

peripheral blood B lymphocytes in the glomerular vascular endothelium and mesangium.<sup>21</sup>

Histopathological features of HCV-associated nephritis are similar to those of typical membranoproliferative glomerulonephritis type I, but the former sometimes show cryoglobulin deposition.<sup>22</sup> In essential cryoglobulinemia and nephrotic syndrome with a rheumatoid factor, HCV-associated nephritis is suspected; therefore, the presence or absence of HCV infection should be determined.

IFN therapy has been reported to be efficacious for HCV-associated nephritis.<sup>23,24</sup> Johnson et al.<sup>23</sup> reported that the administration of IFN to 14 patients with HCV-associated nephritis improved proteinuria, but they observed a relapse of nephritis in association with HCV reexpression after the end of IFN therapy in many patients.<sup>23</sup> Recently, IFN and ribavirin combination therapy, which shows a low relapse rate, has been tested.<sup>25,26</sup> Sabry et al.<sup>26</sup> reported on the effectiveness of IFN and ribavirin combination therapy administered to 16 patients with HCV-associated nephritis for whom IFN monotherapy had proved ineffective; a follow-up study is awaited. Steroid and cyclophosphamide have been used for immunosuppression therapy, but satisfactory results using an immunosuppressant alone have not yet been obtained.<sup>27</sup> Because patients with HCV-associated nephritis have been reported to have a poor prognosis,<sup>11</sup> early establishment of a therapeutic procedure based mainly on IFN and ribavirin combination therapy is desirable.

### Myocardial impairments

Myocardial impairments for which a causal relationship with HCV infection has been suspected to date include dilated cardiomyopathy, hypertrophic cardiomyopathy, arrhythmogenic right ventricular dysplasia cardiomyopathy, and chronic myocarditis.<sup>28-30</sup>

A study by Matsumori<sup>28</sup> observed positivity for serum anti-HCV antibody in 6.3% (42/663) of patients with hypertrophic cardiomyopathy and in 10.6% (74/697) of patients with dilated cardiomyopathy. These positivity rates were higher than the rate (2.4%) observed among age-matched Japanese blood donors.<sup>28</sup> Positive- and negative-strand HCV RNAs were detected in cardiac muscle samples of these patients, indicating potential intramyocardial HCV multiplication.<sup>29,30</sup> HCV RNA has also been detected in cardiac muscle samples of patients with arrhythmogenic right ventricular dysplasia cardiomyopathy and chronic myocarditis, indicating that HCV potentially plays an important role in the onset of myocardial impairments.<sup>32</sup>

With regard to the cause of myocardial impairments associated with HCV, the involvement of host immuno-

logic responses to HCV, particularly that of the human major histocompatibility (MHC) class II antigen, has been suggested.<sup>30</sup> There are many patients with normal liver enzyme levels among hepatitis C patients with a concomitant myocardial impairment.<sup>28</sup> No established therapy is currently available, but the use of IFN-based antiviral therapy should be considered.

### Porphyria cutanea tarda

Porphyria cutanea tarda is an acquired condition in which patients exhibit solar photosensitivity and hepatic damage owing to decreased activity of uroporphyrinogen decarboxylase in the liver.<sup>33</sup> The involvement of alcohol, excess iron, and medications for hepatic impairments in porphyria cutanea tarda was previously considered. However, because HCV infection has been observed in 60%–100% of cases of porphyria cutanea tarda, the involvement of HCV infection in the pathogenesis of porphyria cutanea tarda is suspected.<sup>34</sup>

The mechanism underlying the pathogenesis of porphyria cutanea tarda associated with HCV infection has not yet been clarified. It is assumed, however, that porphyria cutanea tarda results from reduced uroporphyrinogen decarboxylase activity associated with excessive deposition of iron in the liver as a result of HCV infection.<sup>34</sup>

The efficacy of IFN therapy for the treatment of porphyria cutanea tarda has been demonstrated, in addition to avoidance of sun exposure, abstention from alcoholic beverages, and blood letting. Okano et al.<sup>38</sup> reported that IFN therapy given to porphyria cutanea tarda patients with HCV infection led to transaminase normalization, HCV RNA disappearance, and normalization of porphyrin and ferritin levels with improvement of clinical symptoms, including vesicle formation and hypertrichosis. These results demonstrate the efficacy of IFN therapy for porphyria cutanea tarda.

### Sjögren's syndrome

Sjögren's syndrome is an aggregate of symptoms characterized by insufficient tear production by the lacrimal glands and insufficient saliva production by the salivary glands because of exocrine lymphocyte infiltration, causing dryness of the eyes and mouth. Patients with Sjögren's syndrome are classified roughly into two groups, those exhibiting only dryness and those exhibiting both dryness and connective tissue disease symptoms such as arthralgia.<sup>39</sup>

An association of Sjögren's syndrome with viral infection has been reported for some time, and 0%–45% of Sjögren's syndrome patients test positive for

anti-HCV antibody.<sup>40</sup> Differences in the anti-HCV antibody positivity rate are attributed to regional differences in the HCV infection rate. Koike et al.<sup>41</sup> verified that transgenic mice with the 1b HCV envelope genotype developed sialadenitis resembling Sjögren's syndrome. Takamatsu et al.<sup>42</sup> detected HCV RNA in salivary gland tissue from anti-HCV antibody-positive patients with Sjögren's syndrome by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Arreita et al.<sup>43</sup> performed *in situ* hybridization of 19 salivary gland tissue samples obtained from eight anti-HCV antibody-positive patients and 11 anti-HCV antibody-negative patients with chronic sialoadenitis or Sjögren's syndrome, and detected HCV RNA in all salivary gland tissue samples from the anti-HCV antibody-positive patients. Moreover, the HCV-infected salivary gland epithelium showed viral multiplication.<sup>43</sup> These reports indicate that HCV plays some role in the development of sialoadenitis in Sjögren's syndrome, but it has not yet been determined whether HCV itself or immunologic responses to HCV infection induce sialoadenitis.

Current therapies for Sjögren's syndrome mainly aim to alleviate the symptoms. Artificial lacrimal fluid and artificial saliva are used to alleviate dryness, and a non-steroidal anti-inflammatory drug or a steroid is administered for treatment of fever and articular symptoms.<sup>39</sup> There are no reports regarding the efficacy of IFN therapy for HCV-associated sialadenitis,<sup>44</sup> and it is necessary to establish a treatment protocol in the future on the basis of accumulated case reports.

### Lichen planus

Lichen planus is an inflammatory disease associated with abnormal chronic dermal and intraoral keratinization of unknown etiology. The assumed causes of lichen planus include viral or bacterial infection, immunologic responses, circulatory disorder, allergy, mental stress, abnormal autonomic function, medication, and glucose metabolism disorder.<sup>45,46</sup>

There are many reports of a relationship between lichen planus and HCV infection, but the anti-HCV antibody positivity rate in lichen planus shows marked regional differences, ranging from 0% to 65%.<sup>47-53</sup> HCV reproduction in the skin and oral mucosal epithelium has been examined by *in situ* hybridization and RT-PCR analysis.<sup>54-56</sup> HCV-specific T cells are reported to be associated with the pathogenesis of lichen planus,<sup>57</sup> but its pathogenesis is not associated with HCV level, genotype, or pathologic severity.<sup>58,59</sup>

The intravenous administration of a glycyrrhizinate preparation has been demonstrated to have efficacy for treatment of HCV-associated lichen planus.<sup>60</sup> Antiviral

therapy based on IFN has also been attempted recently and has been reported to be effective,<sup>61</sup> but other investigators have reported that IFN is a lichen-planus-inducing factor<sup>62</sup> or that it can be aggravating factor.<sup>63</sup> No definite conclusion on the effectiveness of IFN against lichen planus is possible. Nagao et al.<sup>64</sup> reported that when intraoral lichen planus lesions in chronic hepatitis C patients administered IFN were observed over time, no macroscopic changes were observed in the lesions 1 year after the end of IFN administration, but that macroscopic and histological improvements were observed 3 or more years after the end of IFN administration. They also assumed that, since positive-strand HCV RNA was detected in the oral mucous membrane of some patients despite the demonstration of histological recovery from lichen planus following IFN therapy, host immunologic responses to HCV infection were related to the development of oral lichen planus.<sup>64</sup> The early establishment of a treatment procedure for lichen planus is desired, because lichen planus is also considered to be a precancerous condition.<sup>45,46</sup>

### Oral cancer

A relationship between HCV infection and oral cancer was first reported by Nagao et al.<sup>65</sup> They showed that the HCV infection rate was higher in oral cancer patients than in esophageal, gastric, or colorectal cancer patients.<sup>65</sup> The HCV infection rate has also been found to be higher in patients with cervical squamous cell carcinoma than in controls.<sup>66</sup> When HCV-RNA was examined in cancer tissues from 17 oral cancer patients by RT-PCR analysis, positive-strand HCV RNA was detected in all anti-HCV antibody-positive patients and negative-strand HCV RNA was detected in 71.4% of the anti-HCV antibody-positive patients.<sup>65</sup> These findings interestingly indicate the possibility of HCV multiplication in cancer tissue. No definite conclusion has been arrived at regarding the relationship between oral lichen planus and oral cancer. However, because lichen planus is considered precancerous, as mentioned above, oral examination is also important for patients with chronic hepatitis C.

### Diabetes mellitus

In 1994, Allison et al.<sup>67</sup> reported a relationship between HCV-associated cirrhosis and diabetes mellitus, because the rate of diabetes mellitus complication in patients with both cirrhosis and HCV infection was 50%, which is much higher than that (9%) in patients with cirrhosis but without HCV infection. A large-scale epidemiologic survey showed that the rate of non-insulin-dependent

diabetes mellitus occurring as a complication of chronic hepatic diseases associated with HCV infection was higher than that of other chronic hepatic diseases, and that anti-HCV antibody-positive patients aged 40 years or more had a 3.77-fold higher risk of becoming diabetic than anti-HCV antibody-negative patients.<sup>68</sup> In addition, it has been demonstrated that complication by diabetes mellitus is both a risk factor for hepatocellular carcinoma<sup>69</sup> and a prognostic factor in cirrhosis patients.<sup>70</sup> These reports suggest a correlation between HCV infection and type 2 diabetes. Increased insulin resistance and insulin secretory deficiency are considered to be highly involved in the pathogenesis of type 2 diabetes.<sup>71</sup> Petit et al.<sup>72</sup> reported that insulin resistance increases even in chronic hepatitis C patients with slight hepatic impairment and that the index of impairment (HOMA-IR) correlates with the severity of the liver tissue disorder. Tumor necrosis factor (TNF)- $\alpha$ , which closely correlates with hepatic inflammation and fibrillation in chronic hepatitis C,<sup>73</sup> is considered to enhance glucose uptake in peripheral tissue and to promote gluconeogenesis in the liver, leading to the induction of insulin resistance.<sup>74</sup> Shintani et al.<sup>75</sup> confirmed that in transgenic mice with the 1b HCV core genotype, tyrosyl phosphorylation of the insulin receptor substrate 1 in the insulin signal transduction pathway is disrupted and that this disruption causes gluconeogenesis inhibition by insulin in the liver, leading to the induction of marked insulin resistance. These transgenic mice exhibited a high anti-TNF- $\alpha$  antibody level, and insulin resistance was improved by the administration of an anti-TNF- $\alpha$  antibody. These results indicate a close relationship between HCV infection and the pathogenesis of diabetes mellitus. The relationship between HCV infection and hepatocyte fat modification has also attracted attention.<sup>76</sup> Moriya et al.<sup>77</sup> suggested the possible direct involvement of HCV core protein in hepatocyte fat modification, because they observed hepatocyte fat deposits in transgenic mice expressing the HCV core gene. In summary, the above-described findings strongly indicate that hepatitis C has the characteristics of a metabolic disease, and nutritional management is also considered important in the treatment of chronic hepatitis C.

### **Malignant lymphoma**

HCV reproduces in lymphocytes, and studies of a short-term HCV culture system using lymphocytes have been reported.<sup>78,79</sup> Infected lymphocytes may undergo malignant transformation, leading to the development of malignant lymphoma. HCV infection is considered to be associated with the development of malignant lymphoma, particularly in association with the pathogenesis

of non-Hodgkin B-cell lymphoma, and many reports suggest a relationship between HCV infection and malignant lymphoma.<sup>80-88</sup> It has been assumed that some cryoglobulinemia patients develop non-Hodgkin B-cell lymphoma in association with *myc* gene mutation.<sup>89</sup> The anti-HCV antibody positivity rates in patients with non-Hodgkin B-cell lymphoma range from 0% to 33%.<sup>80-88</sup> These differences in HCV antibody positivity rates are considered to relate to regional differences in the HCV infection rate. The HCV antibody prevalence tends to be higher in Japan and Italy but lower in Britain and Canada. Studies indicating a relationship between HCV infection and malignant lymphoma have been reported by Ferri et al.<sup>90</sup> and De Vita et al.<sup>91</sup> Ferri et al.<sup>90</sup> reported that 14 of 500 patients with chronic hepatitis C were complicated with non-Hodgkin B-cell lymphoma, and they detected HCV RNA in peripheral blood lymphocytes in all of these patients. De Vita et al.<sup>91</sup> detected positive-strand and negative-strand HCV RNAs in the parotid glands of patients with parotid non-Hodgkin B-cell lymphoma associated with HCV infection, and confirmed the presence of HCV in the parotid gland by *in situ* hybridization.<sup>91</sup> As shown by these findings, many patients with HCV-associated non-Hodgkin B-cell lymphoma show involvement of extranodal sites such as the liver and salivary glands.<sup>92</sup>

Treatment of HCV-associated malignant lymphoma is similar to that of HCV-associated non-Hodgkin B-cell lymphoma; however, recently, IFN monotherapy or IFN and ribavirin combination therapy have been reported to be effective.<sup>93-96</sup> Vallisa et al.<sup>96</sup> reported that administration of both pegylated IFN and ribavirin to 13 patients with HCV-associated non-Hodgkin B-cell lymphoma achieved a complete response in seven of these patients. It is interesting that IFN-based antiviral therapy has been demonstrated to be useful for malignant lymphoma associated with HCV-associated non-Hodgkin B-cell lymphoma in addition to conventional chemotherapy.

### **Autoimmune thyroid disease**

The relationship between HCV infection and thyroid disease has been analyzed in many studies,<sup>97,100</sup> and a causal relationship between HCV infection and autoimmune thyroid disease has been particularly suggested.<sup>98-100</sup> Antonelli et al.<sup>98</sup> assessed the incidence of thyroid dysfunction in 630 chronic hepatitis C patients without cirrhosis or hepatocellular carcinoma who had not been treated with IFN by recruiting 389 patients from an iodine-deficient area, 268 patients from an iodine-sufficient area, and 86 patients with chronic hepatitis B aged 40 years or older as study subjects. The chronic hepatitis C patients exhibited a higher thyroid-

stimulating hormone level and lower free thyroxine and triiodothyronine levels than the controls. In addition, the chronic hepatitis C patients exhibited hypothyroidism and tended to have antithyroglobulin antibodies and anti-thyroid peroxidase antibodies. These findings suggest a relationship between HCV infection and thyroid disorder.<sup>98</sup> A possible relationship between HCV infection and thyroid cancer has also attracted attention recently.<sup>99</sup> The mechanism underlying the pathogenesis of thyroid disease associated with HCV infection has not yet been elucidated, but a relationship with liver/kidney microsomal antibody type 1 has been suggested.<sup>99</sup> Many patients with thyroid disorder caused by HCV infection are asymptomatic, requiring no special treatment. Thyroid disorder is also known to be an adverse reaction to IFN- $\alpha$  therapy for chronic hepatitis C.<sup>99,101-104</sup> Thyroid hypofunction caused by the administration of IFN- $\alpha$  is usually transient, and the patient recovers spontaneously after the end of the therapy. Hence, discontinuation of IFN- $\alpha$  is not required in many cases.<sup>103</sup>

#### Idiopathic interstitial pneumonitis

Recently, viral infection has been suggested to be a cause of idiopathic interstitial pneumonitis.<sup>104</sup> With regard to the relationship between HCV infection and idiopathic interstitial pneumonitis, Ueda et al.<sup>106</sup> reported in 1992 that the anti-HCV antibody positivity rate in 66 patients with idiopathic interstitial pneumonitis determined by enzyme-linked immunosorbent assay was 28.8%, which was significantly higher than that in 9464 normal subjects serving as controls.<sup>106</sup> It has not yet been clarified how HCV infection is associated with the pathogenesis of idiopathic interstitial pneumonitis. Kubo et al.<sup>107</sup> suggested that activated T lymphocytes and eosinophils are related to the pathogenesis of idiopathic interstitial pneumonitis associated with HCV infection, because they observed increased activated T-lymphocyte and eosinophil counts in the bronchoalveolar fluid of 13 chronic hepatitis C patients, despite their having the same total cell counts as normal subjects. On the other hand, studies disagree regarding the relationship between HCV infection and idiopathic interstitial pneumonitis,<sup>108</sup> and in-depth studies of this issue are expected. Idiopathic interstitial pneumonitis is also reported to be an adverse reaction to IFN therapy in chronic hepatitis C patients.<sup>109</sup> Such patients often have a high pretreatment KL-6 level, and the potential of their developing idiopathic interstitial pneumonitis is suggested. Recovery from IFN therapy-induced idiopathic interstitial pneumonitis is achieved by the discontinuation of the therapy,<sup>109</sup> but steroid administration is required in some cases.

#### Rheumatoid arthritis

HCV-associated rheumatoid arthritis complicated by cryoglobulinemia or Sjögren's syndrome has been reported.<sup>10,39</sup> For further information, please refer to the cited references.

#### Mooren's ulcer

Mooren's ulcer is a progressive ulcer associated with congestion and pain around the cornea.<sup>110</sup> HCV infection has been suggested to contribute to the development of this disease.<sup>111-113</sup> The effectiveness of IFN therapy for HCV-associated Mooren's ulcer has been reported,<sup>110,111</sup> but the exacerbation of ocular pain following the discontinuation of IFN therapy has also been observed; hence, caution is required.<sup>111</sup> Systemic corticoid administration has also been reported to be effective.<sup>112</sup> However, other investigators reported a negative correlation between HCV infection and Mooren's ulcer.<sup>114-116</sup> It is hoped that further detailed studies will clarify this issue.

#### Conclusion

It is necessary to consider possible complications associated with extrahepatic diseases in the treatment of HCV-infected patients.

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## 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.

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**S**TAPHYLOCOCCUS AUREUS is a major cause of bacterial infection after liver transplantation.<sup>1,2</sup> 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).<sup>1,3-5</sup> Among several centers, 91% (45 of 49 isolates) of all *S aureus* infections after DDLT were caused by MRSA.<sup>4</sup>

Preoperative MRSA carriage is associated with an increased risk for MRSA infection after DDLT.<sup>1,3-5</sup> In addition, postoperative MRSA colonization is prevalent in DDLT.<sup>6</sup> 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.<sup>7</sup> In a prospective study,<sup>7</sup> 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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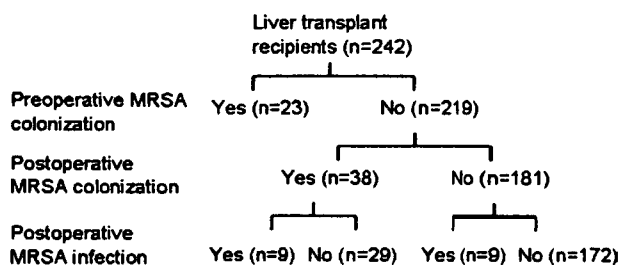


Fig 1. The patient profile of postoperative MRSA colonization and infection. MRSA, methicillin-resistant *S aureus*.

## 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<sup>8</sup> and surgical techniques for recipient and donor operations have been described elsewhere.<sup>9</sup>

### 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).<sup>10</sup>

### 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.<sup>11</sup> 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 incisional 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
<b>Preoperative variables</b>			
Age (y) $\geq 50$	51 (19-67)	48 (24-62)	
	111	8	.46
Gender (male/female)	106/95	13/5	.14
<b>Underlying liver disease</b>			
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)	
$\geq 10$	105	13	.14
MELD score	12.9 (-3.4-48.2)	14.6 (4.3-29.4)	
$\geq 15$	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)	
$\geq 1.7$	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)	
$\geq 1.5$	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
Diabetes mellitus	24	2	1.0
MSSA colonization	100	10	.81
<b>Surgical variables</b>			
Operation time (h)	14.9 (10.7-33.2)	16.3 (12.2-19.3)	
$\geq 16$	64	11	.02
Blood loss (mL)	5240 (830-53835)	4415 (2590-34800)	
$\geq 5000$	106	8	.62
Blood transfusion (mL)	6970 (900-42890)	6385 (4240-26240)	
$\geq 8000$	83	6	.62
GV/SLV ratio (%)	46 (25-88)	42 (36-66)	
$\geq 40$	160	15	1.0
Duct to duct biliary reconstruction	144	14	.78
<b>Postoperative variables</b>			
ICU stay (d)	5 (3-46)	5 (4-26)	
$\geq 10$	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-susceptible *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 ( $\geq 16$  hours;  $P = .02$ ), postoperative apheresis ( $P = .02$ ), and postoperative colonization with MRSA ( $P = .001$ , Table 2). In the multivariate analyses

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) $\geq 16$	3.27 (1.15–9.89)	.03
Colonization with MRSA	7.13 (2.43–21.65)	.0004

MRSA, methicillin-resistant *S aureus*.

(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 ( $\geq 16$  hours; odds ratio, 3.27) and postoperative colonization with MRSA (odds ratio, 7.13) independently predicted postoperative MRSA infection.

#### 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*,<sup>12</sup> other extranasal sites such as skin, perineum, pharynx, gastrointestinal tract, vagina, and axillae can also harbor the organism.<sup>5,12</sup> 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<sup>1,6</sup> 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.<sup>1,3–5</sup> Most studies have reported that preoperative MRSA carriage increased the risk of MRSA infection, but these studies<sup>2,3,5</sup> 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%].<sup>7,13</sup> 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.<sup>13</sup> 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.<sup>14</sup> George et al<sup>15</sup> 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<sup>1</sup> 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,<sup>16</sup> 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.<sup>17</sup> 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.<sup>18</sup> Crowcroft et al<sup>19</sup> 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<sup>4</sup> 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 $\alpha$ 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  $\alpha$  (PPAR $\alpha$ ). In these transgenic mice, spontaneous and persistent PPAR $\alpha$  activation occurred heterogeneously, which was different from that observed in mice treated with clofibrate, a potent peroxisome proliferator. We further demonstrated that stabilization of PPAR $\alpha$  through a possible interaction with HCV core protein and an increase in nonesterified fatty acids, which may serve as endogenous PPAR $\alpha$  ligands, in hepatocyte nuclei contributed to the core protein-specific PPAR $\alpha$  activation. In conclusion, these results offer the first suggestion that HCV core protein induces spontaneous, persistent, age-dependent and heterogeneous activation of PPAR $\alpha$  in transgenic mice, which may contribute to the age-dependent and multicentric hepatocarcinogenesis mediated by the core protein.

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**Key words:** cell-cycle regulator; peroxisome; nuclear stabilization; heterogeneous PPAR $\alpha$  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).<sup>1,2</sup> The prevalence of HCC because of chronic HCV infection has increased over the past two decades,<sup>3,4</sup> 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.<sup>5</sup> For example, HCV core protein transforms rat embryo fibroblasts to a tumorigenic phenotype in cooperation with the *H-ras* oncogene,<sup>6</sup> suppresses *c-myc*-related apoptosis<sup>7</sup> and transcription of the *p53* gene,<sup>8</sup> interacts with a variety of proteins, including helicase, lymphotoxin- $\beta$  receptor, or dead box protein, and modulates their functions.<sup>9</sup> 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.<sup>10</sup> These mice exhibited multicentric hepatic adenomas, and developed HCCs in an age-dependent manner.<sup>11</sup> The livers of these mice were almost free of inflammation, necrosis and fibrosis,<sup>10,11</sup> suggesting that the core protein itself has a hepatocarcinogenic potential *in vivo*. However, the molecular mechanism of the de-

velopment 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,<sup>12</sup> 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.<sup>13,14</sup> For example, in patients with chronic HCV infection, high hepatocyte proliferative activity relative to apoptosis may reliably predict a new development of HCC.<sup>15</sup> 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.<sup>10</sup> Because HCC developed preferentially in male transgenic mice,<sup>11</sup> 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, Millville, 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<sub>2</sub> and 0.25 mM phenylmethylsulfonyl fluoride (PMSF)]. The homogenate was filtered through gauze and centrifuged at 4,500g 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 23,000g for 1 hr at 4°C. The pellet obtained after ultracentrifugation was resuspended in 250  $\mu$ L of nuclei buffer and used as the nuclear fraction. Preparation of nuclear fraction from isolated hepatocytes was performed as described elsewhere.<sup>16</sup>

### Immunoblot analysis

Protein concentration was measured colorimetrically by a BCA<sup>TM</sup> Protein Assay kit (Pierce, Rockford, IL). For analysis of fatty acid-metabolizing enzymes and protein, whole liver lysate (10–20  $\mu$ g protein) was subjected to 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.<sup>17</sup> For analysis of other proteins, hepatocyte nuclear fraction (100  $\mu$ g protein) or whole liver lysate (200–300  $\mu$ g 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.<sup>17</sup> For immunoblot analysis of peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ), a polyclonal anti-mouse antibody<sup>18</sup> 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.).<sup>19</sup> Equal loading of the protein obtained from whole liver lysate and nuclear fraction was confirmed by reprob- ing the membranes with an antibody against  $\beta$ -actin and histone H1, respectively. The band intensity of nuclear PPAR $\alpha$  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<sup>TM</sup> (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 $\times$  SSC buffer (3 M NaCl and 300 mM sodium citrate, pH 7.0) overnight. The membranes were hybridized with <sup>32</sup>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.<sup>17–19</sup> Northern blot of  $\beta$ -actin was used as the internal control. The blot intensity was quantified, normalized to that of  $\beta$ -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.<sup>20</sup> 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  $\mu$ Ci/mL of [<sup>35</sup>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 [<sup>35</sup>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  $\mu$ g/mL aprotinin]. The lysate was incubated for 3 hr at 4°C with purified anti-PPAR $\alpha$  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 PPAR $\alpha$ .

### Affinity chromatography for PPAR $\alpha$ 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 Power Sonic Model 50 (Yamato, Tokyo, Japan), and then centrifuged at 100,000g for 20 min. The supernatant was applied to an immobilized anti-PPAR $\alpha$  IgG column (1.0  $\times$  4.0 cm<sup>2</sup>), prepared with the Affigel HZ Immunoaffinity kit<sup>R</sup> (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 PPAR $\alpha$  and the HCV core protein, respectively. The core protein expressed in COS cells was used as a positive marker.<sup>21</sup> 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.<sup>22</sup> 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- $\mu$ m sections with a Lancer<sup>R</sup> 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<sub>2</sub>O<sub>2</sub>) and postfixed with 1% OsO<sub>4</sub> 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- $\mu$ m 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 $\times$ . 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/ $\mu\text{m}^2$ ) =  $N_p/(A_T - A_{\text{empty}})$ , and volume density (%) =  $A_{TP}/(A_T - A_{\text{empty}}) \times 100$ , where  $N_p$  is the peroxisome number in the test area,  $A_T$  is the test area,  $A_{\text{empty}}$  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<sup>TM</sup> (Sakura Finetek, Torrance, CA) and frozen. Frozen liver 5- $\mu\text{m}$  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)<sup>19</sup> and PPAR $\alpha$  (1:100 dilution),<sup>18</sup> and with mouse monoclonal antibody against proliferating cell nuclear antigen (PCNA) (1:100 dilution).<sup>19</sup> 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, PPAR $\alpha$  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- $\mu\text{m}$  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.<sup>23</sup> For analysis of the nuclear contents of nonesterified fatty acids (NEFAs), ~150  $\mu\text{L}$  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.*<sup>24</sup> and the nuclear content of NEFAs was measured with a NEFA C-test kit<sup>TM</sup> (Wako).

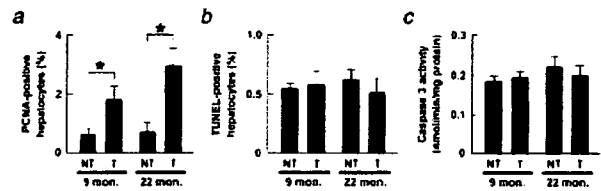
#### Statistical analysis

Statistical analysis was performed by means of Student's *t*-test. The results are expressed as the mean  $\pm$  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. 1a). The increase was more significant in the



**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  $\pm$  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  $\pm$  standard deviation ( $n = 8$ ). (c) Caspase 3 activity. Results are expressed as the mean  $\pm$  standard deviation ( $n = 8$ ).

22-month-old transgenic mice (Fig. 1a). The numbers of PCNA-positive hepatocytes in the 22-month-old transgenic mice corresponded with those in HCV polyprotein-expressing transgenic mice with HCC.<sup>25</sup> 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. 1b and 1c). 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.

#### 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 3a. 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. 3b and 3c). 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 PPAR $\alpha$ 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 PPAR $\alpha$ .<sup>19,26–30</sup> To investigate whether PPAR $\alpha$  is activated in the livers of transgenic mice, the expression of representative PPAR $\alpha$  target genes,<sup>30</sup> acyl-CoA oxidase (AOX), peroxisomal thiolase (PT) and liver-type fatty acid-binding protein (L-FABP), was examined. As demonstrated in Figure 3a, 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 PPAR $\alpha$  targets was based on the increase in the transcriptional activity (Figs. 3b and 3c). The increase in the

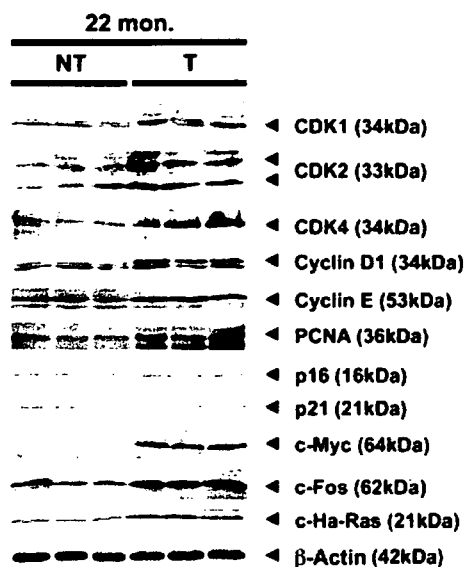


FIGURE 2 – Immunoblot analysis of cell-cycle regulators and oncogene products. Whole liver lysate (200  $\mu$ g) was loaded in each lane. The band of  $\beta$ -actin was used as the loading control. The apparent molecular weight is indicated in parentheses. 22 mon, 22-month-old mice; NT, nontransgenic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

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

#### Histological evaluation of PPAR $\alpha$ activation

An increase in the numbers of peroxisomes is associated with PPAR $\alpha$  activation.<sup>18</sup> 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. 4a). Such hepatocytes were also found in the 22-month-old transgenic mouse livers (Fig. 4a). In contrast, almost all of the hepatocytes in the clofibrate-treated mice showed significant peroxisome proliferation (Fig. 4a). 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. 4b). 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. 4b). The finding that only some hepatocytes in the transgenic mice presented a marked peroxisome proliferation (Fig. 4a) is noteworthy, since it seems to correlate with the finding that intense expression of the core protein was observed only in particular hepatocytes.<sup>10</sup> These histological analyses reveal that spontaneous, continuous and age-dependent peroxisome proliferation and PPAR $\alpha$  activation occur heterogeneously in the transgenic mouse

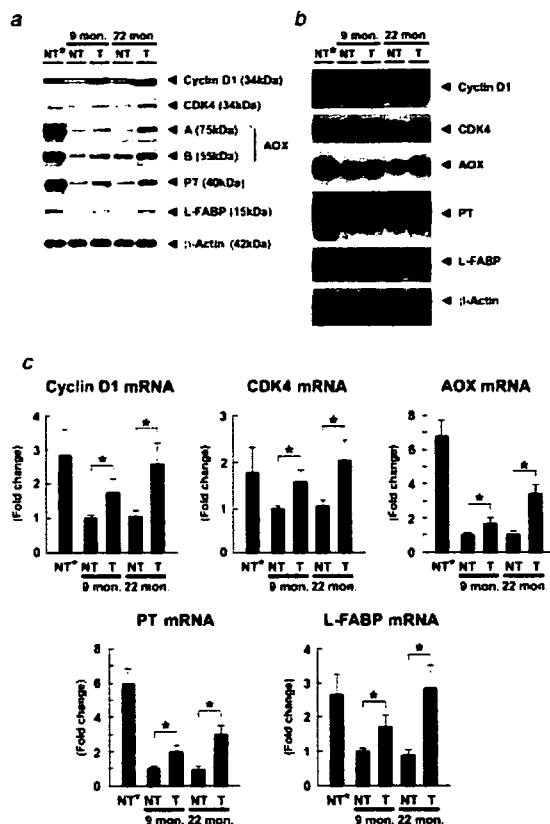


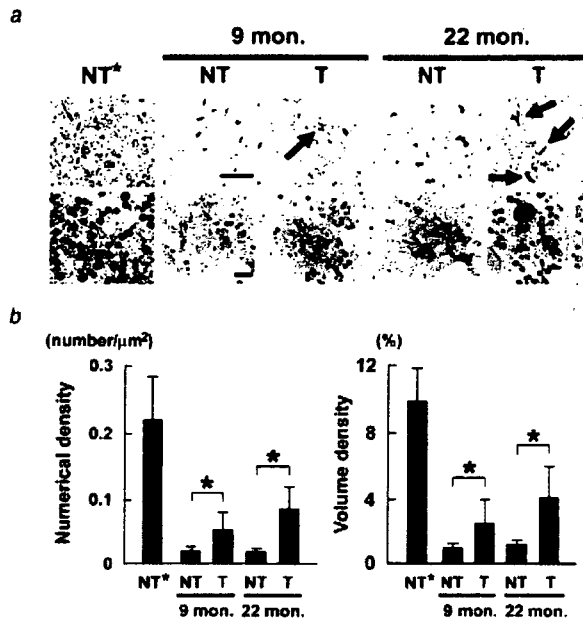
FIGURE 3 – Analysis of PPAR $\alpha$ -regulated proteins. (a) Immunoblot analysis of cell-cycle regulators and fatty acid-metabolizing enzymes and proteins. Since no significant individual differences in the same mouse group were found in the preliminary experiments, 10 mg of liver pieces prepared from each mouse ( $n = 8$ /group) was mixed and homogenized. Whole liver lysate (200  $\mu$ g for cyclin D1 and CDK4, and 20  $\mu$ g for others) was loaded in each lane. The band of  $\beta$ -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  $\mu$ g) was separated on a denaturing gel, transferred to membranes and hybridized with the indicated <sup>32</sup>P-labeled cDNA probes. The blot of  $\beta$ -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  $\beta$ -actin, and subsequently normalized to that of 9-month-old nontransgenic mice. Results were obtained from 4 independent experiments and expressed as the mean  $\pm$  standard deviation. Abbreviations are identical with those in (b). \*,  $p < 0.05$  between the transgenic mice and the nontransgenic mice.

livers, which is different from the response observed in the mice receiving clofibrate treatment.

#### Appearance of PPAR $\alpha$ - and cyclin D1-positive hepatocytes

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



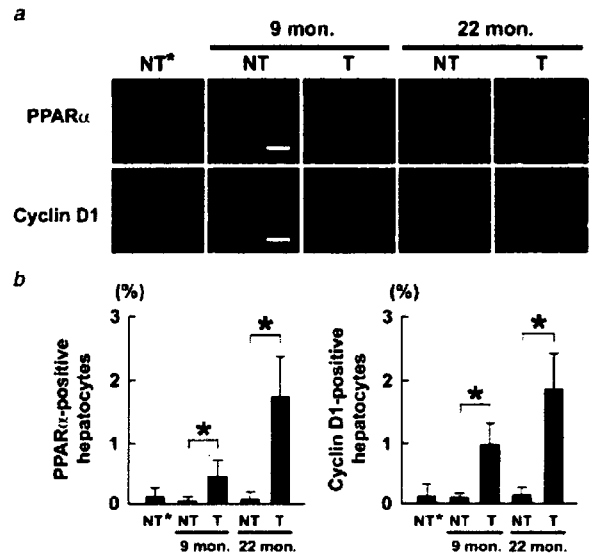


**FIGURE 4** – Cytochemical staining for hepatic peroxisomes. (a) Light and electron photomicrographs of DAB-stained liver tissues. Peroxisomes are detected as darkly stained particles. The arrows in upper panels indicate hepatocytes showing profound peroxisome proliferation. The bars in the light and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

by anti-PPAR $\alpha$  antibody were detected only in the transgenic mice (Fig. 5a). Similar to the case of PPAR $\alpha$ , the hepatocytes having nuclei stained intensively by anti-cyclin D1 antibody were found only in the transgenic mice (Fig. 5a). A few hepatocytes stained by anti-CDK4 antibody were also observed only in the transgenic mice (data not shown). The frequency of appearance of PPAR $\alpha$ -, or cyclin D1-positive hepatocytes was increased with age (Figs. 5a and 5b). 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 PPAR $\alpha$  activation in the transgenic mice.

#### Changes in PPAR $\alpha$ levels

Since the expression of PPAR $\alpha$  is known to be enhanced by its activation,<sup>18,30</sup> the quantitative change in PPAR $\alpha$  was evaluated. The nuclear PPAR $\alpha$  level in the transgenic mice was increased age-dependently, as expected (Figs. 6a, upper panel and 6b), but the PPAR $\alpha$  level in the whole liver lysate remained unchanged (data not shown). The increase in nuclear PPAR $\alpha$  in the transgenic mice was smaller than that in the clofibrate-treated wild-type mice (Figs. 6a, upper panel and 6b). Northern blot analysis revealed a higher PPAR $\alpha$  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. 6a, lower panel and 6b). These results indicate that the increase in



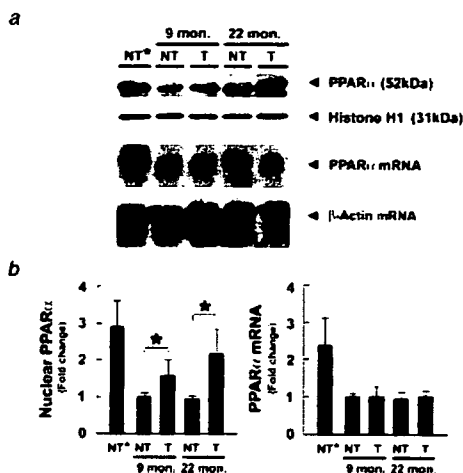
**FIGURE 5** – Immunofluorescence staining for PPAR $\alpha$  and cyclin D1. (a) Immunofluorescence staining using antibodies against PPAR $\alpha$  and cyclin D1. The bars in the photomicrographs of 9-month-old nontransgenic mice indicate 50 μm. 9 mon, 9-month-old mice; 22 mon, 22-month-old mice; NT, nontransgenic mice; T, transgenic mice; NT\*, nontransgenic mice treated with a control diet containing 0.5% clofibrate for 2 weeks. (b) The number of PPAR $\alpha$ -, or cyclin D1-positive hepatocytes. Two-thousand hepatocyte nuclei were examined for each mouse, and the number of nuclei intensively stained with anti-PPAR $\alpha$  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.

nuclear PPAR $\alpha$  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 PPAR $\alpha$ through a possible interaction with HCV core protein in hepatocyte nuclei

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

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



**FIGURE 6** – Analysis of PPAR $\alpha$ . (a) (Upper panels) Immunoblot analysis of nuclear PPAR $\alpha$ . 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 PPAR $\alpha$ . 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 PPAR $\alpha$ . A sample used in Figure 3b was adopted. Hepatic RNA (5  $\mu$ g) was electrophoresed and hybridized with cDNAs for PPAR $\alpha$  and  $\beta$ -actin, respectively. Results are representative of 4 independent experiments. (b) Quantification of nuclear PPAR $\alpha$  levels and PPAR $\alpha$  mRNA levels. The nuclear PPAR $\alpha$  level was quantified densitometrically and normalized to the histone H1 level. The mRNA level of PPAR $\alpha$  was quantified using a phosphorimager and normalized to that of  $\beta$ -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  $\pm$  standard deviation. Abbreviations are identical with those in (a). \*,  $p < 0.05$  between the transgenic mice and the nontransgenic mice.

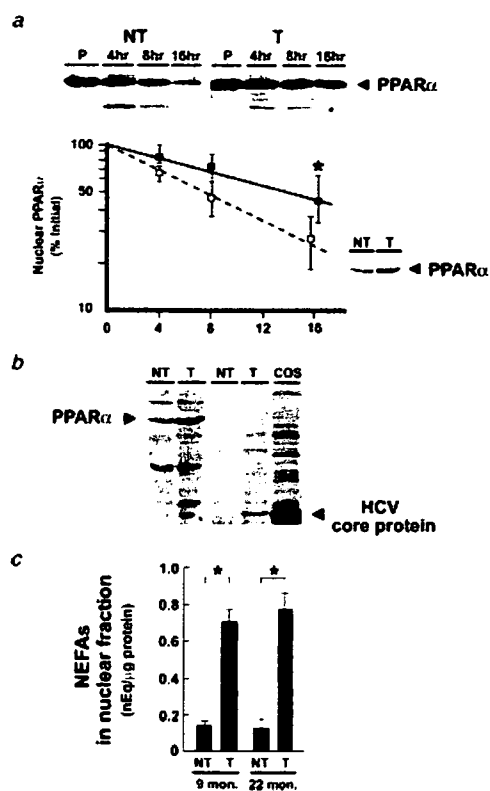
directly or indirectly affect the stability of PPAR $\alpha$  in hepatocyte nuclei.

#### Increase in PPAR $\alpha$ ligands

PPAR $\alpha$  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 PPAR $\alpha$ <sup>33</sup>, 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  $\sim 5$  times higher than those in the control mice at the same age (Fig. 7c). This could account for the higher activation of PPAR $\alpha$  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 PPAR $\alpha$  activation in the transgenic mouse livers for the first time. This study thus advances our understanding of the association



**FIGURE 7** – Analyses of PPAR $\alpha$  stability, interaction between PPAR $\alpha$  with the core protein in hepatocyte nuclei, and nuclear contents of NEFAs. (a) Pulse-label and pulse-chase experiments for nuclear PPAR $\alpha$  using isolated mouse hepatocytes. (Upper panels) Labeled PPAR $\alpha$  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 PPAR $\alpha$  in 5 independent experiments. Values are normalized as a percentage of the values of the pulse-labeled band and expressed as the mean  $\pm$  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 PPAR $\alpha$  and HCV core protein in the nucleus. (Left panel) Immunoblot analysis (PPAR $\alpha$ ) of the eluate on anti-PPAR $\alpha$  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  $\pm$  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 PPAR $\alpha$  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 PPAR $\alpha$  activation.<sup>19,26–30</sup> In particular, cyclin D1, CDK4, PCNA and c-Myc are potent and critical regulators of the G1-S checkpoint and cell-cycle progression,<sup>13,14</sup> and aberrant expression of these proteins is frequently detected in HCV-related HCC.<sup>34–37</sup> These key regulators are known to be induced in a PPAR $\alpha$ -dependent manner in mice<sup>19,30</sup>, the continuous induction of these proteins and the

resultant acceleration of hepatocyte proliferation found in the transgenic mice may be attributed to persistent PPAR $\alpha$  activation. In the current study, we demonstrated that there was a great variety of the intensity of PPAR $\alpha$  activation among different hepatocytes (Fig. 4). This persistent and heterogeneous PPAR $\alpha$  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.<sup>29</sup> 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 PPAR $\alpha$  mRNA (Fig. 6), heterogeneous peroxisome proliferation (Fig. 4) and age-dependent emergence of hepatocytes having nuclei stained intensively by anti-PPAR $\alpha$  or anti-cyclin D1 antibody (Fig. 5). Therefore, the mode of PPAR $\alpha$  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 PPAR $\alpha$  activation in mice is stabilization of PPAR $\alpha$  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 PPAR $\alpha$ -RXR $\alpha$  heterodimer and enhances the transcriptional activation mediated by PPAR $\alpha$  regardless of the presence or absence of its ligands.<sup>31</sup> Since PPAR $\alpha$  is ubiquitinated and degraded *via* the proteasome pathway,<sup>38</sup> 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 PA28 $\gamma$ <sup>39</sup> which is known to combine with steroid receptor coactivator-3 and to accelerate its degradation.<sup>40</sup> Another possible mechanism is an increase in NEFAs in hepatocyte nuclei. The PPAR $\alpha$  activation induced by the core protein enhances the expression of L-FABP,<sup>30</sup> 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.<sup>41</sup> Thus, increased L-FABP expression may facilitate the shuttling of NEFAs into hepatocyte nuclei for donating NEFAs to PPAR $\alpha$ , leading to PPAR $\alpha$  activation and further increase in L-FABP expression. Moreover, the binding of ligands

causes conformational alternation of PPAR $\alpha$ <sup>42</sup> and further stabilizes it in nuclei,<sup>32</sup> resulting in synergistic PPAR $\alpha$  activation. Therefore, these findings concerning spontaneous and persistent PPAR $\alpha$  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,<sup>43</sup> an increase in CDK4, cyclin D1 and E, PCNA, c-Myc and c-Fos,<sup>34-37</sup> and multicentric appearance of HCC.<sup>44</sup> Furthermore, it has been reported that a massive proliferation of peroxisomes was found in human non-tumorous liver tissue adjacent to HCC.<sup>45</sup> Thus the earlier findings, including the unique function of HCV core protein *in vivo* and the diverse and significant roles of PPAR $\alpha$ , 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 PPAR $\alpha$  was impaired in patients with chronic HCV infection,<sup>46</sup> 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 PPAR $\alpha$  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 PPAR $\alpha$  activation and hepatocarcinogenesis may be elucidated by additional experiments in which PPAR $\alpha$  activation is continuously inhibited in the same transgenic mice. Furthermore, the exact relationship may be confirmed when PPAR $\alpha$ -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 PPAR $\alpha$  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 PPAR $\alpha$  and an increase in NEFAs in the hepatocyte nuclei of the transgenic mice, which may have resulted in the HCV core protein-specific PPAR $\alpha$  activation.

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