Table 1. Tumor Incidence of Subcutaneously Inoculated Mixed PLC/PRF/5 Cells

Inoculated Cells	Tumor Incidence (%)
100% parental/0% angiostatin	5/5 (100)
90% parental/10% angiostatin	5/5 (100)
50% parental/50% angiostatin	2/5 (40)
0% parental/100% angiostatin	0/5 (0)

ANG were significant compared with CM-N, CM-Mock, or CM-ANG ΔHis ($P < .01$; Fig. 5B). Absorbance at 492 nm was 0.120 ± 0.017 for CM-N, 0.125 ± 0.013 for CM-Mock, 0.065 ± 0.007 for CM-ANG, and 0.110 ± 0.018 for CM-ANG ΔHis.

In Vivo *Study.* Because PLC/PRF/5 cells are transplantable in athymic mice, the mice were subcutaneously implanted with nontransfected or transfected PLC/PRF/5 cells in the right thigh. When 100% of angiostatin gene stable transfected cells were implanted, no tumor developed in these mice (Table 1). Indeed, tumor could not be detected in mice implanted with these cells even when postmortem examination was performed immediately after the mice were killed 8 weeks after implantation. Therefore, to ensure the antiangiogenic effects of angiostatin gene transduction, the angiostatin gene stable transduced cells and nontransduced parental cells were mixed at various proportions (0%, 10%, or 50% of transfectants) and the mixed cells were subcutaneously implanted. Eight weeks after implantation, tumors grew in 5 of 5 mice (100%) that received 0% transfectant cells, in 5 of 5 of those inoculated with 10% of transfectants, and only 2 of 5 (20%) that were implanted with 50% of transfectants (Table 1). As shown in Fig. 6A, the tumor of 50% transfected cells was pale and slightly whitish with few surface vessels compared with those in mice implanted with 0% and 10% transfected cells. Repeated measurements of the tumor volume showed growth suppression was dependent on the proportion of angiostatin gene–transfected cells. The tumor volume was 1,184 ± 312, 684 ± 97, and 374 ± 112 mm^3 for 0%, 10%, and 50% of transfectants at day 56, respectively (Fig. 6B). To confirm the expression of angiostatin protein in each tumor, immunostaining using anti-mouse angiostatin antibody was performed. The number of angiostatin-expressing cells correlated with the proportion of inoculated angiostatin gene–transfected cells (Fig. 7A). Furthermore, angiostatin-positive cells exhibited a mosaic pattern. The intratumoral vascular density was estimated in each tumor by immunostaining with anti-CD31 antibody (Fig. 7A) and quantified as described previously (Fig. 7B).[17] Briefly, CD31-positive cells were first identified at low magnification, and then CD31-positive vessels were counted at ×200 magnification. The average of 5 areas was recorded as the vascular density in each tumor. The vascular density was also suppressed by angiostatin gene transfection, and such suppression was dependent on the proportion of transfected cells (25 ± 4.8, 12 ± 3.2, and 5 ± 2.1 CD31-positive vessels/fields for 0%, 10%, and 50% of transfectants, respectively). These results indicate that the tumor suppressive effects of angiostatin gene transfection were related to tumor vascularity.

Discussion

Angiostatin is a circulating angiogenesis inhibitor that has been purified from serum and urine of Lewis rats bearing lung carcinoma.[11] The amino acid sequence of angiostatin is identical to first 4 kringle domain of plasminogen, and its molecular weight is 38 kd.[18] In addition to angiostatin, other kringle domains of plasminogen such as kringles 1-3, 1-5, and 5 alone also have antiangio-

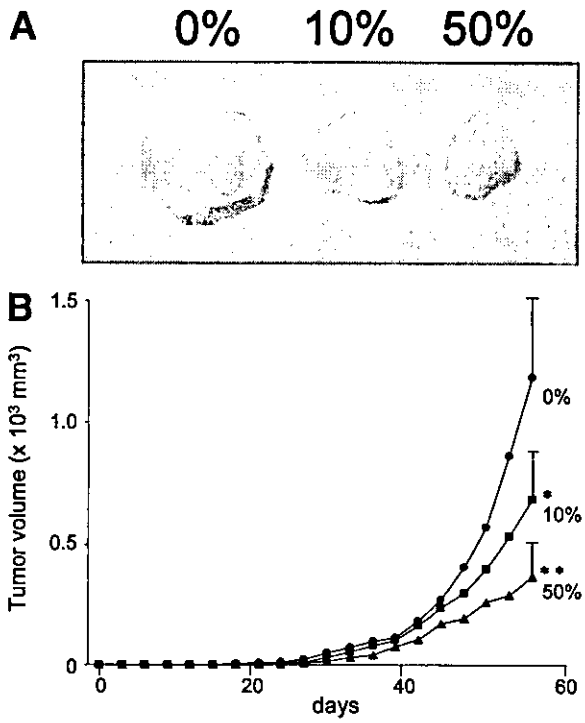

Fig. 6. Growth of implanted PLC/PRF/5 tumor cells in athymic mice. The pSecTag2B-ANG stable transfected PLC/PRF/5 cells and nontransfected PLC/PRF/5 cells were mixed at various proportions, and the mixed cells were subcutaneously implanted into athymic mice as described in Materials and Methods. (A) Representative photographs of harvested tumor. (B) Tumor volume was measured serially, and data represent the mean ± SD tumor volume at day 56. ●, 0%; ■, 10%; ▲, 50% of the pSecTag2B-ANG stable transfected PLC/PRF/5 cells. The depicted percentages indicate the proportion of pSecTag2B-ANG stable transfected PLC/PRF/5 cells against parental PLC/PRF/5 cells. *$P < .01$ vs. 0% of transfectant and CM-Mock. **$P < .01$ vs. 0% and 10% of transfectants.

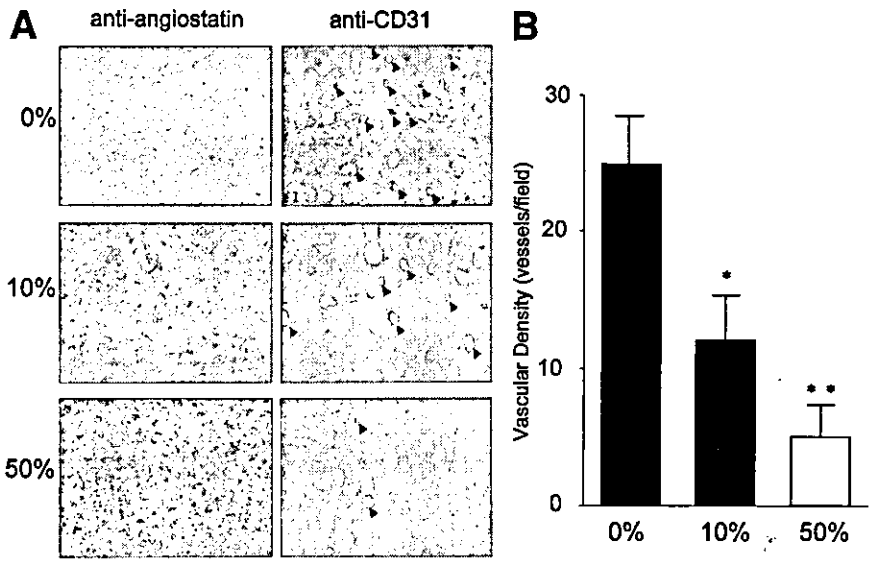

Fig. 7. Immunohistochemical staining for CD31 and angiostatin and evaluation of vascular density in the *in vivo* tumor. (A) Representative photographs of a harvested tumor stained immunohistochemically with anti-mouse angiostatin and anti-CD31 antibodies. **Arrowheads** indicate CD31-positive vessels. (Original magnification ×200.) (B) Vascular density was quantified as described in Materials and Methods. Data are mean ± SD of completely developed tumors. The depicted percentages indicate the proportion of pSecTag2B-ANG stable transfected PLC/PRF/5 cells relative to parental PLC/PRF/5 cells. $*P < .01$ vs. 0% of transfectant. $**P < .01$ vs. 0% and 10% of transfectants.

genic and antitumor properties in animal models.[12] In the process of angiogenic inhibition, angiostatin could inhibit endothelial cell growth by apoptosis,[19] endothelial cell migration in a modified Boyden chamber,[20] and tube formation of endothelial cells on a Matrigel *in vitro*.[21] Although several studies have examined the mechanism of such actions on endothelial cells, including interaction with adenosine triphosphate synthase,[22] inhibition of Erk activity,[23] and increase in ceramid and RhoA activation,[24] the precise mechanism remains unclear. Recently, Gao et al.[16] investigated the mechanism of antiangiogenic activity of plasminogen kringle 5, which down-regulates VEGF and up-regulates PEDF. In the present study, in contrast to the report of Gao et al.[16], expression of angiostatin cDNA in PLC/PRF/5 cells did not influence the expression of both VEGF and PEDF, whereas angiostatin protein was sufficiently detected by Western blotting in the CM derived from those cells. However, proliferation of HUVEC was suppressed in CM derived from angiostatin gene transduced PLC/PRF/5 cells *in vitro*. Because the proliferation of angiostatin gene transduced PLC/PRF/5 cells itself was not altered relative to the parental or control vector transduced PLC/PRF/5 cells, the cell growth suppression seems to be specific to endothelial cells. Furthermore, migration of HUVEC was suppressed in the same CM. Both the suppression of proliferation and migration of HUVEC appeared to be specific to secreted angiostatin, because CM-ANG ΔHis did not exhibit such suppressive effects. These results suggest that the antiangiogenic action of angiostatin does not involve kringle 5 but rather seems to be a direct action on endothelial cells without alteration of expression of other endogenous angiogenic or antiangiogenic agents. Further studies are needed to clarify the precise mechanism of antiangiogenic property of plasminogen kringle domains.

HCC is a hypervascular tumor, and the amount of VEGF or basic fibroblast growth factor in tumor cells closely correlates with the degree of hypervascularity and progression of HCC.[25,26] Therefore, antiangiogenic therapy might be quite effective for HCC. In this regard, several studies have examined the inhibitory effects of angiostatin gene transduction on the growth of various tumors such as glioma,[13] breast cancer,[21] and renal cell carcinoma.[27] The tumor growth suppressive effect of our system seems to be more potent than reported in these studies. When nude mice were subcutaneously implanted with 100% of stable transfectants of pSecTag2B-ANG, none showed tumor growth. Basically, during the progression of solid tumors, the angiogenic phenotype is involved in tumor expansion over 1 to 2 mm of diameter[28] but not in oncogenic initiation or promotion, and the main aim of antiangiogenic therapy for cancer is to halt tumor growth.[29] We presume that the potent effect seen in our study might be due to efficient angiostatin protein secretion of our constructed vector or increased sensitivity of HCC to vascular starvation.

It has been reported that the growth of adenovirus interleukin 12–treated HCC tumor is suppressed by activation of natural killer cells and inhibition of angiogenesis.[30] However, there is no study of genuine antiangiogenic gene therapy for HCC. Gene therapy using endogenous angiogenic inhibitors has several advantages.[8,31] Local concentration of therapeutic agent generated by gene delivery is higher than systemic administration. In addition, systemic administration of antiangiogenic agent has potential side effects because of the high effective dose

and prolonged continuous administration of angiogenesis inhibitor, which is required for cancer treatment to achieve dormant tumor growth.[32] TNP-470 inhibits normal endothelial maturation in nonpregnant mice and growth of embryos in pregnant mice,[33] and angiostatin impairs anastomotic healing in mice.[34] In this regard, 2 general strategies for antiangiogenic cancer gene therapy have been proposed: tumor-directed and systemic gene therapies.[35] A high local concentration of therapeutic agent is achieved by the tumor-directed strategy, although the latter does not seem to be effective against metastatic tumors or multiple lesions. On the other hand, systemic gene therapy requires efficient transgene expression without any adverse effects. In our system, angiostatin protein tagged with hexahistidine was not detected in the sera of mice bearing angiostatin gene transduced tumors as indicated by Western blotting using anti-hexahistidine antibody (data not shown), although the presence of angiostatin protein mixed with transfectant was confirmed in the tumor by immunohistochemistry. However, efficient bystander effect was shown in an *in vivo* experiment. Combined therapy with another antiangiogenic agent, chemotherapy, or radiation might increase the suppressive effect of tumor growth and metastases.[36,37]

Currently available vectors could be classified into viral and nonviral gene delivery systems.[35] For antiangiogenic gene therapy, efficient and permanent transgene expression is important. The nonviral gene delivery system is less efficient at inducing transgene expression, and no virus vector meets those requirements completely.[32,35,38] In the present study, we used the *ex vivo* induction of mammalian expression vector and established stable transfected PLC/PRF/5 cells. This strategy might not match the clinical setting. HCC is often diagnosed in an advanced stage of growth and the tumor presents as an organized and a highly vascular mass, even when detected at an early stage. However, in our *in vivo* study, we showed the suppression of tumor growth even when the transfected cells comprised only 10% of the inoculated cells. Extrapolation of these experimental results to patients with HCC suggests that at least some beneficial therapeutic effects might be obtained when angiostatin gene is introduced into, for example, 10% of cancer cells in HCC *in vivo*. However, further studies are needed before any attempts are made to use antiangiogenic gene therapy in humans, including the development of other vector systems.

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Hepatic Steatosis Is a Risk Factor for Hepatocellular Carcinoma in Patients with Chronic Hepatitis C Virus Infection

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See editorial on pages 2948–50, this issue.

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BACKGROUND. Hepatic steatosis is one of the histopathologic features of chronic hepatitis C. It was reported recently that the expression of hepatitis C virus (HCV) core protein in transgenic mice induced hepatocellular carcinoma (HCC) in association with steatosis. The objective of this study was to determine the relation between hepatic steatosis and hepatocarcinogenesis in patients with chronic HCV infection.

METHODS. The authors studied 161 patients with chronic HCV infection who were diagnosed at Nagasaki University Hospital, Nagasaki, Japan, between January 1980 and December 1999. Age, gender, body mass index (BMI), habitual drinking, diabetes mellitus, serum alanine aminotransferase (ALT) level, HCV serotype, serum level of HCV core protein, interferon (IFN) treatment, hepatic fibrosis inflammation, and hepatic steatosis were studied with regard to their significance in the development of HCC using univariate and multivariate analyses.

RESULTS. The cumulative incidence rates of HCC were 24%, 51%, and 63% at 5 years, 10 years, and 15 years, respectively. Multivariate analysis identified hepatic steatosis, together with aging, cirrhosis, and no IFN treatment, as independent and significant risk factors for HCC ($P = 0.0135$, $P = 0.0390$, $P = 0.0068$, and $P = 0.0142$, respectively). In addition, hepatic steatosis was correlated with BMI, serum ALT levels, and triglyceride levels.

CONCLUSIONS. The findings of the current study indicate that hepatic steatosis is a risk factor for HCC in patients with chronic HCV infection. Patients with chronic HCV and hepatic steatosis should be monitored carefully for HCC. *Cancer* **2003;97: 3036–43.** *© 2003 American Cancer Society.*
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KEYWORDS: core protein, hepatic steatosis, hepatitis C virus, hepatocellular carcinoma.

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world. The incidence of HCC has increased substantially in Japan during the past several decades[1,2] and also has increased slightly in the United Kingdom, France,[3,4] and the U.S.[5] These increases in the incidence of HCC have been attributed to the increased numbers of patients with hepatitis C virus (HCV)-associated liver cirrhosis. Several risk factors for the development of HCV-associated HCC have been reported, such as aging, gender, total alcohol intake,[6,7] cirrhosis,[8] irregular regeneration of hepatocytes,[9,10] and HCV genotype.[11,12] Recent studies have indicated that interferon (IFN) treatment can reduce the incidence of HCC.[13,14] The identification of additional variables associated with changes in the risk of HCC

would be particularly important with regard to optimizing preventive medical programs.

Hepatic steatosis is one of the histopathologic features of chronic hepatitis C.[15–18] According to previous reports, the prevalence of hepatic steatosis ranges from 31 to 72%.[15–20] The pathogenesis of hepatic steatosis in patients with chronic HCV infection has been postulated recently. Both in vitro studies and in vivo studies have shown that HCV core protein expression either in cell cultures or in transgenic mice led to the development of hepatic steatosis, contributing to carcinogenesis.[21–23] In addition, hyperplasia of hepatocytes has been described in ob/ob mice with fatty liver disease, suggestive of malignant changes secondary to hepatic steatosis.[24] These observations suggest that hepatic steatosis may be involved in the development of HCC.

The objective of this study was to determine whether hepatic steatosis is an independent risk factor in the development of HCC in patients with chronic HCV. To this end, univariate and multivariate analyses were performed to identify independent risk factors, including hepatic steatosis, for HCC in 161 patients with chronic HCV infection.

MATERIALS AND METHODS

Patients

Liver biopsies were obtained from 560 patients who had no detectable HCC at the Nagasaki University Hospital, Nagasaki, Japan, between January 1980 and December 1999. Patients with bleeding tendencies or clinically evident cirrhosis associated with ascites or hepatic encephalopathy were excluded from the biopsy procedure. Of these 560 patients, 218 patients who were diagnosed with chronic hepatitis or cirrhosis had confirmed negative results for hepatitis B surface antigen (HBsAg) but positive results for the antibody to HCV (anti-HCV). Of these 218 patients, 161 patients who were followed at the Nagasaki University Hospital for > 6 months were enrolled in this study. Patients who had other causes of liver disease, such as primary biliary cirrhosis or autoimmune hepatitis, were excluded from this study. The body mass index (BMI) was calculated as the patient's weight (kilograms) divided by height (meters) squared (kg/m^2). At the time patients underwent liver biopsy, information regarding alcohol consumption was obtained through an interview by physicians. Habitual drinking was defined as an average daily consumption of an amount equivalent to 80 g per day of pure ethanol over a period of > 5 years. Diabetes mellitus was diagnosed based on fasting serum glucose levels that exceeded 7.8 mmol/L (140 mg/dL), abnormal results for a 75-g oral glucose tolerant test, or the need for insulin or an oral antihyperglycemic drug to control glucose levels. Informed consent was obtained from each patient at the time they underwent liver biopsy.

Serologic Tests

A serum sample was obtained from each patient at the time of liver biopsy and stored at − 40 °C for later analysis of viral markers. Anti-HCV was determined using a second-generatoin or third-generation enzyme-linked immunosorbent assay (Ortho Diagnostics Systems, Tokyo, Japan). HCV serotype was determined using the genotyping enzyme-linked immunosorbent assay (International Reagents Corporation, Tokyo, Japan) reported by Tanaka et al.[25] In this assay, HCV serotypes 1 and 2 correspond to genotypes 1 and 2 of Simmonds classification, respectively. Serum HCV core protein level was measured by enzyme immunoassay (Ortho Clinical Diagnostics, Tokyo, Japan) for quantitative evaluation of HCV viremia.[26]

Follow-Up of Patients

Clinical evaluation and biochemical tests were performed every 1–3 months. Patients received ultrasonography or computed tomography studies of the liver at least every 3–6 months. The diagnosis of HCC was based on the histopathologic findings in tumor tissue or on the characteristic appearance on ultrasonography, computed tomography, and hepatic arteriography. The endpoint used in the current study was the appearance of HCC, and the reference date used was December 31, 2000. The numbers of patients who were followed without showing any appearance of HCC for 5 years, 10 years, and 15 years after liver biopsy were 90 patients, 29 patients, and 16 patients, respectively. Overall, 70 patients were followed until the endpoint of this study. The average observation period was 76.5 months (6.4 years).

Seventy-one patients received IFN treatment during the follow-up period. IFN therapy was initiated within 1 year after liver biopsy, and each patient was followed for at least 48 weeks after the completion of IFN therapy. A sustained response (SR) was defined as negative results for serum HCV RNA according to polymerase chain reaction analysis and normal alanine aminotransferase (ALT) levels for > 24 weeks after completion of IFN therapy. A nonresponse (NR) was defined as any other response.

Histopathologic Examination of the Liver

Liver biopsy specimens were fixed in 10% formalin, embedded in paraffin, cut to thickness of 4 μm, and stained with hematoxylin-eosin and Azan. All liver

TABLE 1
Clinical and Laboratory Characteristics of 161 Patients

Characteristic	No of patients (%)
Median age (yrs; mean ± SD)	53 ± 12
Male:female ratio	106:55
BMI (kg/m^2, mean ± SD)	22.7 ± 0.24
No. of patients with BMI > 25 kg/m^2	31 (19)
Habitual drinking	11 (7)
Diabetes mellitus	26 (16)
ALT (IU/L; mean ± SD)	103.4 ± 94.8
γ-GTP (IU/L; mean ± SD)	78.3 ± 74.9
Triglyceride (mg/dL; mean ± SD)	102.3 ± 53.0
Cholesterol (mg/dL; mean ± SD)	162.9 ± 34.5
Serotype (1:2:undetermined)	106:17:3
HCV core protein(fmol/L mean ± SD)	1623.9 ± 2668.2
IFN (yes/no)	71/90

SD: standard deviation; BMI: body mass index; ALT: alanine aminotransferase; γ-GTP: γ-glutamyltransferase; HCV: hepatitis C virus; IFN: interferon.

TABLE 2
Histologic Characteristics of 161 Patients

Characteristic	No. of patients (%)
Distribution of stage of fibrosis	
1	45 (28)
2	28 (17)
3	25 (16)
4	63 (39)
Distribution of grade of inflammation	
1	79 (49)
2	79 (49)
3	3 (2)
Distribution of grade of steatosis	
0 (absent)	71 (44)
1 (1–10% of hepatocytes affected)	79 (49)
2 (11–30% of hepatocytes affected)	8 (5)
3 (> 30% of hepatocytes affected)	3 (2)

tissue specimens were evaluated by one pathologist (K. T.) who was unaware of the clinical condition of the patient. Liver histology was evaluated according to the degree of fibrosis, necroinflammatory activity, and steatosis. The extent of fibrosis (staging) and the degree of necroinflammatory activity (grading) were classified according to Desmet et al.[27] as follows: F1 (periportal expansion), F2 (portoportal septa), and F3 (portocentral linkage or bridging fibrosis) were categorized as noncirrhosis; and F4 (cirrhosis) was categorized as cirrhosis. In terms of necroinflammatory activity, A1 represented mild activity and A2 or A3 represented severe activity. The severity of steatosis was graded as Grade 0 (absent), Grade 1 (1–10% of hepatocytes affected), Grade 2 (11–30% of hepatocytes affected), or Grade 3 (> 30% of hepatocytes affected).

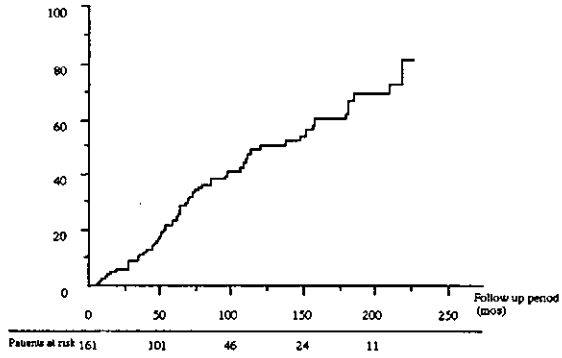

FIGURE 1. Chart illustrating the cumulative incidence of hepatocellular carcinoma (HCC; %) in 161 patients with chronic hepatitis C virus.

Statistical Analysis

Data were expressed as the mean ± standard deviation for continuous variables and as counts for categorical variables. Continuous variables and categorical variables were compared using the Student *t* test and the chi-square test, respectively. Cumulative incidence curves were determined with the Kaplan–Meier method, and the differences between groups were assessed with the log-rank test. Univariate and multivariate analyses of the risk ratios for the occurrence of HCC were conducted by using Cox proportional hazards regression analysis. The factors examined included age, gender, BMI, habitual drinking, diabetes mellitus, serum ALT level, HCV serotype, serum HCV core protein level, IFN treatment during follow-up, histopathologic staging (noncirrhosis or cirrhosis), histopathologic grading (mild activity or severe activity), and hepatic steatosis (absence or presence). In addition, factors that were correlated with hepatic steatosis were analyzed. All *P* values were two-tailed, and *P* values < 0.05 were considered significant. Statistical analysis was performed by using Stat View software (version 5.0; SAS Institute Inc., Cary, NC).

RESULTS

Patients Characteristics

Table 1 shows the clinical features of patients at study entry. The study included 106 males and 55 females with a mean age of 53 years (range, 19–78 years). The mean BMI was 22.7 kg/m^2, and 31 patients had a BMI > 25 kg/m^2 (19%). This study included 11 patients (7%) who were habitual drinkers and 26 patients (16%) with diabetes mellitus. Serotype was available for 126 patients; 106 patients had serotype 1, 17 patients had serotype 2, and serotype 3 was not determined. The mean level of serum HCV core protein in 143 patients was 1623.9 fmol/L (range, 0–20000 fmol/L). During

TABLE 3
Univariate Analysis of Risk Ratios for Hepatocellular Carcinoma

Characteristic	Risk ratio	95% CI	*P* value
Age (compared with ≤ 50 yrs)	5.52	2.51–12.04	0.0001[a]
Gender (compared with female)	1.30	0.76–2.23	0.3435
Diabetes mellitus (compared with absent)	1.46	0.82–2.60	0.2003
BMI (compared with < 24 kg/m^2)	1.04	0.63–1.73	0.8690
Habitual drinking (compared with no habitual drinking)	1.22	0.51–2.88	0.6581
ALT (compared with < 90 IU/L)	1.56	0.96–2.53	0.0725
HCV serotype (compared with serotype 2)	1.02	0.40–2.62	0.9620
HCV core (compared with 500 fmol/L)	0.85	0.51–1.42	0.5365
IFN (compared with no treatment)	0.18	0.09–0.38	0.0001[a]
Cirrhosis (compared with no cirrhosis)	2.65	1.62–4.34	0.0001[a]
Grading (compared with mild)	1.37	0.85–2.23	0.1995
Steatosis (compared with absent)	2.56	1.49–4.41	0.0007[a]

95% CI: 95% confidence interval; BMI: body mass index; ALT: alanine aminotransferase; HCV: hepatitis C virus; IFN: interferon.
[a] Statistically significant.

follow-up, 71 of 161 patients received IFN treatment: Twenty of those 71 patients (28%) had an SR, and 51 patients (72%) had NR. None of the patients with an SR and eight patients with NR developed HCC. Of the 161 patients examined, only 0 patients, 5 patients, and 6 patients had serum total bilirubin levels > 2.0 mg/dL, serum albumin levels < 3.5g/dL, and prothrombin time < 80%, respectively.

Examples of histopathologic findings are shown in Table 2. According to the histopathologic staging of the extent of fibrosis, 45 patients (28%) had F1 fibrosis, 28 patients (17%) had F2 fibrosis, 25 patients (16%) had F3 fibrosis, and 63 patients (39%) had F4 fibrosis. According to the histopathologic grading of necroinflammatory activity, 79 patients (49%) had A1 activity, 79 patients (49%) had A2 activity, and 3 patients (2%) had A3 activity. Hepatic steatosis was present in 91 of 161 patients (56%). The distribution of steatosis showed that 71 patients (43%) had Grade 0 steatosis, 79 patients (49%) had Grade 1 steatosis, 8 patients (6%) had Grade 2 steatosis, and 3 patients (2%) had Grade 3 steatosis.

Analysis of Clinicopathologic Factors that Influence the Development of HCC

Figure 1 shows that the cumulative incidence rates of HCC were 24% 5 years after biopsy, 51% 10 years after biopsy, and 63% 15 years after patients underwent liver biopsy. Recently, Hashem and El-Serag[28] reported that the incidence of HCC in patients with chronic HCV tended to be greater in Japan compared with other countries. In a large retrospective cohort study from Japan, the annual incidence of HCC was 0.5% in patients with HCV who had low-grade fibrosis and 7.9% in patients who had severe fibrosis.[13] Those results are in agreement with the current findings drawn from a cohort in which > 50% of patients had severe fibrosis (F3 or F4).

To determine the factors that may influence the development of HCC, a Cox proportional hazards regression analysis was performed. Based on univariate analysis, the following four factors influenced the incidence of HCC significantly: patient age at the time of liver biopsy (relative risk, 5.52; 95% confidence interval [95% CI], 2.51–12.04 [$P < 0.0001$]), IFN treatment (relative risk: 0.18; 95% CI, 0.09–0.38 [$P < 0.0001$]), histopathologic staging (cirrhosis: relative risk, 2.65; 95%CI, 1.62–4.34 [$P = 0.0001$]), and hepatic steatosis (steatosis present: relative risk, 2.56; 95% CI, 1.49–4.41 [$P = 0.0007$]) (Table 3). Furthermore, a multivariate analysis of determinants of HCC was applied using a Cox regression model. Age at the time of liver biopsy (relative risk, 2.96; 95% CI, 1.06–8.26 [$P = 0.0390$]), IFN treatment (relative risk, 0.30; 95% CI, 0.12–0.79 [$P = 0.0142$]), cirrhosis (relative risk, 3.21; 95% CI, 1.38–7.48 [$P = 0.0068$]), and hepatic steatosis (relative risk, 2.81; 95% CI, 1.24–6.37 [$P = 0.0135$]) were identified as statistically independent risk factors (Table 4). Figure 2 shows the cumulative incidence of HCC based on the prevalence of hepatic steatosis. The cumulative incidence rate of HCC in 90 patients who had steatosis was significantly greater compared with the rate in 71 patients who did not have steatosis ($P = 0.0012$).

Analysis of Factors Associated with Hepatic Steatosis

The characteristics and laboratory features of patients with and without steatosis are summarized in Table 5. The Student *t* test or the chi-square test was performed to evaluate the various factors that affected the presence of hepatic steatosis. The results showed sig-

TABLE 4
Multivariate Analysis of Risk Ratios for Hepatocellular Carcinoma

Characteristic	Risk ratio	95% CI	*P* value
Age (compared with ≤ 50 yrs)	2.96	1.06–8.26	0.0390*
Gender (compared with female)	1.45	0.67–3.13	0.3484
Diabetes mellitus (compared with absent)	1.58	0.62–3.99	0.3351
BMI (compared with < 24 kg/m^2)	1.67	0.80–3.46	0.1700
Habitual drinking (compared with no habitual drinking)	1.56	0.38–6.33	0.5375
ALT (compared with < 90 IU/L)	1.17	0.59–2.33	0.6596
HCV serotype (compared with serotype 2)	1.15	0.38–3.48	0.8073
HCV core (compared with < 500 fmol/L)	1.24	0.60–2.56	0.5698
IFN (compared with no treatment)	0.30	0.12–0.79	0.0142*
Cirrhosis (compared with no cirrhosis)	3.21	1.38–7.48	0.0068*
Grading (compared with mild)	1.01	0.44–2.29	0.9887
Steatosis (compared with absent)	2.81	1.24–6.37	0.0135*

95% CI: 95% confidence interval; BMI: body mass index; ALT: alanine aminotransferase; HCV: hepatitis C virus; IFN: interferon.
* Statistically significant.

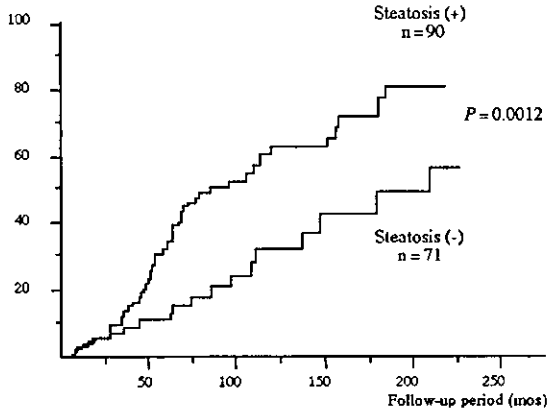

FIGURE 2. Chart illustrating the cumulative incidence of hepatocellular carcinoma (HCC; %) based on the prevalence of hepatic steatosis in patients with chronic hepatitis C virus. The cumulative incidence of HCC in 90 patients with hepatic steatosis was significantly greater compared with the cumulative incidence of HCC in 71 patients who were free of hepatic steatosis (P = 0.0012).

nificant associations between the presence of steatosis and BMI (P = 0.0067), ALT levels (P = 0.026), and triglyceride levels (P = 0.0044) (Table 5). There was no correlation between BMI and the grade of steatosis (Table 6). Diabetes and habitual drinking were not correlated with the presence of steatosis.

DISCUSSION

Several histopathologic features characterize chronic hepatitis C and distinguish it from other forms of hepatitis, including bile duct damage, lymphoid follicles, and steatosis.[15–18] In a comparative study, steatosis was observed in 72% of patients who had chronic hepatitis C, compared with 19% of patients who had autoimmune hepatitis.[16] In another study, fatty changes were observed at a significantly greater rate in patients who had chronic hepatitis C compared with patients who had chronic hepatitis B.[17] The prevalence of steatosis observed in the current study was compatible with previous reports, although most patients with steatosis had the low-grade variety.

In the current study, we analyzed the factors that affected the incidence of HCC in patients with chronic HCV infection who were diagnosed by liver biopsy. Univariate and multivariate analyses identified hepatic steatosis, together with aging, cirrhosis, and no IFN treatment, as significant independent risk factors for HCC. Recently, it was shown that the expression of HCV core protein in transgenic mice induced hepatic steatosis and HCC without inflammation.[23,29] Lerat et al.[30] provided additional evidence in support of the direct causative roles of both the structural and nonstructural proteins of HCV in steatosis and carcinogenesis. In addition, several investigators have revealed that hepatic steatosis, including steatosis induced by HCV core protein, predisposes to lipid peroxidation and excess free-radical activity with the potential risk of genomic mutations.[31] In addition, Marreto et al.[32] reported that nonalcoholic fatty liver disease may be a common underlying liver disease in patients with HCC in the U.S. Those studies support our results showing that hepatic steatosis plays a role in hepatocarcinogenesis in patients with chronic HCV.

Hepatic steatosis is seen frequently in obese patients and in patients with diabetes mellitus.[33–35] Several lines of evidence indicate that diabetes mellitus is a risk factor for HCC in patients with cirrhosis,[36] although the exact correlation remains to be deter-

TABLE 5
Clinical, Laboratory, and Histologic Characteristics of the 161 Patients According to the Prevalence of Steatosis

Variable	Steatosis		*P* value
	Absent	Present	
No. of patients	71	90	—
Median age (yrs; mean ± SD)	51 ± 13.8	55 ± 10.1	0.0540
Male:female	42:29	64:26	0.1100
BMI (kg/m^2; mean ± SD)	21.9 ± 2.83	23.3 ± 3.14	0.0067*
Habitual drinking (%)	5 (7)	6 (7)	0.9056
Diabetes mellitus (%)	11 (16)	15 (17)	0.8200
AST (IU/L; mean ± SD)	77.6 ± 78.5	97.9 ± 89.4	0.1500
ALT (IU/L; mean ± SD)	84.8 ± 72.2	118.2 ± 107.7	0.0260*
γ-GTP (IU/L; mean ± SD)	71.3 ± 85.4	83.9 ± 65.6	0.3000
Triglyceride (mg/dL; mean ± SD)	88.4 ± 30.9	113.2 ± 63.5	0.0044*
Cholesterol (mg/dL; mean ± SD)	161.8 ± 35.1	163.7 ± 34.2	0.7300
Serotype (1:2)	48:9	58:8	0.5567
HCV core (fmol/L; mean ± SD)	2154.1 ± 3990.0	1376.5 ± 1942.2	0.1309
Grading (mild:severe)	38:33	35:55	0.0640
Staging (no cirrhosis:cirrhosis)	48:50	23:40	0.1198

SD: standard deviation; BMI: body mass index; AST: aspartate aminotransferase; ALT: alanine aminotransferase; γ-GTP: γ-glutamyltransferase; HCV: hepatitis C virus.
* Statistically significant.

TABLE 6
Correlation between Body Mass Index and Grade of Hepatic Steatosis

BMI (kg/m^2)	Grade of hepatic steatosis*				Total
	0	1	2	3	
Below normal (BMI < 18.5)	10	4	1	0	15
Normal (BMI 18.5–25.0)	51	58	3	1	113
Overweight (BMI 25.0–30.0)	10	15	4	—	29
Obese (BMI ≥ 30.0)	—	2	—	2	4
Total	71	79	8	3	161

BMI: body mass index.
* The severity of steatosis was graded as follows: Grade 0, absent; Grade 1, 1–10% of hepatocytes affected; Grade 2, 11–30% of hepatocytes affected; and Grade 3, >30% of hepatocytes affected.

mined. Nair et al.[37] also showed that obesity is an independent risk factor for HCC in patients with alcoholic liver disease and cryptogenic cirrhosis, but not in patients with viral hepatitis, based on an analysis of explanted liver specimens in patients with advanced cirrhosis. We did not find direct effects of diabetes or obesity on hepatocarcinogenesis. This may be attributable to the fact that our study did not include patients who had advanced cirrhosis at baseline and/or that the studied population included relatively small numbers of obese patients and patients with diabetes.

Hepatic steatosis has been cited as a characteristic feature of chronic HCV, although it remains uncertain whether it is related directly to the virus or is secondary to host factors. Several investigators have reported that an increase in BMI or visceral obesity is related to steatosis in patients with chronic hepatitis C.[38,39] In concordance with these observations, we found a significant correlation between hepatic steatosis and BMI, although the prevalence of a BMI > 25 kg/m^2 was low in our patients. In addition, higher serum levels of ALT and triglycerides were observed in patients who had steatosis compared with patients who were without steatosis. These results suggest that weight reduction in patients with chronic hepatitis C may be associated with a reduction in steatosis and abnormal liver enzymes. However, it is unclear whether weight reduction leads to a favorable outcome in patients with chronic HCV, because BMI had no significant effect on the development of HCC in our study. The lack of such association also may suggest that additional factors are associated with the presence of steatosis, although other host factors (such as age, gender, habitual drinking, diabetes, and cholesterol levels) were not statistically significant. Polymorphism of one or more host factors may provide an intriguing and likely mechanism. Several such factors have been described in various experimental models of steatosis and steatohepatitis, including tumor necrosis factor[40] and peroxisome proliferator-activated receptor α.[41]

Hourigan et al.[38] showed a significant association between steatosis and hepatic fibrosis, suggesting that steatosis is an important cofactor in accelerating the development of hepatic fibrosis and inflammatory ac-

tivity. In the current study, however, no relation was found between these factors. This discrepancy may be attributable to differences in the background of patients studied, including a lower prevalence of steatosis > Grade 2 in our study compared with previous reports. Alternatively, our results may suggest that steatosis contributes to hepatocarcinogenesis independent of necroinflammatory reaction, as suggested in experimental models.[23]

Recent studies have reported that hepatic steatosis is more likely to be associated with genotype 3 than with other HCV genotypes.[42,43] We could not find an effect for HCV type on hepatic steatosis, because the majority of patients studied were infected with serotype 1, which corresponds to genotype 1. Other studies also demonstrated that viral load was associated with hepatic steatosis in patients infected with HCV genotype 3 but not with genotype 1.[42,43] In agreement with those observations, our results showed no significant difference in serum HCV core protein levels between patients with and without hepatic steatosis, most of whom were infected with serotype 1. Fujie et al.[44] showed that the intrahepatic concentration of HCV, but not the serum concentration, was associated with the development of steatosis in the liver of patients with chronic HCV. Therefore, it is possible that HCV itself or the core protein may play a role in the pathogenesis of steatosis in patients with chronic HCV, although information on intrahepatic HCV RNA was not available in the current study.

The results of the current study demonstrate that hepatic steatosis is an independent risk factor for HCC in patients with chronic HCV infection, although the factors responsible for steatosis could not be identified clearly. Therefore, our data emphasize the need for the careful monitoring of patients with chronic HCV and hepatic steatosis for the development of HCC. Because of the small number of patients studied, the current findings need to be confirmed in a larger population of patients with chronic HCV infection.

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Analysis of anti-HBs levels in healthcare workers over 10 years following booster vaccination for hepatitis B virus

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Abstract

In this study, we analyzed anti-HBs levels in 104 Japanese healthcare workers who received three booster HBV surface antigen (HBsAg) vaccines because 80 became anti-HBs-negative at a mean of 2.4 years after the primary vaccination and 24 did not respond to primary vaccination. Of the re-vaccinees, 96% achieved a level of 10 mIU/ml or more of anti-HBs (i.e. seroprotected), 1 month after booster vaccination. Although anti-HBs levels of re-vaccinees decreased as rapidly as those of primary immunized vaccinees, at 10 years post-booster, 64% of re-vaccinees maintained anti-HBs levels at 10 mIU/ml or higher. Our results suggest that the additional three-dose protocol of booster HBsAg vaccination is beneficial in maintaining a seroprotective level of anti-HBs until new immunogenic vaccination protocols are established.

Keywords: Healthcare worker; Booster vaccination; Anti-HBs

1. Introduction

Healthcare workers represent one of the most important risk groups for hepatitis B virus (HBV) infection, primarily from occupational exposure to infectious body fluids from patients with chronic HBV infection [1]. Therefore, they are advised to be vaccinated with HBV surface antigen (HBsAg) [1]. However, previous studies have shown that the level of anti-HBsAg (anti-HBs) in vaccinees declines rapidly within the first year and more slowly thereafter [2,3], and that nearly 30% of vaccinees have an anti-HBs level below 10 mIU/ml within 15 years of primary vaccination [4,5]. Since 10 mIU/ml is widely considered to be a seroprotective level of anti-HBs [6,7], booster vaccination has been recommended for individuals in whom anti-HBs declines to such a low level [7,8]. On the other hand, the European Consensus Group on Hepatitis B Immunity has recently stated that booster vaccination is not needed for healthcare workers in whom adequate immunological priming has been achieved [4]. Their statement is based on the following evidence: that no obvious breakthrough hepatitis has been reported in vaccinees even with anti-HBs levels below 10 mIU/ml [9], and that in such individuals a booster vaccination, which may mimic exposure to HBV, can produce a rapid and adequate response of anti-HBs because of the presence of memory B cells for HBsAg [10–13]. However, at present, the ultimate lifetime of vaccine-induced immunological memory for HBsAg in healthy subjects is still unknown [4,5,8]. In addition, another important consideration is the cost–benefit evaluation of booster vaccination in healthcare workers.

At the Nagasaki University Hospital, a regimen of three doses of HBsAg vaccine has been administered to healthcare workers since 1989, and an additional three booster doses were given to some individuals whose anti-HBs levels became negative after primary vaccination. Since changes in anti-HBs levels during the decade after booster vaccination are not well understood, in the present study, we analyzed the anti-HBs levels in booster-immunized vaccinees up to 10 years after booster, as well as in primarily immunized vaccinees up to 12 years after primary immunization.

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2. Materials and methods

2.1. Subjects

A total of 1327 healthcare workers (men 562; women 765), aged 21–61 years (mean 36 years) in 2001, who worked at the Nagasaki University Hospital during the period from 1998 to 2001 were subjected to serum HBsAg and anti-HBs measurement. In addition, their HBV vaccination histories were obtained from written records, as was the anti-HBs status for persons working at the hospital before 1998. Post-vaccination periods for vaccinated persons were estimated from the year of last immunization to the year of the study (1998–2001).

2.2. Vaccination protocol

Three 10 μg doses of recombinant HBsAg vaccines, Bimmugen (Kaketsuken, Kumamoto, Japan) or Meinyu (Meiji Dairies Co., Tokyo, Japan), was administered by intramuscular injection in the deltoid region according to a 0, 1, and 6 months schedule, to individuals negative for HBsAg and anti-HBs. Since it was possible that these subjects could become anti-HBs-negative after primary vaccination or show no response to primary vaccination, some individuals received additional booster immunizations with three other doses of HBsAg vaccine.

2.3. HBsAg and anti-HBs measurements

Since 1989, serum levels of HBsAg and anti-HBs in healthcare workers were measured every year. In addition, serum levels of anti-HBs in the newly vaccinated healthcare workers were assayed 1 month after the three doses of vaccination. Prior to 1998, serum levels of HBsAg were determined by reverse passive hemagglutination (SERODIA-HBs; Fujirebio Inc., Tokyo, Japan) or by enzyme-linked immunosorbent assay (ELISA) (Enzygnost HBsAg monoclonal II; Behring Diagnostics, Ontario, Canada), and serum levels of anti-HBs were determined by passive hemagglutination (SERODIA-Anti-HBs; Fujirebio Inc.) or by ELISA (Enzygnost Anti-HBs micro; Behring Diagnostics). These methods were semi-quantitative but sufficient to evaluate whether anti-HBs was positive or negative in each subject. Since 1998, serum levels of HBsAg and anti-HBs were determined by sensitive and quantitative methods such as the chemiluminescent enzyme immunoassay (CLEIA) [14], Lumipulse II HBsAg and HBsAb (Fujirebio Inc.). Lumipulse II HBsAb can detect 0.1 to 1000 mIU/ml of anti-HBs, and a positive level of anti-HBs was regarded as 5 mIU/ml or more. Because it was difficult to analyze the data determined by different methods as one group, analysis of anti-HBs levels was performed on the values determined by Lumipulse II HBsAb during the period from 1998 to 2001, while the data collected prior to 1998 only determined the status of anti-HBs (positive or negative).

Table 1
Subjects

n	1327
Not vaccinated	467 (35.2)
HBsAg-positive	15 (1.1)
Anti-HBs-positive	275 (20.7)
Anti-HBs-negative	177 (13.3)
Vaccinated	860 (64.8)
Once	739 (55.7)
Twice	104 (7.8)
≥3 times	17 (1.3)

Data are numbers (percentages) of healthcare workers.

3. Results

Healthcare workers (n = 1327) who worked at Nagasaki University Hospital during the period between 1998 and 2001 were the subjects of this study (Table 1). Of the 1327 subjects, 467 had not been immunized against HBV. Of the latter group, 15 were HBsAg-positive, and 275 were anti-HBs-positive without a history of vaccination, suggesting that they had naturally acquired immunity for HBsAg, probably through previous exposure to HBV though the dates of natural infection were not clear. The remaining 860 of 1327 had been immunized against HBV. Of these, 739 were administered only three primary doses of HBsAg vaccination, and 104 were administered a further three-dose booster vaccination (re-vaccinees) because 80 became anti-HBs-negative within a mean of 2.4 years after the primary vaccination and 24 did not respond to primary vaccination.

The histogram of anti-HBs levels in primarily immunized vaccinees at 1 month, 1 and 10 years after vaccination is shown in Fig. 1A. The level of anti-HBs was ≥1000 mIU/ml at 1 month after primary vaccination in almost 55% of vaccines. However, at 1 year, the percentage of vaccinees with such high level decreased. In fact, the level of anti-HBs in most vaccinees was <50 mIU/ml. At 10 years after primary vaccination, the level of anti-HBs in almost 50% of vaccinees was <50 mIU/ml. Fig. 1B shows histograms of anti-HBs levels in re-vaccinees at 1 month, 1 and 10 years after booster vaccination. The level of anti-HBs was ≥1000 mIU/ml in almost 40% of re-vaccinees at 1 month after booster vaccination, however, the percentage of re-vaccinees with low anti-HBs levels increased at 1 year after booster vaccination, similar to primarily immunized vaccines. Furthermore, anti-HBs levels were <50 mIU/ml in more than 60% of re-vaccinees at 10 years after booster vaccination. Comparison of the 275 workers who had naturally acquired immunity for HBsAg showed that anti-HBs levels were ≥1000 mIU/ml in 35% of workers in 1998 (Fig. 2). However, the proportion of workers with these anti-HBs levels remained almost stable until 2001 (Fig. 2).

We then analyzed the percentage of vaccinees in whom the level of anti-HBs was maintained at ≥10 mIU/ml after primary or booster vaccination (Tables 2 and 3). One month

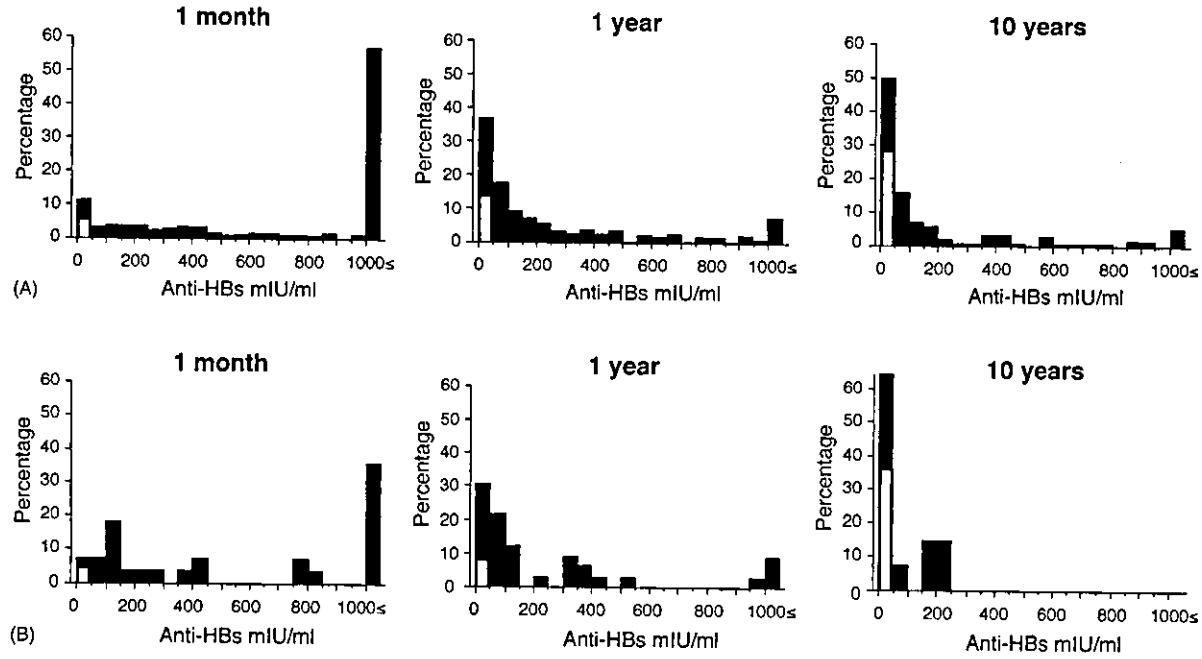

Fig. 1. Distribution of anti-HBs level after vaccination. Anti-HBs levels were grouped into 50 mIU/ml values and ≥1000 mIU/ml, and shown as histograms. (A) Closed columns: percentage of vaccinees (after primary vaccination) with the indicated anti-HBs levels at 1 month ($n = 210$), 1 year ($n = 183$) and 10 years ($n = 170$). Open columns: percentages of vaccinees with anti-HBs <10 mIU/ml. Numbers of subjects are identical to those shown in Table 2. (B) Closed columns: percentages of re-vaccinees (after booster vaccination) with the indicated anti-HBs levels at 1 month ($n = 28$), 1 year ($n = 40$) and 10 years ($n = 14$). Open columns: percentages of re-vaccinees with anti-HBs <10 mIU/ml. Numbers of subjects are identical to those shown in Table 3.

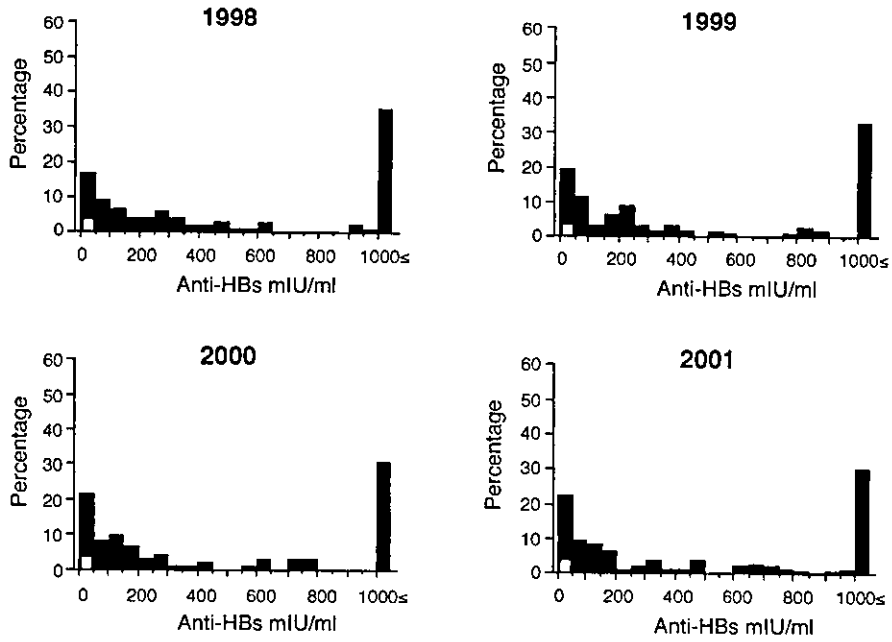

Fig. 2. Distribution of anti-HBs levels in individuals with naturally acquired immunity for HBsAg. Anti-HBs levels in individuals who had naturally acquired immunity for HBsAg in 1998 ($n = 275$), 1999 ($n = 262$), 2000 ($n = 257$) and 2001 ($n = 254$) were grouped into 50 mIU/ml values and ≥1000 mIU/ml, and shown as histograms (closed columns). Open columns: percentages of individuals with 5 mIU/ml ≤ anti-HBs < 10 mIU/ml.

Table 2
Proportion of vaccinees with anti-HBs ≥10 mIU/ml after primary vaccination

Post-primary vaccination period (years)	1 month	4 months	1	2	3	4	5	6	7	8	9	10	11	12
Total number of persons determined anti-HBs during 1998–2001	210	202	183	157	144	124	95	95	94	98	188	170	146	119
Individuals with anti-HBs ≥10 mIU/ml														
n	199	196	159	136	120	102	80	80	75	77	143	123	105	83
Percentage	94.8	97.0	86.9	86.6	83.3	82.3	84.2	84.2	79.8	78.6	76.1	72.4	71.9	69.7

Table 3
Proportion of re-vaccinees with anti-HBs ≥10 mIU/ml after booster vaccination

Post-booster vaccination period (years)	1 month	4 months	1	2	3	4	5	6	7	8	9	10
Total number of persons determined anti-HBs during 1998–2001	28	35	40	38	31	37	33	34	38	31	27	14
Individuals with anti-HBs ≥10 mIU/ml												
n	27	33	37	35	28	33	29	29	30	22	19	9
Percentage	96.4	94.3	92.5	92.1	90.3	89.2	87.9	85.3	78.9	71.0	70.4	64.3

after primary vaccination, the level of anti-HBs in 94.8% of vaccinees was ≥10 mIU/ml. However, the percentage of vaccinees with that level of anti-HBs decreased in parallel with the increase in post-vaccination period, and at 12 years after primary vaccination, the percentage had decreased to 69.7% (Table 2). Similarly, although 96.4% of re-vaccinees achieved ≥10 mIU/ml anti-HBs 1 month after booster vaccination, the percentage of re-vaccinees with ≥10 mIU/ml anti-HBs decreased in parallel with post-booster vaccination period, such that at 10 years after booster vaccination, the percentage was reduced to 64.3% (Table 3). We also compared the proportions of vaccinees with anti-HBs ≥10 mIU/ml after primary and booster vaccination (Tables 2 and 3) at 1 month, 1, 5, and 10 years postvaccination using the chi-square test. There were no significant differences in these proportions between the years.

Finally, anti-HBs levels at 1 month after completion of three doses of booster vaccination in 28 re-vaccinees were stratified by the time intervals between the primary and booster vaccination (Table 4). Twenty-three of 28 responded to the primary vaccination but anti-HBs levels became negative (i.e. <5 mIU/ml) in all these workers before booster vaccination. Of these 28 re-vaccinees, 27 achieved anti-HBs level ≥10 mIU/ml at 1 month after booster vaccination (Table 3). There was no relationship between the period between primary to booster vaccination and anti-HBs level at 1 month after booster vaccination. These results suggest that the degree of immunological memory for HBsAg may be different among each individual although we did not examine anti-HBs levels within days after a single dose of booster vaccination.

4. Discussion

In the present study, we have shown that levels of anti-HBs in primarily immunized vaccinees decreased rapidly in the first year and were <10 mIU/ml in almost 30% of vaccinees at 12 years. These results were consistent with those of previous studies conducted in other countries [4,5,9,15], suggesting that there are no race differences in the decrease

Table 4
Anti-HBs levels in 28 re-vaccinees stratified by the primary to booster vaccination period

Period (years)		Anti-HBs level (mIU/ml)	
Primary to booster vaccination	Anti-HBs-positive after primary vaccination	Before booster vaccination	One month after booster vaccination
2	1	0.9	103.9
2	1	3.6	108.4
2	1	4.6	141.2
2	1	1.4	381.8
2	1	0.9	405.1
3	2	1.1	≥1000
3	2	3.2	≥1000
3	1	3.8	769.6
3	1	0.7	≥1000
5	4	2.3	80.6
5	3	0.5	≥1000
5	3	3.3	≥1000
5	1	3.7	≥1000
5	NR	0.2	≥1000
6	4	0.6	136.6
6	3	4.5	≥1000
7	6	4.3	770.2
7	1	4.2	292.5
8	7	3.6	765.8
8	5	4.9	73.0
8	NR	0.5	≥1000
8	NR	0.6	8.5
10	8	3.6	108.6
11	10	0.9	433.1
11	8	2.4	≥1000
11	3	3.8	≥1000
11	NR	0.1	163.4
11	NR	0.1	≥1000

NR: no response to primary vaccination.

of anti-HBs level after primary vaccination. In re-vaccinees who had lost immunity or failed to express anti-HBs after primary vaccination, the distribution of anti-HBs levels at 1 month after booster vaccination was very similar to that in primarily immunized vaccinees. These results are consistent with several reports indicating that booster vaccination can promote rapid and adequate production of anti-HBs, even in individuals whose anti-HBs became negative after primary vaccination, based on the presence of memory B cells for HBsAg [10–13]. Therefore, we anticipated that the levels of anti-HBs in re-vaccinees might be sustained for much longer periods than in primarily immunized vaccinees. However, anti-HBs levels in re-vaccinees decreased as rapidly as in primarily immunized vaccinees. In contrast, individuals who had naturally acquired immunity against HBV maintained a higher level of anti-HBs than vaccinees, indicating that natural exposure to HBV can evoke a much longer lasting immunological response against HBsAg than regular three-dose HBsAg vaccination.

These findings support the recent efforts to establish more immunogenic vaccination protocols against HBV than the current regular HBsAg vaccination. Of these, the DNA-based or pre-S containing vaccination protocol is a possible candidate [16–18]. However, our results showed that additional booster immunization with three doses of HBsAg vaccines enabled 64.3% of re-vaccinees who had lost or failed to achieve an anti-HBs level after primary vaccination, to maintain a protective anti-HBs level of $\geq$10 mIU/ml for up to 10 years. In our study, we did not determine the serum levels of antibody to HBV core antigen (anti-HBc), which is a good indicator of previous exposure to HBV. In this regard, it should be remembered that an insidious exposure to HBV, which provides a natural booster effect, could influence the results of our study. However, this effect may be small because the percentages of vaccinees with anti-HBs $\geq$10 mIU/ml decreased in parallel with the increase in post-vaccination period as reported previously [4,5,9,15].

Since Japan is an intermediate endemic area of chronic HBV infection [19–21], the incidence of occupational exposure to HBV among healthcare workers is not rare. In fact, 275 of 1327 (20.7%) healthcare workers in our hospital appeared to have been previously exposed to HBV (Table 1). In addition, six healthcare workers in our hospital had needle-stick injuries from HBV carriers in 2001 (unpublished data). However, clinically significant infection, such as acute hepatitis, was not observed during the study period (1999–2001), which may be in part due to the policy used: the serum level of anti-HBs was immediately measured in healthcare workers after injury by needle prick and when it was found to be negative or <10 mIU/ml, they received an immunoglobulin injection containing a high titer of anti-HBs irrespective of the vaccination history. Several studies have indicated that a booster vaccination is not required for healthcare workers in whom adequate immunological priming has been achieved [4,10]. In addition, the fact that clinically significant infection was not observed during the study period could argue against the need for booster vaccination. However, at present, it is not clear whether individuals in whom anti-HBs levels become negative can retain life-time vaccine-induced immunological memory for HBsAg [4,5,8]. Therefore, further prospective studies are needed to answer this issue.

Our results demonstrated that anti-HBs level in re-vaccinees after additional booster vaccination decreased as rapidly as that in primarily immunized vaccinees. However, at least 64% of re-vaccinees maintained an anti-HBs level of 10 mIU/ml or more for up to 10 years. Taken together, these findings indicate that until more immunogenic or more durable vaccination protocols are established, additional booster immunization with three doses of HBsAg vaccine may be beneficial in healthcare workers whose anti-HBs levels have became negative or dropped below 10 mIU/ml after primary vaccination. With regard to the cost–benefit, it should be clarified whether a three-dose protocol of booster vaccination is actually required to maintain the seroprotective level of anti-HBs or whether a single-dose booster vaccination is sufficient, as reported previously [11–13]. In addition, it may be difficult to fix the period at which booster vaccination is given, because anti-HBs levels at 1 month after booster vaccination did not correlate with the period between the primary and booster vaccination, and because anti-HBs levels and persistence of anti-HBs level at $\geq$10 mIU/ml after primary vaccination varied among individuals. Therefore, we recommend monitoring of anti-HBs levels in vaccinees every 1 or 2 years after primary vaccination and that booster vaccination should be given when anti-HBs becomes negative or below 10 mIU/ml.

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Letters to the Editor

Consumption of wild boar linked to cases of hepatitis E

To the Editor:

A zoonotic transmission of hepatitis E virus (HEV) has been suggested, based on indirect [1,2] and direct [3] evidence. However, the extent and impact of zoonotic transmission of HEV infection have not been fully elucidated and should be further clarified.

We encountered two acute hepatitis patients who visited one of our hospitals in April 2003 (Table 1; cases 1 and 2). Serum samples were all negative for Hepatitis A-Immunoglobulin (Ig) M antibody, hepatitis B surface antigen and hepatitis C virus RNA. However, they were positive for HEV-IgM and -IgG antibodies as well as HEV-RNA, indicating a diagnosis of acute hepatitis E.

Careful history taking revealed that the two patients had a single occasion of eating at a barbecue party where they exclusively ate charcoal-grilled, but partially undercooked, wild boar (*Sus scrofa leucomystax*) meat on March 13 (39 days prior to the admission of case 1).

A survey was done on August 26 (166 days after the meeting) for a total of 12 members of a local senior association who participated in the party. All participants were males with a median age of 79 years (Table 1). None of the members had traveled to HEV endemic areas. Surprisingly, eight persons (67%) were positive for HEV-IgM and 11 persons (92%) were positive for HEV-IgG antibodies. These results indicate that at least eight persons were recently infected by HEV, suggesting that the barbecue party was the occasion of the infection. It is even possible that all but one (case 12) became infected at the party in March but HEV-IgM subsequently disappeared, as of case 1. Two individuals (cases 3 and 4) had been admitted to other hospitals because of acute hepatitis. One (case 5) visited a clinic on May 15 where an elevation of liver enzymes was noted. Although no acute phase serum samples of such patients were available, these three patients positive for HEV-IgM, are considered to be infected with acute hepatitis E. All five patients recovered within 3–4 weeks.

A phylogenetic analysis of 317 nucleotide within the open reading frame 1 [4] performed on the HEV isolates obtained from the first two patients sera revealed that the strains belong to genotype III, with 99.4% homology to each other. These isolates were neighbored by, yet with a notable distance, some Japanese isolates, such as JRA1 (AB003430; 92.1% homology) and JJT-Kan (AB091394; 92.1% homology),

Table 1
Data of members who ingested boar meat on March 13

Data obtained in April and May						Case No	Age (year)	Sex	Sx	Survey on August 26 (166 days after boar meat ingestion)			
HEV-IgG	HEV-IgM	HEV-RNA	ALT (IU/mL)	T.Bil (mg/dL)	Time* (Days)					ALT (IU/mL)	HEV-IgG	HEV-IgM	HEV-RNA
+	+	+	1,172	1.9	39	1	69	M	Yes	10	+	−	−
+	+	+	751	0.9	43	2	69	M	Yes	15	+	+	−
			290	2.6	40	3	78	M	Yes	15	+	+	−
			531	3.9	36	4	80	M	Yes	6	+	+	−
			312	1.2	55	5	81	M	Yes	12	+	+	−
						6	80	M	No	13	+	+	−
						7	64	M	No	11	+	+	−
						8	81	M	No	12	+	+	−
						9	78	M	No	36	+	−	−
						10	75	M	No	9	+	−	−
						11	82	M	No	23	+	−	−
						12	80	M	No	18	−	−	−

Sx., Symptoms; ALT, alanine aminotransferase; ALP, alkaline phosphatase; T.Bil, total bilirubin; M, male; +, positive; −, negative.
GenBank accession numbers obtained are: AY427956 for Case 1 (ENK-NGS03) and AY427957 for Case 2 (EMN-NGS03).
* ; Time of blood examination (days after boar meat ingestion) when peak ALT (for cases 1–4) levels were observed. "Yes" indicates existence of fatigue or jaundice. Blanks indicate data not available.