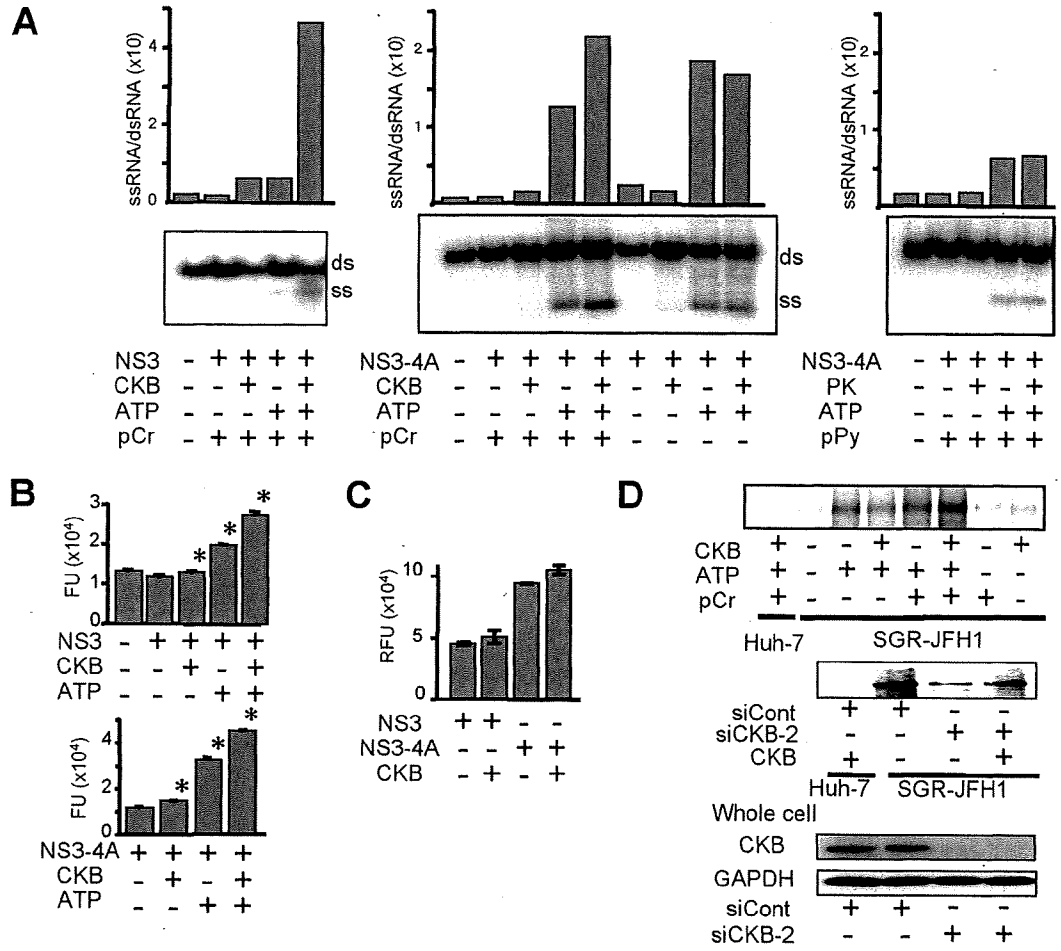

FIG. 5. CKB enhances NS3-4A helicase and HCV replicase activities. (A) In vitro RNA helicase activity of NS3-4A or NS3 was determined by detecting unwound single-strand RNA (ss) derived from the partially dsRNA substrate (ds). Band intensities corresponding to unwound products and those to dsRNA substrates were determined by ImageQuant 5.2 (Molecular Dynamics), and the ssRNA/dsRNA ratios were calculated. The results are representative of three similar experiments. (B) In vitro DNA helicase activity of NS3-4A or NS3 was analyzed by using a commercially available kit. The data represent averages and standard deviations ($n = 3$). *, $P < 0.05$ against the value without supplementation of CKB and ATP. (C) The in vitro HCV protease activity of NS3-4A or NS3 in the presence or absence of CKB was analyzed. Error bars represent standard deviations ($n = 3$). (D) Replicase activity in permeabilized replicon cells. The upper panel shows the activity for synthesis of HCV subgenomic RNA in the digitonin-permeabilized SGR-JFH1 cells with or without supplementation of CKB was measured. The middle panel shows results for SGR-JFH1 or Huh-7 cells that were transfected with siCKB-2 or siCont and permeabilized at 72 h posttransfection. The permeabilized cells with or without supplementation of CKB were subjected to the replicase assay. The lower panel shows the immunoblotting results for whole-cell lysates of siRNA-transfected cells.

DISCUSSION

Viral replication requires energy and macromolecule synthesis, and host cells provide the viruses with metabolic resources necessary for their efficient replication. Thus, it is highly likely that interaction of viruses with host cell metabolic pathways, including energy-generating systems, contributes to the virus growth cycle. In the regulation of HCV genome replication, the functions of the viral NS proteins that comprise the RC might be regulated by association in individual host cell factors. For example, hVAP-A and -B function as cofactors of modulating RC formation via interacting with NS5A and NS5B (13, 18). Cyclophilin B is involved in stimulating viral RNA binding activity via interacting with NS5B (49). FKBP8 (39) and hB-ind1 (45) play an important role in recruiting Hsp90 to RC via interacting with NS5A. However, the association of viral protein(s) with the cellular energy-generating system to directly regulate the activity of the RC has not been well understood.

In the present study, the accumulation of CKB, an ATP-generating enzyme, in the HCV RC-rich membrane fraction of viral replicating cells and its importance in replication of the HCV genome and production of infectious virions have been demonstrated. Enzymatic analyses with semi-intact replicon cells and purified NS3-4A protein revealed that CKB enhances the functional replicase and helicase of HCV. Its enhancing effect was observed in the presence of pCr but not in its absence, suggesting that the catalytic activity of CKB is important for enhancing the replicase and

helicase activities. Moreover, we clearly detected a CKB-NS4A complex using anti-tag antibodies in cotransfection experiments, but the endogenous complex could not be immunoprecipitated from cells expressing only endogenous levels of CKB, probably because of the inefficiency of the available antibodies. Further, a deletion of the NS4A-interacting region within an inactive mutant of CKB (CKB-C283S) resulted in the loss of its dominant-negative effect on HCV replication.

Creatine kinase, an evolutionarily conserved enzyme, is known to be critical for the maintenance and regulation of cellular energy stores in tissues with high and rapidly changing energy demands (48). In mammals, three cytosolic and two mitochondrial isoforms of CK, which share certain conserved regions, are expressed (35). The brain-type CK, CKB, plays a major role in cellular energy metabolism of nonmuscle cells, reversibly catalyzing the ATP-dependent phosphorylation of creatine and, hence, providing an ATP buffering system in subcellular compartments of high and fluctuating energy demand (21, 29). CKB is overexpressed in a wide range of tumor tissues and tumor cell lines, including hepatocellular carcinoma (32), and is used as a prognostic marker of cancer.

Although CK and creatine phosphate have been supplemented to in vitro replicase assays of some RNA viruses (15, 33), understanding of CKB function in the virus life cycle has been limited. One study indicated that the CK substrate analog, Ccr, exhibits antiviral activity against several herpesviruses but not influenza viruses or vesicular stomatitis virus (26). We have demonstrated here that HCV genome replication is downregulated by either treatment with Ccr, siRNA-mediated knockdown of CKB, or the exogenous expression of CKB-C283S. Coimmunoprecipitation experiments revealed that the essential domain within NS4A for the interaction with CKB is the NS4A central domain, aa 21 to 39, which is also responsible for NS3-4A complex formation. However, the NS3-4A interaction was not impaired by overexpression of CKB, and CKB was found to be able to form a complex with NS3-4A (Fig. 3H). Since CKB does not directly interact with NS3 (Fig. 3A), it is likely that NS3-4A-CKB association occurs through two interactions of NS3-4A and NS4A-CKB. We examined whether the formation of the ternary complex affects HCV enzymatic activities, possibly through conformational changes in the viral proteins, and found that CKB has no influence on NS3-4A protease activity (Fig. 5C). With regard to helicase activity, the effect of CKB on RNA unwinding activity by NS3-4A was similar to the effect of NS3 alone in the presence of ATP (Fig. 5A). It is conceivable that interaction with CKB causes no or little global change in the NS3-4A conformation and does not affect the viral helicase and protease activities.

In general, translation initiation in eukaryotes includes an ATP-dependent process such as unwinding the secondary structure in the 5′-untranslated region to permit assembly of 48S ribosomal complexes. It was reported, however, that 48S complex formation on the HCV internal ribosome entry site (IRES) has no requirement for ATP hydrolysis (25). In fact, we found that Huh-7 cells with or without gene silencing of CKB exhibited the same level of HCV IRES activity by transfection with IRES-reporter constructs (data not shown).

Collectively, we conclude that CKB is targeted to the HCV RC through its interaction with NS4A and functions as a positive regulator for the viral replicase by providing ATP. It is likely that the catalytic activity of CKB that associates with the viral RC is important for enhancing the RNA replication. The role of CKB-NS4A interaction in the enhancing effect seems to be limited. Although either knocking down CKB, expression of the dominant-negative mutant of CKB, or Ccr treatment resulted in the reduction of HCV replication (Fig. 2A to C), the total cellular ATP levels were not changed under these conditions (Fig. 2D). This suggests that CKB contributes to enhancing HCV replication through controlling the ATP level in the particular RC compartment. A tight coupling of a fast ATP regeneration and delivery system to the viral RC is advantageous for achieving efficient replication of the viral genome. To our knowledge, the findings presented here provide the first experimental evidence of the involvement of viral protein in recruiting an ATP generating/buffering system to the subcellular compartment for viral genome replication, a site with high-energy turnover. Given that the levels of HCV RNA were not dramatically diminished by the knocking down, dominant-negative mutant or Ccr, CKB may not be absolutely critical for the viral replication. One would argue that energy required for HCV genome replication can be partly complemented from the intracellular ATP pool.

Although there are several isoforms of CK as described above, the most abundant CK species expressed in Huh-7 cells in the present study was CKB, and no other isoenzymes, including mitochondrial CK, were detected by an isoform analysis based on the overlay gel technique (32; data not shown). Thus, the CKB isoenzyme appears to be a key molecule in the energy metabolism of HCV replicating cells. To identify potential HCV RC components, we used a comparative proteome analysis of the DRM fraction in cells harboring HCV subgenomic replicon and the DRM fractions in parental cells and then identified proteins that were more abundant in the fraction of HCV replicating cells. In agreement with similar previously reported approaches using the DRM or lipid raft fraction (30, 53), the functional categories of identified proteins included protein folding or assembly, cell metabolism and biosynthesis, cellular processes, and cytoskeleton organization (Table 1). Interestingly, Mannova et al. found that CKB was upregulated in the fraction of Huh-7 cells carrying the genotype 1b Con1 isolate-derived HCV replicon, as determined using stable isotope labeling by amino acids combined with one-dimensional electrophoresis (30). However, the effect of CKB on regulation of the HCV life cycle was not examined in that study.

In conclusion, CKB interacts with HCV NS4A and is important for efficient replication of the viral genome. Recruitment of CKB to the HCV replication machinery through its interaction with NS4A may have important implications for the maintenance or enhancement of the functional replicase activity in the RC compartment, where high-energy phosphoryl groups are required. A strategy for specific interception of energy supply at the subcellular site of HCV genome replication by disruption of the NS4A-CKB interface may lead to development of a new type of antiviral agent.

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Impacts of Changes in Obesity Parameters for the Prediction of Blood Pressure Change in Japanese Individuals

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Key Words
Waist circumference · Body mass index · Blood pressure · Health screening

Abstract
Aims and Methods: By analyzing data from 2,861 individuals who underwent general health screening 2 years running, we have investigated the impact of changes in waist circumference (WC) and body mass index (BMI) over a 1-year period on systolic blood pressure (BPs). We termed WC, BMI, and BPs at the first visit as WC1, BMI1, and BPs1, respectively, and those at the second visit as WC2, BMI2, and BPs2, respectively. The %dWC, %dBMI, and %dBPs was defined as (WC2 – WC1)/WC1 × 100, (BMI2 – BMI1)/BMI1 × 100, and (BPs2 – BPs1)/BPs1 × 100, respectively. ***Results:*** In multivariate regression analysis using age, BPs1, WC1, and %dWC as independent variables, %dWC was a significant predictor for %BPs only in men. %dBMI was a significant predictor for %BPs in both genders when age, BPs1, BMI1, and %dBMI were used as independent variables. Compared with individuals with both %dWC <0 and %dBMI <0, age-adjusted %dBPs was significantly greater in those with both %dWC <0 and %dBMI ≥0; however, it did not significantly differ in those with both %dWC ≥0 and %dBMI <0. ***Conclusion:*** Our data suggest that the impact of BMI change might be greater than WC change in terms of BPs change during this short period.

Introduction

Much evidence supports a positive association between obesity parameters and hypertension [1–4], although the strength of such an association may differ according to the parameter used [5]. In addition, a loss or gain in body weight may affect blood pressure levels [6, 7], even in relatively lean or non-obese individuals [8, 9]. Therefore, weight control may be an important target for better blood pressure control, leading to a reduction in mortality from heart and cerebrovascular disease [4]. Compared with weight, or body mass index (BMI), less information seems to be available on whether, or to what extent, a loss (or gain) in waist circumference (WC) would result in a change in blood pressure. We previously reported that a reduction or gain in obesity parameters may affect the status of chronic kidney disease in individuals who underwent general health screening [10]. To this end, here we investigated the mode of association be-

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tween changes in WC or BMI over a 1-year period and changes in blood pressure levels in Japanese individuals. We analyzed the data separately for each gender, because there may be gender differences in the strength of the association between various obesity parameters and blood pressure [11].

Subjects and Methods

Study Population

The study was approved by the Ethical Committees of University of Tokyo and Mitsui Memorial Hospital. Between October 2005 and October 2006, 3,312 (1,203 women, 2,109 men) individuals underwent general health screening (visit 1), and they visited our institute again in the following year (visit 2). Among these 3,312 individuals, 2,861 (1,114 women, 1,747 men) who reported not taking antihypertensive drugs at both visits were enrolled in the present study. After about 10 min of rest, systolic blood pressure (BPs) and diastolic blood pressure (BPd) were measured in the sitting position by automated sphygmomanometer, BP-203RVIII (Omron Colin, Tokyo, Japan). Blood pressure was measured twice and the mean of these data were taken. With the subject standing, WC was measured at the umbilical level to the nearest 1 cm by trained physicians and technicians [12]. After changing into a robe from our institute, height and weight were measured, and the weight of the robe was subtracted from the value indicated by the scales. Age, WC, BMI, and BPs at visit 1 were designated age1, WC1, BMI1, and BPs1, respectively. Similarly, WC, BMI, and BPs at visit 2 were designated WC2, BMI2, and BPs2, respectively. %dWC, %dBMI, and %dBPs were defined as (WC2 – WC1)/WC1 × 100, (BMI2 – BMI1)/BMI1 × 100, and (BPs2 – BPs1)/BPs1 × 100, respectively.

Laboratory Analysis

Blood samples were taken from the subjects after an overnight fast. Serum levels of total cholesterol (TC), HDL cholesterol (HDL-C), and triglycerides (TG) were determined enzymatically. Serum uric acid was measured by the uricase-peroxidase method, hemoglobin A_{1C} was determined using the latex agglutination immunoassay. Serum creatinine was measured by TBA-200FR (Toshiba Medical Systems, Tochigi, Japan) using commercially available kits, Accuras Auto CRE (Shino-test, Tokyo, Japan), according to the manufacturer's instructions. Accuracy control was performed every day by constructing X-bar and R charts using commercially available standards. Estimated glomerular filtration rate (eGFR) was calculated by the following equation: $eGFR = 194 \times (\text{serum creatinine})^{-1.094} \times (\text{age})^{-0.287}$ (× 0.739 if female) [13]. Serum insulin was measured by enzyme immunoassay. Homeostasis model assessment insulin resistance (HOMA-IR) was calculated in these individuals according to the following formula: HOMA-IR = [fasting immunoreactive insulin (μU/ml) × fasting plasma glucose (mg/dl)]/405 [14].

Statistical Analysis

Data are expressed as the mean ± SD unless stated otherwise. Analyses of variance with trend analysis, Tukey's post-hoc analysis and multiple regression analysis were conducted as appropriate to assess the statistical significance of differences between groups using computer software Dr. SPSS II (SPSS, Inc., Chicago, Ill., USA). A value of $p < 0.05$ was taken to be statistically significant.

Results

Baseline Characteristics

As described in the Methods section, among the 3,312 individuals who underwent general health screening visited our institute again in the following year; 2,861 (1,114 women, 1,747 men) who reported not taking antihypertensive drugs at both visits were enrolled in the current study (table 1). The mean ± SD of the interval between the two visits of the individuals enrolled was 355 ± 52 days. The mean ± SD age of the enrolled women (51.3 ± 9.9 years) and men (52.5 ± 10.1 years) was significantly smaller than that of the women (60.7 ± 8.3 years) and men (59.0 ± 8.5 years), respectively ($p < 0.001$), who were excluded because of the antihypertensive medication at either or both visits. Similarly, the mean BMI values of enrolled women (21.2 ± 2.9) and men (23.5 ± 2.7) were significantly smaller than those of the excluded women (22.5 ± 3.2) and men (25.0 ± 2.8), respectively ($p < 0.001$).

WC1 ranged between 51.8 and 118.5 cm, and a WC1 ≥90 cm was found in 71/1,114 women (6.4%), and a WC1 ≥85 cm was found in 183/1,114 men (16.4%). BMI1 ranged between 13.1 and 39.4. A BMI1 ≥25 was found in 110/1,114 women (9.9%) and 453/1,747 men (25.9%), and BMI1 ≥30 was found only in 12/1,114 (1.1%) women and 33/1,747 (1.9%) men. The correlation coefficients between %dWC, %dBMI, %dBPs, WC1, BMI1, and BPs1 are described in table 2. The correlation between %dWC and %dBMI was found to be moderate in men (r = 0.476), whereas it was weak in women (r = 0.241). The relationship between %dBMI and %dBPs was found to be statistically significant in the both genders. On the other hand, the relationship between %dWC and %dBPs was statistically significant only in men. Among the study subjects, it was reported that 60 subjects experienced a WC change of –10 cm or less, and 94 subjects experienced a WC change of +10 cm or more. After excluding these 154 individuals from the study population, the results obtained were not essentially changed (data not shown). It was calculated that a 10% weight gain (loss) over a 1-year period was associated with a 3.88 mm Hg BPs gain (loss) in women and a 9.86 mm Hg BPs gain (loss) in men.

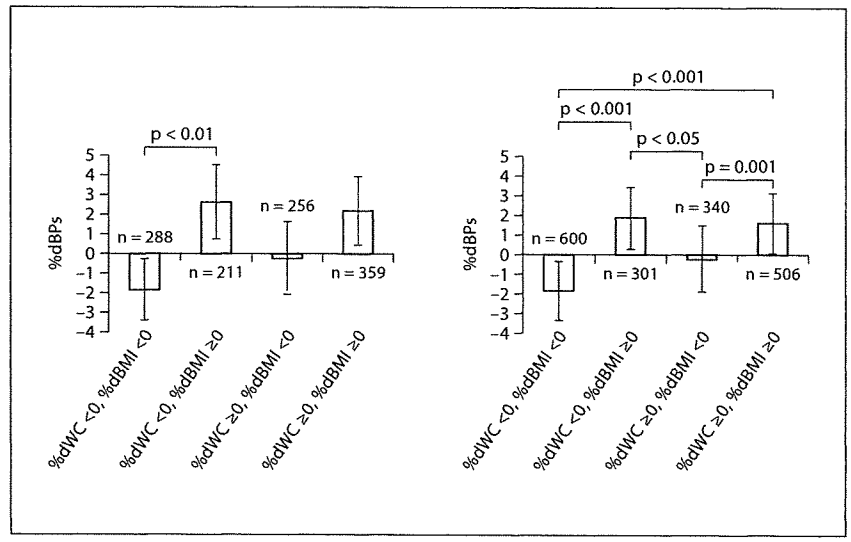

Fig. 1. Comparison of the age-adjusted %dBPs in four subgroups categorized according to the gain or loss of %dWC and %dBMI values. p values were from the result of the Tukey's post-hoc analysis following analyses of variance. Mean ± 95% confidence interval is shown in each group.

Table 1. Clinical characteristics and laboratory data at the first visit

Variables	Whole	%dBPs				p value
		first (range: −40 ~ −7)	second (range: −7 ~ 0)	third (range: +1 ~ +6)	fourth (range: +6 ~ +52)	
Number	2,861	714	809	639	699	
Women/men	1,114/1,747	288/426	314/495	251/388	261/438	0.712
Age, years	52.0 ± 10.1	52.8 ± 10.1	51.4 ± 9.9	51.8 ± 10.0	52.2 ± 10.2	0.047
Height, cm	164.8 ± 8.4	164.5 ± 8.3	165.2 ± 8.5	164.7 ± 8.5	164.7 ± 8.6	0.379
Weight, kg	61.8 ± 11.5	61.8 ± 11.4	62.0 ± 11.6	61.5 ± 11.3	61.8 ± 11.7	0.883
BMI, kg/m^2	22.6 ± 3.0	22.7 ± 3.0	22.6 ± 3.1	22.5 ± 3.0	22.6 ± 3.1	0.781
WC, cm	81.8 ± 9.1	82.0 ± 9.1	81.8 ± 9.3	81.5 ± 9.0	81.9 ± 9.0	0.851
Systolic BP, mm Hg	120.9 ± 18.0	128.7 ± 18.3	121.8 ± 17.0	118.5 ± 16.7	114.2 ± 16.8	<0.001
Diastolic BP, mm Hg	76.4 ± 11.4	79.3 ± 11.3	76.8 ± 10.9	75.5 ± 11.0	73.7 ± 11.5	<0.001
LDL cholesterol, mg/dl	129.2 ± 31.1	131.4 ± 31.5	128.3 ± 29.5	127.1 ± 30.9	130.1 ± 32.4	0.051
HDL cholesterol, mg/dl	61.2 ± 15.3	60.8 ± 15.0	61.8 ± 15.7	61.4 ± 15.6	60.7 ± 15.0	0.465
Triglyceride, mg/dl	109.9 ± 71.4	115.7 ± 69.9	104.7 ± 61.8	109.8 ± 81.0	110.1 ± 73.4	0.030
Uric acid, mg/dl	5.4 ± 1.3	5.4 ± 1.3	5.5 ± 1.3	5.4 ± 1.4	5.5 ± 1.4	0.688
Fasting glucose, mg/dl	95.2 ± 20.0	96.8 ± 20.4	95.1 ± 21.1	94.2 ± 18.0	94.7 ± 20.0	0.072
Hemoglobin A1C, %	5.3 ± 0.7	5.3 ± 0.7	5.3 ± 0.7	5.3 ± 0.7	5.3 ± 0.7	0.506
HOMA-IR	1.5 ± 1.1	1.6 ± 1.1	1.5 ± 1.1	1.4 ± 1.0	1.5 ± 1.0	0.066
Blood urea nitrogen, mg/dl	14.0 ± 3.4	13.8 ± 3.7	14.0 ± 3.2	14.2 ± 3.4	14.1 ± 3.5	0.245
Serum creatinine, mg/dl	0.8 ± 0.3	0.8 ± 0.4	0.8 ± 0.2	0.8 ± 0.2	0.8 ± 0.2	0.764
Estimated glomerular filtration rate	68.6 ± 11.8	68.3 ± 11.4	69.3 ± 12.0	68.4 ± 11.8	68.1 ± 11.8	0.177
Antidiabetic medication, n (%)	51 (1.8)	12 (1.7)	20 (2.5)	10 (1.6)	9 (1.3)	0.335
Current smoker, n (%)	680 (23.8)	179 (25.0)	184 (22.7)	139 (21.8)	178 (25.5)	0.298

Data are means ± SD, unless stated otherwise. BMI = Body mass index; WC = waist circumference; HOMA-IR = homeostasis model assessment of insulin resistance. %dBPs was calculated by the following equation: (BPs at the second visit − BP1 at the second visit)/(BP1 at the second visit) × 100 (%). p value is for trend.

Table 2. Pearson's correlation coefficient of obesity indices and blood pressure parameters

	%dWC	%dBMI	%dBPs	WC1	BMI1	BPs1
Women						
%dWC						
r	–					
p value	–					
%dBMI						
r	0.241	–				
p value	<0.001	–				
%dBPs						
r	−0.014	0.097	–			
p value	0.635	0.001	–			
WC1						
r	−0.317	−0.053	−0.028	–		
p value	<0.001	0.078	0.350	–		
BMI1						
r	−0.026	−0.087	−0.029	0.787	–	
p value	0.393	0.004	0.331	<0.001	–	
BPs1						
r	−0.025	−0.055	−0.325	0.365	0.409	–
p value	0.396	0.064	<0.001	<0.001	<0.001	–
Men						
%dWC						
r	–					
p value	–					
%dBMI						
r	0.476	–				
p value	<0.001	–				
%dBPs						
r	0.116	0.232	–			
p value	<0.001	<0.001	–			
WC1						
r	−0.268	−0.089	−0.031	–		
p value	<0.001	<0.001	0.189	–		
BMI1						
r	−0.054	−0.071	−0.026	0.830	–	
p value	0.023	0.003	0.286	<0.001	–	
BPs1						
r	−0.090	−0.077	−0.327	0.308	0.322	–
p value	<0.001	0.001	<0.001	<0.001	<0.001	–

BPs = Systolic blood pressure; WC = waist circumference; BMI = body mass index. BPs at visit 1 and visit 2 were designated BPs1 and BPs2, respectively. BMI at visit 1 and visit 2 were designated BMI1 and BMI2, respectively, and WC at visit 1 and visit 2 were designated WC1 and WC2, respectively. %dBMI, %dWC, and %dBPs were calculated by the equation (BMI2 − BMI1)/BMI1 × 100 (%), (WC2 − WC1)/WC1 × 100 (%), and (BPs2 − BPs1)/BPs1 × 100 (%), respectively.

Table 3. Multiple regression analysis between %dBPs and age1, WC1, BMI1, %dWC, and %dBMI

	β	95% CI	Standardized β	p value
Women				
Model 1				
BPs1	−0.23	−0.27 to −0.20	−0.38	<0.001
Age1	0.11	0.05 to 0.18	0.10	0.001
WC1	0.11	0.03 to 0.19	0.09	0.005
%dWC	0.01	−0.06 to 0.09	0.01	0.733
Model 2				
BPs1	−0.24	−0.28 to −0.21	−0.40	<0.001
BMI1	0.47	0.25 to 0.70	0.13	<0.001
Age1	0.13	0.07 to 0.19	0.12	<0.001
%dBMI	0.34	0.15 to 0.53	0.10	0.001
Model 3				
BPs1	−0.24	−0.28 to −0.21	−0.40	<0.001
BMI1	0.65	0.28 to 1.03	0.17	0.001
Age1	0.14	0.07 to 0.20	0.13	<0.001
%dBMI	0.39	0.19 to 0.60	0.11	<0.001
WC1	−0.08	−0.21 to 0.05	−0.06	0.244
%dWC	−0.08	−0.17 to 0.01	−0.06	0.071
Men				
Model 1				
BPs1	−0.22	−0.25 to −0.19	−0.35	<0.001
WC1	0.15	0.08 to 0.22	0.11	<0.001
%dWC	0.28	0.17 to 0.39	0.11	<0.001
Age1	0.02	−0.03 to 0.07	0.02	0.467
Model 2				
BPs1	−0.22	−0.25 to −0.19	−0.35	<0.001
%dBMI	0.80	0.64 to 0.96	0.22	<0.001
BMI1	0.41	0.23 to 0.59	0.10	<0.001
Age1	0.05	0.00 to 0.10	0.05	0.035
Model 3				
BPs1	−0.22	−0.25 to −0.19	−0.35	<0.001
%dBMI	0.82	0.63 to 1.00	0.22	<0.001
BMI1	0.38	0.04 to 0.72	0.10	0.027
Age1	0.05	0.00 to 0.10	0.05	0.046
WC1	0.01	−0.11 to 0.14	0.01	0.845
%dWC	−0.03	−0.16 to 0.11	−0.01	0.705

BPs = Systolic blood pressure; WC = waist circumference; BMI = body mass index. Standardized β values are the estimates resulting from an analysis performed on variables that were standardized. BPs at visit 1 and visit 2 were designated BPs1 and BPs2, respectively. BMI at visit 1 and visit 2 were designated BMI1 and BMI2, respectively, and WC at visit 1 and visit 2 were designated WC1 and WC2, respectively. %dBMI, %dWC, and %dBPs were calculated by the equation of (BMI2 − BMI1)/BMI1 × 100 (%), (WC2 − WC1)/WC1 × 100 (%), and (BPs2 − BPs1)/BPs1 × 100 (%), respectively.

Model 1 = Independent variables include age, BPs1, WC1, and %dWC; model 2 = independent variables include age, BPs1, BMI1, and %dBMI; model 3 = independent variables include model 1 + BMI1, and %dBMI.

Multiple Linear Regression Analysis

In multiple regression analysis, in which age1, WC1, BPs1, and %dWC were used as independent variables (model 1), %dWC was found to be an independent predictive value for %dBPs in men, but not in women (table 3). In a model where age1, BMI1, BPs1, and %dBMI were used as independent variables (model 2), %dBMI was found to be an independent predictive value for %dBPs in the both genders. After including all of the age1, BPs1, WC1, BMI1, %dWC, and %dBMI in a model as independent variables (model 3), %dBMI remained to be a predictor for %dBPs in both genders. In model 3, the variance inflation factor scores of all applied independent variables were <10 (data not shown)

Comparison between Individuals with BMI Gain or Loss together with WC Gain or Loss

We then compared the %BPS values between individuals with both WC loss (%dWC <0) and BMI loss (%dBMI <0), those with both WC loss and BMI gain (%dBMI ≥0), both WC gain and BMI loss, and those with both WC gain and BMI gain during a 1-year period (fig. 1). Age-adjusted %dBPs was significantly greater in individuals with both WC loss and BMI gain compared with those with both WC loss and BMI loss. On the other hand, age-adjusted %dBPs did not significantly differ between individuals with both WC loss and BMI loss and those with WC gain and BMI loss in both genders. When the same analysis was performed after excluding 154 subjects who experienced WC change of –10 cm or less or +10 cm or more, the results obtained were not essentially changed (data not shown).

Discussion

By analyzing data from individuals who underwent general health screening for 2 consecutive years, we showed that a percent difference in BMI (%dBMI) was a statistically significant predictor for a percent difference in BPs (%dBPs) in both genders. A percent difference in WC (%dWC) was also found to be a predictor for %dBPs in men; however, it lost statistical significance after further adjustment for BMI at the first visit and %dBMI, and it was not significant in women before and after such further adjustment.

A body of evidence indicates an association between obesity parameters and blood pressure levels [15, 16]. A reduction in body weight may result in a lowering of blood pressure in overweight or obese subjects [17, 18], although the results may not be always uniform. Moore et al. [19] showed that modest weight loss over a 4-year period substantially lowered the long-term risk of hypertension in overweight adults in Framingham. Haung et al. [20] showed that weight loss occurring after 18 years of age was related to a significantly lower risk, whereas weight gain was related to greater risk of hypertension in middle-aged women. In addition, Yang et al. [21] showed that in men aged between 40 and 74 years, weight gain occurring after 20 years of age was significantly associated with prehypertension. Most of the reports studying the potential association between changes in obesity parameters and changes in blood pressure were carried over a follow-up period longer than that in the current study. Furthermore, Truesdale et al. [22] have more recently shown that weight change over a 3-year period resulted in change in blood pressure levels; men who had experienced a 10% weight gain over the previous 3 years had BPs that was 2.6 mm Hg higher. They found, however, that the impact of weight change was, albeit present, less prominent in women. Women who had experienced a 10% weight gain over the previous 3 years had BPs that was only 0.9 mm Hg higher, suggesting the presence of gender difference in the extent of association between weight change and blood pressure change. We also showed here that the magnitude of the effect of changes in obesity parameters on blood pressure changes may vary by gender (table 3).

As compared to changes in weight, and thus in BMI, fewer analyses have focused on the relationship between changes in WC and blood pressure alterations. Considering that reductions in WC have been recommended more strongly than before for the purpose of prophylaxis and/or resolution of metabolic syndrome by the government in our country [23], the impact of WC reduction (gain) in terms of alterations of atherogenic risk factors, including blood pressure and levels of glucose and lipids, is becoming a more important issue to be investigated. Therefore, we also assessed whether changes in WC were reflected by the BPs change, and whether this relationship, if present, was independent of BMI change. We found that WC change was predictive of BPs change in men but not in women. In addition, the association between %dWC and %dBPs in men lost statistical significance after controlling for BMI1 and %dBMI (table 3). In contrast, %dBMI was a predictor for %dBPs in both genders regardless of the control of %dWC, suggesting that a reduction in BMI may represent a more essential target than WC reduction in terms of blood pressure control. This concept may be further supported by our finding that mean %dBPs did

not differ significantly between individuals with %dWC <0 and those with %dWC ≥0 among individuals with %dBMI <0. In reverse, %dBPs reduction was significantly greater in individuals with %dBMI <0 than in those with %dBMI ≥0 among individuals with %dWC <0 (fig. 1).

It has been reported that, in individuals with a mean BMI of 31, change in BMI was significantly correlated with change in BPs in both genders, even after adjusting for change in waist-hip ratio [24]. In the same study, it was reported that change in waist-hip ratio was not significantly correlated with change in BPs after adjusting for BMI change in men, and that the relationship between change in waist-hip ratio and BPs change was not significant before any adjustment in women. The results of Wing et al. [24] can be said to be similar to our current observation although there is a difference between WC and waist-hip ratio.

The current study has several limitations. First, we retrospectively analyzed data on individuals who underwent general health screening at our institute for 2 consecutive years; as a result, individuals who did not visit our institute the second year for unknown reasons were not enrolled in the current study, which may cause some biases. Second, we could not specify the reasons for weight gain or loss in individuals, however, very few individuals would have been taking antiobesity medications because only one individual in each gender had a BMI of 35 kg/m^2 or more at the first visit. Third, this study population included many non-obese subjects; a BMI1 ≥30 was found only in 1.1% of women and 1.9% of men. Fourth, we excluded those subjects who were taking antihypertensive drugs at either visit. We found that BMI was significantly greater in these excluded subjects than in the study population for both genders. Lastly, although change in BMI may seem to be superior for predicting BPs change than changes in abdominal obesity, abdominal fat volume should be measured by more reliable methods, such as computed tomography, before conclusion. In addition, we have to follow the subjects for a longer period, as a recent study has shown that surrogate measures of abdominal obesity are stronger predictors of all-cause and cardiovascular death than BMI in the general population [25].

In conclusion, in individuals who underwent general health screening for consecutive years, percent change in WC was significantly associated with percent change in BPs in men, but not in women; although this association in men lost statistical significance after controlling for percent change in BMI. By contrast, percent change in BMI was significantly associated with percent change in BPs regardless of controlling for percent change in WC. Our data suggest that controlling BMI, and thus controlling body weight, may represent a more essential goal than a reduction in WC in terms of blood pressure lowering among Japanese individuals who are not taking antihypertensive medication.

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Immunopathology and Infectious Diseases

Tacrolimus Ameliorates Metabolic Disturbance and Oxidative Stress Caused by Hepatitis C Virus Core Protein

Analysis Using Mouse Model and Cultured Cells

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Hepatic steatosis and insulin resistance are factors that aggravate the progression of liver disease caused by hepatitis C virus (HCV) infection. In the pathogenesis of liver disease and metabolic disorders in HCV infection, oxidative stress due to mitochondrial respiratory chain dysfunction plays a pivotal role. Tacrolimus (FK506) is supposed to protect mitochondrial respiratory function. We studied whether tacrolimus affects the development of HCV-associated liver disease using *HCV core gene* transgenic mice, which develop hepatic steatosis, insulin resistance, and hepatocellular carcinoma. Administration of tacrolimus to *HCV core gene* transgenic mice three times per week for 3 months led to a significant reduction in the amounts of lipid in the liver as well as in serum insulin. Tacrolimus treatment also ameliorated oxidative stress and DNA damage in the liver of the *core gene* transgenic mice. Tacrolimus administration reproduced these effects in a dose-dependent manner in HepG2 cells expressing the core protein. The intrahepatic level of tumor necrosis factor-α, which may be a key molecule for the pathogenesis in HCV infection, was significantly decreased in tacrolimus-treated core gene transgenic mice. Tacrolimus thus reversed the effect of the core protein in the pathogenesis of HCV-associated liver disease. These results may provide new therapeutic tools for chronic hepatitis C, in which oxidative stress and abnormalities in lipid and glucose metabolism contribute to liver pathogenesis. ***(Am J Pathol 2009, 175:1515–1524; DOI: 10.2353/ajpath.2009.090102)***

Hepatitis C virus (HCV) is a major cause of liver disease; approximately 170 million people are chronically infected worldwide. Persistent HCV infection leads to the development of chronic hepatitis, cirrhosis, and, eventually, hepatocellular carcinoma (HCC), thereby being a serious problem from both medical and socioeconomic viewpoints.[1,2] Recently, a growing amount of evidence showing that HCV infection induces alteration in lipid[3–7] and glucose metabolism has accumulated.[8,9] Augmentation of oxidative stress is also substantiated in HCV infection by a number of clinical and basic studies.[10–13]

We demonstrated previously that the core protein of HCV induces HCC in transgenic mice that have marked hepatic steatosis in the absence of inflammation.[14] In this animal model for HCV-associated HCC, there is augmentation of oxidative stress in the liver during the incubation period.[10] Also noted is an accumulation of lipid droplets that are rich with carbon 18 monounsaturated fatty acids such as oleic and vaccenic acids, which is also observed in liver tissues of patients with chronic hepatitis C com-

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pared with those in patients with fatty liver due to simple obesity.[15] Recently, we have also shown, using the HCV transgenic mouse model, that the ability of insulin to lower plasma glucose levels is impaired in association with HCV infection,[16] which would be the basis for the frequent development of type 2 diabetes in patients with chronic hepatitis C.[8,9]

Disturbances in lipid and glucose metabolism are notable features of HCV infection and may be profoundly involved in the pathogenesis of liver diseases. Although the mechanism underlying these phenomena is not yet well understood, the development of clues to correct these metabolic disturbances occurring in HCV infection, which have been recently connected to the poor prognosis of patients with chronic hepatitis C, is awaited. Moreover, a key role for oxidative stress in the pathogenesis of hepatitis C,[11,12] which may be closely associated with the aforementioned metabolic disorders, has been identified. The association of oxidative stress augmentation in HCV infection with mitochondrial respiratory dysfunction[10,13,17] suggests that one possibility to ameliorate such a condition is the use of agents that can protect the mitochondrial respiratory function.

We have conducted information retrieval and screening for agents that can protect the mitochondrial respiratory function. Tacrolimus (FK506), which is widely used in organ transplantation, is one such agent with evidence showing protection of the mitochondrial respiratory function,[18–21] although it shows no antiviral effect. We explored, using transgenic mouse and cultured cell models that express the HCV core protein, whether tacrolimus improves metabolic disturbances including lipid and glucose homeostases as well as oxidative stress augmentation through a possible involvement of mitochondrial function.

Materials and Methods

Transgenic Mouse and Cultured Cells

The production of *HCV core gene* transgenic mice has been described previously.[6] Mice were cared for according to institutional guidelines with the approval by the institutional review board of the animal care committee, fed an ordinary chow diet (Oriental Yeast Co., Ltd., Tokyo, Japan), and maintained in a specific pathogen-free state. Because there is a sex preference in the development of liver lesions in the transgenic mice, we used only male mice. At least five mice were used in each experiment, and the data were subjected to statistical analysis. HepG2 cell lines expressing the HCV core protein under the control of the CAG promoter (Hep39J, Hep396, and Hep397) or a control HepG2 line (Hepswx) carrying the empty vector were described previously.[22,23] Bulk HepG2 cells were also used as a control.

Reagents

Cholesterol esters and lipid standards were purchased from Sigma-Aldrich (St. Louis, MO), and glycogen and amyloglucosidase were obtained from Seikagaku Kogyo (Tokyo, Japan). Other chemicals were of analytical grade and were purchased from Wako Chemicals (Tokyo, Japan). Tacrolimus (FK506) was kindly provided by Astellas Pharma Inc. (Tokyo, Japan). Cyclosporine A (CyA) was purchased from Sigma-Aldrich.

Administration of Tacrolimus and Cyclosporine A

Tacrolimus (0.1 mg/kg b.wt., suspended in mannitol and hydroxychlorinated caster oil [HCO-60]), or vehicle only was administered to the core gene transgenic or control mice i.p., three times per week for 3 months beginning at 3 months of age. For *in vitro* experiments, tacrolimus was added to the culture medium at the final concentration of 0 nmol/L, 10 nmol/L, 100 nmol/L, or 1 μmol/L. CyA was also added to the culture medium at the same concentrations.

Assessment of Glucose Homeostasis

Blood was drawn at different time points from the tail vein, and plasma glucose concentrations were measured using an automatic biochemical analyzer (DRI-CHEM 3000V, Fuji Film, Tokyo, Japan). The levels of serum insulin were determined by radioimmunoassay (Biotrak, Amersham Pharmacia Biotech, Piscataway, NJ) using rat insulin as a standard. For the determination of the fasting plasma glucose level, the mice were fasted for >16 hours before the study. An insulin tolerance test was performed as described previously.[16]

Lipid Extraction, Measurement of Triglyceride Content, and Analysis of Fatty Acid Compositions

Lipid extraction from the mouse liver tissues or cultured cells was performed as described previously.[15,24] For the analysis of fatty acid compositions, the residue was methanolysed by the modified Morrison and Smith method with boron trifluoride as a catalyst.[25] Fatty acid methyl esters were analyzed using a Shimadzu GC-7A gas chromatograph (Shimadzu Corp., Kyoto, Japan) equipped with a 30-m-long × 0.3-mm diameter support coated with ethylene glycol succinate.[24]

Evaluation of Oxidative and Antioxidative System

Lipid peroxidation was estimated spectrophotometrically using thiobarbituric acid-reactive substances and is expressed in terms of malondialdehyde formed per milligram protein. Reduced glutathione and oxidized glutathione levels were measured as described previously.[10] The total amount of glutathione was calculated by adding the amounts obtained for glutathione and oxidized glutathione. For the evaluation of DNA damage in cells, apurinic/apyrimidinic sites were determined using a DNA Damage Quantification Kit (Dojindo Molecular Technolo-

gies, Inc., Tokyo, Japan) following the manufacturer's protocol.

Determination of Reactive Oxygen Species

Cells were plated onto glass coverslips and examined for reactive oxygen species (ROS) production as a marker for oxidative stress. They were loaded for 2 hours with chloromethyl 2′,7′-dichlorodihydrofluorescein diacetate (Molecular Probes Inc., Eugene, OR) at a final concentration of 10 μmol/L.[26] Results were expressed as relative fluorescence intensity and normalized to the control cells. In some experiments, ROS was measured after the incubation with tacrolimus or CyA.

Measurement of Ketone Body Ratio

For the determination of ketone body ratio (KBR), cells were cultured to confluence on a 3.5-cm dish, and the medium was replaced with 700 μl of fresh medium. For arterial KBR, the mice were fasted for >16 hours, followed by the drawing of arterial blood. After a 24-hour incubation, acetoacetate and β-hydroxybutyrate in the medium were measured by monitoring the production or consumption of NADH with a Ketorex kit (Sanwa Chemical, Nagoya, Japan).[27] The KBR was calculated as the acetoacetate/β-hydroxybutyrate ratio.

Microarray Analysis

An Affymetrix GeneChip analysis cDNA array system (Mouse Genome 430A 2.0, Kurabo, Osaka, Japan) was used for the analysis. Two thousand species of mouse DNA fragments were spotted on the filter. Genes that were 1.5-fold increased or decreased in both of the two tacrolimus-treated mice compared with mice treated with vehicle were defined as up-regulated or down-regulated, respectively.

Real-Time PCR and Western Blotting

RNA was prepared from mouse liver tissues using TRIzol LS (Invitrogen, Carlsbad, CA). The first-strand cDNAs were synthesized with a first-strand cDNA synthesis kit (Amersham Pharmacia Biotech, Franklin Lakes, NJ). The fluorescent signal was measured with an ABI Prism 7000 system (Applied Biosystems, Tokyo, Japan).

The genes encoding mouse *tumor necrosis factor (TNF)-α, sterol regulatory element binding protein (SREBP)-1c, resistin, stearoyl-CoA desaturase (SCD)-1, and hypoxanthine phosphoribosyltransferase* were amplified with the primer pairs 5′-GACAAGGTGGGCTACGGGCTTG-3′ and 5′-TCCCAAATGGGCTCCCTCT-3′, 5′-ACGGAGCCATGGATTGCACATTTG-3′ and 5′-TACATCTTTAAAGCAGCGGGTGCCGATGGT-3′, 5′-GAAGGCACAGCAGTCTTGA-3′ and 5′-GCGACCTGCAGCTTACAG-3′, 5′-TTCCCTCCTGCAAGCTCTAC-3′ and 5′-CGCAAGAAGGTGCTAACGAAC-3′, and 5′-CCAGCAAGCTTGCAACCTTAACCA-3′ and 5′-GTAATGATCAGTCAACGGGGGAC-3′, respectively. The sense and antisense primers were located in different exons to avoid false-positive amplification from contaminated genomic DNA. Each PCR product was confirmed as a single band of the correct size by agarose gel electrophoresis (data not shown).

Reporter Assay for SREBP-1c Promoter Activity

A plasmid encoding firefly luciferase under the control of the *SREBP-1c* promoter (pGL3-srebp-1cPro) and a control plasmid encoding *Renilla* luciferase (Promega, Madison, WI) were transfected into 293T cells. Tacrolimus was added at a final concentration of 100 nmol/L to the culture medium of 293T cells transfected with pGL3-srebp-1cPro with or without an expression plasmid of HCV core protein at 24 hours after transfection. Cells were harvested 24 hours after treatment. Luciferase activity was measured by using the dual-luciferase reporter assay system (Promega). Firefly luciferase activity was standardized with that of *Renilla* luciferase, and the results are expressed as the fold-increase in relative luciferase units.

Statistical Analysis

Data are presented as the mean ± SE. The significance of the difference in means was determined by a Mann-Whitney *U* test wherever appropriate. $P < 0.05$ was considered significant.

Results

Effect of Tacrolimus on Insulin Resistance Induced by HCV

The *core gene* transgenic mice exhibit insulin resistance in the absence of obesity from the age of 2 months.[16] In tacrolimus-treated mice, there was a slight, but not significant, reduction in body weight compared with control mice at the end of tacrolimus administration at 6 months of age (Figure 1A). Tacrolimus administration to the *core gene* transgenic mice restored the plasma glucose levels to within normal limit (Figure 1B) ($P < 0.05$), whereas it caused no significant reduction in the control mice. The plasma glucose levels in the vehicle-treated core gene transgenic mice were higher than those in the core gene transgenic mice reported previously,[16] probably owing to the older age of mice in the current study than in the previous one. The levels of serum insulin were also significantly reduced by the treatment with tacrolimus for 3 months in the core gene transgenic mice, whereas there was no significant change in the control mice (Figure 1C). The reduction in both plasma glucose and serum insulin levels indicates that the administration of tacrolimus restored the resistance to insulin action, which is attributed to the suppression of insulin action in the liver by the core protein.[16] Actually, an insulin tolerance test (1 U/kg b.wt.) demonstrated the improvement of insulin action in the tacrolimus-treated core gene transgenic mice (Figure 1D).

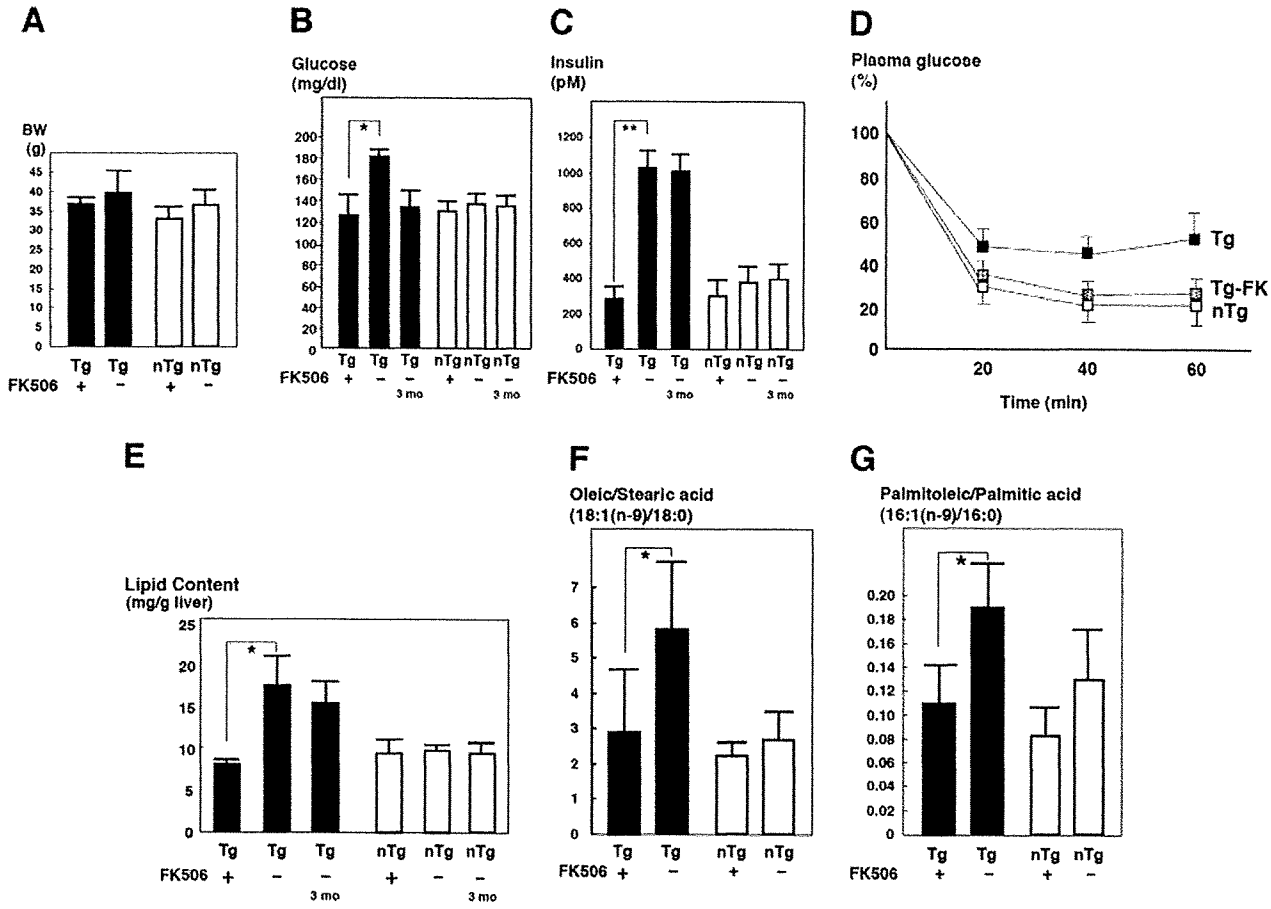

Figure 1. Effect of tacrolimus (FK506) on glucose and lipid metabolism in the core gene transgenic mice. Tacrolimus (0.1 mg/kg b.wt.) or vehicle was administered to core gene transgenic or control mice i.p., three times weekly for 3 months beginning at 3 months of age. **A:** Body weight at the baseline and end of treatment. **B:** Plasma glucose level. **C:** Serum insulin level. **D:** Insulin tolerance test. Black boxes represent core gene transgenic mice; white boxes represent control mice; gray boxes represent core gene transgenic mice treated with tacrolimus (Tg-FK). **E:** Total lipid content in the liver. **F:** Ratio of oleic/stearic acid [18:1(n-9)/18:0]. **G:** Ratio of palmitoleic/palmitic acid [16:1(n-9)/16:0]. black bars represent transgenic mice; white bars represent control mice. Tg 3 mo indicates 3-month-old transgenic mice showing the baseline state just before FK treatment, and Tg indicates 6-month-old transgenic mice, either with or without tacrolimus treatment for 3 months. Values represent the mean ± SE, $n = 5$ in each group. $^{*}P < 0.05$. Tg, transgenic mice; nTg, nontransgenic control mice. $^{**}P < 0.01$.

Tacrolimus Improves Lipid Metabolism Disorders in Mice

We then studied whether tacrolimus administration affects lipid metabolism in the mice. The core gene transgenic mice developed a marked hepatic steatosis.[6,14] In addition, the composition of accumulated lipid was different from that in the fatty liver as a result of simple overnutrition: carbon 18 or 16 monounsaturated fatty acid levels were significantly increased.[15] As shown in Figure 1E, the tacrolimus treatment significantly reduced the lipid content in liver tissues compared with the vehicle treatment of the core gene transgenic mice ($P < 0.05$, $n = 5$ each), whereas there was no change in the control mice. The increased ratios of oleic to stearic acid [18:1 (n-9)/18:0] and palmitoleic to palmitic acid [16:1(n-9)/16: 0] in the core gene transgenic mice returned to levels similar to those in control mice (Figure 1, F and G) ($P < 0.05$). Thus, the administration of tacrolimus for 3 months restored the abnormalities in lipid metabolism that were induced by the core protein of HCV. Histologically, tacrolimus significantly improved steatosis in the liver of core gene transgenic mice, in which micro- and macrovesicular lipid droplets were accumulated in hepatocytes, chiefly around the central veins of the liver (Figure 2A). There was no sign of inflammation in the liver with or without the tacrolimus treatment.

Effect of Tacrolimus on Lipid Metabolism in HepG2 Cells Expressing HCV Core Protein

To further prove the ameliorating effect of tacrolimus on lipid metabolism, we then performed experiments using HepG2 cells that express the core protein.[22,23] HepG2 cells, the lipid metabolism of which is somewhat different from that in normal hepatocytes,[28] show a significant increase in the level of 5,8,11-eicosatrienoic acid [20:3 (n-9)], as a result of activations of the fatty acid enzymes, Δ^9-, Δ^6-, and Δ^5-desaturases, by the core protein (H. Miyoshi and K. Koike, unpublished data). Incubation of the core-expressing HepG2 cells with tacrolimus at 100 nmol/L and 1 μmol/L for 48 hours significantly reduced the accumulation of 20:3(n-9), whereas CyA treat-

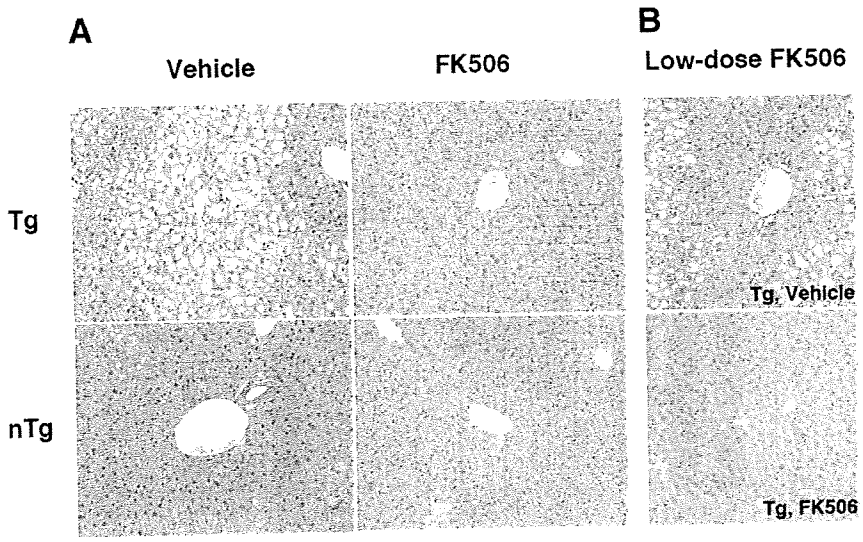

Figure 2. Morphological analysis of the liver of the core gene transgenic mice. Representative cases are shown either treated with tacrolimus (FK506) or vehicle (H&E staining). **A:** There is a prominent improvement of steatosis in the 3-month tacrolimus-treated core gene transgenic mice compared with the vehicle-treated mice. **B:** A prominent improvement in steatosis was also obtained by the administration of one-fifth dose of tacrolimus for 1 month beginning at 3 months of age. For histological analysis, two independent researchers evaluated 40 microscopic fields each, and a representative picture is shown for each category. Original magnification, ×125. Tg, transgenic mice; nTg, nontransgenic control mice.

ment increased the level of 20:3(n-9) in a dose-dependent manner in the core-expressing HepG2 cells (Figure 3, A and B). Neither tacrolimus nor CyA changed the 20:3(n-9) content in HepG2 cells that do not express the core protein.

Low Dose of Tacrolimus Also Ameliorates Steatosis and Insulin Resistance

Because the usual dose of tacrolimus for liver transplantation naturally induces an immunosuppressed state in patients, we conducted a mouse study with a tacrolimus dose lower than that in the aforementioned study. In this low-dose experiment, tacrolimus at 0.02 mg/kg b.wt. (one-fifth of the previous one) was administered to mice for 1 month from the age of 3 months. Similar to the results with the dose of 0.1 mg/kg b.wt., there were significant decreases in the lipid content in the liver (9.5 ± 0.8 [0.02 mg/kg b.wt. tacrolimus] versus 18.7 ± 4.4 [vehicle only] mg/g liver; $P < 0.05$) and serum insulin concentration (96.6 ± 16.9 [0.02 mg/kg b.wt. tacrolimus] versus 1137.1 ± 88.0 [vehicle only] pmol/L; $P < 0.05$) in the core gene transgenic mice treated with tacrolimus. Histological changes are shown in Figure 2B.

Effect of Tacrolimus on Oxidative Stress and Antioxidative System in Mice

We next examined whether the 3-month administration of tacrolimus affects the redox state in the core gene transgenic mice. In the liver of the core gene transgenic mice, the ROS level was higher than that in the liver of control mice as determined by lipid peroxidation.[10] Treatment with tacrolimus significantly reduced the level of thiobarbituric acid-reactive substances in the liver of the core gene transgenic mice (Figure 4A) ($P < 0.05$). As a result of oxidative stress overproduction, there was damage in the DNA of hepatocytes of the core gene transgenic mice from a young age.[10] To evaluate the effect of tacrolimus on the nuclear DNA damage, the apurinic/apyrimidinic site index was determined in liver tissues from the core gene transgenic mice. As shown in Figure 4B, the apurinic/apyrimidinic site index in the liver of the core gene transgenic mice, which was significantly higher

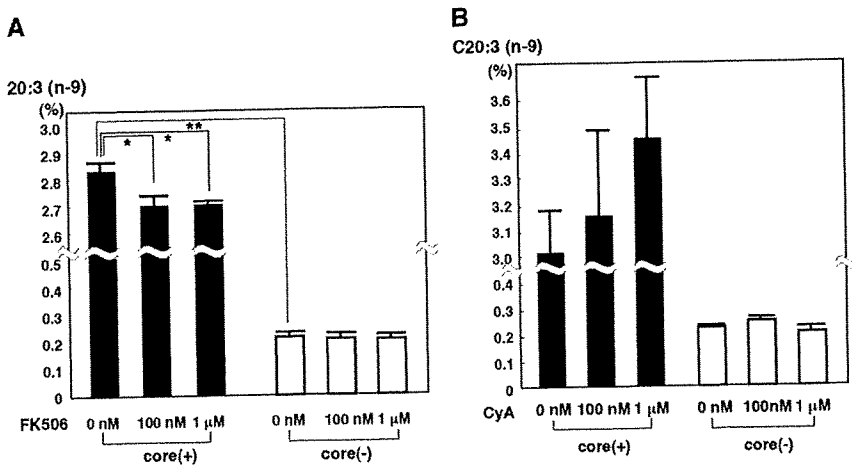

Figure 3. Effect of tacrolimus (FK506) or CyA on fatty acid compositions in HepG2 cells expressing the core protein. The fatty acid compositions of the total cell lipids were analyzed, and the percentage of 5,8,11-eicosatrienoic acid [20:3(n-9)] in the core-expressing and control HepG2 cells was calculated. **A:** Treatment with tacrolimus at 0 nmol/L, 100 nmol/L, or 1 μmol/L. **B:** Treatment with CyA at 0 nmol/L, 100 nmol/L, or 1 μmol/L. Black bars represent core-expressing cells; white bars represent control cells. Because similar results were obtained by using Hep39J, Hep396, and Hep397 cell lines, representative results using the Hep39J cell line are shown. Values represent the mean ± SE; $n = 5$ in each group. $^{*}P < 0.05$ and $^{**}P < 0.01$.

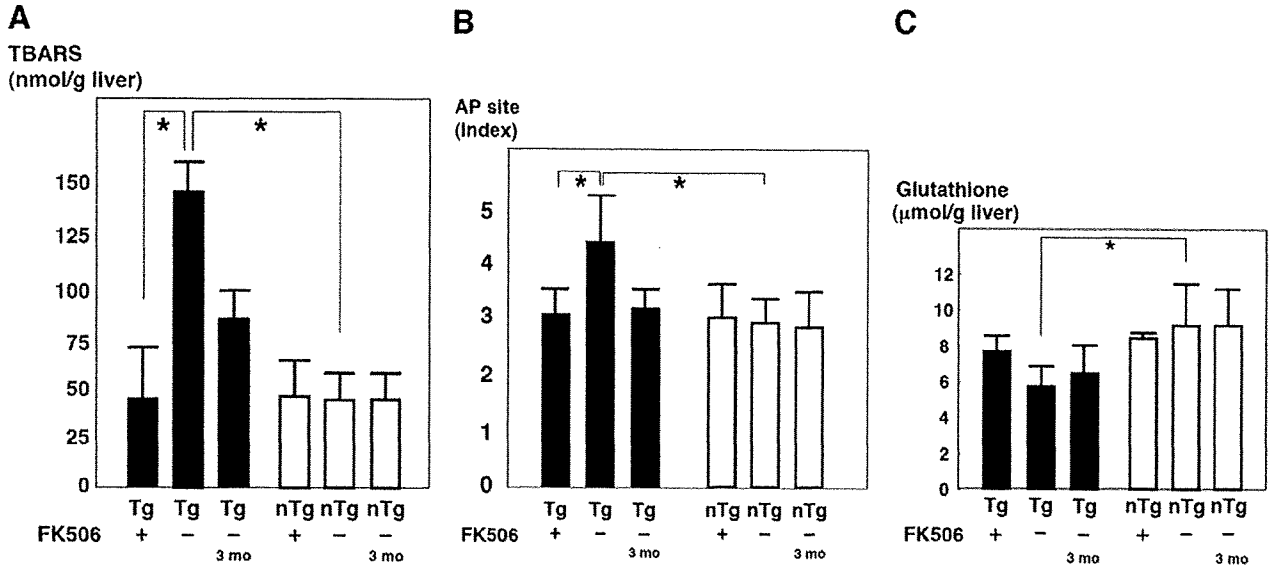

Figure 4. Effect of tacrolimus (FK506) on oxidative stress in the core gene transgenic mice. Tacrolimus (0.1 mg/kg b.wt.) or vehicle only was administered to the core gene transgenic or control mice for 3 months. **A:** Lipid peroxidation in the liver. **B:** apurinic/apyrimidinic (AP) site in the liver as a marker of nuclear DNA damage; **C:** Total glutathione level in the liver. Black bars represent transgenic mice; white bars represent control mice. Tg 3 mo indicates 3-month-old transgenic mice, showing the baseline state just before tacrolimus treatment, and Tg indicates 6-month-old transgenic mice, either with or without 3 months of tacrolimus treatment. Values represent the mean ± SE; $n = 5$ in each group. $^{*}P < 0.05$. Tg, transgenic mice; nTg, nontransgenic control mice. TBARS, thiobarbituric acid-reactive substances.

than that in the control mice, was significantly decreased by the tacrolimus treatment to a level similar to that in the control mice ($P < 0.05$).

The level of glutathione, one of the antioxidant systems, was significantly decreased in the liver of the core gene transgenic mice presumably as a result of oxidative stress overproduction but returned to a level similar to that in the control mice after the 3-month administration of tacrolimus, although the difference was not statistically significant ($P = 0.063$) (Figure 4C). Thus, the oxidative stress augmentation induced by the core protein of HCV was reduced by tacrolimus.

Effect of Tacrolimus on Oxidative Stress in Core-Expressing HepG2 Cells

Evidence for scavenging ROS by the administration of tacrolimus to the mice prompted us to validate this finding using cultured cells. For this purpose, tacrolimus or CyA was added to the culture medium of HepG2 cells that express or do not express the core protein. After 24 hours of incubation, tacrolimus decreased the ROS production level in the core-expressing HepG2 cells in a dose-dependent manner (Figure 5A). In contrast, no decrease but rather an augmentation of ROS production was observed by the treatment with CyA at various concentrations (Figure 5B).

Because dysfunction of the mitochondrial respiratory chain complex 1 is suspected to be the reason for the ROS production associated with HCV infection (H. Miyoshi and K. Koike, unpublished data),[12,13,17] an increase in the NADH/NAD$^+$ ratio, which is caused by the repression of the complex 1 NADH dehydrogenase activity, would be a good marker for the mitochondrial complex 1 dysfunction. Therefore, we evaluated the effect of tacrolimus on the accumulation of NADH in the core-expressing HepG2 cells. The NADH/NAD$^+$ ratio, which is strictly estimated from a reciprocal of KBR,[26,29] was significantly higher in the core gene transgenic mice than in control mice (1/atrial KBR) and in HepG2 cells expressing the core protein than in control cells (1/KBR) (Figure 6A). By the treatment with 1 μmol/L tacrolimus, the ratio significantly decreased compared with the baseline (Figure 6B), whereas CyA treatment caused no effect in the core-expressing HepG2 cells (Figure 6C), as was the

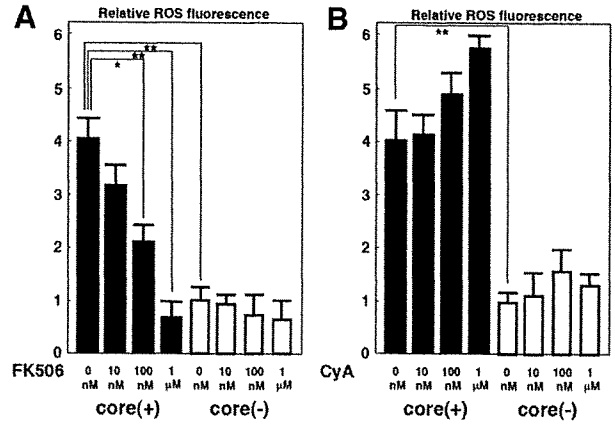

Figure 5. Effect of tacrolimus (FK506) or CyA on ROS production in HepG2 cells expressing the core protein. Results are expressed as relative brightness and normalized to control cells. **A:** Treatment with tacrolimus at 0 nmol/L, 10 nmol/L, 100 nmol/L, or 1 μmol/L. **B:** Treatment with CyA at 0 nmol/L, 10 nmol/L, 100 nmol/L, or 1 μmol/L. Black bars represent transgenic mice; white bars represent control cells. Because similar results were obtained by using Hep39J, Hep396, and Hep397 cell lines, representative results using the Hep39J cell line are shown. Values represent the mean ± SE; $n = 5$ in each group. $^{*}P < 0.05$; $^{**}P < 0.01$.

case in the determination of ROS by chloromethyl 2′,7′-dichlorodihydrofluorescein diacetate.

Changes in Gene Expression by Tacrolimus Treatment of Mice

We then performed a comprehensive microarray analysis of gene expression in the liver, which was up- or down-regulated by tacrolimus. For this analysis, the tacrolimus-treated mice were compared with the vehicle-treated mice, in two pairs of the core gene transgenic and control mice, respectively. Genes that were 1.5-fold increased or decreased in both of the two tacrolimus-treated mice compared with those treated with vehicle were defined as up-regulated or down-regulated, respectively. As shown in Table 1, several genes were found to be up-regulated or down-regulated in both the core gene transgenic and control mice after the treatment with tacrolimus for 3 months. A number of genes including that for TNF-α were up- or down-regulated both in the core gene transgenic and control mice. In contrast, the expressions of some genes including that for resistin were differentially regulated between the core gene transgenic and control mice. The expressions of these genes were confirmed by real-time PCR analysis.

Then, to explore the mechanism by which tacrolimus reverses the pathological effect of the core protein in the liver, we examined, by real-time PCR analysis, the expression of some cellular genes including TNF-α, SREBP-1c, SCD-1, and proteasome activator 28-γ. These genes or gene products have been suggested to play a pivotal role in the pathogenesis of HCV-associated liver disease.[30,31] TNF-α and SREBP-1c genes have been shown to be up-regulated in the liver of the core gene transgenic mice and considered to play a role in the development of insulin resistance and steatosis.[30,31] By the treatment of the core gene transgenic mice with tacrolimus for 3 months, there was a significant decrease in the mRNA level of both TNF-α and SREBP-1c (Figure 7, A–C) ($P < 0.05$). The SCD-1 mRNA level was also reduced in the tacrolimus-treated core gene transgenic mice. Because down-regulation of SREBP-1c expression by tacrolimus was observed only in the core gene transgenic mice but not in control mice, it is estimated that tacrolimus antagonizes the action of core protein in its transactivating function of the SREBP-1c promoter. The down-regulation of SREBP-1c, then, would lead to the suppression of SCD-1 expression and amelioration of steatosis. We confirmed this by conducting luciferase assays using cultured cells. As shown in Figure 7D, tacrolimus cancelled the effect of the core protein on the activation of SREBP-1c gene promoter. The level of the proteasome activator 28-γ protein, which is indispensable for the action of the core protein in the pathogenesis of HCV-associated liver lesion,[31] was determined by Western blotting, but there was no change caused by the tacrolimus treatment (data not shown).

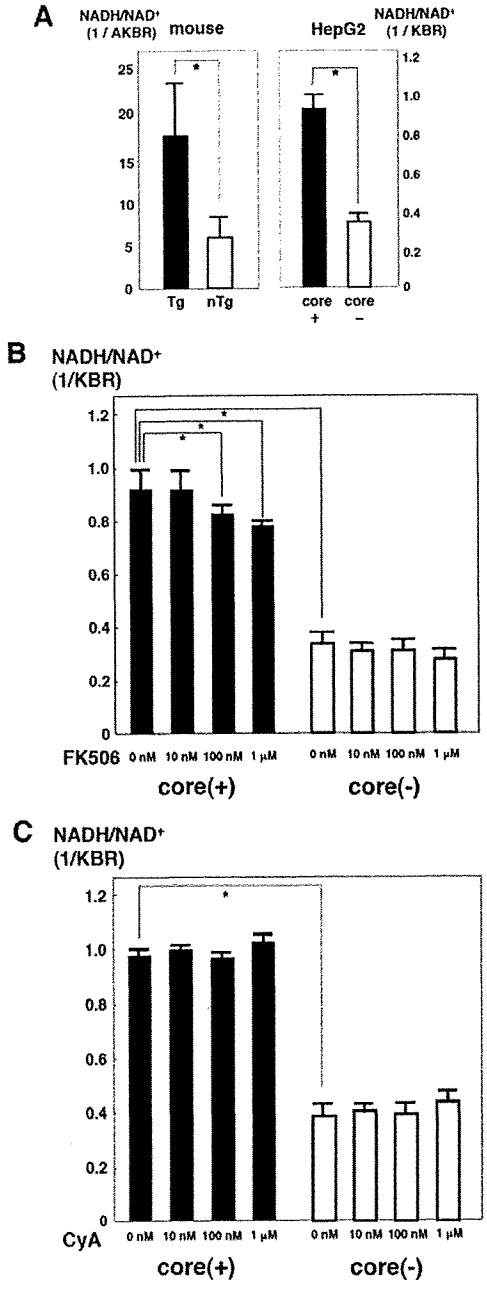

Figure 6. Effect of tacrolimus (FK506) or CyA on NADH accumulation in HepG2 cells expressing the core protein. **A:** NADH/NAD$^+$ was determined in mice (left) or HepG2 cells (right) with or without the core protein. **B:** The ketone body ratio was determined in HepG2 cells with or without the core protein after incubation with tacrolimus for 24 hours at 0 nmol/L, 10 nmol/L, 100 nmol/L, or 1 μmol/L. **C:** The ketone body ratio was determined in HepG2 cells with or without the core protein after incubation with CyA for 24 hours at 0 nmol/L, 10 nmol/L, 100 nmol/L, or 1 μmol/L. Black bars represent transgenic mice; white bars represent control cells. Because similar results were obtained by using Hep39J, Hep396 and Hep397 cell lines, representative results using the Hep39J cell line are shown. Values represent the mean ± SE; $n = 5$ in each group. $^*P < 0.05$. AKBR, arterial KBR; Tg, transgenic mice; nTg, nontransgenic mice.

Discussion

Antiviral treatment for chronic hepatitis C has advanced markedly. Nearly 50% of patients with chronic hepatitis C

Table 1. Genes Whose Expression Levels in the Mouse Liver Were Altered by the Treatment with FK506

	Up-regulated in Tg	Down-regulated in Tg
Up-regulated in nTg	Nuclear factor, erythroid derived 2	Resistin
	DNA segment, human D6S2654E	Resistin like alpha
	Fatty acid binding protein 5	Nuclear receptor subfamily 4, group A, member
	epidermal squalene epoxidase	insulin-like growth factor binding protein 1
	Zinc finger protein 69	calcium and integrin binding family member 3
Down-regulated in nTg	X-linked lymphocyte-regulated 4	Tumor necrosis factor alpha
	Cytochrome P450, family 2,	Cytochrome P450, family 17, subfamily a,
	subfamily b, polypeptide 9	polypeptide 1
	X-linked lymphocyte-regulated 3a	B-cell leukemia/lymphoma 6
	Signal sequence receptor, delta	

Genes with altered expression in Tg (columns) or in nTg (rows) are described in a 4 × 4 table. Genes that were 1.5-fold increased or decreased in both of the two FK506-treated mice compared with those treated with placebo were defined as up-regulated or down-regulated, respectively. Tg, core gene transgenic mouse; nTg, nontransgenic control mouse.

with HCV genotype 1 and high viral loads achieve a sustained virological response as a result of ribavirin/peginterferon combination therapy.[32,33] However, the remaining patients who could not achieve sustained virological response continue to experience progression of chronic hepatitis and have a high probability for development of HCC. Although therapies with new agents such as viral protease or RNA polymerase inhibitors are being developed, there is hope for development of the means to retard the progression of chronic hepatitis.

Recently, evidence showing that hepatic steatosis and insulin resistance are crucial determinants of the progression of liver fibrosis has accumulated.[34–37] Moreover, the importance of oxidative stress, which is closely associated with metabolic disorders such as insulin resistance and steatosis, is implicated in the pathogenesis of HCV-associated liver disease. Given the suggested association of oxidative stress augmentation with the dysfunction of mitochondrial respiration in HCV infection,[12,13,17] one possibility to ameliorate such a condition is the use of agents that can protect the mitochondrial respiratory function. Tacrolimus is one such agent with evidence of providing protection of the mitochondrial respiratory function,[18–21] although it does not show an antiviral effect.

In the current study, the administration of tacrolimus significantly improved the disturbances in lipid and glucose metabolism both *in vivo* and *in vitro*. As disorders of lipid metabolism associated with HCV infection, hepatic steatosis and increases in monounsaturated fatty acid levels have been demonstrated.[3,4,6,7,15] The latter is caused by the activation of fatty acid enzymes such as Δ^9- or Δ^6-desaturase, resulting in increases in 18:1(n-9)/18:0 and 16:1(n-9)/16:0 ratios (H. Miyoshi and K. Koike, unpublished data).[15] Tacrolimus ameliorated these lipid alterations associated with HCV infection with no impact on mouse body weight. Tacrolimus also improved the insulin resistance in the HCV mouse model, in which tyrosine phosphorylation of insulin receptor substrate-1 is impaired by the HCV core protein.[16]

Moreover, tacrolimus treatment ameliorated oxidative stress augmentation, which is considered to play a pivotal role in the progression of liver disease or the development of HCC in HCV infection.[10–13] In mice transgenic for the HCV core gene, in which DNA damage develops because of oxidative stress augmentation,[13] tacrolimus decreased the levels of peroxylipid and DNA damage formations. Dysfunction of the mitochondrial respiratory chain complex 1 is suspected to be a source of ROS overproduction in HCV infection.[12,13,17] To assess changes in mitochondrial complex 1 function caused by tacrolimus, the NADH/NAD^+ ratio, which reflects the complex 1 NADH dehydrogenase activity, was determined in HepG2 cells expressing the core protein. The NADH/NAD^+ ratio, which is strictly estimated from a reciprocal of KBR (1/atrial KBR),[26,29] was significantly re-

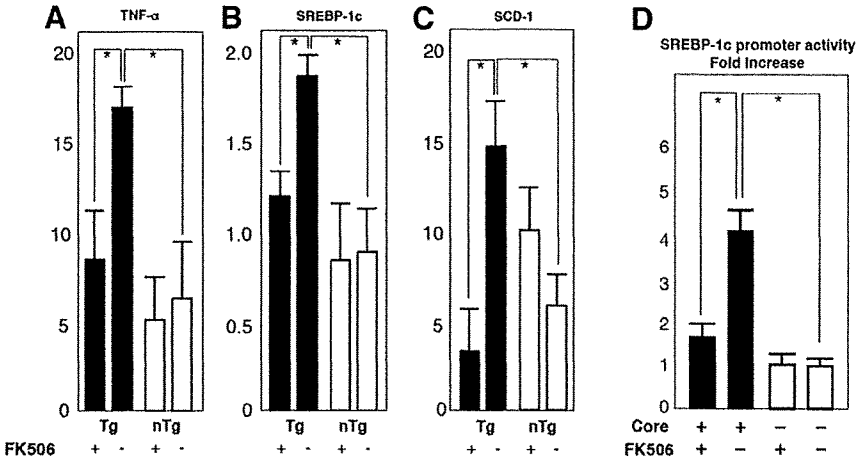

Figure 7. A–C: Effect of tacrolimus (FK506) on mRNA levels of cellular genes. The mRNA levels of TNF-α (**A**), SREBP-1c (**B**), and SCD-1 (**C**) genes were determined by real-time PCR analysis in the tacrolimus- or vehicle-treated mouse livers. The transcriptions of the genes were normalized with that of hypoxanthine phosphoribosyltransferase, and the values are expressed as relative activities. **D:** Effect of tacrolimus on the transactivating function of the core protein on the SREBP-1c promoter. A luciferase assay was performed using a plasmid encoding firefly luciferase under the control of the SREBP-1c promoter with or without the expression of HCV core protein. Tacrolimus was added at a final concentration of 100 nmol/L to the culture medium. Black bars represent transgenic mice; white bars represent control cells. Values represent the mean ± SE; $n = 5$ in each group. $^*P < 0.05$. Tg, transgenic mice; nTg, nontransgenic mice.

duced by the addition of tacrolimus but not CyA. Thus, tacrolimus protected the mitochondrial respiratory chain complex 1 function from the impact of the core protein, decreased oxidative stress, and improved steatosis and insulin resistance.

Some of features induced by the core protein including steatosis, insulin, and DNA damage were already present in the core gene transgenic mice at 3 months of age as the baseline, and those were improved by tacrolimus treatment. This fact indicates that tacrolimus is not only preventing the development of core-induced features but also reversing such changes in the mouse liver.

The tacrolimus dose used in the current study was 0.1 mg/kg b.wt. This is the same dose as that used in recipients of liver or kidney transplantation. The result of a subexperiment with a lower tacrolimus dose of 0.02 mg/kg b.wt. was similar to that with the dose of 0.1 mg/kg b.wt. This finding is promising because it indicates that the "anti-core protein effect" may be achievable at such a low dose of tacrolimus without provoking strong immunosuppression. The tacrolimus concentration (100 nmol/L) that caused the anti-core protein effect in the cultured cell study is similar to that in the blood of recipients of liver transplantation and much lower than those used in previous studies.[19,38] In the current study, tacrolimus was administered only i.p., although it tacrolimus is administered i.v. or p.o. in humans. Therefore, a concern may arise regarding the administration route. Because the bioavailability of tacrolimus is approximately 25% (range from 5 to 93%) in human patients,[39] a difference in the concentrations of tacrolimus may be possible between i.p. and p.o. administration. However, in human patients, target levels of tacrolimus concentration are generally achieved by p.o. administration as the maintenance therapy. Therefore, the target concentration would be achieved in mouse models by p.o. administration for 3 months as it is in human patients. Our current results strongly support the notion that tacrolimus can protect the mitochondrial respiratory function, resulting in a reduction of ROS production.

There is also a controversy concerning the effect of tacrolimus on glucose homeostasis. Post-transplantation diabetes is a complication in kidney or liver transplantation.[40,41] *In vivo* and *in vitro* studies have shown that tacrolimus may inhibit insulin secretion from the pancreatic β-cells.[40] Thus, tacrolimus may have a potential to induce diabetes. However, there have been no well designed studies on this specific point: in one study, corticosteroid withdrawal from tacrolimus-based immunosuppression reduced insulin resistance without changing insulin secretion.[41] In our study using the HCV mouse model, tacrolimus administration at the dose similar to those in organ transplant recipients decreased serum insulin levels without increasing plasma glucose levels. These results point toward the future use of tacrolimus *in vivo* for the amendment of metabolic abnormalities, such as steatosis and insulin resistance, associated with HCV infection. However, it should be noted that there is a difference between our mouse model and human patients. Organ transplant recipients generally have injury to other bodily organs after a prolonged course of illness, whereas the mouse model we have exploited does not. In addition, our mouse model originally has insulin resistance with the presence of hyperplasia of Langerhans islands.[16] Therefore, the effect of tacrolimus on glucose homeostasis in the current mouse study may not be exactly applicable to human patients.

The results of the gene expression analysis by microarray and subsequent real-time PCR were of considerable interest. Tacrolimus reduced the mRNA levels of TNF-α, SCD-1, and SREBP-1c genes, which are elevated in both patients with chronic hepatitis C and HCV core gene transgenic mice.[30,31] The elevation in the TNF-α level causes insulin resistance *in vivo*, which is also observed in HCV core gene transgenic mice.[16] The elevations in SREBP-1c and SCD-1 gene mRNA levels cause the overproduction of triglycerides, leading to the development of steatosis. The reductions in the expression levels of these genes may explain the effect of tacrolimus on the improvement of steatosis, insulin resistance, and oxidative stress in these HCV models. Although recent investigations have shown that the immunosuppressive drugs tacrolimus and rapamycin inhibit the expression of different inflammatory mediators,[42,43] the anti-inflammatory functions of these drugs are not well established. Our *in vitro* and *in vivo* experiments confirmed that tacrolimus inhibited the induction of ROS generation, which is mediated by the core protein. Our data indicate that the inhibition of ROS formation may explain part of the favorable effect of immunosuppressive agents on inflammatory conditions.

In conclusion, our results demonstrate that tacrolimus has protective potential against damage caused by the HCV core protein including the induction of steatosis, insulin resistance, and oxidative stress, both in mice and cultured cells. Although more studies are required to elucidate the precise mechanism underlying the potential of tacrolimus in reversing the pathogenesis in HCV infection, these results may provide new therapeutic tools for chronic hepatitis C, in which oxidative stress and abnormalities in lipid and glucose metabolism contribute to liver pathogenesis.

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