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Short Communication

Prevalence of hepatitis B virus infection in Japanese patients with HIV

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Patients with HIV infection are frequently infected with hepatitis viruses, which are presently the major cause of mortality in HIV-infected patients after the widespread use of highly active antiretrovirus therapy. We previously reported that approximately 20% of HIV-positive Japanese patients were also infected with hepatitis C virus (HCV). Hepatitis B virus (HBV) infection may also be an impediment to a good course of treatment for HIV-infected patients, because of recurrent liver injuries and a common effectiveness of some anti-HIV drugs on HBV replication. However, the status of co-infection with HIV and HBV in Japan is unclear. We conducted a nationwide survey to determine the prevalence of HIV–HBV co-infection by distributing a questionnaire to the hospitals belonging to the HIV/AIDS Network of Japan. Among the 5998 patients reported to be HIV positive, 377 (6.4%) were positive for the hepatitis B surface antigen. Homosexual men accounted for two-thirds (70.8%) of the HIV–HBV co-infected patients, distinct from HIV–HCV co-infection in Japan in which most of the HIV–HCV co-infected patients were recipients of blood products. One-third of HIV–HBV co-infected patients had elevated serum alanine aminotransferase levels at least once during the 1-year observation period. In conclusion, some HIV-infected Japanese patients also have HBV infection and liver disease. A detailed analysis of the progression and activity of liver disease in co-infected patients is needed.

Key words: co-infection, hepatitis B, HIV, liver disease.

INTRODUCTION

HEPATITIS B VIRUS (HBV) infection is a major public health problem worldwide, along with hepatitis C virus (HCV) and HIV infections. In the USA, the estimated prevalence of HBV is less than 1%, but approximately 1 million people are persistently infected.[1] The prevalence of HIV in the USA is also <1%, and the virus is estimated to have infected approximately 800 000 people.[2] Because of the common transmission routes, that is, parenteral transmission routes, many people with HIV infection are also infected with HBV. Among the HIV-positive people in the USA, the prevalence of HBV co-infection is 6–14%.[1,2] Before the introduction of highly active antiretroviral therapy (HAART) in 1996, most patients with HIV infection died of HIV-associated opportunistic infections, such as *Pneumocystis jiroveci* pneumonia and cytomegalovirus infection. Since the widespread use of HAART, the mortality associated with HIV infection has declined. However, the reduction in mortality due to opportunistic infection, has left patients co-infected with HIV and hepatitis viruses faced with the menace of progressive liver diseases due to HBV infection,[3,4] in addition to HCV infection.[5]

HBV co-infection or superinfection of HIV-infected patients leads to several problematic situations. First, HBV infection tends to develop into persistent infection in HIV-infected patients,[1,6,7] which is a rare event in healthy adults, although it substantially depends on the genotype of HBV.[8] It results in the acceleration of the development of cirrhosis and eventually hepatocellular carcinoma. Second, some nucleoside reverse transcriptase inhibitors (NRTI) used in HAART also have

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inhibitory effects on the replication of HBV [9–12] A careless administration or discontinuation of NRTI on HIV–HBV co-infected patients may cause reactivation and/or aggravation of hepatitis B. In addition, the administration of anti-HBV drugs in HIV–HBV co-infection may lead to the development of drug resistance.[11,12] Third, liver injury occurs more frequently in patients on HAART who are co-infected with HIV and HBV than those infected with HIV only [9,10]

Importantly, co-infection with HIV and HCV increases the morbidity and mortality of HIV-infected patients in Japan,[13] where the prevalence of HIV infection is increasing linearly, and is exceptionally high among developed countries.[14] There are more than 14 000 HIV-positive people in Japan as of 2006, according to the AIDS National Survey in Japan,[14] and approximately 0.8 million chronic HBV carriers.[15] However, the prevalence of co-infection with HIV and HBV in Japan has not been clarified to date. Therefore, we conducted a nationwide study by distributing a postal mail-based questionnaire to the hospitals belonging to the HIV/AIDS Network of Japan.

PATIENTS AND METHODS

IN THE QUESTIONNAIRE, the following information was obtained from the hospitals regarding the number of patients who visited the hospitals at least once between January and December in 2006: (i) the number of HIV-positive patients; (ii) the number of hepatitis B surface antigen (HBsAg)-positive patients among (i); (iii) the number of patients among (ii) who were determined at least once to have a serum alanine aminotransferase (ALT) level higher than 100 IU/L; (iv) the number of HIV-positive patients that contracted HIV from blood products; (v) the number of HBsAg-positive patients among (iv), (vi) the number of patients among (v) who were determined at least once to have a serum ALT level higher than 100 IU/L; (vii) the number of HIV-positive patients among homosexual men, (viii) the number of HBsAg-positive patients among (vii), (ix) the number of patients among (viii) who were determined at least once to have a serum ALT level higher than 100 IU/L; (x) the number of HIV-positive patients that contracted HIV through intravenous drug use (xi) the number of HBsAg-positive patients among (x), (xii) the number of patients among (xi) who had at least one determination of a serum ALT level more than 100 IU/L; (xiii) the number of HIV-positive patients whose transmission routes were classified as "others", (xiv) the number of HBsAg-positive patients among (xiii); and (xv) the number of patients among (xiv) who were determined at least once to have a serum ALT level higher than 100 IU/L.

The questionnaire was sent to the 372 hospitals belonging to the HIV/AIDS Network of Japan by mail. Answers were mostly returned by mail and in some cases by fax. The list of the hospitals in the HIV/AIDS Network of Japan can be viewed at http://www.acc.go.jp/mLhw/mLhw_frame.htm.

RESULTS

THE QUESTIONNAIRE WAS sent to all 372 hospitals that were on the list of the hospitals in the HIV/AIDS Network of Japan in January 2006. Two hundred and seven hospitals (55.6%) responded within the indicated period. In total, 5998 patients were reported to be HIV positive. The collection rate of 55.6% was higher than that (47.8%) for a questionnaire HIV–HCV co-infection study carried out in 2003.[15] It may appear rather low, particularly considering the number of reported HIV-positive people in 2006, which was approximately 14 000, according to the AIDS National Survey in Japan.[14] However, not all of the HIV-positive people were going to hospitals, and the answers to the questionnaire were obtained from most of the major hospitals in the HIV/AIDS Network in big cities around Japan. This suggests that not all, but a majority of HIV-positive Japanese patients were enrolled in the study

Among the 5998 patients reported to be HIV positive, 377 (6.3%) patients were positive for HBsAg (Table 1). Of these 377 patients, 122 (32.4%) had elevated serum ALT levels at least one time during the 1-year observation period.

The HBV prevalence rates, when fractionated by the routes of transmission, were as follows: among the 508 HIV-positive patients who contracted HIV from blood products, such as unheated concentrated coagulation factors, only 30 (5.9%) were HBsAg positive, which shows a marked contrast to the prevalence of HCV in this cohort (Fig. 1).[16] Among the 23 intravenous drug users, three (13.0%) were HBsAg positive. Among the 3213 HIV-positive patients who were homosexual men, 267 (8.3%) were HBsAg positive. In the remaining 2254 patients who were HIV-positive and whose route of HIV transmission was classified as "others", most contracted HIV heterosexually. This number (2254) showed a substantial increase from the 1316 obtained in the questionnaire for the HIV–HCV co-infection study in 2003, while the total number of HIV-positive patients increased from 4877 to 5998.[16] Among these, 77 (3.4%)

Table 1 Prevalence rates of hepatitis B virus infection among HIV-positive patients

Routes of transmission	No. patients	HBsAg positive (% in HIV positive according to route)	ALT >100 IU/L (% in HBsAg positive according to route)
Blood products	508 (5.9%)	30 (40.0%)	12
Homosexual men	3213 (8.3%)	267 (32.2%)	86
Drug addicts	23 (13.0%)	3 (66.7%)	2
Others (heterosexual etc.)	2254 (3.4%)	77 (28.6%)	22
Total	5998	377 (6.3%)	122 (32.4%)

ALT, serum alanine aminotransferase; HBsAg, hepatitis B surface antigen.

were HBsAg positive. In terms of the route of HIV infection, 267 (70.8%) of the 377 patients were homosexual men among the HIV–HBV co-infected patients. This shows a contrast to the status of HIV–HCV co-infection, in which the majority of HIV–HCV co-infected Japanese patients contracted both viruses from blood products.[16]

There were one or more HIV-positive patients in 154 (74.4%) of the 207 hospitals in the HIV/AIDS Network of Japan (Table 2). Twenty four (11.6%) of 207 hospitals had 20–49 HIV-positive patients, and 16 (7.7%) hospitals had 50 or more HIV-positive patients. There were one or more patients who were co-infected with HIV and HBV in 64 (30.9%) of the 207 hospitals. There were 10 or more HIV–HBV co-infected patients in nine (4.3%) hospitals, all of which had 50 or more HIV-positive patients (Table 2). HIV–HBV co-infected patients were concentrated in specific hospitals in big cities around Japan. In particular, in the Kanto area, HIV–HBV co-infected patients were concentrated in the HIV/AIDS Network hospitals in the Tokyo city area.

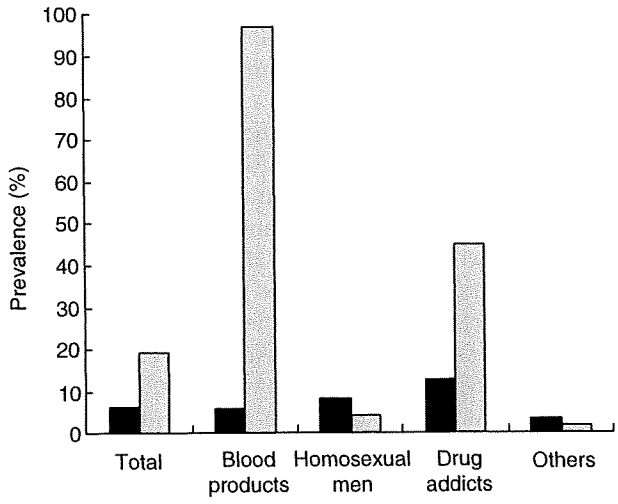

Figure 1 Prevalence rates of persistent hepatitis B virus and hepatitis C virus infections in the HIV-positive population sorted by the HIV risk group. (■), HBsAg, hepatitis B surface antigen; (□), anti-HCV, antibody to hepatitis C virus. *Prevalence rates of anti-HCV are obtained from Koike K *et al.*[16]

DISCUSSION

ALONG WITH THE increase in the number of HIV-infected patients in Japan, co-infection with HIV and hepatitis viruses has become a major medical issue. HBV infection of HIV-positive patients raises several difficult problems: HBV infection tends to develop into persistent infection, even in adults; some NRTI used in HAART also have inhibitory effects on the replication of HBV, the improper administration, or discontinuation of which may lead to drug resistance; and HIV–HBV co-infected patients on HAART have liver injuries more frequently than HIV-monoinfected patients. It is important to determine the status of HBV infection in HIV-positive patients.

According to the statistics of the Ministry of Health, Labor, and Welfare of Japan, the number of reported HIV-positive people was slightly over 14 000 in 2006.[14] In the present study, 6.4% of HIV-positive patients were positive for HBsAg, the most reliable marker for ongoing HBV infection. It might have been advantageous if

Table 2 Number of hospitals categorized according to the number of patients infected with HIV and those co-infected with HIV and hepatitis B virus (HBV)

No. HIV (+)/ HBV (+)	No. HIV(+)				
	0	1–19	20–49	50+	Total
0	53	76	13	1	143
1–9	0	38	11	6	55
10+	0	0	0	9	9
Total	53	114	24	16	207

serum HBV-DNA levels were determined, but unfortunately, HBV-DNA level determination was not a routine laboratory test in most hospitals. In addition, considering that the antibody to the hepatitis B core antigen might be the only marker of ongoing HBV infection in some immuno-compromised patients, it would also be advantageous if this viral marker were available. These issues should be investigated in future studies. Comments from hospitals to the questionnaire included one indicating that not all HIV-positive patients underwent a test for serum HBsAg, suggesting the actual prevalence of HBsAg in HIV-infected patients might be higher than 6.4%.

In a previous questionnaire study of HIV–HCV co-infection, the prevalence of HCV infection among HIV-infected patients was 19.2%;[16] the prevalence of HBV infection (6.4%), is one-third of it. The lower positivity for HBsAg than for the anti-HCV antibody among those who contracted HIV through blood products accounts for this difference: almost all (96.9%) of the patients who contracted HIV through blood products were also anti-HCV antibody positive.[16] It should be noted that among the homosexual male patients who were HIV positive, 8.3% were HBsAg positive, which is twice as high as that of the anti-HCV antibody in these populations. A higher prevalence of HBV infection as a sexually transmitted infection than that of HCV[17] may explain the high prevalence of HBV infection in HIV-positive homosexual men. Similarly, a HBV prevalence of 3.4% in heterosexually transmitted HIV-positive patients is higher than that of the general Japanese population of the same age.[15]

Of the 377 patients who were HBsAg positive, 122 (32.4%) had elevated serum ALT levels at least once in the 1-year observation period. In this type of study using a questionnaire, it is difficult to obtain the details of patients' data, including age, body weight, and the degrees of liver injuries and fibrosis. If detailed items were included in the questionnaire, then the collection rate would be low. This time, to obtain a high collection rate, we asked whether the patients with HBsAg showed an elevated ALT level higher than 100 IU/L at least once during the 1-year observation period. We thereby do not have details on liver disease in HIV–HBV co-infected patients in the current study. Nonetheless, one-third of HIV–HBV co-infected patients have moderate liver injuries, either chronic hepatitis B or adverse effects of drugs, and are waiting for an aid for the amelioration of liver disease. A detailed analysis of the progression and activity of liver disease in HIV–HBV co-infected patients is expected.

The collection rate of the present questionnaire from the hospitals belonging to the HIV/AIDS Network was 55.6% (207 of 372). This was higher than that (47.8%) in the HIV–HCV co-infection questionnaire study carried out in 2003. The reason for this increase is not clear, but presumably the questionnaire conducted in 2003 has raised awareness among hospital staff regarding the relevance of hepatitis virus and HIV co-infection in clinical practice.

In the current study, both Japanese patients and those of other nationalities/ethnicities were included in the study. Although the ratio of newly diagnosed HIV-positive foreign people has been declining to approximately 10% in 2006, the one in total HIV positive still accounts for approximately 25% in Japan. Because the rates of the HBV carrier are different among countries, it is ideal to analyze the HBV prevalence separately according to the nationalities/ethnicities. However, in the current survey to the hospitals in HIV/AIDS Network of Japan, nationality/ethnicity was not itemized in order to make the questionnaire simple. If we would attempt to obtain such data under the approval of the ethical committee in each hospital, the response rate to questionnaire would be extremely lowered.

To establish measures that decrease the morbidity and mortality of HIV–HBV co-infected patients, it is essential to determine the current status of co-infection. In the present study, the number and transmission routes of HIV–HBV co-infected patients in Japan were determined for the first time, although detailed information on the severity and progression of liver disease in HIV–HBV co-infected patients has not been obtained yet. Undoubtedly, this will be the first step towards improving the prognosis and quality of life of Japanese patients co-infected with HIV and HBV.

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Hepatitis C virus core protein induces spontaneous and persistent activation of peroxisome proliferator-activated receptor α in transgenic mice: Implications for HCV-associated hepatocarcinogenesis

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Persistent infection of hepatitis C virus (HCV) can lead to a high risk for hepatocellular carcinoma (HCC). HCV core protein plays important roles in HCV-related hepatocarcinogenesis, because mice carrying the core protein exhibit multicentric HCCs without hepatic inflammation and fibrosis. However, the precise mechanism of hepatocarcinogenesis in these transgenic mice remains unclear. To evaluate whether the core protein modulates hepatocyte proliferation and apoptosis *in vivo*, we examined these parameters in 9- and 22-month-old transgenic mice. Although the numbers of apoptotic hepatocytes and hepatic caspase 3 activities were similar between transgenic and nontransgenic mice, the numbers of proliferating hepatocytes and the levels of numerous proteins such as cyclin D1, cyclin-dependent kinase 4 and c-Myc, were markedly increased in an age-dependent manner in the transgenic mice. This increase was correlated with the activation of peroxisome proliferator-activated receptor α (PPARα). In these transgenic mice, spontaneous and persistent PPARα activation occurred heterogeneously, which was different from that observed in mice treated with clofibrate, a potent peroxisome proliferator. We further demonstrated that stabilization of PPARα through a possible interaction with HCV core protein and an increase in nonesterified fatty acids, which may serve as endogenous PPARα ligands, in hepatocyte nuclei contributed to the core protein-specific PPARα activation. In conclusion, these results offer the first suggestion that HCV core protein induces spontaneous, persistent, age-dependent and heterogeneous activation of PPARα in transgenic mice, which may contribute to the age-dependent and multicentric hepatocarcinogenesis mediated by the core protein.

Key words: cell-cycle regulator; peroxisome; nuclear stabilization; heterogeneous PPARα activation

Hepatitis C virus (HCV) is one of the major causes of chronic hepatitis, and persistent infection with this virus can lead to a high incidence of hepatocellular carcinoma (HCC).[1,2] The prevalence of HCC because of chronic HCV infection has increased over the past two decades,[3,4] and chronic HCV infection has therefore been recognized as a serious disease. However, the precise mechanism of hepatocarcinogenesis during chronic HCV infection remains unclear.

Many experiments using cell culture systems have suggested the possibility that HCV core protein itself can modulate various cellular functions and can be directly linked to the development of HCV-related HCC.[5] For example, HCV core protein transforms rat embryo fibroblasts to a tumorigenic phenotype in cooperation with the H-*ras* oncogene,[6] suppresses *c-myc*-related apoptosis[7] and transcription of the *p53* gene,[8] interacts with a variety of proteins, including helicase, lymphotoxin-β receptor, or dead box protein, and modulates their functions.[9] We further established transgenic mouse lines carrying the HCV core gene, in which the core protein is constitutively expressed in the liver at levels similar to that found in chronic hepatitis C patients.[10] These mice exhibited multicentric hepatic adenomas, and developed HCCs in an age-dependent manner.[11] The livers of these mice were almost free of inflammation, necrosis and fibrosis,[10,11] suggesting that the core protein itself has a hepatocarcinogenic potential *in vivo*. However, the molecular mechanism of the development of HCC in the transgenic mice has not been fully understood.

In the livers of HCV core gene transgenic mice, an age-dependent increase in oxidative stress and resultant DNA damage were found,[12] and these effects may contribute to or facilitate the development of HCC. Another possible mechanism of hepatocarcinogenesis is continuous enhancement of hepatocyte proliferation. Cell proliferation and apoptosis are highly regulated processes for maintaining homeostasis in many organs, and during the carcinogenic process, sustained imbalance generally precedes cancer.[13,14] For example, in patients with chronic HCV infection, high hepatocyte proliferative activity relative to apoptosis may reliably predict a new development of HCC.[15] However, there is no information about whether or not hepatocyte proliferation accelerates persistently in mice carrying the HCV core gene, and no information about how the core protein promotes hepatocyte proliferation *in vivo*. In the current study, we began to examine changes in the parameters of hepatocyte proliferation and apoptosis in the transgenic mice.

Material and methods

Animals and treatments

HCV core gene transgenic mice on a C57BL/6N genetic background were produced as described earlier.[10] Because HCC developed preferentially in male transgenic mice,[11] 9- and 22-month-old male mice ($n = 8$ for either age group) were adopted. Sex- and age-matched nontransgenic mice ($n = 8$ for either age group) were used as controls. These mice were fed an ordinary diet and were treated in a specific pathogen-free state according to the institutional guidelines. For additional experiment, male wild-type mice fed a control diet containing 0.5% clofibrate for 2 weeks ($n = 8$) were used. All mice were killed by cervical dislocation and the livers were excised. When a hepatic tumor was present, it was removed and the remaining liver tissue was used. All experiments were performed in accordance with animal study protocols approved by the Shinshu University School of Medicine.

Abbreviations: AOX, acyl-CoA oxidase; CDK, cyclin-dependent kinase; DAB, 3,3'-diaminobenzidine; FITC, fluorescein isothiocyanate; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; L-FABP, liver-type fatty acid-binding protein; NEFA, nonesterified fatty acid; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; PMSF, phenylmethylsulfonyl fluoride; PPAR, peroxisome proliferator-activated receptor; PT, peroxisomal thiolase; RXR, retinoid X receptor; SDS, sodium dodecyl sulfate; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling.

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Preparation of hepatocyte nuclear fraction

Approximately 200 mg of liver tissues was transferred to a chilled Dounce homogenizer (Wheaton, Milliville, NJ) and homogenized on ice by 30 strokes in 1.2 mL of nuclei buffer [300 mM sucrose in 10 mM Tris-HCl, pH 7.4, 15 mM NaCl, 5 mM $MgCl_2$ and 0.25 mM phenylmethylsulfonyl fluoride (PMSF)]. The homogenate was filtered through gauze and centrifuged at 4,500*g* for 5 min at 4°C. The resulting pellet was resuspended, layered over 2 mL of nuclei buffer containing 2 M sucrose, and centrifuged at 23,000*g* for 1 hr at 4°C. The pellet obtained after ultracentrifugation was resuspended in 250 μL of nuclei buffer and used as the nuclear fraction. Preparation of nuclear fraction from isolated hepatocytes was performed as described elsewhere.[16]

Immunoblot analysis

Protein concentration was measured colorimetrically by a BCA™ Protein Assay kit (Pierce, Rockford, IL). For analysis of fatty acid-metabolizing enzymes and protein, whole liver lysate (10–20 μg protein) was subjected to 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.[17] For analysis of other proteins, hepatocyte nuclear fraction (100 μg protein) or whole liver lysate (200–300 μg protein) was subjected to electrophoresis. After electrophoresis, the proteins were transferred to nitrocellulose membranes, which were incubated with the primary antibody, followed by alkaline phosphatase-conjugated goat anti-rabbit or anti-mouse IgG. The origin of the primary rabbit polyclonal antibodies against fatty acid-metabolizing enzymes and protein was described earlier.[17] For immunoblot analysis of peroxisome proliferator-activated receptor α (PPARα), a polyclonal anti-mouse antibody[18] or commercial antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used. The antibodies against cell-cycle regulators and oncogene products were purchased commercially (Santa Cruz Biotech.).[19] Equal loading of the protein obtained from whole liver lysate and nuclear fraction was confirmed by reprobing the membranes with an antibody against β-actin and histone H1, respectively. The band intensity of nuclear PPARα was quantified densitometrically, normalized to that of histone H1, and subsequently expressed as the fold changes relative to that of 9-month-old nontransgenic mice.

mRNA analysis

Total liver RNA was extracted with an RNeasy Mini Kit™ (Qiagen, Valencia, CA). Five microgram of RNA was electrophoresed on 1.1 M formaldehyde-containing 1% agarose gels and transferred to nylon membranes by capillary blotting in 20× SSC buffer (3 M NaCl and 300 mM sodium citrate, pH 7.0) overnight. The membranes were hybridized with ^{32}P-labeled cDNA probes. The blots were exposed to a phosphorimager screen cassette and were analyzed using a Molecular Dynamics Storm 860 Phosphorimager system (Sunnyvale, CA). The origin of the cDNA probes has been described elsewhere.[17–19] Northern blot of β-actin was used as the internal control. The blot intensity was quantified, normalized to that of β-actin and subsequently expressed as the fold changes relative to that of 9-month-old nontransgenic mice.

Pulse-label and pulse-chase experiment

Parenchymal hepatocytes were isolated from transgenic and control mice by the modified *in situ* perfusion method.[20] After perfusion with 0.05% collagenase solution (Wako, Osaka, Japan), the isolated hepatocytes were washed thrice by means of differential centrifugation and the dead cells removed by density gradient centrifugation on Percoll (Amersham Pharmacia Biotech, Buckinghamshire, UK). The live hepatocytes were washed and suspended in William's E medium containing 5% fetal bovine serum. When the viability of the isolated hepatocytes exceeded 85% as determined by the trypan blue exclusion test, the following experiments were conducted. The isolated hepatocytes were washed twice and incubated in methionine-free medium containing 5% dialyzed fetal bovine serum for 1 hr at 37°C. The medium was replaced with the same medium containing 300 μCi/mL of [^{35}S]methionine (Amersham Pharmacia Biotech.). After 3-hr of incubation, the labeled medium was changed to the standard medium and the preparation was chased for 4, 8 or 16 hr. The labeled cells were washed, homogenized and centrifuged for preparation of the nuclear fraction. The levels of radioactivity in the homogenates of the pulse-labeled preparations were similar between the transgenic and the nontransgenic mice, suggesting that the [^{35}S]methionine uptake capacity in the former hepatocytes is similar to that in the latter. The nuclear fraction was lysed in RIPA buffer [10 mM Tris-HCl, pH 7.4, 0.2% sodium deoxycholate, 0.2% Nonidet P-40, 0.1% SDS, 0.25 mM PMSF, 10 μg/mL aprotinin]. The lysate was incubated for 3 hr at 4°C with purified anti-PPARα antibody. The immune complexes were precipitated with *Staphylococcus aureus* protein A bound to agarose beads. After the precipitates had been washed in RIPA buffer, the labeled proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. The nuclear fractions of the pulse-labeled preparations were also used for immunoblot analysis of PPARα.

Affinity chromatography for PPARα complex

All procedures were performed at 4°C. The nuclear fraction from the mouse liver was mixed with a 4-fold volume of a solution containing 12.5 mM potassium phosphate, pH 7.5, 25 mM NaCl, 0.25% Tween 20 and 0.1 mM PMSF. The mixture was briefly sonicated with a microsonicator, the Powersonic Model 50 (Yamato, Tokyo, Japan), and then centrifuged at 100,000*g* for 20 min. The supernatant was applied to an immobilized anti-PPARα IgG column (1.0 × 4.0 cm^2), prepared with the Affigel HZ Immunoaffinity kit^R (Bio-Rad, Hercules, CA) and equilibrated with 10 mM potassium phosphate, pH 7.5, 20 mM NaCl and 0.2% Tween 20. The solution was again passed through the column and this was repeated at least thrice. The column was washed and the elution performed with 150 mM sodium citrate, pH 3.0, and 200 mM NaCl, in a total volume of 2 mL. The eluate was resolved by 10 and 15% SDS-polyacrylamide gel electrophoresis for PPARα and the HCV core protein, respectively. The core protein expressed in COS cells was used as a positive marker.[21] The monoclonal antibody against the core protein was purchased commercially (ViroGen, Watertown, MA).

Cytochemical staining of peroxisomes

Liver peroxisome proliferation was evaluated by using 3,3′-diaminobenzidine (DAB) staining for catalase according to the method of Novikoff and Goldfischer with minor modifications.[22] Small pieces of liver were fixed with 2% glutaraldehyde in 100 mM sodium cacodylate buffer, pH 7.2, for 3 hr at 4°C, rinsed with sodium cacodylate buffer and cut into 100-μm sections with a Lancer^R Vibratome 1000 (Lancer, Bridgeton, MO). These sections were then incubated for 1 hr at 37°C in the DAB reaction medium (0.2% DAB tetrahydrochloride in 50 mM propanediol, pH 9.7, 5 mM KCN, 0.05% H_2O_2) and postfixed with 1% OsO_4 in 100 mM sodium phosphate, pH 7.4 for 1 hr. The sections were dehydrated through a graded series of ethanol and acetone treatments and embedded in Epok 812 (Oken, Tokyo, Japan). One micrometer sections were prepared, counterstained with 0.1% toluidine blue solution and examined by light microscopy. For electron microscopic examination, 0.1-μm sections were cut with a diamond knife, collected on grid meshes, stained with lead citrate and uranyl acetate and visualized with a JEM 1200EX II electron microscope (JEOL, Tokyo, Japan) at an accelerating voltage of 80 keV

Morphometry of hepatic peroxisomes

Morphometric analysis of DAB-stained peroxisomes was carried out using electron photomicrographs. For each mouse, 10 independent fields in the pericentral area of liver lobuli were photomicrographed at an original magnification of 4,000×. At this magnification, peroxisomes smaller than 450 nm were clearly

identified. Peroxisomes were easily detected because of their high contrast because of the positive DAB reaction. In each frame, the number of peroxisomal profiles and the area of each individual profile were determined. The numerical density and volume density of peroxisomes were calculated using the following equations: numerical density (number/μm^2) = $N_P/(A_T - A_{empty})$, and volume density (%) = $A_{TP}/(A_T - A_{empty}) \times 100$, where N_P is the peroxisome number in the test area, A_T is the test area, A_{empty} is the area of the vascular and biliary lumens and that of the hepatocyte nuclei and lipid droplets, and A_{TP} is the area of total peroxisomal profiles in the test area. The area was measured with a Luzex AP image analyzer (Nireco, Tokyo, Japan).

Immunofluorescence staining

Liver samples were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), embedded in Tissue-Tek O.C.T compound™ (Sakura Finetek, Torrance, CA) and frozen. Frozen liver 5-μm sections were prepared, washed with PBS, blocked with bovine serum albumin for 1 hr and incubated overnight with rabbit polyclonal antibodies against cyclin D1 (1:50 dilution)[19] and PPARα (1:100 dilution),[18] and with mouse monoclonal antibody against proliferating cell nuclear antigen (PCNA) (1:100 dilution).[19] After 5 washes with PBS, these sections were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) or donkey anti-mouse IgG (Dako). The sections were mounted and viewed with an Olympus Fluoview confocal laser scanning microscope (Olympus, Tokyo, Japan). Two-thousand hepatocyte nuclei were examined for each mouse, and the number of hepatocyte nuclei stained with the antibodies against cyclin D1, PPARα and PCNA was counted and expressed as a percentage.

Assessment of apoptotic hepatocytes

Liver samples were cut into small pieces and then fixed in 4% paraformaldehyde in PBS. These samples were dehydrated, embedded in paraffin and cut into 4-μm sections. The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay was performed using a MEBSTAIN Apoptosis Kit II (Medical and Biological Laboratories, Nagoya, Japan). The number of apoptotic hepatocytes in 2,000 hepatocytes was counted for each mouse, and expressed as a percentage.

Other methods

Hepatic caspase 3 activity was measured as described elsewhere.[23] For analysis of the nuclear contents of nonesterified fatty acids (NEFAs), ~150 μL of the hepatocyte nuclear fraction, containing 1–2 mg of protein, was treated with a microsonicator. Lipid extraction was performed according to a modification of the method developed by Folch *et al.*[24] and the nuclear content of NEFAs was measured with a *NEFA* C-test kit™ (Wako).

Statistical analysis

Statistical analysis was performed by means of Student's *t*-test. The results are expressed as the mean ± standard deviation. A probability value of less than 0.05 was considered to be statistically significant.

Results

Accelerated hepatocyte proliferation in HCV core gene transgenic mice

To evaluate hepatocyte proliferative activity, PCNA-positive hepatocytes were counted in male transgenic mice and nontransgenic mice. Although hepatic inflammation and hepatocyte necrosis were not detected in either group, the numbers of PCNA-positive hepatocytes were significantly increased in the 9-month-old transgenic mice compared with the 9-month-old nontransgenic mice (Fig. 1*a*). The increase was more significant in the 22-month-old transgenic mice (Fig. 1*a*). The numbers of PCNA-positive hepatocytes in the 22-month-old transgenic mice corresponded with those in HCV polyprotein-expressing transgenic mice with HCC.[25] On the other hand, the parameters of apoptosis, *i.e.*, the numbers of TUNEL-positive hepatocytes and hepatic caspase 3 activity, remained unchanged between the 2 groups at the same ages (Figs. 1*b* and 1*c*). These results suggest that spontaneous hepatocyte proliferation occurs as early as the age of 9 months and persists for a long time in HCV core gene transgenic mice.

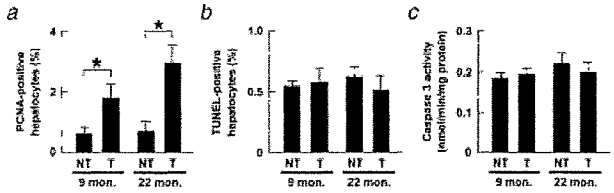

FIGURE 1 – Increase in hepatocyte proliferative activity. (*a*) The number of PCNA-positive hepatocytes. Two-thousand hepatocyte nuclei were examined for each mouse, and the number stained with anti-PCNA antibody was counted. Results are expressed as the mean ± standard deviation (n = 8). *, $p < 0.05$ between the transgenic mice and the nontransgenic mice: NT, nontransgenic mice; T, transgenic mice; 9 mon, 9-month-old mice; 22 mon, 22-month-old mice. (*b*) The number of apoptotic hepatocytes. The number of TUNEL-positive hepatocytes in 2,000 hepatocytes was determined for each mouse. Results are expressed as the mean ± standard deviation (n = 8). (*c*) Caspase 3 activity. Results are expressed as the mean ± standard deviation (n = 8).

Simultaneous induction of cell-cycle regulators and oncogene products in HCV core gene transgenic mouse livers

To examine the changes in the expression of proteins associated with hepatocyte division, the livers of the 9- and 22-month-old mice were subjected to immunoblot analysis. The levels of many proteins including cell-cycle regulators [cyclin-dependent kinase (CDK) 1, 2 and 4, cyclin D1 and E, and PCNA], and oncogene products (c-Myc, c-Fos and c-Ha-Ras) were significantly higher in the 22-month-old transgenic mice than in the control mice (Fig. 2). The levels of CDK inhibitors such as p16 and p21 were similar between the 2 groups. Similar results were obtained from the 9-month-old transgenic mice (data not shown). Time course changes in the expression of key G1-S checkpoint regulators, cyclin D1 and CDK4, are shown in Figure 3*a*. The simultaneous increase in the expression of cyclin D1 and CDK4 in the transgenic mice was continuous and more pronounced with age. Northern blot analysis revealed that the increase of these proteins occurred at the transcriptional level (Figs. 3*b* and 3*c*). Thus, these results reveal that various proteins which accelerate cell-cycle progression were induced simultaneously, persistently and age-dependently in the transgenic mice.

Correlative induction of PPARα targets in HCV core gene transgenic mouse livers

As shown in Figure 2, the expression of many kinds of cell-cycle regulators and oncogene products is known to be induced by the functional activation of PPARα.[19,26–30] To investigate whether PPARα is activated in the livers of transgenic mice, the expression of representative PPARα target genes,[30] acyl-CoA oxidase (AOX), peroxisomal thiolase (PT) and liver-type fatty acid-binding protein (L-FABP), was examined. As demonstrated in Figure 3*a*, the levels of AOX, PT, and L-FABP were increased in the 9-month-old transgenic mice compared with the nontransgenic mice, and the increase was more pronounced in the 22-month-old transgenic mice. Northern blot analysis demonstrated that the increase in these PPARα targets was based on the increase in the transcriptional activity (Figs. 3*b* and 3*c*). The increase in the

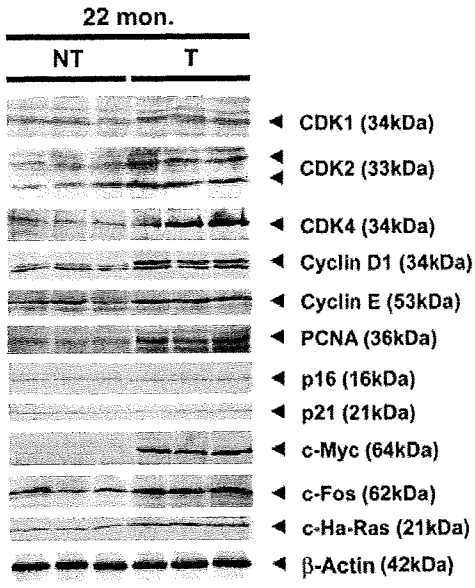

FIGURE 2 – Immunoblot analysis of cell-cycle regulators and oncogene products. Whole liver lysate (200 μg) was loaded in each lane. The band of β-actin was used as the loading control. The apparent molecular weight is indicated in parentheses. 22 mon, 22-month-old mice; NT, nontransgenic mice; T, transgenic mice.

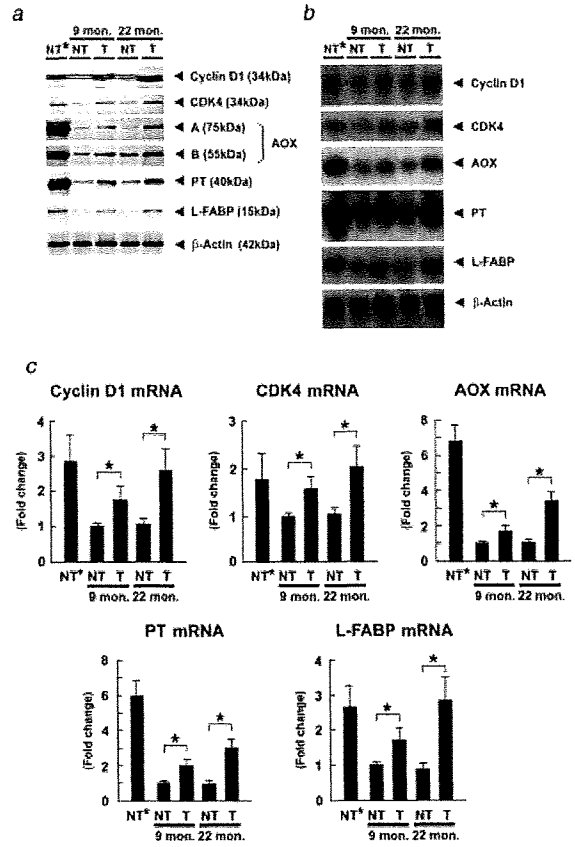

FIGURE 3 – Analysis of PPARα-regulated proteins. (*a*) Immunoblot analysis of cell-cycle regulators and fatty acid-metabolizing enzymes and proteins. Since no significant individual differences in the same mouse group were found in the preliminary experiments, 10 mg of liver pieces prepared from each mouse (n = 8/group) was mixed and homogenized. Whole liver lysate (200 μg for cyclin D1 and CDK4, and 20 μg for others) was loaded in each lane. The band of β-actin was used as the loading control. Results are representative of 4 independent experiments. The apparent molecular weight is indicated in parentheses. 9 mon, 9-month-old mice; 22 mon, 22-month-old mice; NT, nontransgenic mice; T, transgenic mice; NT*, nontransgenic mice treated with a control diet containing 0.5% clofibrate for 2 weeks; A and B, full-length and truncated AOX, respectively. (*b*) Northern blot analysis concerning the proteins in (*a*). Ten milligram of liver pieces from each mouse (n = 8/group) was mixed and homogenized, and total liver RNA was extracted. Hepatic RNA (5 μg) was separated on a denaturing gel, transferred to membranes and hybridized with the indicated ^{32}P-labeled cDNA probes. The blot of β-actin was used as the internal control. Results are representative of 4 independent experiments. (*c*) Quantification of hepatic mRNA levels. The mRNA level was quantified using a phosphorimager, normalized to that of β-actin, and subsequently normalized to that of 9-month-old nontransgenic mice. Results were obtained from 4 independent experiments and expressed as the mean ± standard deviation. Abbreviations are identical with those in (*b*). *, $p < 0.05$ between the transgenic mice and the nontransgenic mice.

mRNA expression of AOX, PT and L-FABP corresponded exactly with that of cyclin D1 or CDK4 (Figs. 3*b* and 3*c*). Therefore, these results demonstrate the strong correlation between continuous and age-dependent induction of cell-cycle regulators and functional activation of PPARα in these transgenic mice. Furthermore, the induction of these 5 proteins was also observed in wild-type mice treated with clofibrate, a potent PPARα activator; however, the degree of the induction of AOX and PT in the transgenic mice was smaller than that in the clofibrate-treated wild-type mice (Fig. 3), suggesting that the PPARα activation found in the transgenic mice was not as intense as that in the mice treated with clofibrate.

Histological evaluation of PPARα activation

An increase in the numbers of peroxisomes is associated with PPARα activation.[18] To determine whether peroxisome proliferation occurs in the HCV core gene transgenic mice, cytochemical staining for peroxisomal catalase was performed. A scattered distribution of hepatocytes with numerous peroxisomes was observed in the 9-month-old transgenic mice (Fig. 4*a*). Such hepatocytes were also found in the 22-month-old transgenic mouse livers (Fig. 4*a*). In contrast, almost all of the hepatocytes in the clofibrate-treated mice showed significant peroxisome proliferation (Fig. 4*a*). To quantitatively evaluate the degree of peroxisome proliferation, morphometric analysis of peroxisomes was conducted. The numerical density and volume density were significantly increased in the transgenic mice compared with those in the nontransgenic mice (Fig. 4*b*). The volume density, the most reliable parameter of peroxisome proliferation, was increased age-dependently in the transgenic mice, but the degree of the increase was not as prominent as that observed in mice with clofibrate administration (Fig. 4*b*). The finding that only some hepatocytes in the transgenic mice presented a marked peroxisome proliferation (Fig. 4*a*) is noteworthy, since it seems to correlate with the finding that intense expression of the core protein was observed only in particular hepatocytes.[10] These histological analyses reveal that spontaneous, continuous and age-dependent peroxisome proliferation and PPARα activation occur heterogeneously in the transgenic mouse livers, which is different from the response observed in the mice receiving clofibrate treatment.

Appearance of PPARα-, and cyclin D1-positive hepatocytes

We tried to detect abnormal hepatocytes to clarify the mechanism of hepatocarcinogenesis in the transgenic mice. On PPARα immunofluorescence staining, PPARα was primarily detected in the cytoplasm of the nontransgenic mice and the clofibrate-administered mice. Some hepatocytes having nuclei positively stained

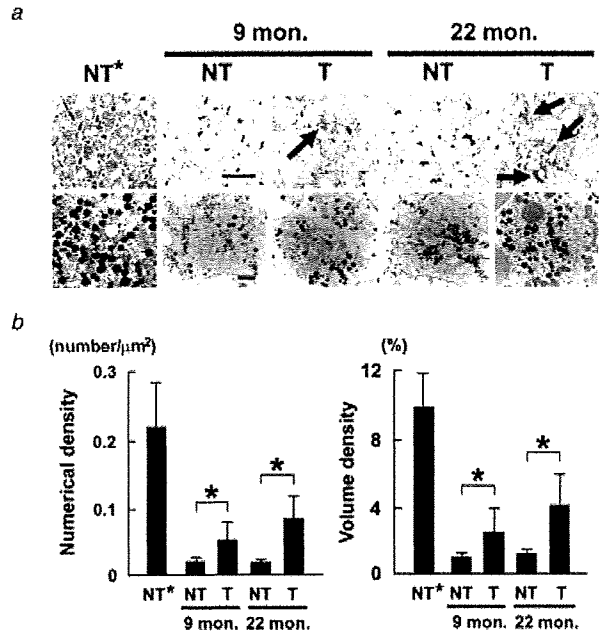

FIGURE 4 – Cytochemical staining for hepatic peroxisomes. (*a*) Light and electron photomicrographs of DAB-stained liver tissues. Peroxisomes are detected as darkly stained particles. The arrows in upper panels indicate hepatocytes showing profound peroxisome proliferation. The bars in the light and electron photomicrographs of 9-month-old nontransgenic mice indicate 50 and 2 μm, respectively. 9 mon, 9-month-old mice; 22 mon, 22-month-old mice; NT, nontransgenic mice; T, transgenic mice; NT*, nontransgenic mice treated with a control diet containing 0.5% clofibrate for 2 weeks. (*b*) Morphometric analysis of hepatic peroxisomes. The number of peroxisomes and the area of each individual peroxisome profile were measured in 10 photomicrographs for each mouse, and morphometric parameters such as numerical density and volume density were calculated. Results are expressed as the mean ± standard deviation ($n = 8$). Abbreviations are identical with those in (*a*). *, $p < 0.05$ between the transgenic mice and the nontransgenic mice.

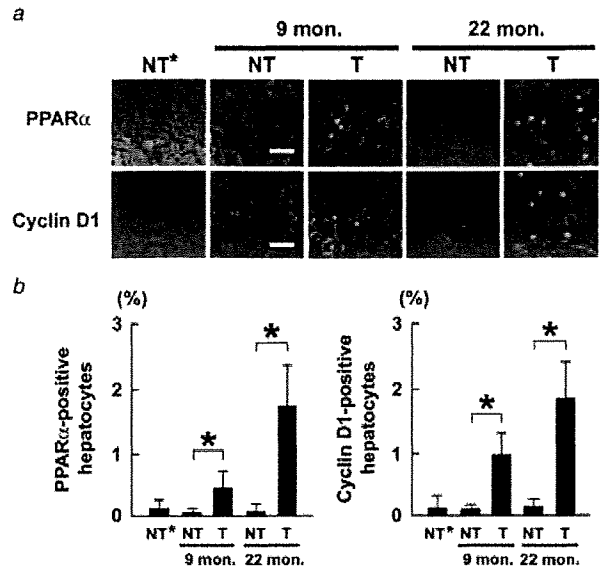

FIGURE 5 – Immunofluorescence staining for PPARα and cyclin D1. (*a*) Immunofluorescence staining using antibodies against PPARα and cyclin D1. The bars in the photomicrographs of 9-month-old nontransgenic mice indicate 50 μm. 9 mon, 9-month-old mice; 22 mon, 22-month-old mice; NT, nontransgenic mice; T, transgenic mice; NT*, nontransgenic mice treated with a control diet containing 0.5% clofibrate for 2 weeks. (*b*) The number of PPARα-, or cyclin D1-positive hepatocytes. Two-thousand hepatocyte nuclei were examined for each mouse, and the number of nuclei intensively stained with anti-PPARα or anti-cyclin D1 antibody was counted. Results are expressed as the mean ± standard deviation ($n = 8$). Abbreviations are identical with those of (*a*). *, $p < 0.05$ between the transgenic mice and the nontransgenic mice.

by anti-PPARα antibody were detected only in the transgenic mice (Fig. 5*a*). Similar to the case of PPARα, the hepatocytes having nuclei stained intensively by anti-cyclin D1 antibody were found only in the transgenic mice (Fig. 5*a*). A few hepatocytes stained by anti-CDK4 antibody were also observed only in the transgenic mice (data not shown). The frequency of appearance of PPARα-, or cyclin D1-positive hepatocytes was increased with age (Figs. 5*a* and 5*b*). Thus, the appearance of these specific hepatocytes in the transgenic mice seemed to be, at least in part, associated with sustained, age-dependent and heterogeneous PPARα activation in the transgenic mice.

Changes in PPARα levels

Since the expression of PPARα is known to be enhanced by its activation,[18,30] the quantitative change in PPARα was evaluated. The nuclear PPARα level in the transgenic mice was increased age-dependently, as expected (Figs. 6*a*, upper panel and 6*b*), but the PPARα level in the whole liver lysate remained unchanged (data not shown). The increase in nuclear PPARα in the transgenic mice was smaller than that in the clofibrate-treated wild-type mice (Figs. 6*a*, upper panel and 6*b*). Northern blot analysis revealed a higher PPARα mRNA level in the clofibrate-treated mice than in the controls, although this parameter in the transgenic mouse groups of each age was similar to that in the controls (Figs. 6*a*, lower panel and 6*b*). These results indicate that the increase in nuclear PPARα in the transgenic mice occurs mainly at the post-transcriptional level, which is distinct from that observed in the clofibrate-treated wild-type mice.

Stabilization of PPARα through a possible interaction with HCV core protein in hepatocyte nuclei

The increased stability of PPARα in hepatocyte nuclei is thought to be one of the possible causes of a disproportional increase in the nuclear PPARα level. To examine this possibility, a pulse-chase experiment was performed using isolated hepatocytes. The half-life of nuclear PPARα was ~7 hr in the control mice and 12.5 hr in the transgenic mice (Fig. 7*a*). In addition, the intensity of the labeled PPARα band (P in Fig. 7*a*, upper panels) in the control mice was similar to that in the transgenic mice. The finding that the [^{35}S]methionine uptake in the hepatocytes from the control mice was similar to that from the transgenic mice suggests that the increase in nuclear PPARα in the hepatocytes from the transgenic mice (Fig. 7*a*, lower right panel), as well as that *in vivo* (Fig. 6*a*, upper panel), is not because of the increased PPARα transfer into the nucleus.

In the transgenic mice, HCV core protein accumulated in the nuclei, as evidenced by immunoelectron microscopy,[11] suggesting a possible interaction of the core protein with PPARα in the nuclei. We therefore examined this possibility by anti-PPARα IgG affinity chromatography. When proteins combining with PPARα in hepatocyte nuclei were subjected to immunoblot analysis, the core protein was clearly detected (Fig. 7*b*). This result suggests the possibility of complex formation between the HCV core protein and PPARα, which is consistent with an interaction of the core protein with retinoid X receptor (RXR) α,[31] an essential heterodimeric partner of PPARα.[32] Thus, HCV core protein may

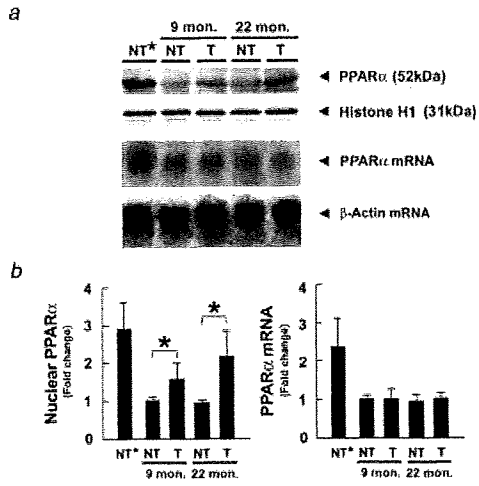

FIGURE 6 – Analysis of PPARα. (*a*) (Upper panels) Immunoblot analysis of nuclear PPARα. Since few individual differences in the same mouse group were found in the preliminary experiments, 30 mg of liver pieces from each mouse (n = 8/group) was mixed and homogenized to prepare the nuclear fraction. One-hundred microgram of nuclear protein was separated on 10% SDS-polyacrylamide gel, transferred to nitrocellulose membranes and reacted with antibody against PPARα. The band of histone H1 was used as the loading control. Results are representative of 4 independent experiments. The apparent molecular weight is indicated in parentheses. 9 mon, 9-month-old mice; 22 mon, 22-month-old mice; NT, nontransgenic mice; T, transgenic mice; NT*, nontransgenic mice treated with a control diet containing 0.5% clofibrate for 2 weeks. (Lower panels) Northern blot analysis of PPARα. A sample used in Figure 3*b* was adopted. Hepatic RNA (5 μg) was electrophoresed and hybridized with cDNAs for PPARα and β-actin, respectively. Results are representative of 4 independent experiments. (*b*) Quantification of nuclear PPARα levels and PPARα mRNA levels. The nuclear PPARα level was quantified densitometrically and normalized to the histone H1 level. The mRNA level of PPARα was quantified using a phosphorimager and normalized to that of β-actin. Values were subsequently normalized to those of 9-month-old nontransgenic mice. Results were obtained from 4 independent experiments and expressed as the mean ± standard deviation. Abbreviations are identical with those in (*a*). *, $p < 0.05$ between the transgenic mice and the nontransgenic mice.

directly or indirectly affect the stability of PPARα in hepatocyte nuclei.

Increase in PPARα ligands

PPARα is a ligand-activated transcription factor. Since the transgenic mice were fed a standard laboratory chow, endogenous substances such as NEFAs would serve as ligands of PPARα[33]; therefore, the contents of NEFAs in hepatocyte nuclei were compared between the 2 groups. The levels of NEFAs in hepatocyte nuclei in the transgenic mice were ~5 times higher than those in the control mice at the same age (Fig. 7*c*). This could account for the higher activation of PPARα in the transgenic mice than in the controls.

Discussion

A large number of variables are involved in the induction of HCC by HCV core protein. While the precise mechanism underlying hepatocarcinogenesis in HCV core gene transgenic mice cannot be fully elucidated from this study, our results could provide some clues to explain this phenomenon. We found spontaneous, persistent, age-dependent and heterogeneous PPARα activation in the transgenic mouse livers for the first time. This study thus advances our understanding of the association

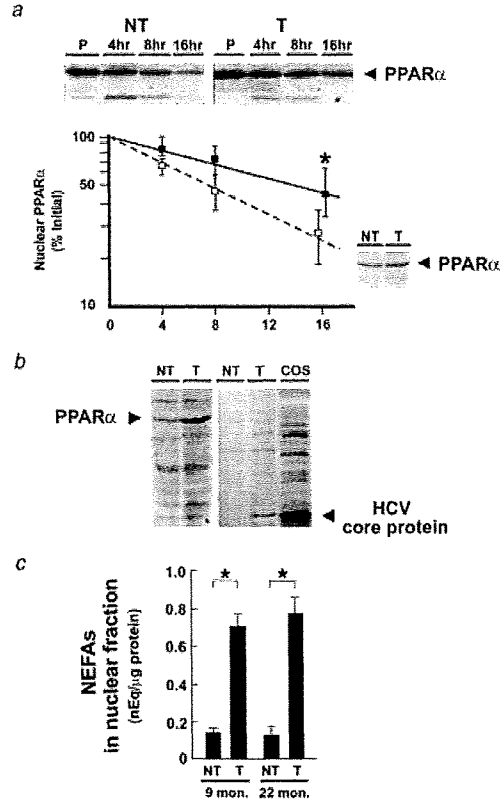

FIGURE 7 – Analyses of PPARα stability, interaction between PPARα with the core protein in hepatocyte nuclei, and nuclear contents of NEFAs. (*a*) Pulse-label and pulse-chase experiments for nuclear PPARα using isolated mouse hepatocytes. (Upper panels) Labeled PPARα bands on X-ray film. Pulse-label and pulse-chase experiments were performed as described in the Material and methods. NT, nontransgenic mice; T, transgenic mice; P, pulse-label; 4, 8, 16 hr, pulse-chase for 4, 8, 16 hr, respectively. (Lower left panel) Intensity plot of PPARα in 5 independent experiments. Values are normalized as a percentage of the values of the pulse-labeled band and expressed as the mean ± standard deviation. Open square, nontransgenic mice; black square, transgenic mice; *, $p < 0.05$ between the transgenic mice and the nontransgenic mice. (Lower right panel) Immunoblot analysis of an isolated hepatocyte nuclear fraction. NT, nontransgenic mice; T, transgenic mice. (*b*) Interaction between PPARα and HCV core protein in the nucleus. (Left panel) Immunoblot analysis (PPARα) of the eluate on anti-PPARα IgG affinity column chromatography. (Right panel) Immunoblot analysis (HCV core protein) of the same eluate. NT, nontransgenic mice; T, transgenic mice; COS, HCV core protein-overexpressing COS cell lysate. (*c*) Nuclear contents of NEFAs. The levels of NEFAs were measured using a hepatocyte nuclear fraction. Results are expressed as the mean ± standard deviation (n = 8). *, $p < 0.05$ between the transgenic mice and the nontransgenic mice; NT, nontransgenic mice; T, transgenic mice; 9 mon, 9-month-old mice; 22 mon, 22-month-old mice.

between HCV core protein-mediated hepatocarcinogenesis and persistent PPARα activation.

Hepatocyte proliferation is influenced by various factors, such as mitogenic chemicals, cytokines, growth factors and transcription factors. It has been reported that various kinds of cell-cycle regulators and oncogene products are induced by PPARα activation.[19,26–30] In particular, cyclin D1, CDK4, PCNA and c-Myc are potent and critical regulators of the G1-S checkpoint and cell-cycle progression,[13,14] and aberrant expression of these proteins is frequently detected in HCV-related HCC.[34–37] These key regulators are known to be induced in a PPARα-dependent manner in mice[19,30]; the continuous induction of these proteins and the

resultant acceleration of hepatocyte proliferation found in the transgenic mice may be attributed to persistent PPARα activation. In the current study, we demonstrated that there was a great variety of the intensity of PPARα activation among different hepatocytes (Fig. 4). This persistent and heterogeneous PPARα activation found especially in the transgenic mice may be linked with the age-dependent and multicentric hepatocarcinogenesis induced by the core protein.

It is well-known that the long-term administration of potent peroxisome proliferators such as fibrate drugs can induce hepatocarcinogenesis in rodents.[29] The findings observed in the transgenic mice markedly differ from those in mice with long-term treatment of peroxisome proliferators in several ways. Namely, the transgenic mice show no intense increase in AOX and PT (Fig. 3), no increase in PPARα mRNA (Fig. 6), heterogeneous peroxisome proliferation (Fig. 4) and age-dependent emergence of hepatocytes having nuclei stained intensively by anti-PPARα or anti-cyclin D1 antibody (Fig. 5). Therefore, the mode of PPARα activation and the mechanism of hepatocarcinogenesis caused by HCV core protein expression are indeed unique.

One of the mechanisms involved in the core protein-specific PPARα activation in mice is stabilization of PPARα in hepatocyte nuclei through a possible interaction with the core protein. In cultured cells expressing the core protein, it has been demonstrated that the core protein interacts with the PPARα-RXRα heterodimer and enhances the transcriptional activation mediated by PPARα regardless of the presence or absence of its ligands.[31] Since PPARα is ubiquitinated and degraded *via* the proteasome pathway,[38] it may be postulated that HCV core protein directly or indirectly influences the degradation pathway. It has been reported that the core protein binds to the proteasome activator PA28γ[39] which is known to combine with steroid receptor coactivator-3 and to accelerate its degradation.[40] Another possible mechanism is an increase in NEFAs in hepatocyte nuclei. The PPARα activation induced by the core protein enhances the expression of L-FABP,[30] which serves as a transporter of NEFAs into nuclei. Indeed, real-time confocal and multiphoton laser scanning microscopy has shown that L-FABP expression significantly increased the total uptake of medium- and long-chain fluorescent fatty acids into the nuclei of living cells.[41] Thus, increased L-FABP expression may facilitate the shuttling of NEFAs into hepatocyte nuclei for donating NEFAs to PPARα, leading to PPARα activation and further increase in L-FABP expression. Moreover, the binding of ligands causes conformational alternation of PPARα[42] and further stabilizes it in nuclei,[32] resulting in synergistic PPARα activation. Therefore, these findings concerning spontaneous and persistent PPARα activation induced by the core protein enable us to partially explain the precise molecular mechanism of hepatocarcinogenesis in HCV core gene transgenic mice.

The results obtained from the current study are consistent with the findings observed in chronically HCV-infected patients in several ways. That is, like the transgenic mice in the present study, chronically HCV-infected patients have been reported to show accelerated hepatocyte proliferation,[43] an increase in CDK4, cyclin D1 and E, PCNA, c-Myc and c-Fos,[34–37] and multicentric appearance of HCC.[44] Furthermore, it has been reported that a massive proliferation of peroxisomes was found in human nontumorous liver tissue adjacent to HCC.[45] Thus the earlier findings, including the unique function of HCV core protein *in vivo* and the diverse and significant roles of PPARα, may help to partially understand the onset and development of HCC in patients with chronic HCV infection. It has been demonstrated that the function of hepatic PPARα was impaired in patients with chronic HCV infection,[46] which is different from our results. Since HCC had not yet developed in the patients in the report, this discrepancy might derive from differences in the stage of the hepatocarcinogenic process.

The interpretation based on persistent activation of PPARα pertains to only one possible mechanism of hepatocarcinogenesis induced by the effects of HCV core protein. We cannot rule out the presence of other mechanisms. The exact relationship between PPARα activation and hepatocarcinogenesis may be elucidated by additional experiments in which PPARα activation is continuously inhibited in the same transgenic mice. Furthermore, the exact relationship may be confirmed when PPARα-null mice bearing the core protein gene do not represent development of HCC.

In conclusion, we demonstrated for the first time that spontaneous, persistent, age-dependent and heterogeneous activation of PPARα occurred in HCV core protein transgenic mice and caused continuous enhancement of hepatocyte proliferation, which may have contributed to the age-dependent and multicentric hepatocarcinogenesis observed in these mice. In addition, we observed nuclear stabilization of PPARα and an increase in NEFAs in the hepatocyte nuclei of the transgenic mice, which may have resulted in the HCV core protein-specific PPARα activation.

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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.

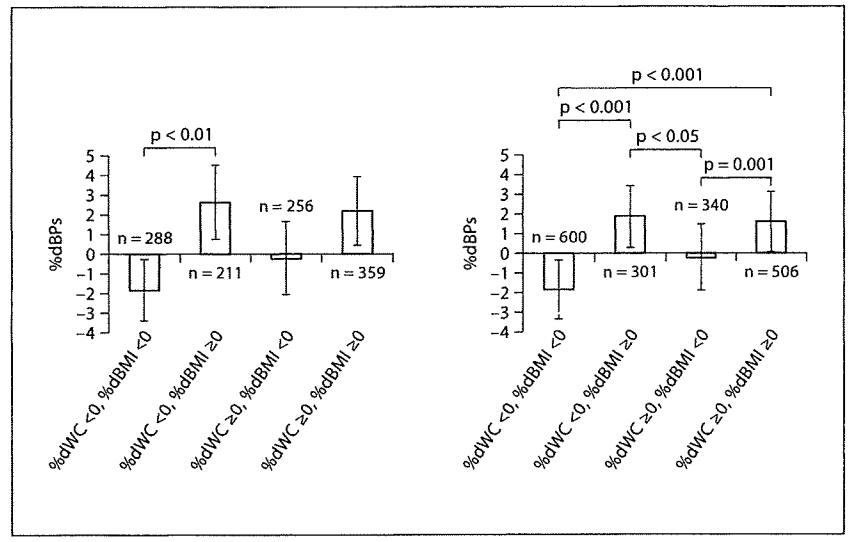

Fig. 1. Comparison of the age-adjusted %dBPs in four subgroups categorized according to the gain or loss of %dWC and %dBMI values. p values were from the result of the Tukey's post-hoc analysis following analyses of variance. Mean ± 95% confidence interval is shown in each group.

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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