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ELSEVIER

Impact of New Methicillin-Resistant *Staphylococcus aureus* Carriage Postoperatively After Living Donor Liver Transplantation

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ABSTRACT

Background. Preoperative carriage of methicillin-resistant *Staphylococcus aureus* (MRSA) is associated with an increased risk of MRSA infection after liver transplantation. It is not known, however, whether new MRSA carriage postoperatively also increases the risk of MRSA infection after liver transplantation.

Methods. We retrospectively reviewed the data from 242 adult patients who underwent living donor liver transplantation (LDLT) including microbiological and medical records from admission to 3 months after LDLT. Uni and multivariate analyses were performed to identify independent risk factors for postoperative MRSA infection among preoperative noncarriers of MRSA.

Results. Postoperative MRSA infection occurred in 18 of 219 preoperative noncarriers of MRSA by median postoperative day 26. Operation time of at least 16 hours and postoperative colonization with MRSA independently predicted postoperative MRSA infection.

Conclusion. Postoperative surveillance cultures should be performed periodically after liver transplantation to identify high-risk candidates for postoperative MRSA infection, even among preoperative noncarriers of MRSA.

STAPHYLOCOCCUS AUREUS is a major cause of bacterial infection after liver transplantation.[1,2] Isolates of *S aureus* causing clinical nosocomial infection can be divided into two groups: methicillin-susceptible *S aureus* and methicillin-resistant *S aureus* (MRSA). MRSA infection frequently complicates the postoperative course after deceased donor liver transplantation (DDLT).[1,3–5] Among several centers, 91% (45 of 49 isolates) of all *S aureus* infections after DDLT were caused by MRSA.[4]

Preoperative MRSA carriage is associated with an increased risk for MRSA infection after DDLT.[1,3–5] In addition, postoperative MRSA colonization is prevalent in DDLT.[6] Positive MRSA cultures on both postoperative and preoperative surveillance is considered important because increased MRSA colonization in a patient during hospitalization increases the risk of MRSA infection.[7] In a prospective study,[7] the relative risk of developing an MRSA infection among patients with MRSA colonization was greater than among patients who were not colonized with *S aureus*. In this particular study, 12 of 394 patients had MRSA colonization during hospitalization, and 4 of the 12 later developed MRSA infection.

It is not known, however, whether new MRSA carriage postoperatively following liver transplantation also increases the risk of MRSA infection. Moreover, MRSA in cases of living donor liver transplantation (LDLT), in which operations are scheduled in a more selective manner, is not well described. The aim of the present study was to assess the details of postoperative MRSA infection among preoperative noncarriers of MRSA and to analyze whether new MRSA carriage postoperatively increased the risk of MRSA infection after LDLT using multivariate analysis.

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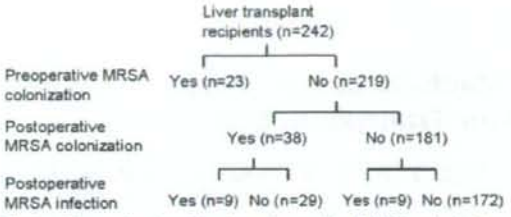

Fig 1. The patient profile of postoperative MRSA colonization and infection. MRSA, methicillin-resistant *S aureus*.

PATIENTS AND METHODS

Patients

We reviewed the 242 patients who underwent LDLT between 1996 and 2004, including 23 colonized with MRSA preoperatively, who were excluded from the study. Of the remaining patients, 119 were men and 100 were women of median age 50 years (range, 19 to 67). The indications included hepatitis C ($n = 62$), followed by primary biliary cirrhosis ($n = 48$) and hepatitis B ($n = 31$). The median Child-Pugh and model for end-stage liver disease (MELD) scores of those patients were 10 (range, 5 to 14) and 13 (range, −3 to 48), respectively. Our donor selection criteria[8] and surgical techniques for recipient and donor operations have been described elsewhere.[9]

Perioperative Management

Antimicrobial prophylaxis consisted of intravenous cefotaxim (1.0 g just before surgery, followed by 1.0 g every 6 hours intraoperatively and thereafter), ampicillin/sulbactam (1.0 g just before surgery, followed by 1.5 g every 12 hours intraoperatively and thereafter), and gentamicin, 60 mg every 12 hours after surgery) for 5 days. Fluconazole (200 mg every 24 hours) was administered intravenously for 7 days after surgery. All patients received the same immunosuppressive regimens using tacrolimus (Prograf, Fujisawa Pharmaceutical Corporation, Tokyo, Japan) and methylprednisolone (Solu-Medrol, Pfizer Inc, New York, NY, USA).[10]

Microbiological Data Collection

All patients were screened preoperatively for *S aureus* after admission for LDLT. Follow-up specimens were collected twice a week during the first month after LDLT and thereafter once a week during the hospital stay. Screened specimens consisted of swabs of the anterior nares, pharynx, sputum, urine, stool, swabs of wound or skin lesions, bile, and abdominal cavity discharge. A catheter or blood sample was also submitted when infection was suspected.

Specimens were plated onto mannitol-salt agar or sheep blood agar. *S aureus* was identified using standard microbiological methods. Methicillin resistance was determined using a disk diffusion test performed on Mueller-Hinton agar after incubation for 24 to 48 hours at 30°C. The strains with an oxacillin minimum inhibitory concentration value of at least 4 μg/mL were defined as MRSA colonization. Patients colonized with *S aureus* at any site and at any time during the hospital stay were considered carriers, and contact precautions were taken in cases with MRSA.

Definition of MRSA Infection

The medical and microbiological records of the patients were reviewed for the occurrence of MRSA infection in the 3 months following LDLT. Only the first MRSA infection was recorded for each patient.

Nosocomial infections were defined according to the reports from the Centers for Disease Control and Prevention in 1988 and in 1992, as described elsewhere.[11] Surgical site infection included superficial incisional, deep incisional, and organ/space infections that occurred within 30 days after surgery. Wound and intra-abdominal cavity infections that occurred more than 1 month after the operation were defined as a gastrointestinal system infection. When an organism isolated from blood culture was compatible with a related nosocomial infection at another site, the bloodstream infection was classified as a secondary bloodstream infection. When MRSA was isolated from culture samples in the presence of nosocomial infection including surgical site infection and other pathogenic organisms were absent, MRSA infection was diagnosed. An MRSA-positive culture sample without the presence of clinical symptoms was diagnosed as MRSA colonization.

Statistical Analysis

Background and clinical data collected for each patient included preoperative, surgical, and postoperative variables. Quantitative variables are presented as medians and ranges. Categorical variables are presented as absolute counts. Univariate analysis was used to identify associations between each of the variables and postoperative MRSA infection. Chi-square test or Fisher exact test was used to compare categorical data.

For multivariate analysis, only variables with a $P < .20$ in the univariate analysis were entered into a logistic regression model by the backward-elimination procedure. The final regression model included covariates associated with a likelihood ratio of $P < .1$. The results of the logistic regression were reported as odds ratios with 95% confidence intervals. A P value of less than .05 was considered statistically significant. All statistical analyses were performed using the JMP5.1 software package (SAS institute Inc, Cary, NC, USA).

RESULTS

Postoperative MRSA Colonization and Infection (Fig 1)

Postoperative MRSA infection occurred in 18 patients among the preoperative noncarriers of MRSA: nine patients were new MRSA carriers postoperatively, and nine

Table 1. Postoperative MRSA Infection in 18 Patients

	Colonized with MRSA ($n = 9$)	Noncarriers with MRSA ($n = 9$)	Total ($n = 18$)
Onset of MRSA infection (postoperative day)	16 (7–54)	40 (9–64)	26 (7–64)
Duration between colonization and infection	13 (2–21)	0	1 (0–21)
During hospitalization infection	9	8	17
SSI	6	3	9
Deep incisonal SSI	6	0	6
Organ/space SSI	0	3	3
Gastrointestinal system infection	2*	4	6
Intra-abdominal infection	2	4	6
Pneumonia	0	1	1
Lower respiratory infection	1	0	1
Primary BSI	0	1	1
Laboratory-confirmed BSI	0	1	1

*One patient had secondary surgical site infection. SSI, surgical site infection; BSI, bloodstream infection.

Table 2. Association Between Postoperative MRSA Infection and Preoperative, Surgical, and Postoperative Variables

Variables	MRSA Infection (−) (*n* = 201)	MRSA Infection (+) (*n* = 18)	*P* Value
Preoperative variables			
Age (y) ≥50	51 (19–67)	48 (24–62)	
	111	8	.46
Gender (male/female)	106/95	13/5	.14
Underlying liver disease			
Hepatitis C	55	7	
Primary biliary cirrhosis	46	2	
Hepatitis B	30	1	
Fulminant hepatitis	19	3	
Biliary atresia	10	1	
Autoimmune hepatitis	8	1	
Primary sclerosing cholangitis	8	1	
Metabolic disease	9	0	
Cryptogenic cirrhosis	6	0	
Alcoholic cirrhosis	2	2	
Others	8	0	
Hepatocellular carcinoma	59	5	1.0
Child-Pugh score	10 (5–14)	11 (5–12)	
≥10	105	13	.14
MELD score	12.9 (−3.4–48.2)	14.6 (4.3–29.4)	
≥15	67	6	1.0
Ascites	95	12	.14
Use of diuretics	109	12	.34
Encephalopathy	32	4	.51
Preoperative apheresis	38	4	.76
PT-INR	1.61 (0.89–7.48)	1.60 (1.23–2.35)	
≥1.7	80	6	.80
Serum bilirubin (mg/dL)	4.1 (0.3–38.6)	7.3 (1.2–32.4)	
>3.0	134	13	.80
Serum albumin (g/dL)	2.9 (1.5–4.4)	2.8 (1.8–3.8)	
>2.8	71	9	.31
Serum creatinine (mg/dL)	0.71 (0.2–7.7)	0.62 (0.4–2.4)	
≥1.5	11	2	.29
Steroid pulse therapy	23	2	1.0
Use of antimicrobials	46	8	.08
Beta lactam	37	7	.06
Glycopeptide	2	0	1.0
Fluroquinolone	13	3	.13
Amynoglycoside	5	1	.41
Others	2	0	1.0
History of abdominal surgery	93	8	1.0
Diabetus mellitus	24	2	1.0
MSSA colonization	100	10	.81
Surgical variables			
Operation time (h)	14.9 (10.7–33.2)	16.3 (12.2–19.3)	
≥16	64	11	.02
Blood loss (mL)	5240 (830–53835)	4415 (2590–34800)	
≥5000	106	8	.62
Blood transfusion (mL)	6970 (900–42890)	6385 (4240–26240)	
≥8000	83	6	.62
GV/SLV ratio (%)	46 (25–88)	42 (36–66)	
≥40	160	15	1.0
Duct to duct biliary reconstruction	144	14	.78
Postoperative variables			
ICU stay (d)	5 (3–46)	5 (4–26)	
≥10	18	4	.09
Apheresis	23	6	.02
Reoperation	72	4	.31
Acute rejection	58	5	1.0

Table 2. (continued)

Variables	MRSA Infection (−) (n = 201)	MRSA Infection (+) (n = 18)	P Value
Cytomegalovirus infection	87	5	.22
Fungal infection	6	1	.46
Colonization with MRSA	29	9	.001

PT-INR, the international normalized ratio of prothrombin time; MSSA, methicillin-susuceptible *S aureus*; MRSA, methicillin-resistant *S aureus*; GV, graft volume; SLV, standard liver volume; ICU, intensive care unit.

patients were MRSA noncarriers until the onset of infection. During the study period, 29 patients were asymptomatic carriers of MRSA. Among the nine patients who were colonized with MRSA postoperatively and subsequently developed infection, the MRSA-colonized sites before the onset of infection were sputum in six, stool in six, nares in five, pharynx in five, urine in two, discharge from an abdominal drain in two, and ascites in one patient.

Details of Postoperative MRSA Infection (Table 1)

The median days to onset of MRSA infection in all the patients with infection, in patients colonized with MRSA before infection, and in patients colonized concurrently with infection were postoperative days 26, 16, and 40, respectively. Among patients who were colonized with MRSA before infection, the median duration between the onset of colonization and infection was 13 days. During the study period, median length of hospital stay after LDLT was 50 (range, 6 to 90) days for patients without MRSA infection and 68 (range, 46 to 90) days for those with MRSA infection. MRSA infection occurred during hospitalization in 17 patients and after discharge in one patient.

Surgical site infection was detected in nine patients. One patient with gastrointestinal system infection had a secondary bloodstream infection. We treated MRSA infection with intravenous vancomycin in 12 patients, reoperation and intravenous vancomycin in two, reoperation alone in two, lavage of the intra-abdominal cavity through the surgical drain in one, and debridement of the wound in one. None of the 18 patients with MRSA infection died during the 3 months after LDLT.

Risk Factors for Postoperative MRSA Infection (Tables 2, 3)

Postoperative MRSA infection was significantly associated with operation time (≥16 hours; P = .02), postoperative apheresis (P = .02), and postoperative colonization with MRSA (P = .001, Table 2). In the multivariate analyses (Table 3), 10 risk factors with P values of less than .20 were entered into a logistic regression model using the backward-elimination procedure. In the final model, operation time (≥16 hours; odds ratio, 3.27) and postoperative colonization with MRSA (odds ratio, 7.13) independently predicted postoperative MRSA infection.

Table 3. Multivariate Analysis of Risk Factors for MRSA Infection After LDLT

Variable	Odds Ratio (95% Confidence Interval)	P Value
Preoperative use of beta lactam	3.03 (0.95–9.37)	.06
Operation time (h) ≥16	3.27 (1.15–9.89)	.03
Colonization with MRSA	7.13 (2.43–21.65)	.0004

MRSA, methicillin-resistant *S aureus*.

DISCUSSION

We have shown the impact of postoperative colonization with MRSA on subsequent MRSA infection after LDLT. Among patients with MRSA infection, 9 of 18 (50%) in the present study were colonized with MRSA before the onset of infection. MRSA infection occurred soon after the operation in patients who were new MRSA carriers postoperatively. Of 18 patients with MRSA infection, 10 developed the infection within 1 month after LDLT, among whom seven were colonized with MRSA before the onset of infection. In addition, patients who were colonized with MRSA developed MRSA infection soon after colonization with MRSA. Of nine patients with MRSA colonization and subsequent infection, all developed infections within 3 weeks after colonization with MRSA.

Postoperative surveillance cultures should be performed at multiple sites, including the nares, after LDLT. Although the anterior nares is the most frequent carriage site for *S aureus*,[12] other extranasal sites such as skin, perineum, pharynx, gastrointestinal tract, vagina, and axillae can also harbor the organism.[5,12] Among nine patients who were colonized with MRSA postoperatively and subsequently developed infection, nasal colonization was detected in 5 (56%). If surveillance culture is performed for only the nares as reported in previous studies in DDLT, new postoperative carriers of MRSA at sites other than the nares[1,6] might be overlooked, thereby delaying the administration of appropriate antimicrobials such as vancomycin in patients suspected of MRSA infection.

The results of the present study indicated that postoperative MRSA colonization and prolonged operative time independently increased the risk of postoperative MRSA infection. MRSA infection is well described in previous studies of DDLT.[1,3–5] Most studies have reported that preoperative MRSA carriage increased the risk of MRSA infection, but these studies[2,3,5] were not focused on the impact of new postoperative MRSA carriage on subsequent infection. Of 38 patients, 9 (24%) who were colonized with MRSA subsequently developed MRSA infection in the present study. This rate is comparable to that of the previous reports [around 30%].[7,13] In one recent retrospec-

tive study, 60 of 209 (29%) patients developed subsequent MRSA infection in the 18-month period after the initial MRSA-positive culture.[13] Postoperative surveillance culture should be performed periodically after LDLT to identify new MRSA carriers who are high-risk candidates for subsequent MRSA infection.

Prolonged operative time increased the risk of MRSA infection in the present study. Prolonged surgical duration indicates technically more difficult surgical procedures in which the risk of complication is increased.[14] George et al[15] used multivariate analysis to demonstrate that prolonged surgery duration increased the risk of bacterial infection among in liver transplant recipients. In contrast, Singh et al[1] reported that there was no such association.

Intense antimicrobial use, measured by the administration of preoperative antimicrobials, during the month before LDLT did not correlate with postoperative MRSA infection among preoperative noncarriers of MRSA in the present study. Although there is little doubt that widespread use of antimicrobials provides multidrug-resistant strains of MRSA with a selective survival advantage,[16] the relationship between MRSA and antimicrobials seemed more complex in the current series. Some studies using multivariate analysis have failed to show such an association.[17] In other studies, exposure to specific antimicrobials, such as third-generation cephalosporins, amoxicillin with clavulanic acid, quinolones, and broad-spectrum antibiotics, increased the risk of MRSA infection or colonization.[18] Crowcroft et al[19] found no association between total antimicrobial use and MRSA colonization or infection, suggesting that the problem was inappropriate rather than excessive use of antimicrobials. This discrepancy is probably due to the fact that in the present study, all patients received multiple antimicrobials, resulting in broad coverage as perioperative prophylaxis, per protocol, and it is difficult to detect the effect of a specific antimicrobial.

One limitation to the present study is that we could not differentiate specific MRSA strains. Pulsed-field gel electrophoresis analysis was not accessible. Therefore, we could not analyze the impact of MRSA transmission, such as patient-to-patient transmission by transient carriage on the hands of the medical staff. Similarly, it was not possible to determine whether infection was due to the same strain as that of the colonization or to a newly acquired strain when the infection occurred. Chang et al[4] analyzed isolates from infected sites and those from the anterior nares in seven patients with MRSA infection, reporting detection of the same isolates. Such a detailed analysis might yield further information to elucidate the relationship between new postoperative MRSA carriage and subsequent infection following LDLT.

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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.

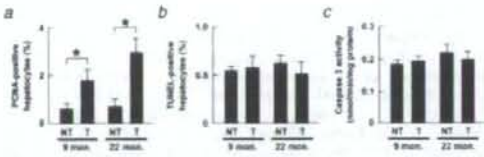

FIGURE 1 – Increase in hepatocyte proliferative activity. (*a*) The number of PCNA-positive hepatocytes. Two-thousand hepatocyte nuclei were examined for each mouse, and the number stained with anti-PCNA antibody was counted. Results are expressed as the mean ± standard deviation ($n = 8$). *, $p < 0.05$ 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

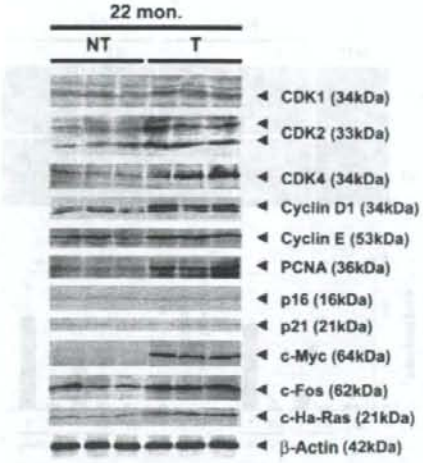

FIGURE 2 – Immunoblot analysis of cell-cycle regulators and oncogene products. Whole liver lysate (200 μg) was loaded in each lane. The band of β-actin was used as the loading control. The apparent molecular weight is indicated in parentheses. 22 mon, 22-month-old mice; NT, nontransgenic mice; T, transgenic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

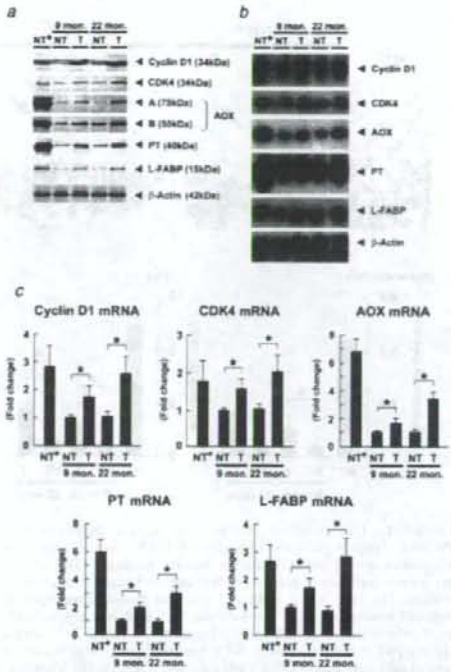

FIGURE 3 – Analysis of PPARα-regulated proteins. (*a*) Immunoblot analysis of cell-cycle regulators and fatty acid-metabolizing enzymes and proteins. Since no significant individual differences in the same mouse group were found in the preliminary experiments, 10 mg of liver pieces prepared from each 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

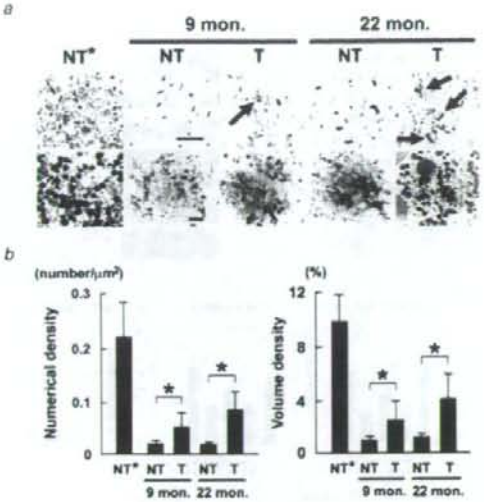

FIGURE 4 – Cytochemical staining for hepatic peroxisomes. (*a*) Light and electron photomicrographs of DAB-stained liver tissues. Peroxisomes are detected as darkly stained particles. The arrows in upper panels indicate hepatocytes showing profound peroxisome proliferation. The bars in the light and 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.

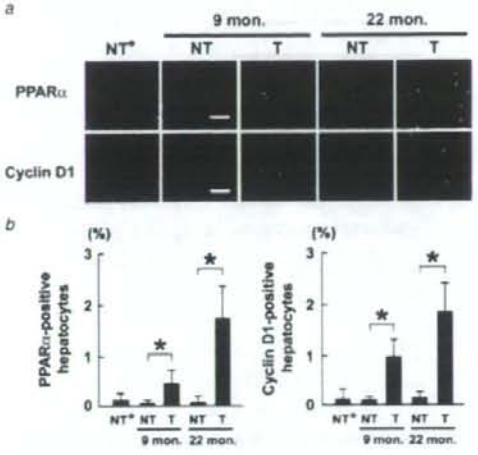

FIGURE 5 – Immunofluorescence staining for PPARα and cyclin D1. (*a*) Immunofluorescence staining using antibodies against PPARα and cyclin D1. The bars in the photomicrographs of 9-month-old nontransgenic mice indicate 50 μm. 9 mon, 9-month-old mice; 22 mon, 22-month-old mice; NT, nontransgenic mice; 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

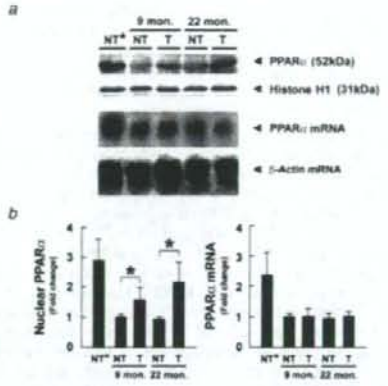

FIGURE 6 – Analysis of PPARα. (*a*) (Upper panels) Immunoblot analysis of nuclear PPARα. Since few individual differences in the same mouse group were found in the preliminary experiments, 30 mg of liver pieces from each mouse ($n = 8$/group) was mixed and homogenized to prepare the nuclear fraction. 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.

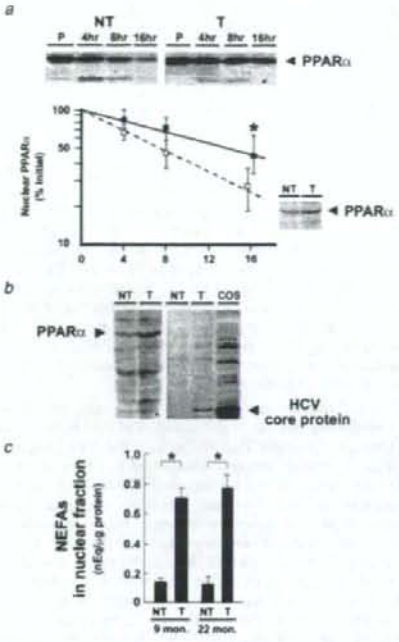

FIGURE 7 – Analyses of PPARα stability, interaction between PPARα with the core protein in hepatocyte nuclei, and nuclear contents of NEFAs. (*a*) Pulse-label and pulse-chase experiments for nuclear PPARα using isolated mouse hepatocytes. (Upper panels) Labeled PPARα bands on X-ray film. Pulse-label 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.

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 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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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 cytomegaloviral 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,[1,4] 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.

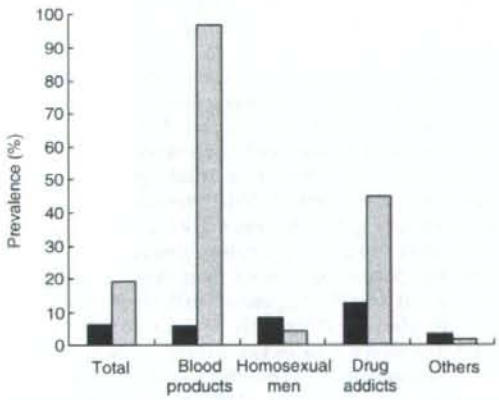

Figure 1 Prevalence rates of persistent hepatitis B virus and hepatitis C virus infections in the HIV-positive population sorted by the HIV risk group. (■), HBsAg, hepatitis B surface antigen; (□), anti-HCV, antibody to hepatitis C virus. *Prevalence rates of anti-HCV are obtained from Koike K *et al.*[16]

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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