Table 2 Univariate and multivariate analysis of the risk ratios (RR) for hepatocellular carcinoma

	Univariate		Multivariate	
	RR (95% CI)	*P*-value	RR (95% CI)	*P*-value
Age (compared with <35 years)	12.82 (1.72–100.00)	0.013*	8.20 (1.03–66.67)	0.047*
Sex (compared with female)	2.10 (0.80–5.53)	0.134	2.28 (0.53–9.84)	0.269
Habitual heavy drinking† (compared with no drinking)	1.32 (0.17–10.10)	0.787	1.96 (0.22–17.24)	0.544
Alanine aminotransferase (compared with <80 IU/L)	2.18 (0.85–5.64)	0.107	2.72 (0.73–10.15)	0.136
Interferon (compared with no therapy)	2.20 (0.29–16.56)	0.444	1.78 (0.21–15.06)	1.776
Hepatitis B virus DNA (compared with <6.0 LGE/mL)	3.44 (1.07–7.46)	0.035*	3.08 (1.03–9.17)	0.045*
Staging (compared with mild)	6.80 (1.59–29.41)	0.010*	7.87 (1.54–40.00)	0.013*
Grading (compared with mild)	1.44 (0.59–3.53)	0.423	1.54 (0.45–5.21)	0.491

*Statistically significant. †Habitual drinking was defined as an average daily consumption of an amount equivalent to 80 g/day of pure ethanol over a period of more than 5 years. CI, confidence interval; LGE, logarithm of the genome equivalent.

Table 3 Clinicopathological findings in patients according to viral load

	Viral load		
Characteristic	High (≥6.0 LGE/mL)	Low (<6.0 LGE/mL)	*P*-value
Age (years; mean ± SD)	38 ± 10	40 ± 12	0.542
Sex (male/female)	8/27	5/23	0.625
Habitual heavy drinking (yes/no)†	3/32	2/26	0.835
Alanine aminotransferase (≥80/<80 IU/L)	22/13	9/19	0.015*
Staging (mild/severe)	12/23	15/13	0.124
Grading (mild/severe)	11/24	16/12	0.040*
Interferon (yes/no)	5/30	1/27	0.130

*Statistically significant. †Habitual drinking was defined as an average daily consumption of an amount equivalent to 80 g/day of pure ethanol over a period of more than 5 years.

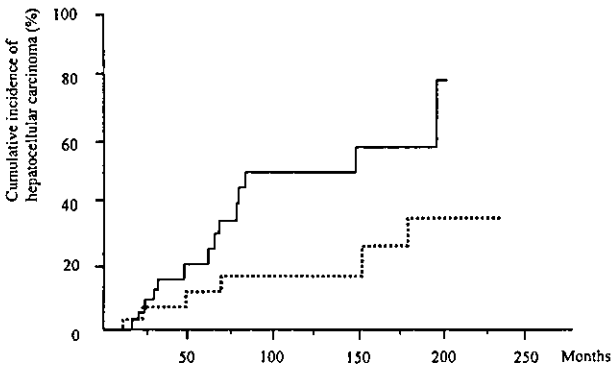

Figure 2 Cumulative incidence of hepatocellular carcinoma (HCC) based on the hepatitis B virus (HBV)-DNA levels in patients with chronic hepatitis B. The cumulative incidence of HCC in (—) 28 patients with high viral load (≥6.0 LGE/mL) was significantly higher than in (---) 35 patients with low viral load (<6.0 LGE/mL; $P = 0.0285$).

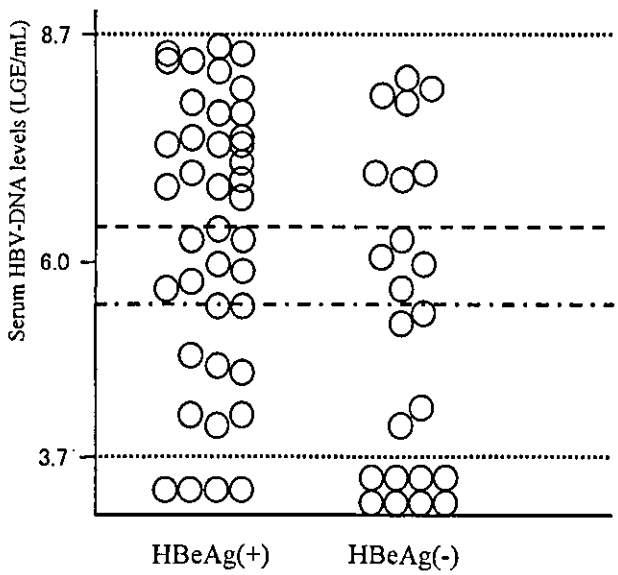

Figure 3 Relationship between the presence or absence of hepatitis B early antigen (HBeAg) and hepatitis B virus (HBV)-DNA levels. Mean serum level of HBV-DNA in patients who were HBeAg-positive was much higher than the value in those patients who were HBeAg-negative (6.4 ± 1.6 LGE/mL *vs* 5.5 ± 1.8 LGE/mL; $P = 0.032$).

DISCUSSION

To identify the relationship of HCC with HBV-related chronic liver disease, including cirrhosis, we investigated the development of HCC in patients infected with

HBV and assessed the associated risk factors for HCC. The results may prove useful in the development of strategies in preventive medicine programs for HCC, and also for identifying screening approaches for the early detection of HCC.

In the current study, the cumulative incidence of HCC in 73 patients with HBV-associated liver disease was almost the same as that found in a previous study.[2] Among the risk factors for HCC that were analyzed, a high viral load of HBV-DNA, together with age and histological fibrosis, were found to be linked to the occurrence of HCC, based on both univariate and multivariate analyses.

It has been reported that the viral load of HBV-DNA is often associated with the presence or absence of HBeAg.[9,11,17] We also found that the viral load of the HBeAg-positive group was higher than that of the HBeAg-negative group (Fig. 3) and that viral load is closely related to the serum ALT level and the grade of histological inflammation (Table 3). These results are in accordance with other published studies.[9]

During the course of chronic HBV infection, the presence of HBeAg is often associated with active and continuing liver disease, whereas HBeAg seroconversion to anti-HBe often coincides with loss of serum HBV-DNA, normalization of liver biochemistry, clinical remission and subsidence of hepatic inflammatory activity.[12,13,18–21] Another study suggests that spontaneous HBeAg seroconversion confers a favorable long-term outcome in the majority of patients. However, HBeAg reversion or HBeAg-negative hepatitis may develop in some patients who subsequently show an increased risk of cirrhosis or HCC, as compared with patients with sustained remission.[22]

Most patients with HBeAg-negative, anti-HBe-positive and HBV-DNA positive chronic hepatitis B have HBV variants with mutations in the precore or core promoter region. In patients with HBV variants, progressive liver damage occurs in parallel with relatively high levels of viremia.[14,22] Ohkubo *et al.* reported that the baseline level of serum HBV-DNA did influence the survival time of patients with HBV-associated HCC, even for patients with HBeAg-negative disease.[23] However, the current study did not show a statistically significant difference in the occurrence of HCC between the high and low viral load groups, perhaps because of the small number of HBeAg-negative patients ($P = 0.13$, data not shown). In HBV-related cirrhotic patients, HBV-DNA was the strongest predictive factor for the development of HCC[24] and a high viral load was found to be associated with a high recurrence rate of HCC after surgical resection in patients with HBV.[9] Thus, it is conceivable that patients with a high viral load may have a high potential for hepatocarcinogenesis.

Chronic infection with HBV is involved in hepatocarcinogenesis by induction of a long-term process of liver cell necrosis and inflammation,[25,26] as well as by *cis*- and *trans*-activation effects on cellular genes.[27] However, there are many theories to explain hepatocarcinogenesis in HBV infection, and the detailed mechanism of hepatocarcinogenesis remains unclear. Even though HCC develops in some HBV-infected patients in the absence of cirrhosis, a direct carcinogenic role of HBV has been suggested. Integration of the HBV genome into cellular DNA has been found in the majority of HBV-induced HCC patients.[28] In addition, it has been recently reported that, compared with hepatitis C patients, cirrhotic patients infected with HBV are at a higher risk of developing an infiltrative and more aggressive type of HCC that is independent of duration or stage of cirrhosis and may reflect more direct viral carcinogenesis.[29]

It is generally agreed that IFN therapy is effective in patients with chronic HBV infection in terms of virological and histological remission.[6,30,31] Antiviral treatment with nucleoside analogs, such as lamivudine or adefovir, has recently been implemented in patients with HBV. In clinical trials, treatment with lamivudine or adefovir improved histological liver abnormalities, reduced serum HBV-DNA levels and normalized ALT levels.[32–36] In the current study, it was found that high viral load is a risk factor for hepatocarcinogenesis in patients with chronic HBV. However, it remains unclear whether antiviral therapeutic agents, such as IFN or nucleoside analogs, prevent HCC with chronic HBV because of the limited number of clinical studies. It has been suggested that the integration of viral DNA into the host genome is essential for carcinogenesis through the activation or suppression of several genes,[37] and that treatment with IFN or nucleoside analogs cannot affect viral DNA that has already been integrated. To clarify the effectiveness of antiviral therapy in carcinogenesis further studies and follow up are required.

In the current study, viral load was found to be an independent risk factor for HCC in patients with chronic HBV. Although the independent role of the level of viremia observed in the current study must be validated by further investigations, more intensive monitoring of HCC should be considered in chronic HBV patients with a high viral load.

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LIVER

Bone marrow engraftment in a rodent model of chemical carcinogenesis but no role in the histogenesis of hepatocellular carcinoma

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Background and aim: Recent studies indicated that hepatic stem cells in the bone marrow could differentiate into mature hepatocytes, suggesting that bone marrow cells could be used for replacement of damaged hepatocytes in a variety of liver diseases. Hepatocellular carcinoma (HCC) is thought to arise from hepatic stem cells. In this study, we investigated the malignant potential of hepatic stem cells derived from the bone marrow in a mouse model of chemical hepatocarcinogenesis.

Methods: Bone marrow cells were obtained from the male β-galactosidase (β-gal) transgenic mouse and transplanted into female recipient mice. Hepatocarcinogenesis was induced by a year of treatment with diethylnitrosamine and phenobarbital (NDEA/PB). One year later, the liver was removed from each treated mouse and evaluated by x-gal staining, immunohistochemistry, and fluorescence in situ hybridisation (FISH).

Results: Forty per cent of recipient mice survived and developed multiple HCC. Clusters of β-gal positive mature hepatocytes were detected sporadically in the entire liver of NDEA/PB treated mice who underwent bone marrow transplantation (BMT) with while no such hepatocytes were identified in the liver of BMT mice that were not treated with NDEA/PB. The Y chromosome was detected with the same frequency as the donor male liver in clusters of β-gal positive mature hepatocytes by FISH. However, no HCC was positive for β-gal or the Y chromosome. Immunohistochemically, β-gal positive mature hepatocytes did not express CD34 or α-fetoprotein.

Conclusions: Our results suggest that hepatic stem cells derived from the bone marrow have low malignant potential, at least in our model.

Although patients at high risk of hepatocellular carcinoma (HCC) are well defined in the clinical setting, the process of hepatocarcinogenesis is not well understood.[1 2] Hepatitis B or C virus can induce chronic hepatitis and potentially results in liver cirrhosis and HCC, and patients with these viral infections are the most frequent among HCC patients.[3] However, the consequences of viral infection in the process of hepatocarcinogenesis are not clearly understood. In this regard, the original cell that develops into a cancerous cell in HCC is debated.[4] Two cell lineages have been considered as candidates: the first is the hepatic stem cell and the second is the mature hepatocyte. However, there is no clear evidence on which cell is directly involved in the development of HCC.[5 6]

Extensive studies have been conducted on the hepatic stem cell but the results are controversial. Several candidate hepatic stem cells have been described in a rodent experimental model (including oval cells, liver epithelial cells, and small hepatocytes).[7–9] Previous studies indicated that bone marrow cells can differentiate into oval cells in rodents and that a similar process could possibly take place in humans.[10–12] If this scenario is correct, isolation of hepatic stem cells from bone marrow cells could be a valuable strategy for future replacement therapy of damaged or malfunctioned hapatocytes.[10] However, the safety and efficacy of hepatic stem cells derived from bone marrow cells should be adequately confirmed before any such therapies are tested in humans.

In an attempt to assess hepatic stem cells, in the present study we investigated the malignant potential of hepatic stem cells derived from bone marrow in vivo. To identify hepatic stem cells, bone marrow cells of β-galactosidase (β-gal) transgenic male mice were transplanted into recipient female mice. After bone marrow transplantation (BMT), HCC was induced in the recipients by chemical hepatocarcinogenic compounds and the presence of β-gal and the Y chromosome were evaluated in HCC.

MATERIALS AND METHODS

Bone marrow transplantation

Six week old male B6-129S-Gtrosa and female B6-129-F2 mice were purchased from Jackson Laboratory (Bar Habor, Maine, USA). B6-129S-Gtrosa mice express transgenic β-gal in the whole body and B6-129-F2 mice are the parental strain of B6-129S-Gtrosa.[13] Animal experiments were performed in accordance with institutional guidelines, and the study was approved by the ethics committee of Nagasaki University. BMT was performed as reported previously.[14] Briefly, the thigh was removed from a dead male B6-129S-Gtrosa, and bone marrow cells were harvested and suspended in phosphate buffered saline at a density of 1×10^7 cells/ml. Female B6-129-F2 mice were irradiated sublethally and 5×10^6 of the above bone marrow cells were injected via the tail vein. To confirm successful transplantation of donor bone marrow cells, a sample of recipient bone marrow cells was obtained from B6-129-F2 mice after sacrifice and examined for x-gal staining.

Abbreviations: AFP, α-fetoprotein; β-gal, β-galactosidase; BMT, bone marrow transplantation; FISH, fluorescence in situ hybridisation; HCC, hepatocellular carcinoma; NDEA/PB, diethylnitrosamine/phenobarbital; H&E, haematoxylin-eosin

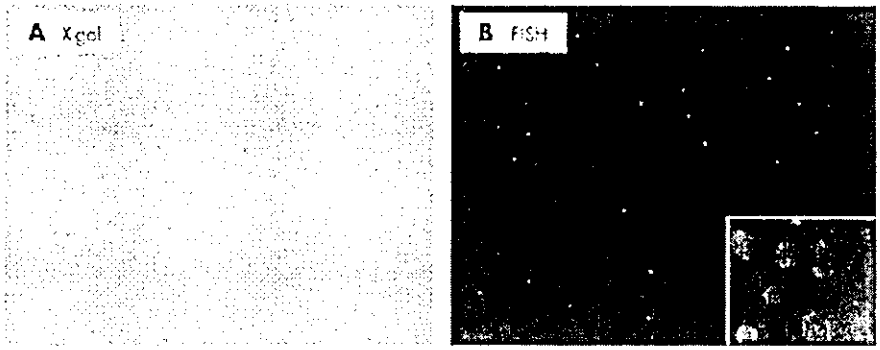

Figure 1 X-gal staining and fluorescence in situ hybridisation (FISH) for the Y chromosome in the liver of the donor mouse. To confirm expression of β-galactosidase and status of the Y chromosome in donor mouse, x-gal staining and FISH were performed. (A) X-gal staining (magnification ×100). (B) FISH for the Y chromosome (magnification ×100). Inset; high magnification of FISH with counterstaining by DAPI (magnification ×400).

Chemically induced hepatocarcinogenesis

To induce HCC, two weeks after BMT, 80 mg/l diethylnitrosamine (NDEA; Sigma, St Louis, Missouri, USA) was added to the drinking water of B6-129-F2 recipient mice for six weeks to initiate the process of hepatocarcinogenesis. This was followed by administration of 700 mg/l phenobarbital (PB; Wako, Tokyo, Japan) as a tumour promoting agent, until sacrifice. Ten bone marrow transplanted and 10 non-transplanted mice were each treated with NDEA/PB, and four mice who underwent BMT but not NDEA/PB treatment and four mice who received no treatment were maintained under the same conditions as controls.

X-gal staining

One year after the start of NDEA, mice who survived were sacrificed and the liver and bone marrow harvested. The liver was fixed in 10% formalin for 24 hours and paraffin embedded. Simultaneously, fresh frozen sections were also prepared. Routine histology was performed with haematoxylin-eosin (H&E) staining of both formalin fixed/paraffin embedded and fresh frozen sections. To identify bone marrow derived cells in the liver specimens, every fifth slide of 5 μm thick serial sections (total 125 slides) was stained for x-gal. X-gal staining kit (Gene Therapy Systems, San Diego, California, USA) was used with fresh frozen sections of the liver removed from each group according to the instructions provided by the manufacturer.

Immunohistochemistry

Immunohistochemistry was performed using antimouse CD34 or antimouse α-fetoprotein (AFP) antibody as the primary antibody. Tissue samples of the fresh frozen liver sections were cut into 5 μm thick sections and mounted on aminopropyltriethoxysilane coated glass slides. The streptavidin-biotin method (Histofine Staining Kit; Nichirei Company, Tokyo, Japan) was used for immunohistochemical detection, as described previously.[15] X-gal and immunohistochemically treated slides were counterstained with haematoxylin. Numbers of clusters or cells positively stained in each section were counted and mean (SD) values were calculated for each sample.

Table 1 Survival rate and success of BMT

Treatment	Survival [n]	β-gal positive/negative bone marrow cells
(1) BMT(−), NDEA/PB(−)	100% (4/4)	0%
(2) BMT(+), NDEA/PB(−)	100% (4/4)	95%
(3) BMT(−), NDEA/PB(+)	50% (5/10)	0%
(4) BMT(+), NDEA/PB(+)	40% (4/10)	91%

BMT, bone marrow transplantation, NDEA, diethylnitrosamine, PB, phenobarbital; β-gal, β-galactosidase.

Fluorescence in situ hybridisation

Because BMT was performed from male donor mice to female recipient mice, the transplanted bone marrow derived cells could be recognised in the recipient by the presence of the Y chromosome in the nucleus. Therefore, fluorescence in situ hybridisation (FISH) for the mouse Y chromosome was conducted to detect the transplanted bone marrow derived cells. Liver specimens of fresh frozen sections were pretreated using the Pretreatment Reagent Kit (Vysis, Downers Grove, Illinois, USA) according to the instructions supplied by the manufacturer, and hybridised with a mouse Y chromosome probe (Cambio, Cambridge, UK). Treated slides were observed under fluorescent microscopy.

RESULTS

X-gal staining and FISH of donor mice

In this study, we used the male B6-129S-Gtrosa mouse as a donor. It has been reported previously that this mouse expresses β-gal throughout the whole body.[13] To confirm this, we performed x-gal staining and FISH for the Y chromosome in the liver. All hepatocytes were positive for x-gal staining (fig 1A) and 98% of bone marrow cells were x-gal stained (data not shown). FISH revealed that 28% of hepatocyte nuclei were positive for the Y chromosome (fig 1B). A positive FISH signal was detected in the nucleus which was confirmed by counterstaining with DAPI (fig 1B, inset). In addition, no nucleus showed two or more signals.

Survival rate

We first evaluated the survival rate of mice that underwent BMT and/or NDEA/PB treatment (table 1). All (100%, 4/4 each) control mice and mice that underwent BMT only were still alive at the end of the study. However, only five of 10 (50%) mice in the NDEA/PB treated group that did not undergo BMT were still alive at the end of the one year study period. The survival rate of mice subjected to BMT and NDEA/PB treatment was the lowest (4/10 mice, 40%).

Bone marrow transplantation

To confirm bone marrow replacement with β-gal gene transduced cells, bone marrow cells derived from each group of recipient mice were subjected to x-gal staining (fig 2A, 2B). Red blood cells were excluded by careful microscopic observation and only nuclear cells were examined. No x-gal stained bone marrow nuclear cells were found in mice that did not receive BMT. In contrast, 91–95% of bone marrow

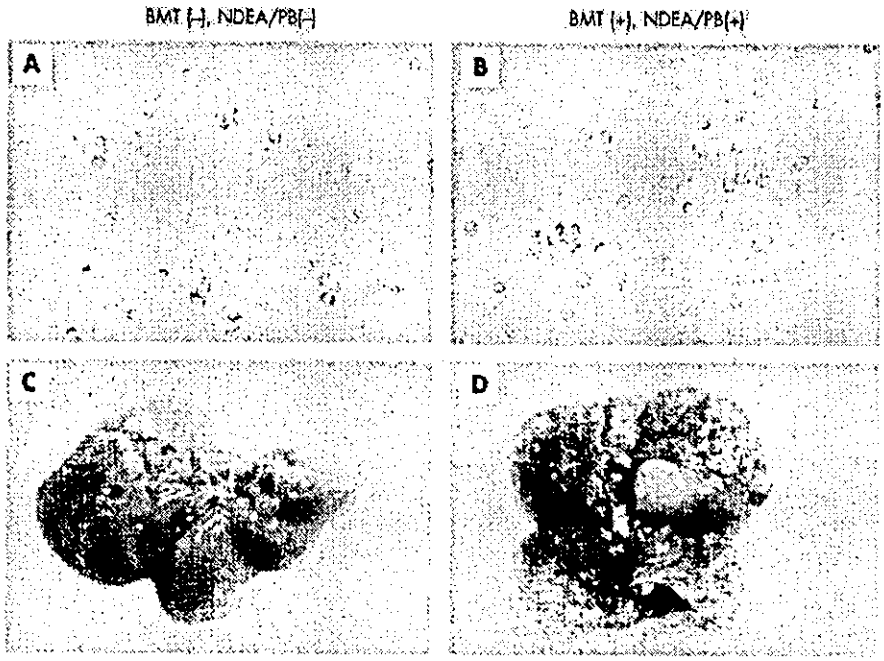

Figure 2 X-gal staining of bone marrow cells and hepatocellular carcinoma development in mice that underwent bone marrow transplantation (BMT) and diethylnitrosamine/phenobarbital (NDEA/PB) treatment. One year after the start of treatment, bone marrow cells were removed from each mouse and stained with x-gal. β-Gal positive cells appear as blue stained cells. Representative photographs are shown. (A) Bone marrow cells derived from a non-treated control mouse. (B) BMT and NDEA/PB treated mouse. (C) The liver removed from a non-treated control mouse. (D) BMT and NDEA/PB treated mice (magnification A and B, ×200).

cells were x-gal stained in mice that received BMT with or without NDEA/PB treatment (table 1). These results indicated successful BMT in this study.

Histopathological analysis of the liver of recipient mice

NDEA/PB treatment resulted in the appearance of multiple macroscopic tumours in the liver after one year of treatment in both BMT and non-BMT mice (fig 2C, 2D). H&E stained sections of these tumours confirmed they were HCC (see fig 6A), based on the criteria defined previously.[16] No other types of liver tumours, such as hepatoblastoma or cholangiocellular carcinoma, were noted in our experiment. Apart from HCC, abundant denatured hepatocytes, oval cells, cysts, and bile duct proliferation were observed in NDEA/PB treated mice compared with control livers.

Phenotypic analysis by x-gal staining, immunohistochemistry, and FISH

In a preliminary study, we sacrificed two BMT mice and liver samples were removed before NDEA/PB treatment. In these samples, β-gal positive hepatocytes were not identified (data not shown). Furthermore, no β-gal positive mature hepatocytes were detected in the livers of BMT mice that were not treated with NDEA/PB one year after BMT except for hematic cells in blood vessels (fig 3). However, x-gal staining of fresh frozen liver samples of four BMT mice treated with NDEA/PB showed sporadic clusters of β-gal positive mature hepatocytes (fig 4A, 4B). The number of these clusters in four mice was mean 0.18 (SD 0.10) in 53 (15) mm^2 sections (0.34 (0.10) cluster/cm^2, n = 125 slides). Furthermore, 17 (4.2) hepatocytes were present within the diameter of a cluster. Immunohistochemical analysis showed that β-gal positive

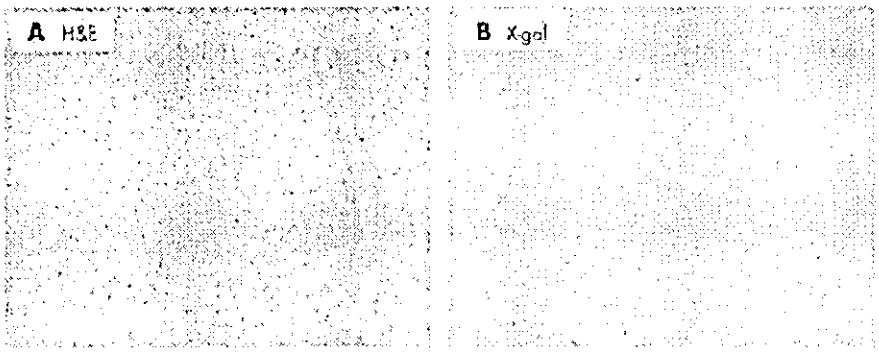

Figure 3 Haematoxylin-eosin (H&E) and x-gal stained liver section obtained from a mouse who underwent bone marrow transplantation (BMT) but not diethylnitrosamine/phenobarbital treatment. One year after BMT, the mouse was sacrificed and the liver removed and stained, as described in materials and methods. (A) H&E staining. (B) X-gal staining (magnification ×100).

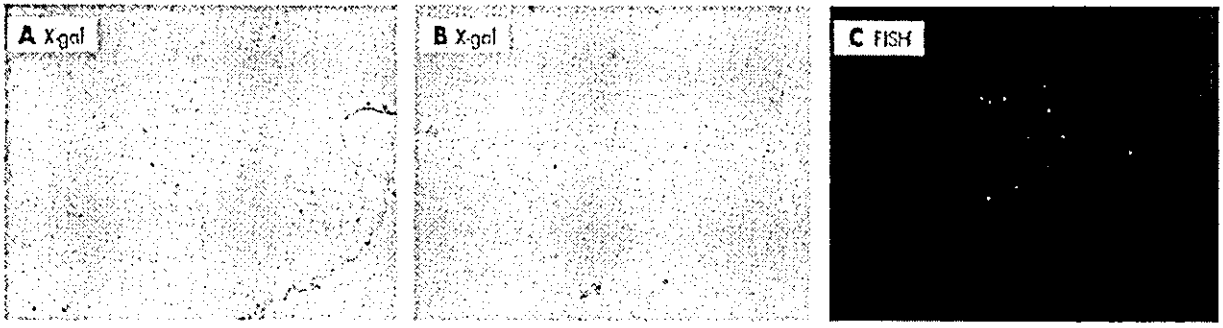

Figure 4 Appearance of β-galactosidase (β-gal) positive cell clusters and confirmation of the Y chromosome. (A) Numbers of β-gal positive cell clusters were counted in each x-gal stained slide examined under low magnification (×40). (B) Representative photograph of β-gal positive cell cluster under high magnification (×100). (C) Fluorescence in situ hybridisation (FISH) was performed as described in materials and methods to confirm the cell origin (magnification ×100).

mature hepatocytes were negatively stained for CD34 and AFP (fig 5). In addition, FISH showed that β-gal positive mature hepatocytes were positive for the Y chromosome with the same frequency as the liver of donor male mice (26%, fig 4C). However, no HCC was positive for x-gal staining or FISH for the Y chromosome (fig 6, n = 10 tumours).

DISCUSSION

The liver is classified as a conditionally renewing tissue and under normal circumstances nearly all hepatocytes proliferate quiescently, with only 0.3–0.5% of cell showing active cell division.[17] Under normal physiological conditions, there is no need for hepatic stem cells, and they only become evident on demand (for example, in circumstances associated with severe hepatic liver damage and coexistent impaired hepatocyte division). Previous studies demonstrated that oval cells, which are considered hepatic stem cells, appear after chemically induced liver injury.[18] Although the oval cell clearly appears in such conditions, its precise origin and existence under normal conditions remains unclear. Recent studies reported that bone marrow cells could differentiate into oval cells.[12 19] This finding indicates not only the origin of the oval cell but also the feasibility of in vivo labelling, at least in part, of oval cells.

In this study, we labelled bone marrow cells by β-gal staining. All hepatocytes were positive for β-gal in donor mice. Also, sensitivity of FISH for the Y chromosome was almost the same as that reported previously[11 12] while this sensitivity was inadequate for the detection of donor cells. Thus we believe that our methods for tracing bone marrow cells of donor mice were acceptable in the present study. After BMT, clusters of β-gal positive cells appeared only in BMT mice treated with NDEA/PB and not in BMT mice who did not receive NDEA/PB. These results suggest that in our

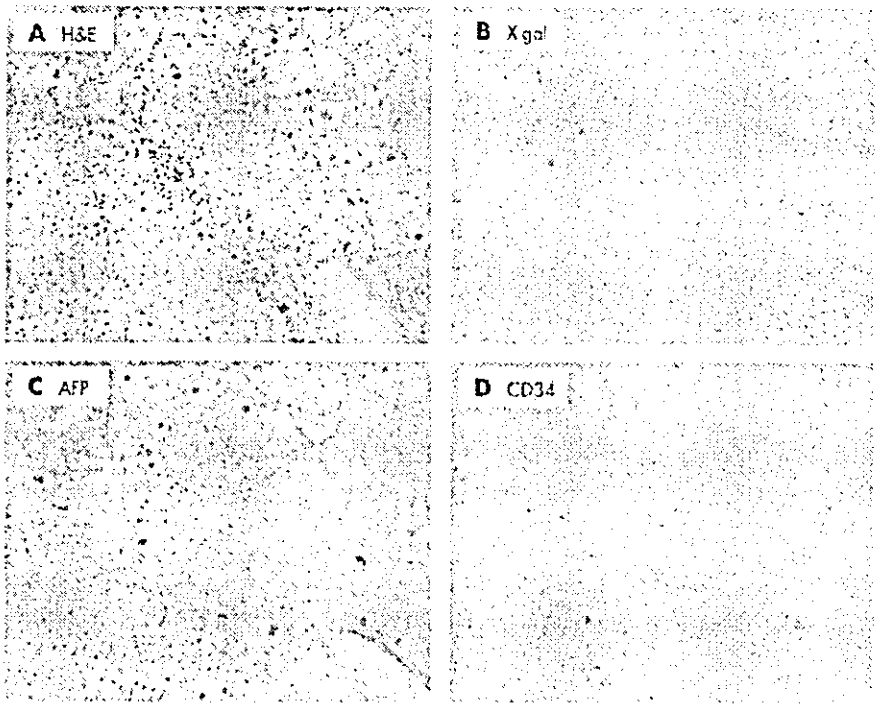

Figure 5 Immunohistochemical analysis of β-galactosidase (β-gal) positive cell clusters in bone marrow transplantation and diethylnitrosamine/phenobarbital treated mice. The phenotype of the β-gal positive cluster was determined by immunostaining. (A) Haematoxylin-eosin (H&E) staining. (B) X-gal staining. (D) Immunostaining with antimouse CD34 antibody. (C) Antimouse α-fetoprotein (AFP) (magnification ×100).

Figure 6 Histopathological analysis and x-gal staining of the tumour. Hepatocellular carcinoma (HCC) was induced by one year of treatment with diethylnitrosamine/phenobarbital (NDEA/PB), as described in materials and methods. Extracted livers were observed histopathologically under the microscope (×100). (A) Representative photograph of haematoxylin-eosin (H&E) stained liver section obtained from a bone marrow transplantation and NDEA/PB treated mouse. T, HCC tumour; N, adjacent normal liver tissue. (B) X-gal staining of the serial section without any counterstaining. β-Galactosidase positive cells appear as blue stained cells (×100). (C) Fluorescence in situ hybridisation (FISH) of the serial section (magnification ×100).

model, bone marrow cells can differentiate into mature hepatocytes under limited conditions. Bone marrow derived hepatic stem cells do not seem to be required for normal hepatocyte substitution. In addition, all hepatic stem cells derived from bone marrow cells may not have been labelled by this method and pre-existent hepatic stem cells, the origin of which is not clear, may be present in the liver. It has been reported that a stem cell of a particular tissue can differentiate into another tissue.[20 21] A stem cell network may exist in the whole body and involve each other in variant tissues. Recently, some investigators have reported that cell fusion between a hepatic lineage cell and a bone marrow cell was the source of bone marrow derived hepatocytes in the $FAH^{-/-}$ mouse model.[22 23] However, other studies have shown that such fusion does not always play a role in haematopoietic to epithelial cell engraftment.[24] Therefore, cell fusion may occur under specific conditions such as in the $FAH^{-/-}$ mouse model.[25] Indeed, in the present study, we found that in β-gal positive hepatocytes, no nucleus had two or more Y chromosomes by FISH. This finding indicates that transdifferentiation, rather than cell fusion, was the main process in our model.

In this study, our interest was focused on the original cell lineage of HCC. Two major hypotheses (the "stem cell theory" and the "hepatocytic theory") have been discussed for almost 20 years.[6 26 27] Debate has centred on whether hepatocytes in dysplastic nodules are responsible for HCCs through a process of dedifferentiation and proliferation, or whether oval cells are the prime targets for malignant changes after a differentiative "block", as proposed in some animal models.[5 28] Possible involvement of oval cells in hepatocarcinogenesis is based on the following: (1) massive existence of oval cells in an animal rodent hepatocarcinogenic model[29]; (2) development of HCC after transformation of oval cells[30 31]; and (3) occurrence of mixed hepatocellular and cholangiocarcinomatous tumours (oval cell exhibits bipotential developmental ability).[32 33] However, the relationship between oval cells and cancer is only circumstantial. In this study, no HCC was positive for β-gal, and all β-gal positive cell clusters were oval cells or mature hepatocytes at the point of sacrifice, even after long term carcinogenic induction. These results allow exclusion of the stem cell theory and acceptance of the hepatocytic theory in hepatocarcinogenesis. However, as all hepatic stem cells might not have been labelled by our method, as mentioned above, we cannot completely exclude the stem cell theory. Although our results may be limited to BMT mice treated with NDEA/PB, we can state that the malignant potential of the hepatic stem cell derived from bone marrow seems to be low. Patients at high risk of HCC are clearly defined in the clinical settings, and viral hepatitis and/or related cirrhosis are the most critical factors.[3 34] We selected the NDEA/PB model in the present study as it is a useful model of human hepatocarcinogenesis caused by viral hepatitis as nitrate and nitrosamine synthesis is increased in viral hepatitis.[35 36] Further studies are needed to clarify the precise interaction of bone marrow cells with hepatic regeneration and carcinogenesis using other animal models or human studies.

Liver transplantation has already been introduced for the treatment of patients with chronic or acute hepatic failure and congenital or malignant liver disease, and a large proportion of such patients have previously been reported to benefit from this procedure.[37] However, there are some problems with liver transplantation, including shortage of donors.[38] Cell based therapy is being developed for replacement of damaged or malfunctioned hepatocytes.[39] Bone marrow cells may potentially be used in cell based replacement therapy or gene delivery systems. Under these circumstances, our results indicate that stem cell therapy is safe.

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Diverse efficacy of vaccination therapy using the α-fetoprotein gene against mouse hepatocellular carcinoma

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Abstract. Antitumor vaccination therapy approaches using naked plasmid DNA or recombinant viruses encoding tumor-associated antigens are currently in development. In the present study, we examined the therapeutic efficacy of vaccination using the mouse α-fetoprotein (AFP) gene in mouse hepatocellular carcinoma (HCC) cells. C57L/J or C3H/HeN mice were primed with an injection of naked plasmid DNA expressing mouse AFP followed by a booster of replication-defective adenovirus expressing mouse AFP (plasmid-AFP prime/adenovirus-AFP booster vaccination). The mice were then challenged with high AFP-producing Hepa1-6 cells or low AFP-producing MH134 cells, respectively, and the tumor growth rate was monitored. Plasmid-AFP prime/adenovirus-AFP booster vaccination promoted protective immunity against Hepa1-6 cells, and significantly increased the number of interferon-γ-producing splenic cells in C57L/J mice. In addition, this vaccination protocol repressed the growth of pre-established Hepa1-6 tumors in C57L/J mice. However, plasmid-AFP prime/adenovirus-AFP booster vaccination did not induce protective immunity against MH134 cells in C3H/HeN mice. These results suggest that vaccination with the AFP gene is a promising strategy to treat HCC, but its outcome may be affected by the level of AFP expression in HCC or by the immunological response of the host.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common fatal malignancies worldwide, and is especially common in several parts of Asia and Africa (1). Although advances in medical technology have permitted the early recognition and treatment of HCC (2,3), the 5-year survival rate for HCC patients has barely reached 40% and the annual death rate from HCC exceeds 30,000 in Japan (3,4). Therefore, there is an urgent need to develop new approaches to treat HCC.

In recent years, increasing efforts have been made to use antitumor vaccination strategies, including genetically modified tumor cells (5,6), dendritic cells (DC) that are either pulsed or transduced with tumor-associated antigens (7-9), synthetic peptides containing tumor-specific epitopes (10,11), and naked plasmid DNA or recombinant viruses encoding tumor-associated antigens (12,13). All of these antitumor vaccination approaches aim to induce specific immunological responses to tumor-associated antigens, resulting in the destruction of tumor cells and the protection of patients from relapses.

α-fetoprotein (AFP) is an oncofetal protein that is expressed in fetal liver and down-regulated after birth, but it is frequently re-expressed in HCC (14). Because of its specific expression in HCC, the enhancer/promoter region of the AFP gene has been used in gene therapy approaches to HCC (15-18). Recently, it has been reported that AFP-specific immune responses can be detected in patients with AFP-expressing HCC (19). This finding suggests that AFP is a possible target for HCC-specific vaccination therapy. Vollmer *et al* first reported that vaccination in mice with dendritic cells (DC) transduced with the AFP gene generated AFP-specific and protective immunity against lymphoma cells expressing an exogenous AFP gene (20). In addition, using a similar model, the same group reported that vaccination with an AFP-expressing naked plasmid followed by a booster vaccination with an AFP-expressing adenovirus also promoted AFP-specific and protective immunity, but that vaccination with a naked plasmid alone did not (21). In contrast, Grimm *et al* (22) reported that vaccination with AFP-expressing naked plasmid DNA together with plasmids expressing interleukin-12 (IL-12) and granulocyte-macrophage colony stimulating factor (GM-CSF) significantly repressed the growth of pre-established Hepa1-6 hepatoma, which produces

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Key words: hepatocellular carcinoma, α-fetoprotein gene, vaccination

large amounts of AFP endogenously. Taken together, these results suggest that immunotherapy with the AFP gene could be a possible approach to the treatment of HCC.

Clinically, the amount of AFP production in each HCC is different, and the immunological background in each patient is not the same. Therefore, in the present study, we examined whether combined vaccination with naked DNA and adenovirus expressing AFP, which is reported to promote antitumor immunity against AFP-expressing lymphoma cells, can induce effective antitumor immunity in two different mouse hepatoma models. One of these used C57L/J mice inoculated with highly AFP-producing Hepa1-6 cells, while the other used C3H/HeN mice inoculated with low AFP-producing MH134 cells.

Materials and methods

Mice and cell lines. C57L/J mice and C3H/HeN mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and Charles River Japan (Osaka, Japan), respectively. All mice were kept in the animal facility of the Nagasaki University and handled in accordance with guidelines for animal experimentation. Mice aged 6-8 weeks old were used for experiments. The mouse hepatoma cells, Hepa1-6 and MH134, and the human HeLa cervical cancer cell line were maintained in DMEM (Life Technologies Inc., San Diego, CA) supplemented with 10% fetal bovine serum (FBS).

Plasmid and adenoviral vectors. The mouse AFP (mAFP) cDNA clone, MAFP (B557), was a generous gift from Professor Shinzo Nishi (Hokkaido University, Sapporo, Japan). To construct an mAFP expression plasmid vector, pmAFP, the mAFP cDNA was inserted into an expression vehicle, pBudCE4, that contained the cytomegalovirus (CMV) promoter (Invitrogen, San Diego, CA). Plasmid vectors expressing mouse GM-CSF, pRx-mGHCSF-bsr, and macrophage colony stimulating factor (M-CSF), pCAhMCSF, were purchased from the RIKEN GeneBank (Tsukuba, Japan). All plasmids were prepared using an EndoFree Mega Prep Kit (Qiagen, Tokyo, Japan) to eliminate endotoxin contamination. The mAFP expression adenoviral vector, AdmAFP, was constructed using an *in vitro* ligation method that was recently established (23). In brief, mAFP cDNA was inserted into a shuttle plasmid vector, pHMCMV6, which contains the CMV promoter, and digested with I-CeuI and PI-SceI. The resulting fragment was inserted into an I-CeuI/PI-SceI-digested adenoviral plasmid vector, pAdHM4, which contains a complete E1/E3-deleted adenovirus type 5 genome. The resultant AFP-expressing adenoviral plasmid vector was digested with PacI, and transfected into 293 human embryonal kidney (HEK) cells to yield AdmAFP. The pHMCMV6 and pAdHM4 plasmids were kindly provided by Dr H. Mizuguchi (National Institute of Health Sciences, Tokyo, Japan). Adenovirus was propagated in HEK293 cells and purified using two rounds of CsCl gradient centrifugation.

AFP detection. HeLa cells were transfected with pmAFP by the lipofection method using Superfect (Qiagen, Tokyo, Japan) or infected with AdmAFP. These cells were lysed and subjected to Western blotting using a goat polyclonal anti-mAFP antibody (also provided by Professor Shinzo Nishi) and a horseradish peroxidase-labelled anti-goat antibody (Chemicon International Inc., Temecula, CA). The lysates from Hepa1-6 and MH134 cells were also analyzed for the presence of AFP.

The total RNA from HeLa cells transfected with pmAFP or infected with AdmAFP and that from MH134 cells was extracted using the guanidium isothiocyanate method and subjected to reverse transcription PCR (RT-PCR). The primers used for the PCR were 5'-TCCAGGCAACAACCAT TATTA-3' and 5'-TTTCCTCGTGTAACCAATAAG-3'.

Immunization protocol. pmAFP, pCAhMCSF (pM-CSF) and pRx-mGHCSF-bsr (pGM-CSF) were diluted in PBS/25% sucrose to a concentration of 100 μg/100 μl and injected into the right posterior tibialis muscle of the mice. Three days before injection of the plasmids, 12.5% bupivacaine (100 μl) was injected to induce muscle inflammation for efficient gene expression (24). Two weeks after injection of the plasmids, AdmAFP was diluted in PBS to a concentration of $1x10^9$ pfu/100 μl and injected into the same location as a booster vaccination.

In vivo studies. Tumor challenge was performed 2 weeks after the last immunization. Hepa1-6 cells ($1x10^7$ cells/mouse) and MH134 cells ($5x10^5$ cells/mouse) were suspended in 100 μl of serum-free medium and were subcutaneously (s.c.) injected into the right flank of C57L/J mice and C3H/HeN mice, respectively, and the tumor growth rate was monitored. The tumor was measured in two dimensions, and its size was calculated using the formula; (width^2 x length)/2. Alternatively, Hepa1-6 cells ($1x10^7$ cells/mouse) were s.c. injected into the right flank of control mice. Seven days after tumor inoculation, the immunization protocol was started.

ELISPOT assay. An ELISPOT assay was performed to measure the number of splenic cells secreting IFN-γ, as described previously (25). Briefly, 1 week after the last immunization, the C57L/J or C3H/HeN mice were sacrificed, and their splenic cells were harvested. The isolated splenic cells were incubated for 2 days with RPMI-1640 medium (Life Technologies Inc., San Diego, CA) containing10% FBS, 10 IU/ml of recombinant mouse IL-2 (R&D systems, Minneapolis, MN) and 50 μM of 2-mercaptoethanol. These splenic cells were then co-cultured with 20 Gy irradiated Hepa1-6 cells or MH134 cells. Twenty-four hours later, an ELISPOT assay was performed with the mouse IFN-γ ELISPOT assay kit (R&D systems) according to the manufacturer's instructions.

Statistical analysis. The statistical significance in all the experiments was calculated using an unpaired Student's t-test.

Results

Detection of AFP. A Western blot analysis was performed to determine the levels of AFP expression in HeLa cells transfected with pmAFP or infected with AdmAFP, as well as the endogenous levels of AFP expression in Hepa1-6 and MH134 cells. As shown in Fig. 1A, HeLa cells infected

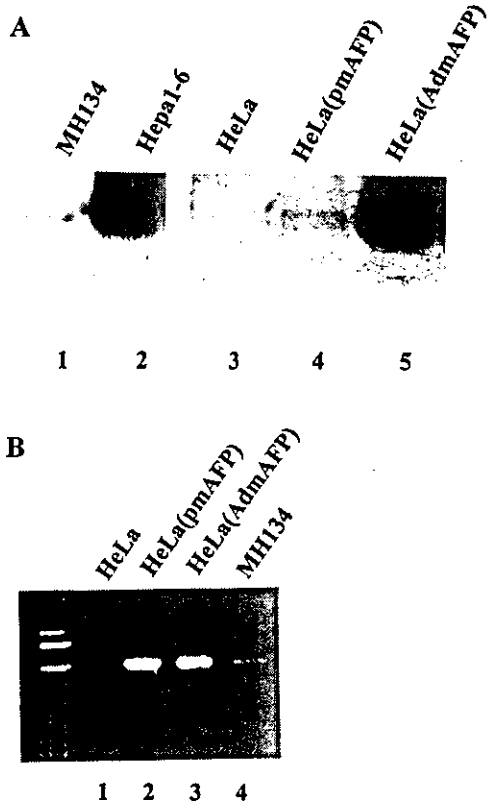

Figure 1. AFP expression in HeLa cells transduced with the mAFP gene and in mouse hepatoma cells. (A), HeLa cells were transfected with (lane 4) or without (lane 3) pmAFP or infected with AdmAFP at a multiplicity of infection (MOI) of 100 (lane 5), and a Western blot analysis was performed using anti-mAFP antibody. Cell lysates from Hepa1-6 cells (lane 1) and MH134 cells (lane 2) were also analyzed. (B), HeLa cells were transfected with (lane 2) or without (lane 1) pmAFP or infected with AdmAFP at a MOI of 100 (lane 3), and RT-PCR was performed to detect AFP mRNA expression. Total RNA from MH134 cells (lane 4) was also subjected to RT-PCR.

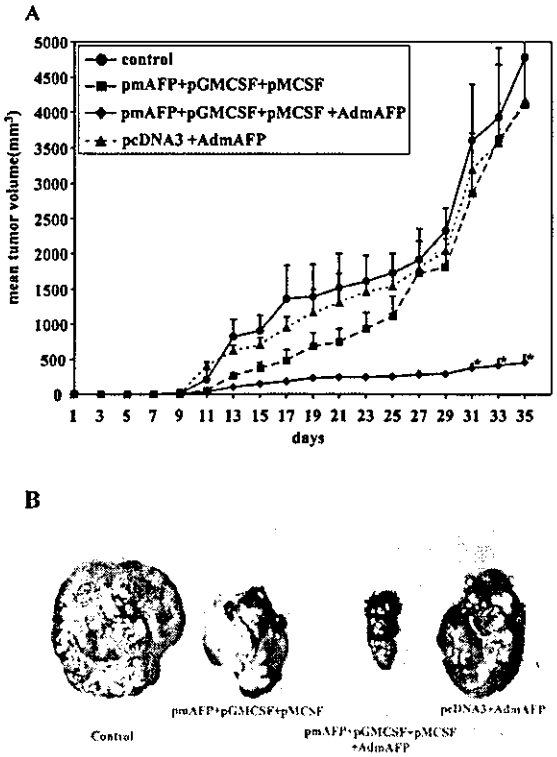

Figure 2. Effect of plasmid-AFP prime/adenovirus-AFP booster immunization on the growth of Hepa1-6 cells in C57L/J mice. (A), pmAFP (100 μg/mouse), pGM-CSF (50 μg/mouse) and pM-CSF (50 μg/mouse) were injected into two groups of C57L/J mice. pcDNA3, a vehicle plasmid, was injected into one group of mice. Two weeks later, AdmAFP ($1x10^9$ pfu/mouse) was injected into two groups of mice as a booster immunization. Control mice were not given any immunization. Two weeks after the last immunization, Hepa1-6 cells ($1x10^7$ cells/mouse) were s.c. inoculated into the right flank of the mice. Tumor volume was determined as described in Materials and methods. Data are expressed as mean ± SE (n=6). *$p<0.05$ versus other groups of mice. (B), A representative Hepa1-6 tumor in each group that was removed at the end of study (day 35).

with AdmAFP produced large amounts of AFP, but those transfected with pmAFP produced lesser amounts of AFP. Hepa1-6 cells produced more abundant amounts of AFP than MH134 cells, as described previously (22). In addition, the expression of AFP mRNA in HeLa cells transduced with the mAFP gene and in MH134 cells was confirmed by RT-PCR (Fig. 1B).

Plasmid-AFP prime/adenovirus-AFP booster vaccination generates protective immunity against Hepa1-6 cells in C57L/J mice, but not against MH134 cells in C3H/HeN mice. We determined whether AFP plasmid vaccination with or without an AFP adenovirus booster could promote protective immunity against Hepa1-6 and MH134 cells in C57L/J and C3H/HeN mice, respectively (Figs. 2 and 3). These mice were primed with an injection of pmAFP, pM-CSF and pGM-CSF, or with pcDNA3 (vehicle) alone, followed 2 weeks later by a booster of AdmAFP, or the lack thereof. Another 2 weeks later, the C57L/J and C3H/HeN mice were challenged with Hepa1-6 and MH134 cells, respectively, and the tumor growth rate was monitored. Two of the six C57L/J mice immunized with a pmAFP prime/AdmAFP booster were completely protected from the challenge of Hepa1-6. In the remaining four mice, the growth of Hepa1-6 was significantly delayed, compared with the other groups ($p<0.05$). However, vaccination with pmAFP, pM-CSF and pGM-CSF or AdmAFP alone did not induce such protective immunity against Hepa1-6 cells in C57L/J mice (Fig. 2A). A representative Hepa1-6 tumor in each group, which was removed at the end of study, is shown in Fig. 2B. In contrast, no protective immunity against MH134 cells was observed in C3H/HeN mice using any vaccination protocol, including pmAFP prime/AdmAFP booster vaccination (Fig. 3).

Plasmid-AFP prime/adenovirus-AFP booster vaccination increases the frequency of IFN-γ-producing splenic cells in C57L/J mice. The frequency of IFN-γ-producing splenic cells from immunized C57L/J mice was determined using an ELISPOT assay (Fig. 4). Similarly to the *in vivo* study (Fig. 2A), the frequency of IFN-γ-producing splenic cells

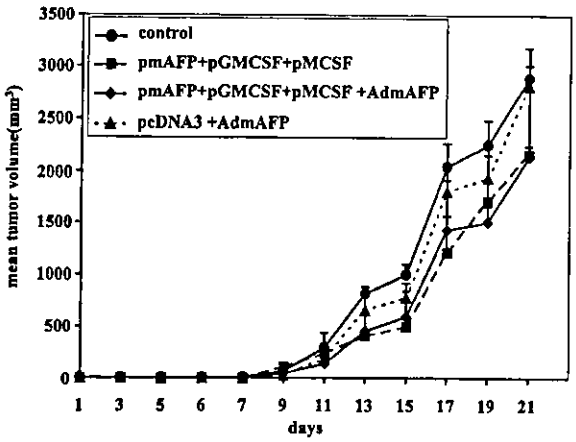

Figure 3. Effect of plasmid-AFP prime/adenovirus-AFP booster immunization on the growth of MH134 cells in C3H/HeN mice. C3H/HeN mice were immunized as described in the Fig. 2 legend. Two weeks after the last immunization, MH134 cells ($5x10^5$ cells/mouse) were s.c. inoculated into the right flank of C3H/HeN mice. Tumor volume was determined as described in Materials and methods. Data are expressed as mean ± SE (n=6).

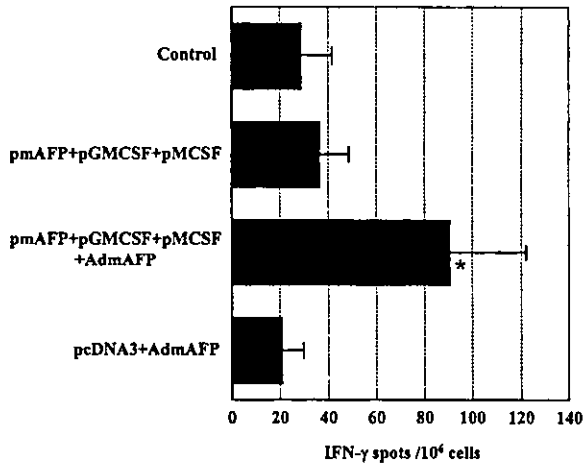

Figure 4. IFN-γ ELISPOT assay. Splenic cells derived from immunized C57L/J mice or control mice were cultured with irradiated Hepa1-6 cells for 24 h, and a mouse IFN-γ ELISPOT assay was performed. The spots in each well were counted under a microscope. Values are expressed as the number of spot-forming cells relative to the number of spleen cells added to each well at the start of the culture. Data are expressed as mean ± SD (n=4).

from C57L/J mice immunized with a pmAFP prime/AdmAFP booster was significantly higher than that using other protocols ($p<0.05$). These results suggested that immunization of C57L/J mice using the pmAFP prime/AdmAFP booster vaccination might elicit T-cell responses to Hepa1-6 cells. We also performed similar experiments using splenic cells from immunized C3H/HeN mice, but could not detect similar responses (data not shown).

Plasmid-AFP prime/adenovirus-AFP booster vaccination shows an antitumor effect against pre-established Hepa1-6 cells in C57L/J mice. Finally, we elucidated the antitumor effect of immunization with a pmAFP prime/AdmAFP booster against pre-established Hepa1-6 tumors. As shown in Fig. 5, the growth of Hepa1-6 tumors was significantly retarded by immunization with a pmAFP prime/AdmAFP booster, although rejection of the Hepa1-6 tumor was not observed.

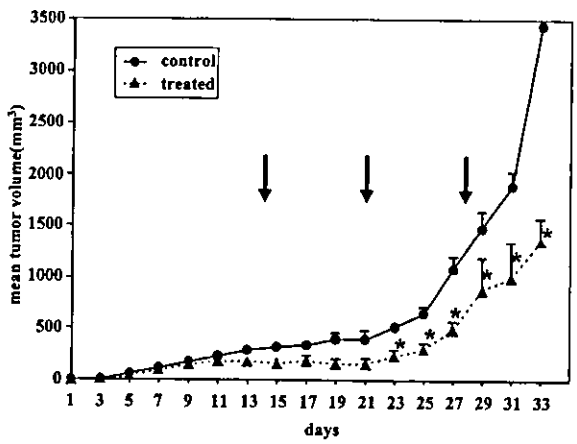

Figure 5. Antitumor effect of pmAFP prime/AdmAFP booster immunization on pre-established Hepa1-6 tumors in C57L/J mice. Hepa1-6 cells ($1x10^7$ cells/mouse) were s.c. inoculated into the right flank of C57L/J mice. Seven days after tumor inoculation, the mice were immunized with pmAFP (100 μg/mouse), pGM-CSF (50 μg/mouse) and pM-CSF (50 μg/mouse). At this point, the tumors had reached an average volume of 100 mm^3. At days 14, 21 and 28, AdmAFP ($1x10^9$ pfu/mouse) was injected into the mice as a booster immunization (arrow). Control mice were not given any immunization. Tumor volume was determined as described in Materials and methods. Data are expressed as mean ± SE (n=6). *$p<0.05$ versus control.

Discussion

In the present study, we injected naked plasmids expressing M-CSF and GM-CSF together with an AFP-expressing plasmid as the primary vaccination protocol. This was based on the observations that co-immunization of M-CSF and GM-CSF genes with the HIV-1 genome enhanced the CTL response against HIV-1 (26), and that Hepa1-6 cells transduced with the membrane form of the M-CSF gene were rejected and this led to CTL immunity in C57L/J mice (27). However, in our experiments, single (Fig. 2) and even repeated vaccination (data not shown) with plasmids expressing AFP, M-CSF and GM-CSF failed to induce protective immunity against Hepa1-6 tumors in C57L/J mice. Similar results suggesting that vaccination using naked plasmids expressing AFP and GM-CSF cannot induce protective immunity against lymphoma cells expressing exogenous AFP in C57BL/6 mice have been reported (20). In contrast to these observations, Grimm *et al* (22) have reported that vaccination with a plasmid encoding AFP and plasmids expressing IL-12 and GM-CSF led to rejection of pre-established Hepa1-6 tumors in C57L/J mice. IL-12 is known to stimulate NK cells, promote maturation of CTL, differentiate the Th1-type immune response and induce antiangiogenic effects (28,29). Since we used M-CSF, rather than IL-12, this may in part explain the difference between our results and those of

Grimm *et al*. Taken together, the kinds of cytokine or growth-factor genes that are chosen for co-immunization with the tumor-associated antigen gene could be crucial in achieving a favorable outcome for antitumor vaccination therapy.

A booster vaccination with adenovirus-AFP, in addition to the primary plasmid-AFP vaccination, generated protective immunity against Hepa1-6 tumors in C57L/J mice, as shown in a previous report using an AFP-expressing lymphoma model (21), but adenovirus-AFP vaccination alone did not promote such immunity. In addition, the adenovirus-AFP booster vaccination in C57L/J mice significantly increased the frequency of IFN-γ-producing splenic cells, probably including T-cells, but an adenovirus-AFP vaccination alone did not achieve a similar result. These results suggest that a booster immunization with adenovirus-AFP greatly enhances the immunological responses against Hepa1-6 cells in C57L/J mice and that the plasmid-AFP primary vaccination is needed prior to the adenovirus booster. Furthermore, this vaccination protocol significantly repressed the growth of pre-established Hepa1-6 tumors, which are commonly used as a model in clinical vaccination therapy. Recently, Nagayama *et al* reported a similar observation whereby the repeated injection of adenovirus expressing the thyrotropin receptor, but not that of plasmid DNA encoding the same receptor, successfully induced Graves' hyperthyroidism in mice (30). At present, it is not clear why an adenovirus booster is superior to a plasmid DNA booster. However, Ambriovic *et al* have suggested that the injection of adenovirus rather than plasmid DNA induces a local inflammation and stimulates cytokine production, which provides a benefit for immunization efficacy (31). In addition, as shown in Fig. 1A, the level of AFP expression was much greater in the adenovirus infected cells than in the plasmid DNA transfected cells. Therefore, the higher level of AFP expression with adenovirus injection, compared to injection of naked plasmid DNA, may account for the advantages shown by the adenovirus booster.

In contrast, plasmid-AFP prime/adenovirus-AFP booster vaccination could not induce protective immunity against MH134 tumors in C3H/HeN mice. This vaccination protocol also failed to increase the frequency of IFN-γ-producing splenic cells in C3H/HeN mice (data not shown). Since MH134 cells produce a much lower level of AFP than Hepa1-6 cells (Fig. 1A), the AFP epitope may not be presented adequately to stimulate a T-cell response, although MH134 cells express class 1 MHC molecules (32) to the same extent as Hepa1-6 cells (22,33). In addition, Ribas *et al* recently reported that C3H/HeN mice, but not C57BL/6 mice, receiving multiple vaccinations with DCs expressing the MART-1 tumor antigen show decreased protection against melanoma, which is associated with a change from a type 1 to a type 2 cytokine response in C3H/HeN mice (34). Taken together, these observations and our results suggest that it may be possible that the plasmid-AFP prime/adenovirus-AFP booster vaccination induces a similar phenomenon in C3H/HeN mice, through which the protective immunity against MH134 cells was diminished.

In conclusion, we have shown that immunotherapy using a plasmid/adenovirus expressing AFP induced an effective antitumor immunity against highly AFP-producing Hepa1-6 cells in C57L/J mice, but not against low AFP-producing MH134 cells in C3H/HeN mice. These results suggest that although AFP-mediated vaccination therapy is a promising approach to treat HCC, its efficacy is probably dependent on the level of AFP expression in HCC or on the immunological response of patients.

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