific antibodies. (D) Promoter activity of TNF- in the presence or absence of the HCV core protein was determined by luciferase assay in the PA28 -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 / 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 / CoreTg mice but not in those of PA28 / , PA28 / , or PA28 / 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 impairs the insulin-signaling pathway through the suppression of both the tyrosine phosphorylation of IRS1 and the expression of IRS2.

PA28 is required for activation of the TNF- promoter by HCV core protein. TNF- is an adipokine (54) and suppresses the signaling pathway of IRS1 and IRS2 (14, 42). Several reports suggested that the serum TNF- level is higher in HCV patients than in healthy individuals (19, 37). Elevations of TNF- levels have also been demonstrated in the livers of PA28 / CoreTg mice (47). To determine the involvement of PA28 in the enhancement of TNF- expression, the expression of TNF- in the livers of each genotype was determined by ELISA and real-time PCR (Fig. 7A and B). Transcription and translation of TNF- were increased in the livers of PA28 / CoreTg mice but were restored in the livers of PA28 / CoreTg mice to levels comparable to those of PA28 / and PA28 / mice. To determine the effect of PA28 expression on the promoter activity of TNF- in human liver cells, PA28 -knockdown human hepatoma cell lines HepG2 and FLC4 were established by the introduction of a plasmid encoding a short hairpin RNA targeting the PA28 gene in the cell lines. The expression of PA28 was clearly suppressed in the cell lines (Fig. 7C). The expression of HCV core protein in the hepatoma cell lines potentiated TNF- promoter activity, whereas the promoter activation by the HCV core protein was suppressed in the PA28 -knockdown cell lines (Fig. 7D). These results suggest that PA28 is required for the activation of the TNF- promoter induced by the expression of the HCV core protein in human hepatoma cell lines.

DISCUSSION

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

knockout of the IRS1 gene has been shown to lead to growth retardation and insulin resistance but not overt diabetes (5, 52). The double knockdown of IRS1 and IRS2 genes in the liver induces hyperinsulinemia and insulin resistance in mice (53). The reduction of both IRS1 and IRS2 under conditions of insulin resistance and hyperinsulinemia (3) and in the livers of *ob/ob* mice, an obese diabetic mouse model (20), has been reported previously. In the present study, the expression of the HCV core protein reduced the phosphorylation of tyrosine on IRS1 and the production of IRS2 in the livers of mice but did not completely abolish the activities of these genes, suggesting that residual activities of IRS transfer a faint signal to the downstream region of IRS. Therefore, PA28 / CoreTg mice may exhibit a milder phenotype than IRS1- and/or IRS2-knockout mice. In this study, knockout of the PA28 gene restored the insulin sensitivity and signaling of IRS1 and IRS2 in PA28 / CoreTg mice, suggesting that the expression of the HCV core protein leads to the dysfunction of both IRS1 and IRS2 through a PA28 -dependent pathway.

Our previous study suggested that the induction of TNF- by the HCV core protein plays a role in insulin resistance (47). An increase in TNF- levels has been correlated with obesity and insulin resistance in animal models and humans (14, 42). However, the mechanism by which TNF- induces insulin resistance is not completely known. The expression of TNF- has been shown to be increased in PA28 / CoreTg mice, resulting in the suppression of phosphorylation of IRS1, and insulin sensitivity in PA28 / CoreTg was improved by the administration of an anti-TNF- antibody (47). In the present study, the expression level of TNF- in PA28 / CoreTg mice was similar to that in PA28 / mice or their normal littermates. The expression of the HCV core protein enhanced the promoter activity of the TNF- gene in human liver cell lines but not in those with a knockdown of the PA28 gene by RNA interference (Fig. 7D). These data suggest that PA28 plays a crucial role in HCV core-induced expression of TNF- . 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- (17, 45). Therefore, it might be feasible to speculate that the HCV core protein may cooperate with PA28 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- 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 / CoreTg mice was similar to that in PA28 / CoreTg mice (data not shown). TNF- 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 / CoreTg mice.

How does the HCV core protein induce TNF- production? Our previous report suggests that the HCV core protein is degraded through a PA28 -dependent pathway (32). Recently, PA28 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 -dependent pathway may regulate the promoter activity of the TNF- 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 -dependent pathway activates the promoter of the TNF- 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- and suppresses the phosphorylation of tyrosine on IRS1 and the production of IRS2 through a PA28 -dependent pathway, thereby leading to insulin resistance. PA28 may be a novel target for the treatment of HCV-induced diabetes.

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