strain (AY090457) belonging to the genotype H group at the nucleotide level. By comparison of the deduced amino acid sequences among the HBV full genome, we identified 24 amino acid residues that were specific for genotype H in each region (Fig. 1). Specifically, 9 strains of genotype H consisting of IM806-2 and 8 isolates from the database showed the consensus sequences of amino acids at 16N, 90Q, 213T, 255S, 270T, 273T, 280E, 306T, 311T, 367S, 461V, 480H, 584A, 743D and 821P in the P gene; 32W, 60A, 102P and 127L in the X gene; 8A, 90P and 219P in the S gene; and 180Q and 184A in the core gene. Furthermore, the HBV-IM806-2 isolate was grouped into genotype H of HBV by phylogenetic analysis based on the full genome sequence (Fig. 2). IM806-2 was found to be more closely related to the Los Angeles strain than to the Nicaragua isolate.

Although extensive studies on HBV have been conducted, the nature of HBV and its true pathogenic role remain controversial. It is possible that HBV genotypes influence the severity of liver diseases and the replication of HBV. Recently, eight different genotypes, A-H, of HBV have been classified. HBV genotypes have been shown to have a distinct geographical distribution and to correlate with the severity of liver diseases (6). It has been reported that genotype A is prevalent in northern and central Europe but is also common in North America and sub-Saharan Africa. Genotypes B and C are confined to Asia. Genotype D is widespread but is the predominant type in the Mediterranean region, while genotype E is found mainly in West Africa. Genotype F shows the highest divergence among the genotypes and is indigenous to aboriginal populations of the Americas (7). The newly described genotype G has been found in the U. S. and France (8). Some of these genotypes have been split into subgroups. Most recently, genotype H has been identified in two Nicaraguans and one American living in Los Angeles (3). Genotype H has been encountered in Nicaragua, Mexico and California, and it seems that its distribution may be restricted to the northern part of Latin America, including Central America and Mexico. Arauz-Ruiz et al. (3) suggest that the genotype H strain from Los Angeles might be an import from Mexico. Nevertheless, the nature of HBV genotype H throughout the world remains obscure. Regarding the genotypic distribution of HBV in Japan, our data showed that genotype C (74%) was the most prevalent, followed by genotype B (17%) and genotype A (4%) among 100 liver disease patients in the Tokyo area (9). In the present study, we coincidentally found a strain of HBV belonging to the genotype H in a Japanese patient with chronic hepatitis and noted that the isolate recovered was more closely related to the prototype of the Los Angeles strain than to that of the Nicaragua strain. Genotype H is very rare in Japan; in fact, the Japanese Red Cross NAT Screening Research Group recently reported that HBV genotype H was found in only 1 of 328 (0.3%) HBV DNA-positive blood donors in Japan and confirmed that it showed high homology with the strain from Los Angeles (10,11). In addition, Shibayama et al. (12) also reported that genotype H was detected in a Japanese patient co-infected with HIV who had a history of traveling to South America and had had sexual intercourse there. Even though this genotype is very rare in Japan, it is important to survey the infection route of HBV in such patients. In our case, the patient had a history of acute hepatitis B after returning to Japan from Thailand, suggesting that infection had occurred in Thailand. Interestingly, the amino acid changes specific to genotype H were concentrated in the P gene. The P gene product is needed for the encapsidation of viral RNA into core particles and for the conversion of the pregenomic viral RNA molecule into genomic viral RNA. These findings may have an impact on the viral replication, immunological and genetic diagnosis of HBV, as well as on treatment options for the ubiquitous disease it causes. Elucidation of the relationship among genotype H, its pathogenicity in chronic liver diseases and its effects against therapy is awaited with great

Genotype			P gene															C gene		X gene				S gene		
		Amino acid	16	90	213	255	270	273	280	306	311	367	461	480	584	743	821	180	184	32	60	102	127	8	90	219
A	AB076678	Malawi	D	N	C	F	S	S	A	S	S	A	L	D	N	K	S	R	S	G	V	A	I	P	A	S
A	M57663	Philippine	D	N	C	F	S	S	A	S	S	A	L	D	N	K	S	R	S	R	V	A	I	P	A	A
A	AB126580	Russia	D	N	C	F	N	S	A	C	S	A	L	D	N	K	S	R	S	G	V	A	I	P	T	S
A	AF090838	France	D	N	C	F	N	S	A	C	S	A	L	D	N	K	S	R	S	G	V	A	T	P	T	S
B	AB033554	Indonesia	D	D	C	V	N	S	A	H	S	A	L	D	Q	K	S	R	S	G	V	A	I	P	T	T
B	AB115551	Cambodia	D	D	C	V	S	S	A	H	S	A	L	D	H	K	S	R	S	G	V	A	I	P	A	T
B	AF121249	Vietnam	E	D	C	V	S	S	A	H	S	A	L	N	H	K	S	R	S	G	V	A	V	P	A	T
B	AB010291	Japan	D	D	C	V	S	S	A	H	S	A	L	N	H	K	S	R	S	G	V	V	M	P	A	T
B	D00329	Japan	D	D	C	V	S	S	A	H	S	A	L	N	N	N	S	R	S	G	V	A	I	P	A	T
B	D50521	Japan	D	D	C	V	S	S	A	H	S	A	L	N	H	K	S	R	S	G	V	A	I	P	A	T
C	AB112063	Vietnam	D	N	G	F	S	S	A	H	S	A	L	D	T	K	S	R	S	G	V	A	T	P	A	A
C	AB112066	Myanmar	D	N	S	F	S	S	A	H	S	A	L	E	N	K	S	R	S	G	V	A	I	P	A	A
C	AB112471	Thailand	D	N	S	F	S	S	A	H	S	A	L	E	N	K	S	R	S	G	V	A	I	P	A	A
C	AB049609	Japan	D	N	G	F	S	S	A	H	S	A	L	D	N	K	S	R	S	G	V	S	I	P	A	A
C	AB049610	Japan	D	N	C	F	S	S	A	N	S	A	L	D	N	K	S	R	S	R	V	A	N	P	A	A
C	AB115417	Japan	D	N	R	F	S	S	A	N	S	A	L	D	N	N	S	R	T	R	V	A	I	P	A	T
D	AB033559	Papua New Guinea	D	K	S	F	S	A	P	N	S	A	L	D	N	N	S	R	S	G	V	A	I	P	A	T
D	AB126581	Russia	D	K	S	F	S	A	P	N	S	A	L	D	N	N	S	R	S	G	V	V	I	P	A	T
D	Z35716	Poland	D	K	S	F	S	A	P	N	S	A	L	D	N	N	S	R	S	G	V	V	V	P	A	T
E	AB091255	Cote d'Ivoire	E	N	S	F	S	A	A	N	S	A	L	D	N	K	S	R	S	G	V	A	I	P	A	A
E	AB106564	Ghana	E	N	S	F	S	A	A	N	S	A	L	D	N	K	S	R	S	G	V	A	I	P	A	A
E	X75657	France	E	N	S	F	S	A	A	N	S	A	L	D	N	K	S	R	S	G	V	A	I	P	A	A
F	AB166850	Bolivia	D	S	S	V	S	A	A	P	P	A	L	N	S	N	S	R	S	G	V	G	T	T	A	L
F	AY090459	Costa Rica	D	H	S	A	S	A	A	S	S	A	L	N	S	K	S	R	S	G	V	G	I	T	A	T
F	X69798	Brazil	D	S	A	A	S	A	A	P	S	A	L	N	S	T	S	R	S	G	V	G	I	T	A	L
G	AB056513	USA	E	T	S	F	S	A	S	S	S	A	L	D	N	K	S	R	S	G	V	V	S	P	A	V
G	AB064310	USA	E	T	S	F	S	A	S	S	S	A	L	D	N	K	S	R	S	G	V	V	S	P	A	V
G	AF160501	France	E	T	S	F	S	A	S	S	S	A	L	D	N	K	S	R	S	G	V	V	S	P	A	V
H	AY090454	Nicaragua	N	Q	T	S	T	T	E	T	T	S	V	H	A	D	P	Q	A	W	A	P	L	A	P	P
H	AY090457	Nicaragua	N	Q	T	S	T	T	E	T	T	S	V	H	A	D	P	Q	A	W	A	P	L	A	P	P
H	AY090460	USA	N	Q	T	S	T	T	E	T	T	S	V	H	A	D	P	Q	A	W	A	P	L	A	P	P
H	AB179747	Japan	N	Q	T	S	T	T	E	T	T	S	V	H	A	D	P	Q	A	W	A	P	L	A	P	P
H	IM806-2	Japan	N	Q	T	S	T	T	E	T	T	S	V	H	A	D	P	Q	A	W	A	P	L	A	P	P

Fig. 1. Comparison of amino acid sequences among different genotypes of HBV. Genotype H-specific 24 amino acid residues can be seen in each region of HBV. Codon numbers indicate the number of position in each region.

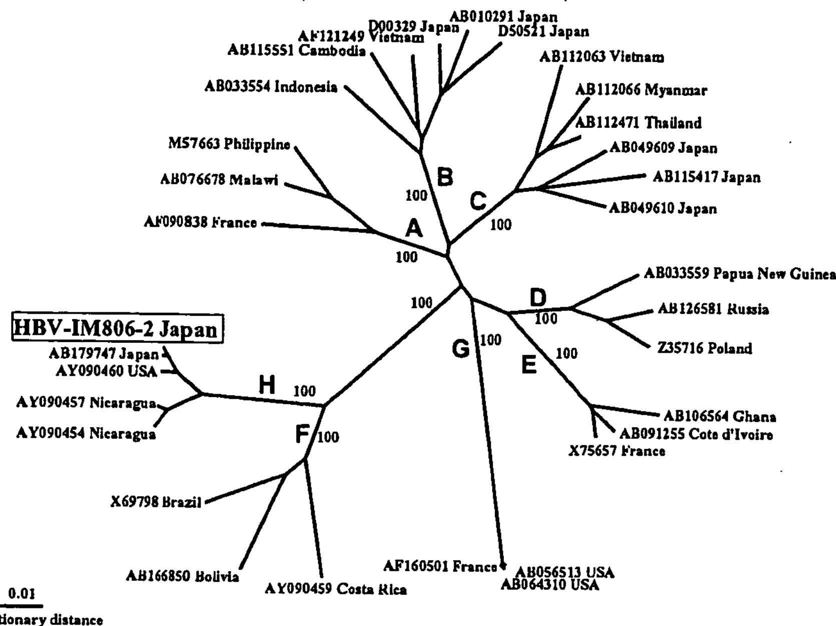

Fig. 2. Phylogram generated by neighbor-joining analysis of genetic distances in the full-length sequence of HBV.

interest. To clarify the virological differences of genotype H, we are now carrying out functional analyses of this genotype by in vitro transfection using the HBV isolate recovered in this study. Recently, it has been reported that HBV shows recombination comprising different genotypes, such as a B/C or A/D recombination. We tested for possibility of recombination using the SimPlot and bootscanning programs, but no recombination was found in the IM806-2 isolate (data not shown).

In conclusion, we identified and cloned the full-length nucleotide sequence of HBV (denoted HBV-IM806-2) recovered from a Japanese patient with chronic hepatitis. Based on phylogenetic analysis of the full genome sequence, it was confirmed that IM806-2 belonged to genotype H and was most closely related to the prototype of the Los Angeles strain.

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Clinical benefits of hepatocellular carcinoma surveillance: A single-center, hospital-based study

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Abstract. Although there is no definitive evidence that hepatocellular carcinoma (HCC) screening in high-risk groups improves survival, many physicians screen high-risk populations with various tools such as α-fetoprotein (AFP) and ultrasonography (USG). The aim of this study was to clarify clinical differences between HCC patients diagnosed by surveillance and those with incidentally-detected HCC. Two hundred and seventy-one Japanese patients with HCC diagnosed between January 1991 and December 2001 were recruited. They were categorized into two groups: 178 patients (group 1) had subclinical HCC diagnosed by surveillance and 93 patients (group 2) presented with incidentally detected HCC. The tumor size was significantly smaller in group 1 compared to that of group 2 (2.8 cm vs. 5.6 cm; P<0.0001). A significantly higher proportion of patients in group 2 had multiple HCC and portal vein infiltration when compared to group 1. Eighty-six (48.3%) group 1 patients and 16 (17.2%) group 2 patients underwent local ablation treatment, which is a curative treatment available for small HCCs (P<0.0001). The cumulative actuarial survival rate was significantly higher in group 1 than in group 2 (P=0.0091). Early detection of HCC by surveillance may contribute to a greater chance of receiving effective treatment and prolonged survival, although a further prospective, randomized study is needed.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world. The incidence of HCC has substantially increased in Japan during the past several decades, and also slightly increased in the United Kingdom, France and the United States (1-4). The prognosis for patients with HCC is still poor. Surgical resection and liver transplantation are the standard forms of curative treatment available. Recently, radio-frequency ablation (RFA) and percutaneous ethanol injection (PEI) have also been recognized as effective methods to achieve complete tumor necrosis for small HCCs (5). However, the chance of curative treatment is often limited by several features of HCC. HCCs are usually large in size before they give rise to symptoms. Bilobar or multifocal tumors are common. The incidence of associated cirrhosis is also high, being over 80% in most series (6-8). Transcatheter intra-arterial chemoembolization (TACE), by which it is thought to be difficult to achieve complete necrosis of HCCs, also depends on the above factors (9). To increase the chance of intervention and to improve survival, early detection of HCC by surveillance has been implemented in many countries. Though studies of Asian populations show promising results, these are not substantiated in European studies (10-13). The impact on survival remains controversial. The aim of this study was to examine whether detection of subclinical HCC by screening methods has any significant clinical and survival benefits.

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Key words: hepatocellular carcinoma, screening, α-fetoprotein, imaging techniques

Patients and methods

Patients. A total of 271 patients with HCC diagnosed between January 1991 and December 2001 in the The First Department of Internal Medicine, Nagasaki University School of Medicine, were recruited into this study. The diagnosis of HCC was based on AFP levels and imaging techniques including ultrasonography (USG), computerized tomography (CT), magnetic resonance imaging (MRI), hepatic angiography (HAG), and/or liver biopsy. The diagnostic criteria for HCC was either a confirmative liver biopsy or elevated AFP (>20 ng/ml) together with neovascularization in HAG and/or CT. They were categorized into two groups: group 1 included 178 patients with subclinical HCC diagnosed by surveillance; group 2 included 93 patients who were incidentally found due to related symptoms at our hospital. Group 1 patients had AFP levels and liver function biochemistry measured every 3-6 months of follow-up and USG every 3-12 months over a

Table I. The demographic data and other basal parameters of the 271 patients with HCC.

	Group 1	Group 2	P-value
Number of patients	178	93	
Average age at diagnosis (range)	64.9 (44-82)	64.3 (32-86)	NS
Sex ratio (male : female)	127:51	79:14	0.0130
Median follow-up in months (range)	41.3 (0-148)	29.6 (0-114)	0.0006
Etiology liver disease (%)			
HBV	28 (15.8)	14 (15.0)	NS
HCV	133 (74.7)	65 (69.9)	NS
HBV + HCV	7 (3.9)	1 (1.1)	NS
Alcohol	3 (1.7)	4 (4.3)	NS
Unknown	7 (3.9)	9 (9.7)	NS
Child-Pugh staging (%)			
A	124 (69.7)	69 (74.2)	NS
B	43 (24.2)	19 (20.4)	NS
C	11 (6.1)	5 (5.4)	NS
Albumin (g/dl) (SD)	3.7 (0.6)	3.8 (0.6)	NS
Total bilirubin (mg/dl) (SD)	1.3 (1.0)	1.4 (2.6)	NS
Alanine transaminase (IU/l) (SD)	71.9 (47)	85.8 (67)	NS
Asparate transaminase (IU/l) (SD)	60.3 (38)	64.6 (42)	NS
Prothrombin time (%) (SD)	81.8 (15.8)	82.1 (16.9)	NS

Mann-Whitney U test.

Table II. The characteristics of the 271 patients with HCC.

	Group 1	Group 2	P-value
Number of patients	178	93	
Median tumor size (range)	2.8 (0.6-15.0)	5.6 (1.0-20.0)	<0.0001
Tumor size <3 cm (%)	115 (64.6)	21 (22.6)	<0.0001
Tumor size <5 cm (%)	168 (94.4)	48 (51.6)	<0.0001
Number of tumor lesion (%)			
1	101 (56.7)	39 (41.9)	0.0208
2	33 (18.6)	12 (12.9)	NS
>3, including diffuse HCC	44 (24.7)	42 (45.2)	0.0006
Portal vein infiltration (%)	7 (3.9)	25 (26.9)	<0.0001
AFP level in ng/ml (%)			
<20	79 (44.4)	25 (26.9)	0.0050
20-200	63 (35.4)	33 (35.5)	NS
>200	36 (20.2)	35 (37.6)	0.0020
Patients with surgical resection (%)	5 (2.8)	3 (3.2)	NS
Patients with RFA and/or PEI (%)	86 (48.3)	16 (17.2)	<0.0001
Patients with TACE (%)	73 (41.0)	55 (59.2)	0.0046
Only palliative care (%)	14 (7.9)	19 (20.4)	0.0027

Mann-Whitney U test.

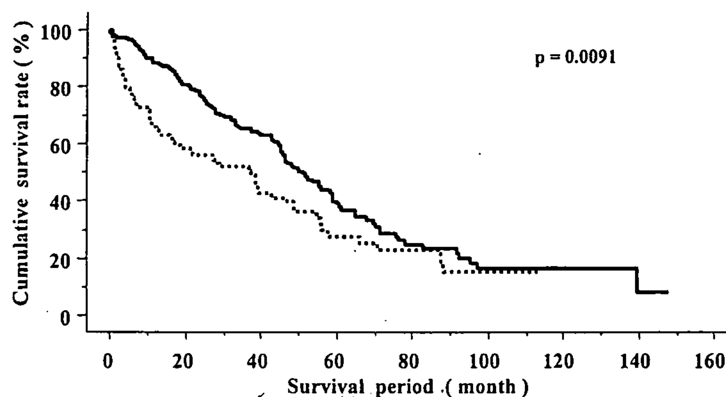

Figure 1. The cumulative survival rate in patients with subclinical HCC (group 1, thick line) and in patients with symptomatic HCC (group 2, thin line).

period of at least 12 months prior to diagnosis of HCC. Patients with AFP >20 ng/ml or a solid mass on USG were further evaluated by CT and/or MRI. Thirteen group 1 patients underwent tumor surveillance at other hospitals and were referred to us after HCC was diagnosed. Group 2 patients presented with clinical symptoms of abdominal pain, discomfort, nausea, or weight loss that led to evaluation and diagnosis of HCC.

Etiology of HCC. Viral hepatitis B infection and hepatitis C infection were diagnosed by testing for serum hepatitis B surface antigen (HBsAg) (enzyme-linked immunosorbent assay; Abbott Laboratories, Chicago, IL) and hepatitis C virus antibodies (HCVAb) (microparticle enzyme immunoassay; Abbott Laboratories), respectively. The serum AFP was measured by a radioimmunoassay (Abbott Laboratories). The history of alcohol intake was noted from medical records. Habitual drinking was defined as an average daily consumption of an amount equivalent to 80 g of pure ethanol over a period of more than 10 years.

Modalities of treatment. All patients were assessed for surgical resection once the HCCs were diagnosed. The assessment was based on lobar involvement and liver function status. The lobar involvement was evaluated by a combination of USG, CT, MRI and HAG. Patients were considered not suitable for resection based on the following criteria: i) bilobar involvement; ii) evidence of main portal vein infiltration/thrombosis; iii) evidence of extrahepatic metastases; iv) Childs C cirrhosis; v) poor cardiac and respiratory performance status. If the patients were not suitable for operation or did not agree to the operation, RFA or PEI would be the second choice of treatment offered to patients with HCCs of <3 cm in size and <3 in number, unless the main portal vein was thrombosed and there were extrahepatic metastases. The other patients without main portal vein thrombosis and extrahepatic metastases were recommended to receive TACE irrespective of size and number of tumors.

Statistical analysis. The time of survival was measured from the time of HCC diagnosis to the time of death or until the time of writing. The data were analyzed by the Mann-Whitney test for the continuous ordinal data, the χ^2 test with Yates' correction and the Fisher's exact test for the association between two qualitative variables, and the Kaplan-Meier method for the calculation of survival. The standard error was calculated based on the binomial model for the response proportion. P<0.05 was considered statistically significant.

Results

Demographic data and liver function. A total of 271 patients with HCC were diagnosed during the study period. One hundred and seventy-eight patients were diagnosed with HCC in the surveillance (group 1), and 93 patients presented with incidentally detected HCC (group 2). The average age was 65 years (range, 32-86 years), and the male:female ratio was 206:65. The underlying causes of HCC were as follows: 42 (15.5%) patients were positive for HBsAg; 198 (73.0%) were positive for HCVAb; 8 (3.0%) were positive for both HBsAg and HCVAb; 7 (2.6%) had a history of significant alcohol intake and were negative for HBsAg and anti-HCV, and 16 (5.9%) had no known etiology. The demographic data and other basal parameters for group 1 and group 2 are listed in Table I. Compared to group 1, group 2 patients had a higher sex ratio and a shorter median follow-up in months.

Features and treatment of HCC. The characteristics of the HCC in the two groups as assessed by US, CT, MRI and/or HAG are listed in Table II. The tumor size of the index HCC (the largest one if more than one focus of HCC was present) was significantly smaller in group 1 compared to that of group 2 (P<0.0001). Group 1 had a higher proportion of patients with small HCCs of <3 cm and 5 cm compared to those of group 2. A significantly higher proportion of multifocal HCC and portal vein infiltration in group 2 was found when compared to group 1. Group 1 had a higher proportion of patients with the AFP level of <20 ng/ml compared to those of group 2 (p=0.005).

Five patients in group 1 and 3 patients in group 2 received surgical resection. For group 1, 86 (48.3%) patients underwent RFA and/or PEI. The number of group 1 patients with RFA and/or PEI was significantly higher compared to group 2 [16 patients (17.2%); P<0.0001]. One hundred and twenty-eight (47.2%) patients [73 (41.0%) in group 1 and 55

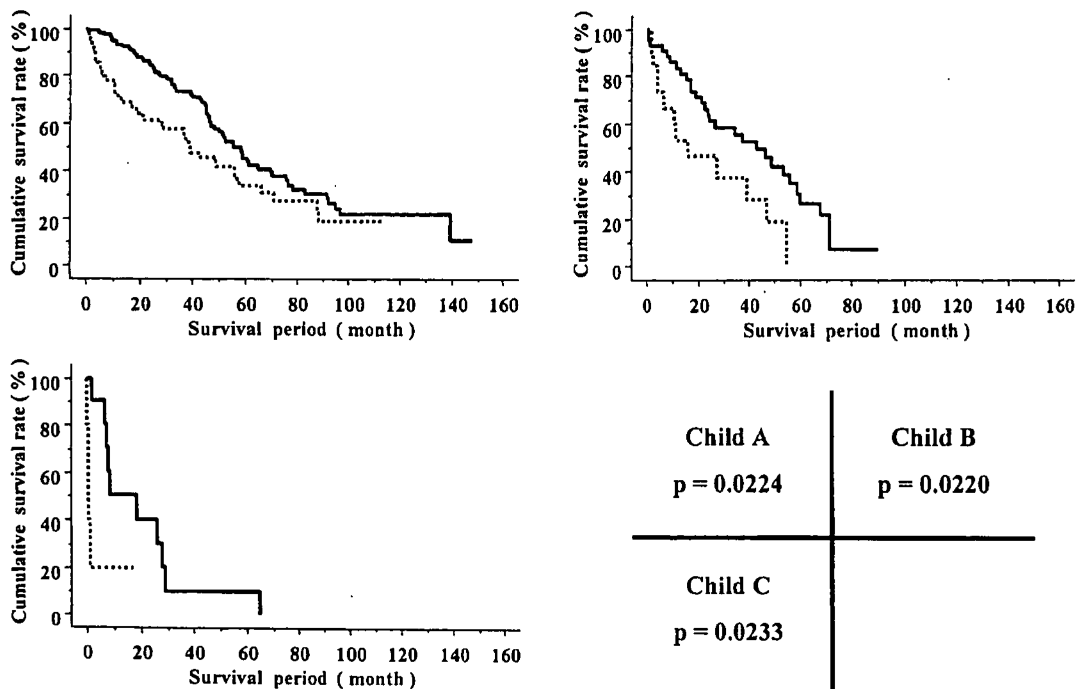

Figure 2. The cumulative survival rate in patients with subclinical HCC (group 1, thick line) and in patients with symptomatic HCC (group 2, thin line) according to Child-Pugh staging.

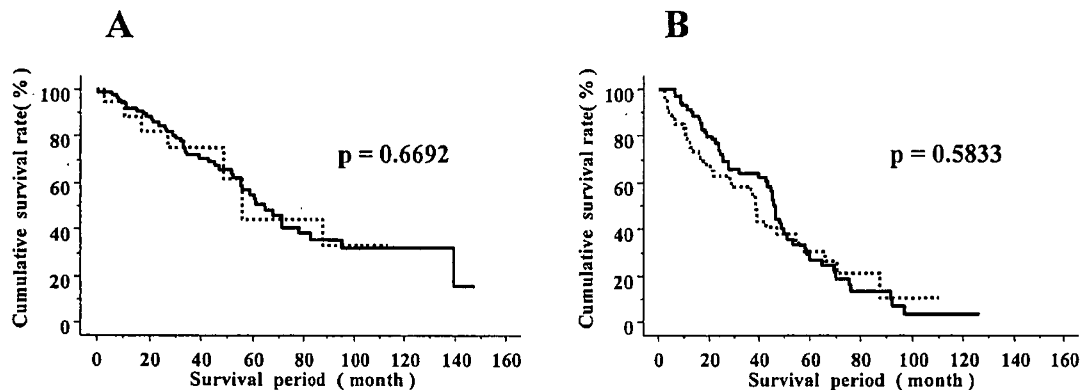

Figure 3. The cumulative survival rate in patients with subclinical HCC (group 1, thick line) and in patients with symptomatic HCC (group 2, thin line) who received treatment. A, Patients with RFA and/or PEI; B, Patients with TAC.

(59.2%) in group 2] received TACE. A significantly higher proportion of patients in group 2 received TACE treatment compared to those in group 1 (P=0.0046). Fourteen (7.9%) and 19 (20.4%) patients in group 1 and group 2 received only palliative care, respectively.

Survival. The median survival of all 271 patients was 37.3 months. The cumulative actuarial survival rate was significantly higher in group 1 than in group 2 (Fig. 1; P=0.0091). By stratifying according to the Child-Pugh score, patients in group 1 with Childs A, Childs B and Childs C had higher cumulative survival rates than those in group 2 (Fig. 2; P=0.0224, P=0.0220, and P=0.0233, respectively) (Fig. 2). For those who had RFA/PEIT or TACE treatment, group 1 and group 2 patients did not show significant differences in survival (Fig. 3). Surgical resection could not be evaluated between group 1 and group 2 because of the small number of patients.

Discussion

More than 90% of the patients in the present study were HBsAg-positive and/or HCV-positive. Validation of the usefulness of surveillance for HCC is of importance in areas such as East Asia, where the incidence of chronic viral

hepatitis is of high prevalence (14-16). However, it is difficult to conduct a prospective, randomized trial on screening programs for HCC in areas in which facilities are readily available to perform AFP and USG because of the ethical implications and possible patient noncompliance.

We have screened high-risk HCC populations with AFP and USG, the most widely used tools (17-19). Group 1 patients had a significantly smaller tumor size compared to group 2 patients. In addition, a higher proportion of group 1 patients had tumors of <3 cm and 5 cm. Group 2 patients had a high AFP level and higher proportions of other adverse features of HCC including multifocal HCC and portal vein infiltration. These findings indicate that patients with HCCs diagnosed by surveillance were at an earlier stage of the disease, obtaining a higher chance of receiving effective treatment. The most important aspect for any screening program is whether it can improve patient survival (12,13,17, 20-23). In this study, there was a significant improvement in survival among patients with HCC surveillance irrespective of Childs staging, although there were no significant differences between groups 1 and 2 for patients with RFA/PEIT or TACE treatment. We recognize that our data was retrospectively analyzed and that improved survival in group 1 patients may be related to lead-time bias. Nevertheless, our data agree with the results of previous studies that surveillance for HCC may increase the rate of early detection and eligibility for curative therapies, which may translate into improved survival.

Although patients with surveillance had smaller tumors, 35.4% of them had >3 cm, and 43.3% had multiple tumors. The high proportion of patients with advanced HCC despite surveillance may be associated with heterogeneity in the frequency of surveillance (every 3-12 months), diversity on the experience of the USG examiners and differences in the equipment used. In addition, 55.6% of group 1 patients had an AFP level of <20 ng/ml at HCC diagnosis. The low sensitivity of AFP for the early detection of HCC may also account for our disappointing results with surveillance. Clearly, there is a pressing need for newer tests with greater accuracy in diagnosis of early HCC.

In conclusion, surveillance for HCC in chronic hepatitis B and hepatitis C carriers by AFP and USG can identify tumors at an early stage, resulting in a higher chance of receiving effective treatment. Further studies are necessary to clarify usefulness of HCC surveillance on survival.

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Vaccination with dendritic cells pulsed with apoptotic cells elicits effective antitumor immunity in murine hepatoma models

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Abstract. Dendritic cell (DC)-based vaccine is a developing strategy to treat cancer including hepatoma. We evaluated the antitumor efficacy of vaccination with DCs pulsed with apoptotic cells, as compared to vaccination with DCs pulsed with cell lysates, in murine hepatoma models. Murine hepatoma cells, Hepa1-6, MH134 and BNL1ME.A.7R.1, and their syngeneic mice, C57BL/6, C3H/HeN and BALB/c, respectively, were used in the study. Protective and therapeutic antitumor effects of vaccination with bone marrow-derived DCs pulsed with irradiation or sulindac-induced apoptotic cells or cell lysates were analyzed. Immature DCs efficiently phagocytosed apoptotic cells and increased expression of CD86, a cell surface maturation marker. Vaccination with apoptotic cell-pulsed, but not cell lysate-pulsed, DCs promoted significant protective immunity against parental hepatoma *in vivo*. Spleen cells from mice vaccinated with apoptotic cell-pulsed DCs showed higher cytolytic activity and contained higher number of IFN-γ producing cells against parental hepatoma cells than those from mice vaccinated with cell lysate-pulsed DCs *in vitro*. Polyriboinosinic polyribocytidylic acid [poly (I:C)], double strand RNA, further enhanced CD86 expression and the therapeutic efficacy of vaccination with DCs pulsed with apoptotic cells for pre-established hepatoma. These results suggest that vaccination with DCs pulsed with apoptotic cells and treated with poly (I:C) appears to be a promising approach as a new therapeutic means for hepatoma.

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common fatal malignancies worldwide, especially in several parts of Asia and Africa (1). Although advances in medical technology have permitted the early recognition and treatment of HCC (1,2), the annual death rate from HCC exceeds 30,000 in Japan (3). Therefore, there is a need to develop new strategies to treat HCC.

Increasing efforts have been made to develop antitumor vaccine strategies, of which dendritic cell (DC)-based vaccine is a promising approach (4-7). DCs are potent antigen presenting cells capable of initiating primary immune responses (8). DCs express high levels of major histocompatibility complex (MHC) class I and II, and co-stimulatory and adhesion molecules that provide secondary signals for stimulation of naïve T cell populations (8). Vaccination with DCs pulsed with tumor-associated peptides or proteins, or transfected with genes encoding tumor-associated antigen (TAA) lead to the induction of antitumor immunity (4-7). However, number of TAAs so far identified in human tumors is limited and tumor cells may escape from immune recognition through downregulation or alteration of TAA. Therefore, several approaches to introduce multiple and unidentified TAAs into DCs have been investigated, including fusions between DCs and tumor cells, or DCs pulsed with tumor cell lysates, peptides derived acid-eluates from tumor cells, tumor-derived exsomes or mRNA of tumor cells (5-7,9). In fact, vaccination with DCs pulsed with tumor cell lysates with or without an adjuvant cytokine therapy has been reported to induce antitumor immunity in murine hepatoma models (10-12). However, phase I trials of vaccination with tumor lysate-pulsed DCs in the treatment of advanced human HCCs have shown limited antitumor effects (13,14), suggesting the necessity to improve the protocols including the introduction of hepatoma-derived antigens into DCs and the stimulation of DCs maturation after capturing antigens.

It has been reported that DCs pulsed with apoptotic tumor cells induced by irradiation more effectively stimulate tumor-

specific cytotoxic T lymphocytes (CTLs) than DCs pulsed with tumor cell lysates *in vitro* (15-18). Therefore, in the present study, we determined whether vaccination with DCs pulsed with apoptotic cells is superior to that with DCs pulsed with cell lysates to promote protective and therapeutic antitumor immunity in murine hepatoma models.

Materials and methods

Mice and cell lines. C57BL/6, C3H/HeN and BALB/c mice were purchased from the Jackson Charles River Japan (Osaka, Japan). All mice were kept in the animal facility of the Nagasaki University and handled in accordance with guidelines for animal experimentation. Female mice aged six to eight weeks were used for experiments. The murine hepatoma cells, Hepa1-6, MH134 and BNL1ME.A.7R.1 were maintained in DMEM medium with 10% fetal bovine serum (FBS).

Preparation of immature DCs. Immature DCs were obtained from murine bone marrow precursors as described previously (19). Briefly, bone marrow cells were harvested from femurs and tibias of mice and plated in RPMI-1640 medium with 10% FBS, 50 μM 2-mercaptoethanol, 25 ng/ml recombinant murine granulocyte macrophage-colony stimulating factor (GM-CSF) (R&D systems, Minneapolis, MN) and 5 ng/ml murine interleukin-4 (IL-4) (R&D Systems). The medium was changed every other day. Seven days later, non-adherent cells (immature DCs) were harvested by gentle washing with warm PBS. To detect the purity of DCs and its maturation status, CD11c and CD86 expression were analyzed by a flow cytometer (Epics XL, Beckman Coulter, Hialeah, FL) using FITC-labeled anti-CD11c and anti-CD86 monoclonal antibodies. These expression were also analyzed in DCs after phagocytosis of apoptotic tumor cells and treatment with poly (I:C) (Sigma, St. Louis, MO).

Induction of apoptosis and preparation of lysates. The cells were irradiated by 40 Gy at room temperature using an EXS-300 X-irradiator (Toshiba, 200 kV, 15 mA, 0.85 Gy/min) and cultured for two days, or treated with 2 mM sulindac (Sigma) for two days. These cells were subjected to analysis of apoptosis. DNA fragmentation was quantified by the percentage of cells with hypodiploid DNA. In brief, cells were fixed with 70% ethanol and treated with RNase (100 μg/ml, Sigma) and stained with propidium iodide (100 μg/ml, Sigma) for 30 min on ice. The stained cells were analyzed by a flow cytometer to detect the presence of cells with hypodiploid DNA.

Tumor cell lysates were prepared by five freeze/thaw cycles. Lysis was monitored by light microscopy. Larger particles were removed by centrifugation, and the supernatants were passed through a 0.2 μm filter. The resulting tumor cell lysates were stored at -80°C in aliquots until use.

Phagocytosis of apoptotic cells. Hepa1-6 cells dyed red using PKH26GL (Sigma) were treated with 40-Gy irradiation to induce apoptosis. These cells were cocultured with DCs at a ratio of 1:1 for 24 h, and then analyzed by a flow cytometer. The phagocytic uptake was defined by the percentage of double positive ($CD11c^+$ and $PKH26GL^+$) cells.

Vaccination protocols and in vivo studies. PBS (50 μl) containing 1×10^6 DCs pulsed with apoptotic tumor cells or tumor cell lysates, or unpulsed DCs were subcutaneously injected into the planta pedis of mice. Control mice were injected with PBS alone. The same vaccination schedule was performed twice at one week interval. Tumor challenge was performed two weeks after the last vaccination. Hepa1-6 cells (1×10^6 cells/mouse), MH134 cells (5×10^5 cells/mouse) and BNL1ME.A.7R.1 cells (1×10^6 cells/mouse) were suspended in 100 μl of serum-free DMEN medium and subcutaneously injected into the right flank of respective syngeneic mice, and the tumor growth was monitored. Tumor size was determined from caliper measurement using the standard formula (length x $width^2/2$). Alternatively, BNL1ME.A.7R.1 cells (3×10^6 cells/mouse) were subcutaneously injected into the right flank of BALB/c mice. Four weeks after tumor cells inoculation when the tumor diameter became more than 5 mm, mice underwent vaccination twice at one week interval. Some mice were vaccinated with DCs both pulsed with apoptotic cells and treated with 20 μg/ml poly (I:C) for 24 h.

Cytolytic assay. Cytolytic assay was performed two weeks after the last vaccination. Spleen cells from vaccinated mice were cocultured with 20 Gy irradiated Hepa1-6 cells in RPMI-1640 medium containing 10% FBS, 50 μM 2-mercaptoethanol and 10 ng/ml of recombinant mouse IL-2. Cytotoxic effector lymphocytes were harvested after 5 days of incubation and subjected to cytolytic assay. The ^{51}Cr release assay was used to test the cytolytic activity of the effector cells. Target cells (Hepa1-6 cells) were labeled with ^{51}Cr and incubated with effector cells at 37°C in 5% CO_2 for 6 h at various effector:target cell (E:T) ratios. Supernatants were obtained after incubation and subjected to gamma-counting. The maximum or spontaneous release was defined as counts from samples incubated with 5% Triton X-100 or medium alone, respectively. Cytolytic activity was calculated using the following formula: percentage of specific ^{51}Cr release = (experimental release - spontaneous release) x 100/ (maximum release - spontaneous release).

IFN-γ ELISPOT assay. An ELISPOT assay was performed to measure the number of spleen cells secreting IFN-γ. Two weeks after the last vaccination, spleen cells were isolated and cocultured with 20 Gy irradiated Hepa1-6 cells in RPMI-1640 medium containing 10% FBS, 50 μM 2-mercaptoethanol and 10 ng/ml recombinant mouse IL-2. 48 h later, an ELISPOT assay was performed with the mouse IFN-γ ELISPOT assay kit (R&D systems) according to the manufacturer's instructions.

Results

Irradiation and sulindac induce apoptosis in mouse hepatoma cells. To detect irradiation- and sulindac-induced apoptosis in Hepa1-6, MH134 and BNL1ME.A.R.1 cells, we examined the content of hypodiploid DNA in these cells.

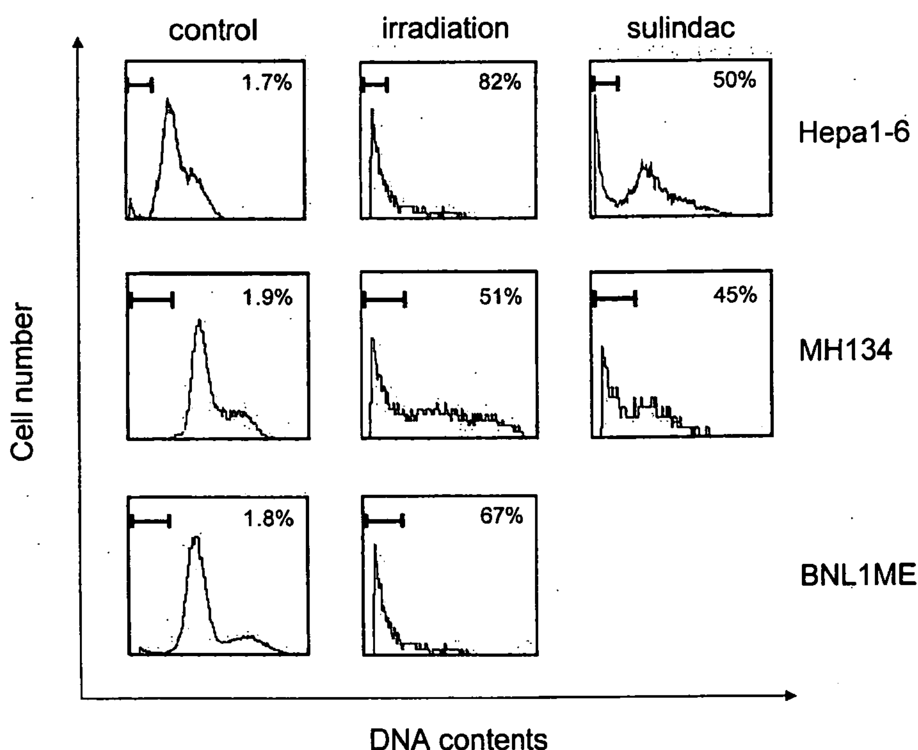

Figure 1. Apoptosis induced by irradiation and sulindac in murine hepatoma cells. Hepa1-6, MH134 and BNL1ME.A.7R.1 cells were irradiated by 40 Gy, or Hepa1-6 and MH134 cells were treated with 2 mM sulindac. Cells were stained with propidium iodide and subjected to DNA content analysis by a flow cytometer. The percentages of cells with hypodiploid DNA are indicated. Results shown are from one representative experiment from a total of four performed.

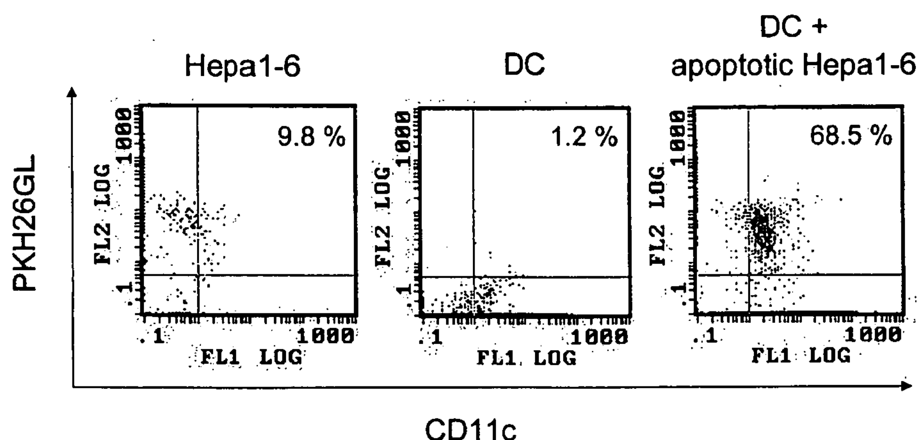

Figure 2. Uptake of apoptotic hepatoma cells by DCs. Hepa1-6 cells were dyed red using PKH26GL, induced apoptosis by 40 Gy irradiation, and cocultured with DCs at a ratio of 1:1. CD11c positive and PKH26GL positive cells were analyzed by a flow cytometer. The percentages of cells with double positive (CD11c$^+$ and PKH26GL$^+$) are indicated. Left, PKH26GL-labeled Hepa1-6 cells; middle, unpulsed DCs; right, DCs pulsed with PKH26GL-labeled apoptotic Hepa1-6 cells. Results shown are from one representative experiment from a total of three performed.

As shown in Fig. 1, 40 Gy of irradiation induced apoptosis in 50-80% of the cells and 2 mM of sulindac in 45-50% of the cells.

DCs efficiently take up irradiation-induced apoptotic hepatoma cells. Phagocytosis of irradiation-induced apoptotic Hepa1-6 cells by DCs was assessed by flow cytometric analysis based on the ability of CD11c positive DCs to take up apoptotic materials from red PKH26GL-labeled apoptotic cells. DCs were cocultured with PKH26GL-labeled apoptotic Hepa1-6 cells for 24 h, and then phagocytic uptake was determined by the percentage of double positive (CD11c$^+$ and PKH26GL$^+$) cells. As shown in Fig. 2, more than 65% of DCs engulfed apoptotic materials.

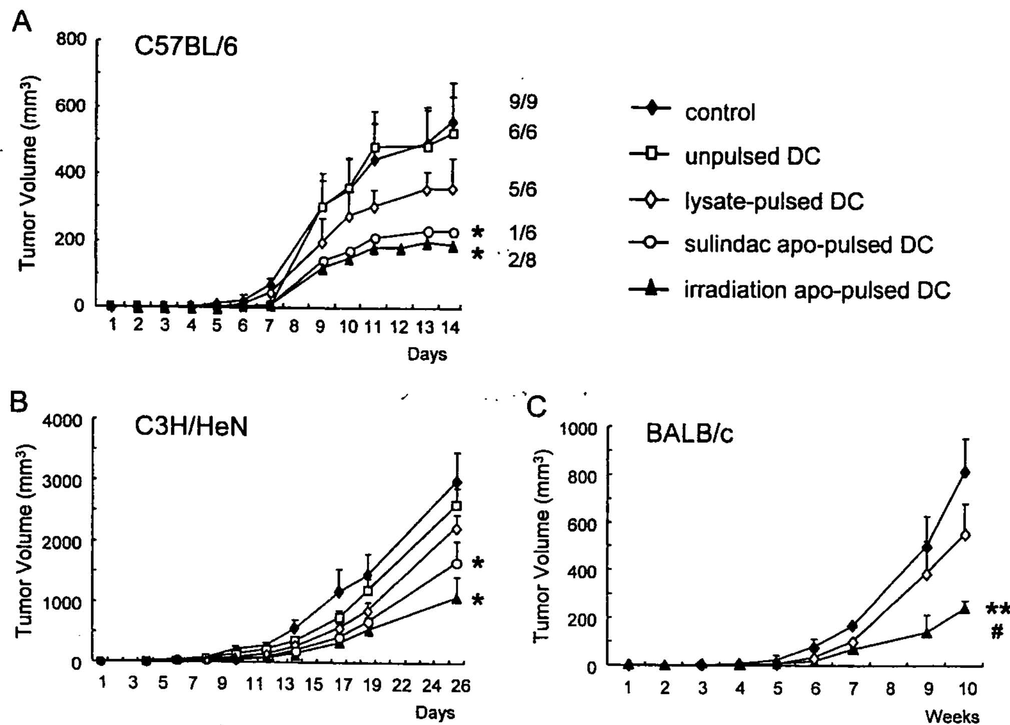

Figure 3. Protective antitumor effect of vaccination with DCs pulsed with apoptotic hepatoma cells *in vivo*. Mice were vaccinated twice with a one week interval with the injection of PBS alone (control), unpulsed DCs, DCs pulsed with hepatoma cell lysates, and DCs pulsed with sulindac- or irradiation-induced apoptotic hepatoma cells, respectively. Two weeks after the last vaccination, mice were challenged with syngeneic hepatoma cells. Tumor volume was determined as described in Materials and methods. Data are expressed as mean ± SE (n=6-9). *$p<0.05$ versus both control and vaccinated with unpulsed DCs group; **$p<0.01$ versus control; #$p<0.05$ versus vaccinated with lysate-pulsed DCs group. (A), C57BL/6 mice with Hepa1-6 cells, the ratio of the number of mice bearing a tumor/the number of mice challenged with Hepa1-6 cells in each group is indicated. (B), C3H/HeN mice with MH134 cells, (C), BALB/c mice with BNL1ME.A.7R.1 cells.

Vaccination with DCs pulsed with apoptotic hepatoma cells promotes protective antitumor immunity. We determined whether vaccination with DCs pulsed with apoptotic cells is superior to that with DCs pulsed with cell lysates to promote protective immunity against parental hepatoma *in vivo*. Mice were vaccinated twice in one week interval with DCs pulsed with apoptotic cells or freeze-thawed cell lysates, or unpulsed DCs. Two weeks after the last vaccination, mice were challenged with syngeneic hepatoma cells, and the tumor growth was monitored (Fig. 3). In C57BL/6 mice, five of six mice vaccinated with DCs pulsed with sulindac-induced apoptotic Hepa1-6 cells and six of eight mice vaccinated with DCs pulsed with irradiation-induced apoptotic Hepa1-6 cells were completely protected from the challenge of Hepa1-6 cells. In the remaining mice vaccinated with DCs pulsed with apoptotic Hepa1-6 cells, the growth of Hepa1-6 tumor was significantly delayed as compared with control. However, vaccination with DCs pulsed with Hepa1-6 cell lysates did not induce significant protective immunity (Fig. 3A). Similarly, in C3H/HeN mice, vaccination with DCs pulsed with sulindac- and irradiation-induced apoptotic MH134 cells but not MH134 cell lysates significantly retarded the growth of MH134 tumors although the complete protection was not observed (Fig. 3B). In addition, vaccination with DCs pulsed with irradiation-induced apoptotic BNL1ME.A.R.1 cells also significantly repressed the growth of BNL1ME.A.R.1 tumors in BALB/c mice (Fig. 3C).

Vaccination with DCs pulsed with apoptotic hepatoma cells induces antitumor cellular immunity. To examine whether vaccination with DCs pulsed with apoptotic hepatoma cells induces systemic cytolytic activity of lymphocytes against parental cells, C57BL/6 mice were twice vaccinated with DCs pulsed with sulindac- and irradiation-induced apoptotic Hepa1-6 cells or DCs pulsed with Hepa1-6 cell lysates. Spleen cells obtained two weeks after the last vaccination were cocultured with 20 Gy irradiated Hepa1-6 cells for five days to obtain the cytotoxic effector lymphocytes. The lymphocytes derived from mice vaccinated with DCs pulsed with sulindac- and irradiation-induced apoptotic Hepa1-6 cells but not Hepa1-6 cell lysates displayed enhanced cytotoxicity to Hepa1-6 cells at E:T ratios of 40:1 and 80:1 as compared with control (Fig. 4A). Since IFN-γ is a Th1-associated cytokine critically involved in the development of cell-mediated immune response, the frequency of IFN-γ-producing spleen cells from vaccinated C57BL/6 mice was determined by ELISPOT assay. The frequency of IFN-γ-producing spleen cells from C57BL/6 mice vaccinated with DCs pulsed with sulindac- and irradiation-induced apoptotic Hepa1-6 cells was significantly higher than that vaccinated with DCs pulsed with Hepa1-6 cell lysates (Fig. 4B). These results suggest that vaccination of mice with DCs pulsed with apoptotic cells promotes much stronger cellular immunity against parental hepatoma cells than that with DCs pulsed with cell lysates.

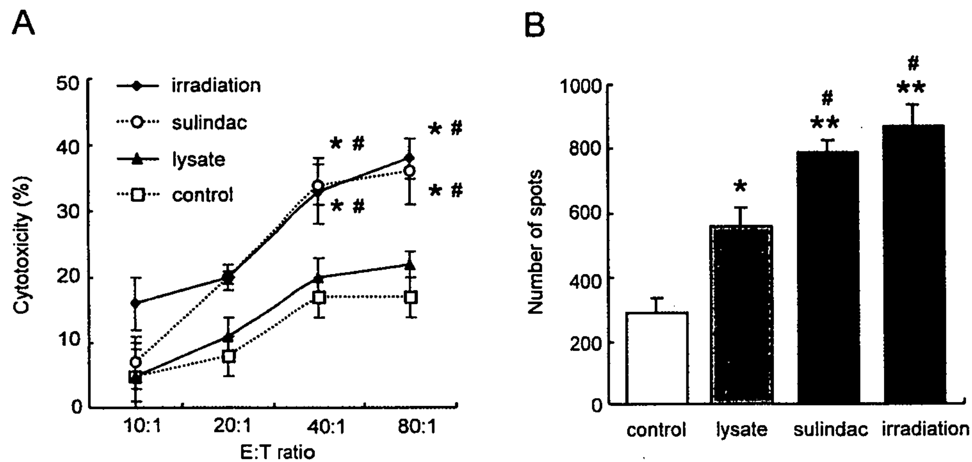

Figure 4. Antitumor cellular immunity induced by vaccination with DCs pulsed with apoptotic hepatoma cells. (A), Cytolytic activity of spleen cells against Hepa 1-6 cells. Spleen cells from vaccinated C57BL/6 mice with PBS alone (control), DCs pulsed with Hepa1-6 cell lysates, and DCs pulsed with sulindac- or irradiation-induced apoptotic Hepa1-6 cells were cocultured with 20 Gy irradiated Hepa1-6 cells. Cytotoxic effector lymphocytes were harvested after 5 days of incubation and the ^{51}Cr release assay was performed at indicated E:T ratios. Data are expressed as mean ± SD (n=6). *p<0.05 versus control; #p<0.05 versus lysate-pulsed DCs. (B), IFN-γ ELISPOT assay. Spleen cells derived from vaccinated C57BL/6 mice were cultured with irradiated Hepa1-6 cells for 48 h, and IFN-γ ELISPOT assay was performed. The spots in each well were counted under a microscope. Data are expressed as mean ± SD (n=6). *p<0.05 versus control; **p<0.01 versus control; #p<0.05 versus lysate-pulsed DCs.

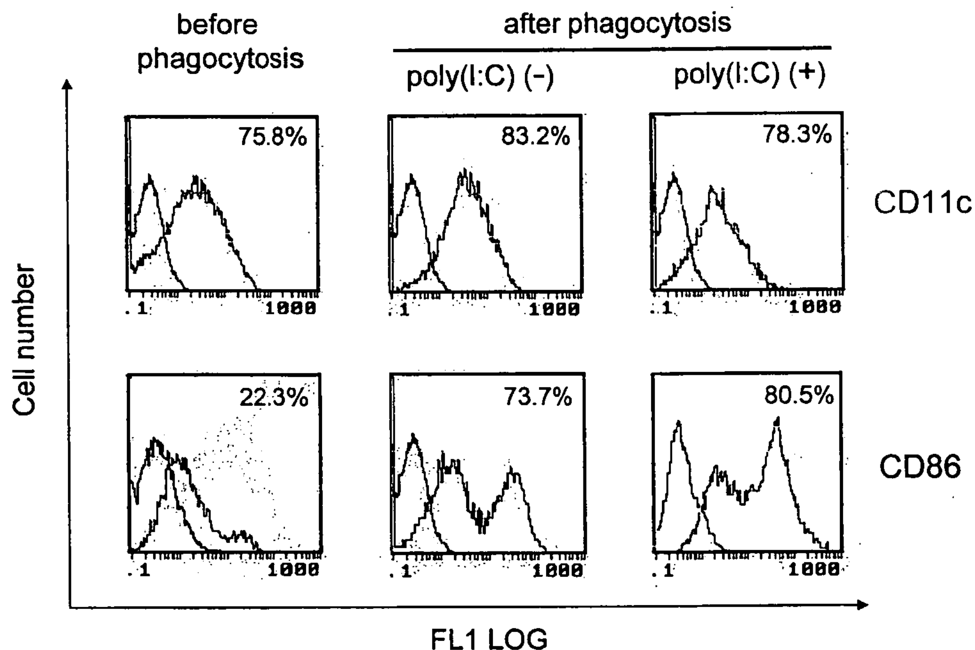

Figure 5. DC maturation after phagocytosis of apoptotic cells and poly (I:C) treatment. CD11c and CD86 expression in DCs after phagocytosis of apoptotic BNL1ME.A.7R.1 cells and poly (I:C) treatment were analyzed by a flow cytometer. The horizontal and vertical bars indicate the fluorescence intensity and the relative number of cells, respectively. Left histograms in each experiment represent negative controls. The percentages of positive cells are indicated. Results shown are from one representative experiment from a total of three performed.

Vaccination with DCs pulsed with apoptotic hepatoma cells represents antitumor effect against pre-established hepatoma. Finally, we elucidated the antitumor effect of vaccination with DCs pulsed with apoptotic BNL1ME.A.R.1 cells against pre-established BNL1ME.A.R.1 tumors. In addition, we examined whether DC maturation induced by poly (I:C) treatment influenced the antitumor effect of vaccination. CD86 expression, one of the maturation phenotypes of DCs (8,15), was upregurated after phagocytosis of apoptotic BNL1ME.A.R.1 cells, and addition of poly (I:C) further enhanced it (Fig. 5) as described previously (20). The growth of pre-established BNL1ME.A.R.1 tumors was significantly

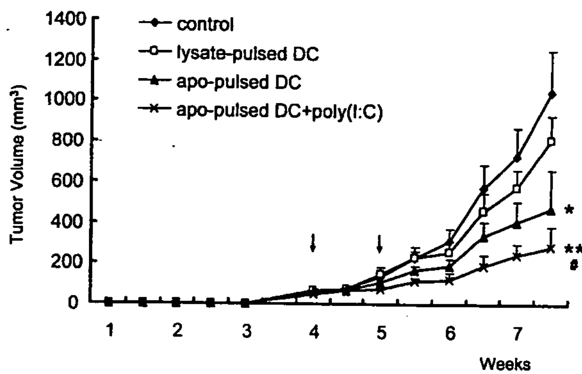

Figure 6. Antitumor effect of vaccination with DCs pulsed with apoptotic hepatoma cells on pre-established BNL1ME.A.7R.1 tumors in BALB/c mice. BNL1ME.A.7R.1 cells (3×10^6 cells/mouse) were subcutaneously injected into the right flank of BALB/c mice. Four weeks after tumor cells inoculation when the tumor diameter became more than 5 mm, mice underwent vaccination twice with a one week interval with the injection of PBS alone (control), lysate-pulsed DCs and DCs pulsed with irradiation-induced apoptotic cells with or without treatment of 20 μg/ml of poly (I:C), respectively. Arrows indicate the day of vaccination. Tumor volume was determined as described in Materials and methods. Data are expressed as mean ± SE (n=6). *$p<0.05$ versus control; **$p<0.01$ versus control; #$p<0.05$ versus vaccinated with lysate-pulsed DCs group.

retarded by vaccination with DCs pulsed with irradiation-induced apoptotic BNL1ME.A.R.1 cells ($p<0.05$ versus control) but not by vaccination with DCs pulsed with BNL1ME.A.R.1 cell lysates. In addition, vaccination with DCs pulsed with apoptotic cells and treated with poly (I:C) more efficiently repressed the tumor growth ($p<0.01$ versus control, $p<0.05$ versus vaccination with lysate-pulsed DCs), although rejection of tumors was not observed (Fig. 6).

Discussion

Albert *et al* have reported that DCs acquire antigens from apoptotic cells and stimulate MHC class I-restricted CTLs (21), and that immature DCs efficiently phagocytose apoptotic cells through αvß5 integrin and CD36, and cross-present antigens to CTLs (22). On the basis of their findings, several *in vitro* studies have shown that DCs pulsed with apoptotic tumor cells, which is induced by UV-B, γ-irradiation or anti-fas antibody, effectively stimulate tumor-specific CTLs (15-18,23), but DCs pulsed with tumor cell lysates cannot (15-18). In addition, it has been reported that protective antitumor immunity is induced by vaccination with DCs pulsed with apoptotic tumor cells in murine leukemia and melanoma models as compared to vaccination with unpulsed DCs (24,25). Taken together, it is likely that adequate cross-priming of CTLs with antitumor activity is achieved by DCs pulsed with apoptotic tumor cells rather than tumor cell lysates. However, there are few *in vivo* studies determining whether vaccination with DCs pulsed with apoptotic tumor cells is superior to that with DCs pulsed with tumor cell lysates to promote therapeutic antitumor immunity although DCs pulsed with tumor cell lysates are commonly used in phase I trials of DC-based cancer immunotherapy (5-7,13,14).

In the present study, we show that: i) immature DCs derived from murine bone marrow cells efficiently phagocytosed apoptotic hepatoma cells as reported previously (15-18,22), ii) vaccination with DCs pulsed with apoptotic hepatoma cells but not vaccination with DCs pulsed with hepatoma cell lysates induced protective antitumor immunity in three murine hepatoma models, iii) spleen cells from mice vaccinated with DCs pulsed with apoptotic hepatoma cells showed higher cytolytic activity and contained higher number of IFN-γ producing cells than those from mice vaccinated with DCs pulsed with hepatoma cell lysates, and moreover, iv) vaccination with DCs pulsed with apoptotic hepatoma cells but not hepatoma cell lysates significantly repressed the growth of pre-established hepatoma. These results indicate that vaccination with DCs pulsed with apoptotic tumor cells elicits more effective antitumor immunity than vaccination with DCs pulsed with tumor cell lysates in murine hepatoma models.

After capturing antigens from apoptotic cells, DCs start to mature (6,8). Matured DCs not only process antigens and present it to MHC molecules but also produce cytokines and chemokines and express costimulatory molecules, which further enhance cellular immunity (8). Therefore, maturation status of DCs could influence the efficacy of DC-based immunotherapy. Recently, it has been reported that heat-stressed apoptotic tumor cells express heat-shock proteins (HSPs), which act as danger signals, stimulate DCs maturation and elicit tumor-specific immunity (26,27). UV, γ-irradiation and certain chemotherapeutic agents are also known to induce HSPs in tumor cells (28-30). In addition, damaged DNA itself in leukemia cells, induced by alkylating agents but not by other chemotherapeutic agents, has been reported to stimulate DCs maturation and antigen presentation (31). Taken together, it is possible that apoptotic stimuli of tumor cells influence the maturation status of DCs uptaked tumor cell-derived antigens. In our study, irradiation or sulindac which is reported to induce apoptosis in human hepatoma cells (32) induced apoptosis in mouse hepatoma cells, and vaccination with apoptotic cell-pulsed DCs induced significant cytolytic activity against parental hepatoma cells. Therefore, loading of irradiated or sulindac-treated hepatoma cells could stimulate DCs maturation. In fact, after phagocytosis of irradiated hepatoma cells, expression of CD86 which is one of the maturation phenotypes of DCs was upregulated. DC maturation is also achieved by several internal stimuli such as TNF-α, IL-1ß, prostaglandins, interferons, and CD40 ligand and by foreign materials such as lipopolysaccharide, dsRNA, CpG-oligonucleotides via Toll-like receptors (6). Accordingly, we also examined the effects of poly (I:C) (dsRNA) on DC maturation and antitumor immunity in pre-established hepatoma models. Addition of poly (I:C) further enhanced the expression of CD86 in DCs, which was stimulated by phagocytosis of apoptotic cells. Vaccination with DCs both pulsed with apoptotic cells and treated with poly (I:C) showed the highest antitumor effect. These results suggest that maturation stimuli of DCs play a key role in DC-based cancer immunotherapy.

Recently, it has been reported that the combined intra-tumor injection of immature DCs and systemic chemotherapy or γ-irradiation induces effective antitumor response in

tumor-bearing mice (33-36). These are possible strategies to treat unresectable cancer since several studies including ours indicate that immature DCs efficiently phagocytose apoptotic tumor cells (15-18) and certain chemotherapeutic agents and γ-irradiation not only induce apoptosis but also stimulate the production of danger signals which stimulate DCs maturation (28-31).

In conclusion, we have shown that DCs pulsed with apoptotic hepatoma cells elicit effective antitumor immunity *in vivo*, which is further enhanced by addition of a DC maturing stimulus. Our results indicate that combination of apoptotic cells as TAAs and a DC maturation signal may be a promising strategy for DC-based immunotherapy for HCC.

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• CASE REPORT •

An autopsy case of acute pancreatitis with a high serum IgG4 complicated by amyloidosis and rheumatoid arthritis

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Abstract

We report an autopsy case of acute pancreatitis with a high serum IgG4 concentration complicated by systemic amyloid A amyloidosis and rheumatoid arthritis (RA). The patient was a 42-year-old Japanese female with a 22-year history of rheumatoid arthritis. She was diagnosed with myasthenia gravis when she was 31-year old. At the onset of pancreatitis, the patient was anti-nuclear antibody-positive, and had high serum gamma globulin and IgG4 levels. Dexamethasone and conventional therapy induced clinical remission and significantly decreased the serum IgG4 and gamma globulin. However, despite the decreased disease parameters, the patient developed a bleeding pseudocyst and died of cardiac failure. In the autopsy examination, it was determined that pancreatitis was probably caused by ischemia due to vascular obstruction caused by amyloid deposition in the pancreas. Even though acute pancreatitis is a rare complication in RA patients, we speculate that an autoimmune pancreatitis-related mechanism and ischemia due to vascular obstruction by amyloid deposition might be attributable to a single source that leads to acute pancreatitis in our particular case.

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Key words: Acute pancreatitis; Rheumatoid arthritis; IgG4; Systemic amyloidosis; Autoimmune pancreatitis

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INTRODUCTION

Acute pancreatitis is a relatively mild disease and has a low-associated morbidity. However, 4-7% of acute pancreatitis patients suffer from a severe illness that has been associated with mortality rates approaching 20-50%[1]. Etiologies of acute pancreatitis are bile duct stones, alcohol abuse, various toxins, drugs, other obstructive causes, metabolic abnormality, trauma, ischemia, infection, autoimmune disease and idiopathic causes[2]. In previous reports, pancreatitis occurring simultaneously with rheumatoid arthritis (RA) has been associated with Sjögren's syndrome (SjS)[3], anti-RA drugs[4], and amyloidosis[5]. It has been reported that a patient who developed amyloidosis following ankylosing spondylitis died of pancreatitis[6]. Occasionally, autoimmune disease is complicated by pancreatitis, which has led to the concept of an autoimmune-related pancreatitis and autoimmune pancreatitis has been correlated with other autoimmune diseases such as SjS, primary sclerosing cholangitis, ulcerative colitis, systemic lupus erythematosus[7] and myasthenia gravis[8]. Patients with autoimmune pancreatitis/sclerosing pancreatitis have several characteristic autoantibodies[7], and high serum gamma globulin and IgG4 concentrations[9]. In this paper, we report a rare case of acute pancreatitis in a patient with RA, who had a high serum IgG4 concentration and a condition complicated by systemic AA amyloidosis.

CASE REPORT

The patient was a 42-year-old Japanese female with a 22-year history of RA. In 1989, she was diagnosed with myasthenia gravis. In 1992, a stomach mucosal biopsy was carried out and amyloid deposition was confirmed. In December 1998, she had occasional epigastralgia after food intake. She had no history of habitual alcohol consumption and she had taken low-dose oral prednisolone (7.5 mg/d).

The patient presented with clinical pancreatitis with severe epigastralgia, back pain, nausea and vomiting and she was admitted to our hospital on January 7, 1999. On admission, she had bilateral joint deformity of the elbow, wrist, MP, PIP and DIP joints but no arthralgia and myasthenia. She had a fever of 38 ℃, tenderness of the epigastrium region and diminished bowel sound. Laboratory data showed the following values: 8 058 IU/L serum amylase, 1 800 ng/dL serum elastase 1, 1.946 g/dL gamma globulin, 1/160 ANA and 156 mg/dL IgG4. Ultrasound (US) examination and abdominal computed tomography (CT) scan revealed a slightly thickened gallbladder wall with a small stone and without biliary duct dilatation, and mild swelling of the pancreatic head consistent with effusion of the peripancreatic space. A left pleural effusion was identified by chest x-ray. Treatment for pancreatitis with an intravenous protease inhibitor, antibiotics and intramuscular dexamethasone

(2 mg/d) was commenced on admission. On the 9th hospital day, the patient's condition became complicated with congestive heart failure and bacterial pneumonia. From a cardiac US examination, we suspected a case of cardiac amyloidosis. On the 25th d, abdominal pseudocysts were found in hilum of the spleen and mesentery of the transverse colon by abdominal CT (Figure 1). The severity of abdominal symptoms was decreased by the treatment for pancreatitis and additionally, serum IgG4 and gamma globulin concentrations were decreased (31 mg/dL and 0.665 g/dL, respectively) on the 67th d. However, serum amylasemia continued to be severe, and we tried a new therapy using an intrasubcutaneous somatostatin analogue on the 75th d. Following this treatment, the serum amylase level gradually decreased and the patient started to eat on the 119th d.

On the 103rd d, hemorrhage occurred in a pseudocyst of the anterior spleen region. The patient complained of epigastralgia, but the hemorrhage was diminished by conservative treatment. However, re-bleeding in the same pseudocyst occurred on the 150th d, and anemia and hemorrhagic shock developed. The patient's condition recovered slightly following blood transfusion, but she then developed congestive heart failure. Several treatments for heart failure did not seem to be effective and the patient developed massive fresh blood stools and died on the 170th hospital day. We were permitted to perform an autopsy.

The autopsy findings were as follows: A marked amyloid deposition (AA type) was found mainly in the vascular wall with stenotic and obstructive changes, and in stroma of the heart, kidney, gastro-intestinal tract, liver, skin, pancreas and other organs. Pancreatic pseudocysts of 5 and 10 cm in diameter retaining blood and necrotic tissue were present in the mesentery and peritonitis with yellowish turbid ascites was identified in the mesentery and retroperitonea. In the pancreas, extensive fibrosis with lymphocyte infiltration, atrophy of acinar cells, dense pancreatic juice retention and dilatation of the pancreatic duct were observed. The small arteries and arterioles in the pancreas were obstructed and narrowed by marked amyloid deposition on the artery walls (Figure 2). There was no inflammatory cell infiltration in the common bile duct of the pancreas. Heart enlargement (500 g) occurred and the right and left ventricle extension was most unsatisfactory. Histopathological examination revealed diffuse amyloid deposition in myocardium and vascular wall, and an intramural thrombus in the right atrium. Dense amyloid deposition in the stroma and vascular wall was observed throughout the gastrointestinal tract. Multiple ulceration of the rectum reached the muscle layer. These results confirmed cardiac failure with systemic amyloidosis as the cause of death of the patient.

DISCUSSION

We have reported an autopsy case of necrotizing pancreatitis in a RA patient. In a postmortem histopathological examination, amyloid deposition was observed on walls of the small arteries and arterioles in the pancreas. Because we did not observe inflammation in the pancreatic common bile duct, the etiology of acute pancreatitis was not bile duct stones. Several cases of pancreatitis in RA patients have previously been reported[5,10], and in one of these cases, amyloidosis was found. Oishi *et al.*[5], described that fatal pancreatitis is associated with systemic amyloidosis in RA patients. In their report, a pancreatic biopsy is performed and AA amyloid deposition is observed on the vascular wall of pancreatic arteries and arterioles. It was speculated that the impairment of microcirculation of the pancreatic vasculature reduces the blood perfusion and causes more rapid deterioration, compared to the natural course of acute pancreatitis[5]. However, acute pancreatitis is a rare complication in RA patients, and we strongly suggest that pancreatitis is likely to be caused by ischemia due to vascular obstruction by amyloid deposition in the pancreas, which is brought about by RA-induced secondary AA amyloidosis.

Occasionally, autoimmune disease has been reported to be complicated by pancreatitis, which has led to the concept of an autoimmune-related pancreatitis[7]. In our case, some of the clinical characteristics (presence of an autoantibody, increased levels of gamma globulin and IgG4, effectiveness of steroid therapy and association with another autoimmune

Figure 1 Enhanced abdominal CT shows the presence of pseudocysts.

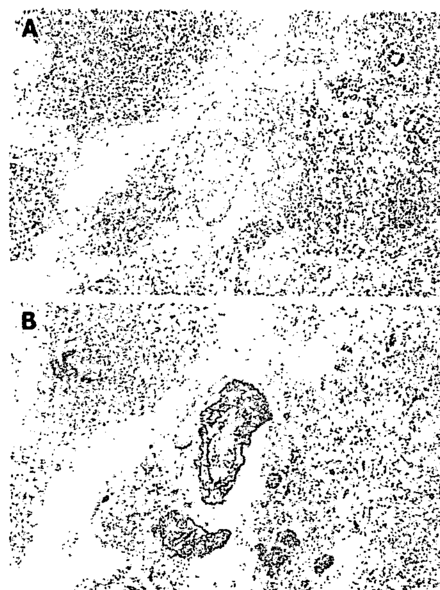

Figure 2 Histological findings of pancreas stained with hematoxylin and eosin (A) and Congo red (B).

disease, myasthenia gravis) correspond to autoimmune pancreatitis. Hamano *et al.*[9], reported that a high serum IgG4 concentration provides a useful means of distinguishing autoimmune pancreatitis/sclerosing pancreatitis from other diseases of the pancreas and biliary tract. In our case, dexamethasone and conventional therapy induced clinical remission and significantly decreased the serum IgG4 and gamma globulin levels. These results indicate that acute pancreatitis might be triggered by the same mechanism of autoimmune pancreatitis that was present in our case. However, acute pancreatitis and pancreatic cysts are unusual findings in autoimmune pancreatitis[7] and autopsy examination does not definitely indicate autoimmune pancreatitis. Hence, we speculate that an autoimmune pancreatitis-related mechanism may exist, which might be attributable to a single source that can also lead to acute pancreatitis.

In summary, acute pancreatitis is a rare complication in RA patients, but RA patients with systemic AA amyloidosis can develop acute pancreatitis from ischemia due to vascular obstruction by amyloid deposition. Such cases might be considered to be autoimmune-related pancreatitis because of the presence of autoantibodies, a high serum IgG4 concentration and an association with other autoimmune diseases.

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Cytokine gene polymorphisms in Japanese patients with hepatitis B virus infection—association between TGF-β1 polymorphisms and hepatocellular carcinoma

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***Background/Aims*: In this study, we determined the frequencies of the genotypes associated with the polymorphism of the cytokines genes, and investigated their association with the risk of hepatocellular carcinoma (HCC) in hepatitis B virus (HBV) carriers.**

***Methods*: Genetic polymorphism in the cytokines TNF-α, IFN-γ, TGF-β1, IL-6, and IL-10 were studied in 236 Japanese patients with HBV infection. The genetic polymorphisms of these cytokines were analyzed by polymerase chain reaction-sequence-specific primer (SSP).**

***Results*: There was no statistically significant difference in the genetic polymorphisms of TNF-α, IFN-γ, and IL-10 genes between HBV carriers with HCC and those without HCC. However, the TGF-β1+29 (codon 10) C/C genotype was lower in HBV carriers with HCC than in those without HCC (HCC 14.6% vs non-HCC 31.9%). The association of HCC was significantly lower in HBV carriers with C/C genotype than in those with T/C or T/T genotype in position + 29 of the TGF-β1 gene.**

***Conclusions*: Our findings suggest that the genetic polymorphism in codon 10 of the TGF-β1 gene may play a role in HCC development in patients with chronic HBV infection.**

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***Keywords*: Cytokines; Hepatitis B virus; Hepatocellular carcinoma; Polymorphism; Transforming growth factor-β1**

1. Introduction

Hepatitis B virus (HBV) infection is a major cause of progressive liver disease such as chronic hepatitis and liver cirrhosis in most industrialized countries [1]. An association between HBV infection and hepatocellular carcinoma (HCC) has also been established [2]. The factors involved in the progression of chronic HBV infection to HCC require investigation. The risk for developing HCC increases with severity of inflammation and fibrosis [3]. However, the host genetic factors that affect the HCC association remain unclear. A strong genetic component determining the outcome of HBV infection has been suggested in family studies [4]. Cytokines, as the product of host responses to inflammation, play an important role in the defense against viral infections and carcinogenesis [5]. An individuals' capacity for cytokine production has a major genetic component, and the variation among individuals can be striking [6,7]. This variation has been considered to be associated with polymorphisms within the promoter lesion or signal sequence of cytokine genes [8]. For example, the promoter of the IL-10 gene contains three biallelic polymorphisms at positions −1082, −819, and −592 from the transcription start site, and these influence the capacity to produce IL-10 [9]. These genetic polymorphisms may

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affect the development of inflammation, fibrosis, and carcinogenesis. The aim of the present study was to characterize cytokine gene polymorphisms in chronic HBV infection and their associations with HCC in a Japanese population.

2. Patients and methods

2.1. Patients

Of consecutive Japanese patients with chronic HBV infection who consulted the outpatient clinic of the National Nagasaki Medical Center and Nagasaki University Hospital between 2000 and 2004, we studied 236 patients. They were regularly followed with measurements of serum ALT and HBV markers such as HBsAg, HBeAg, and anti-HBeAb using commercially available radioimmunoassay kits (Dainabot, Tokyo, Japan) every month, and ultrasonography or computed tomography of the liver every 3 months. All patients were positive for HBsAg and did not have any other types of liver diseases such as chronic hepatitis C, alcoholic liver diseases, autoimmune liver diseases, or metabolic liver diseases. Total 236 patients were divided into two groups; patients with HCC and without HCC. The following clinical parameters of patients were obtained at the time of whole blood collection; age, gender, serum alanine aminotransferase (ALT) levels, and platelet counts. The diagnosis of HCC was made by several imaging modalities and confirmed histologically by sonography-guided fine-needle biopsy specimens in all patients.

The study protocol was approved by the Ethics Committees of both National Nagasaki Medical Center and Nagasaki University Hospital and informed consent was obtained from each individual.

2.2. DNA extraction

Genomic DNA was isolated from whole blood using the QIAamp DNA blood protocol according to the manufacturer's instruction (Qiagen Ltd, UK).

2.3. PCR sequence-specific primer typing

Single nucleotide mutations were analyzed in five different cytokines, leading to the genotype and phenotype assignment (Table 1).

In PCR sequence-specific primer (SSP) typing, oligonucleotide primers were designed to obtain amplification of specific alleles or groups of alleles. This typing method is based on the principle that a completely matched primer will be used more efficiently in the PCR reaction than with a primer with one or more mismatches. This means that the specificity of the typing system is a part of the PCR reaction. Assignment of alleles is then based on the presence or absence of amplified product detected by agarose gel electrophoresis. In this study, PCR amplification of selected TNF-α, TGF-β1, IL-10, IL-6, and IFN-γ alleles and an internal control, the human β-globulin gene were carried out according to the manufacturer's instruction (One Lambda, VH Bio Ltd, Gateshead, Tyne and Wear, UK). Briefly, after addition of the appropriate primer pairs, salts, buffer, and Taq polymerase, the samples were subjected to PCR in a 9600 Perkin-Elmer Thermocycler. The sequences of the primer pair have been described previously [9]. Amplification conditions were 1 cycle of 130 s at 96 °C dropping at 62 °C for an additional 60 s, nine cycles of 10 s at 96 °C and 60 s at 63 °C, and then the final 20 cycles included a three-temperature ramp annealing for 10 s at 96 °C, 50 s at 59 °C and finally 30 s at 72 °C. The amplified products were then separated by agarose gel electrophoresis and visualized by staining with ethidium bromide and exposure to ultraviolet light. Interpretation of PCR results was based on the presence of the internal control band together with the presence or absence of a specific amplified fragment.

2.4. Statistical analysis

Results are expressed as mean ± SD. Comparisons were made by Student's t test, Fisher's extract probability test, and the χ^2 test. All P values were two-tailed, and P values <0.05 were considered to indicate statistical significance.

3. Results

3.1. Patient characteristics

As shown in Table 2, there was no significant difference in gender and serum ALT levels between HBV carriers with HCC and those without HCC. In HBV carriers with HCC, age and proportion of cirrhosis were higher and platelet counts were lower than those without HCC.

Table 1
Characteristics of the cytokine gene polymorphisms

Gene	Position of the polymorphism	Allele	Haplotype	Phenotype	Reference
TNF-α	Promoter −308	A,G	A/A	High	[9]
			G/A	High	
			G/G	Low	
TGF-β	Codon 25	C,G	G/G	High	[9,10,11]
			G/C	Intermediate	
			C/C	Low	
IL-10	Promoters −1082; −819 and −592	A,G;T,C and A,C	GCC/GCC	High	[9]
			GCC/ACC	Intermediated	
			GCC/ATA	Intermediate	
			ACC/ACC	Low	
			ACC/ATA	Low	
			ATA/ATA	Low	
IL-6	Promoter −174	C,G	G/G	High	[12]
			G/C	High	
			C/C	Low	
IFN-γ	Intron +874	T,A	T/T	High	[9]
			T/A	Intermediate	
			A/A	Low	

TNF-α, tumor necrosis factor-α; TGF-β, transforming growth factor-β; IL-10, interleukin-10;IL-6, interleukin-6; INF-γ, interferon-γ.

Table 2
Baseline characteristics of HBV carriers

	Total HBV (n=236)	Without HCC (n=188)	With HCC (n=48)	Statistical analysis
Mean age (yr)	53.7±15.2	51.5±15.6	62.5±8.9	P<0.001. Student's t-test
Sex (M/F)	160/76	127/67	39/9	NS, χ^2
Mean ALT (U/L)	70.4±104.2	73.6±112.2	57.8±63.8	NS, Student's t-test
Mean PLT (10^3/μl)	158.2±71.8	169.7±63.7	119.2±83.8	P<0.001. Student's t-test
Mean albumin (g/dl)	4.3±.6	4.4±0.5	3.6±0.6	P<0.001. Student's t-test
Cirrhosis (%)	45.8	34.6	91.7	P<0.001. χ^2

HCC, hepatocellular carcinoma; ALT, alanine aminotransferse; PLT, platelet; NS, not significant.

3.2. Polymorphism in the TNF-α and IFN-γ genes

There was no polymorphism at the position +74 (codon 25) of the TGF-β1 gene and the position of -174 of the IL-6 gene in Japanese population studied. In the genotype frequencies at position −308 of the TNF-α genes and position of +874 of the IFN-γ gene, no statistically significant difference was found between HBV carriers with HCC and those without HCC (Table 3).

3.3. IL-10 gene promoter polymorphisms in hepatitis B virus carriers

We examined the three biallelic polymorphisms in the IL-10 gene promoter, at positions −1082, −819, and −592 from the transcription start site, respectively, which produce three different haplotypes: GCC, ACC, and ATA. The genotype frequencies are shown in Table 3, and the haplotype frequencies in Table 4. The frequencies of the ACC haplotype were increased in HBV carriers with HCC (33.3%) compared to those without HCC (30.3%), though the difference was not statistically significant.

3.4. TGF-β1 gene codon 10 polymorphisms

The distributions of the genotype of the polymorphism at the position +29 (codon 10) in HBV carriers with or without HCC are shown in Table 3. The genotype distributions were different in HBV carriers with HCC and those without HCC. In HBV carriers without HCC, the genotype frequencies were 18.6% for T/T, 49.5% for T/C, and 31.9% for C/C. In HBV carriers with HCC, on the other hand, the genotype frequencies were 22.9% for T/T, 62.5% for T/C, and 14.6% for C/C. As shown in Table 5, the association with HCC was significantly lower in HBV carriers with C/C genotype than in those with T/T or T/C genotype (P=0.028, odds=0.36, 95% CI; 0.154–0.859). Associations between cytokine gene polymorphism and the development of virus hepatitis were also reported. Ben-Air et al. reported that A/A genotype in the position +874 of IFN-γ gene was associated the development of HBV infection [13]. Gewaltig et al., reported that the presence of proline at TGF-β1gene codon 10 (C/C or C/T genotype) was associated with higher stage of fibrosis in HCV-infected patients [11]. Therefore, we assessed the genetic polymorphism of TGF-β1gene codon10 and the position +874 of IFN-γ gene in HBV carriers with or without liver cirrhosis (LC). As shown in Table 6 and 7, there was no significant association between these genes polymorphisms and the presence of LC.

Table 3
Genotype frequencies in HBV carriers

Variables	Patients with HBV		
	Total (n=236) (%)	Without HCC (n=188) (%)	With HCC (n=48) (%)
TNF-α			
G/G	230(97.5)	183(97.3)	47(97.9)
G/A	6(2.5)	5(2.7)	1(2.1)
A/A	0	0	0
TGF-β1-10			
C/C	67(28.4)	60(31.9)	7(14.6)
T/C	123(52.1)	93(49.5)	30(62.5)
T/T	46(19.5)	35(18.6)	11(22.9)
TGF-β1-25			
G/G	236(100)	188(100)	48(100)
G/C	0	0	0
C/C	0	0	0
IL-10			
ATA/ATA	102(43.2)	85(45.2)	17(35.4)
ACC/ATA	91(38.6)	72(38.3)	19(39.6)
ACC/ACC	25(10.6)	19(10.1)	6(12.5)
GCC/ATA	10(4.2)	6(3.2)	4(8.3)
GCC/ACC	5(2.1)	4(2.1)	1(2.1)
GCC/GCC	3(1.3)	2(1.1)	1(2.1)
IL-6			
G/G	236(100)	188(100)	48(100)
G/C	0	0	0
C/C	0	0	0
IFN-γ			
A/A	198(83.9)	157(83.5)	41(85.4)
T/A	38(16.1)	31(16.5)	7(14.6)
T/T	0	0	0

Note. The genotypes are shown as frequency (percentage). Abbreviations: HBV; hepatitis B virus, HCC; hepatocellular carcinoma, TNF-α; tumor necrosis factor-α, TGF-β; transforming growth factor-β, IL-6; Interleukin-6, IL-10; interleukin-10, IFN-γ; interferon-γ.