quantitative defect of both CD4+ and CD8+ immune response appears as the main determinant of viral persistence [5].

A seemingly unexpected strategy to enhance the immune function has emerged to be selective depletion of excess and activated granulocytes and monocytes [12,13]. The Adacolumn [12] was originally developed for selective depletion of excess and activated granulocytes and monocytes/macrophages in cancer patients who were found to have raised neutrophil counts, but low lymphocyte counts and impaired anti-tumour cytotoxic T-cell function [12,14]. The Adacolumn leucocytapheresis carriers adsorb granulocytes and monocytes/macrophages (FcγR and complement receptors bearing leucocytes [12]. The carriers typically adsorb 65% of granulocytes and 55% of monocytes from the blood in the column [12,15,16]. Studies in patients with rheumatoid arthritis revealed sustained increases in lymphocyte counts ($CD4^+$ T cells) and downregulation of inflammatory cytokines produced by leucocytes [12]. These effects suggested that it might enhance the efficacy of anti-HCV drug therapy. Further, granulocytes and monocytes/macrophages are extrahepatic replication sites and reservoirs for HCV dissemination [17–19] which can be eliminated. Additionally, the column carriers generate complement activation fragments including C3a, C5a, SC5b-9 complex and the opsonin C3bi [12,20] which theoretically should contribute to HCV clearance via opsonization of viral particles and enhanced cell-mediated immunity.

METHODS

Objectives

Adsorptive granulocyte and monocyte apheresis (GMA) was expected to benefit patients with HCV by depleting infected leucocytes and enhancing the immune function against the virus. Therefore, one major objective was to assess its effects on the efficacy of IFN/RBV combination in patients with high plasma HCV viraemia, mostly of genotype 1b. We were also interested to see the effects of GMA on peripheral blood leucocytes.

Patients selection

The demography of 24 patients of this study is shown in Table 1. Major inclusion criteria included: (i) a diagnosis of chronic hepatitis during the past 6 months as confirmed by liver biopsy; (ii) plasma HCV-RNA at least 100 kIU/mL by reverse transcription nested polymerase chain reaction (RT nested-PCR) within the past 3 months; (iii) abnormally high ALT level during 1 month prior to the initiation of the study; (iv) negative for hepatitis B virus and autoimmune hepatitis; (v) not receiving antiviral drugs or immunosuppressants within 3 months prior to the study; (vi) negative for HCC; (vii) neutrophil count >2000/μL, platelet count >80 000/μL, haemoglobin (Hb) >8.5 g/dL; (viii) absence of severe cardiovascular disease or renal failure; (ix) weight >35 kg; age above 12 and under 76 years; and (x) no history of adverse reaction to anticoagulants used during GMA (heparin).

Adsorptive GMA

GMA was performed with Adacolumn which is a new adsorptive carrier-based selective leucocytapheresis device [12]. The unit was provided by Japan Immunoresearch Laboratories (Takasaki, Japan). This medical device is CE marked (validated) by TUV (notified body). The column is filled with cellulose acetate beads of 2 mm in diameter as the column adsorptive carriers, bathed in sterile saline. The column was placed in an extracorporeal setting with a perfusion rate of 30 mL/min, duration 60 min similar to the settings used in patients with rheumatoid arthritis and ulcerative colitis [12,15,16]. Each patient received five GMA, one session/day over five consecutive days.

Drug therapy

IFN-α2b in combination with ribavirin (IFN/RBV) was started within 24 h after five GMA sessions. The dose of IFN was daily 6 million units six times/week for 2 weeks and then 6 million units three times/week for 22 weeks together with 600–800 mg ribavirin/patient/day (600 mg for patients weighing <60 kg and 800 mg for patients weighing 60 kg and higher). Patients were then followed for a further 24 weeks (total 49 weeks) without any anti-HCV medication during follow-up.

Ethics

The study protocol was reviewed and approved by the Institutional Review Board for ethics of clinical studies involving humans at each institution. Further, prior to GMA, informed consent was obtained from all patients verbally and in writing after they were informed of the purpose of the study and the nature of the procedures involved. Patients were advised that they were free to withdraw from the study at anytime without jeopardizing their subsequent care and treatment.

Assessment of efficacy and safety

To monitor treatment efficacy and safety, plasma HCV-RNA, serum ALT, aspartate aminotransferase (AST), total bilirubin, alkaline phosphatase, gamma-glutamyl transpeptidase, lactic dehydrogenase, total protein, creatinine, iron, ferritin, white blood cell counts (WBC), Hb and platelet counts were measured before first, third, fifth session, and once a month up to the end of the follow-up period or otherwise as shown in Figs 1–3.

Table 1 Patients' background and results of granulocyte and monocyte adsorptive apheresis (GMA) followed by IFN-α2b + ribavirin combination therapy. IFN-α2b + ribavirin was given after five GMA sessions over five consecutive days

Patient no.	Genotype	Liver biopsy	HCV-RNA (kU/mL)	Past IFN result	24 weeks IFN + ribavirin therapy								24 weeks observation					
					1	2	4	8	12	16	20	24	28	32	36	40	44	48
1	1b	A1F2-3	220	Relapsed	NT	–	→	→	→	+	+	+	+	+	+	+	+	+
2	1b	A2F2	>850	Relapsed	NT	–	NT	0.9	–	→	→	→	+	+	+	+	+	+
3	1b	A2F2	590	Relapsed	–	→	→	→	→	→	→	→	→	→	→	→	→	→
4	1b	A2F3-4	260	Relapsed	–	→	→	→	→	→	→	→	→	→	→	→	→	→
5	1b	A1F3	790	Naive	NT	–	→	→	→	→	→	→	→	+	+	+	+	+
6*	1b	A2F2	430	Relapsed	+	–	–	–	–	+	NT	NT	NT	NT	NT	NT	NT	NT
7	1b	A2F3	490	Relapsed	+	+	+	NT	+	+	+	+	+	+	+	+	+	+
8	1b	–	>850	Naïve	1.5	–	→	→	→	→	→	→	→	+	+	+	+	+
9	1b	A2F1	700	Naïve	–	→	→	→	→	→	→	→	→	→	→	→	→	→
10	1b	A1F1	600	Naïve	–	→	→	→	→	→	→	→	→	→	→	→	→	→
11	1b	A2F1	370	Naïve	340	62	84	3.8	–	→	→	→	+	+	+	+	+	+
12†	1b	A2F2	>850	Naïve	+	+	+	+	–	→	→	→	→	→	→	→	→	→
13	1b	A1F1	>850	Relapsed	NT	NT	0.7	–	→	→	→	→	→	+	+	+	+	+
14	1b	A1F0	>850	Naïve	NT	NT	–	→	→	→	→	→	→	+	+	+	+	+
15	1b	A1F1	160	Naïve	–	→	→	→	→	→	→	→	→	→	→	→	→	→
16	1b	A2F3	>850	Relapsed	NT	1.8	NT	+	+	–	→	→	→	→	→	→	→	→
17	1b	A1F3	>850	Relapsed	340	NT	440	630	850	850	850	850	850	850	850	850	850	850
18	2a	A2F3	>850	Relapsed	–	→	→	→	→	→	→	→	→	→	→	→	→	→
19	2a	A2F1	110	Naïve	–	→	→	→	→	→	→	→	→	→	→	→	→	→
20	2a	A2F2	540	Relapsed	NT	–	→	→	→	→	→	→	→	→	→	→	→	→
21	2a	A1F1	>850	Naïve	–	→	→	→	→	→	→	→	→	→	→	→	→	→
22	2b	A2F2	100	Naïve	–	→	→	→	NT	→	NT	NT	→	→	→	→	→	→
23	2b	A2F2	>850	Relapsed	NT	NT	–	→	→	→	→	→	→	→	→	→	→	→
24	2b	A2F1	>850	Relapsed	NT	NT	–	→	→	→	→	→	→	→	→	→	→	→

*Withdrew at week 12 because of a broken leg.
†Received IFN after week 24.
NT, not tested; –, absent; → , negative HCV-RNA was maintained; A1–4, slight, mild, moderate and extensive inflammatory cell infiltration respectively; F1–4, slight, mild, moderate and extensive liver fibrosis respectively.

Assays of HCV-RNA, ALT, AST and C3a

Plasma HCV-RNA was measured by RT nested-PCR [21]. ALT, AST and complement activation fragment C3a (by radioimmunoassay) were measured in serum samples. All assays were performed at a special research laboratory (SRL) using the most sensitive assay kits available (R&D Systems, Minneapolis, MN, USA) according to the package insert.

Measurement of TNF-α and IL-8

Tissue necrosis factor-alpha (TNF-α), a major inflammatory cytokine produced by peripheral blood mononuclear leucocytes and the chemokine, interleukin-8 (IL-8) produced by peripheral blood leucocytes were measured to test the effects of GMA on inflammatory factors. TNF-α assay was according to Chofflon et al. [22] while IL-8 was according to DeForge et al. [23]. Blood samples were taken from the Adacolumn inflow (peripheral blood) at the start of GMA and from the outflow towards the end of the 60-min GMA session. Tests at about 5×10^5/mL leucocytes were stimulated with 1.5 μg/mL lipopolysaccharide and the supernatants were sent to an SRL for assay of TNF-α and IL-8 by ELISA as indicated above.

Statistical analysis

The data are presented as the mean ± SD values and ranges unless stated otherwise. Comparisons between sets of data were carried out with the Mann–Whitney *U*-test on raw data by using Stat View Software. A significance level of 0.05 was used for all statistical tests, and two-tailed tests were applied when appropriate.

RESULTS

Changes in HCV-RNA during therapy

Table 1 shows entry HCV-RNA levels, liver biopsy outcomes, previous response to IFN and HCV-RNA profiles during the

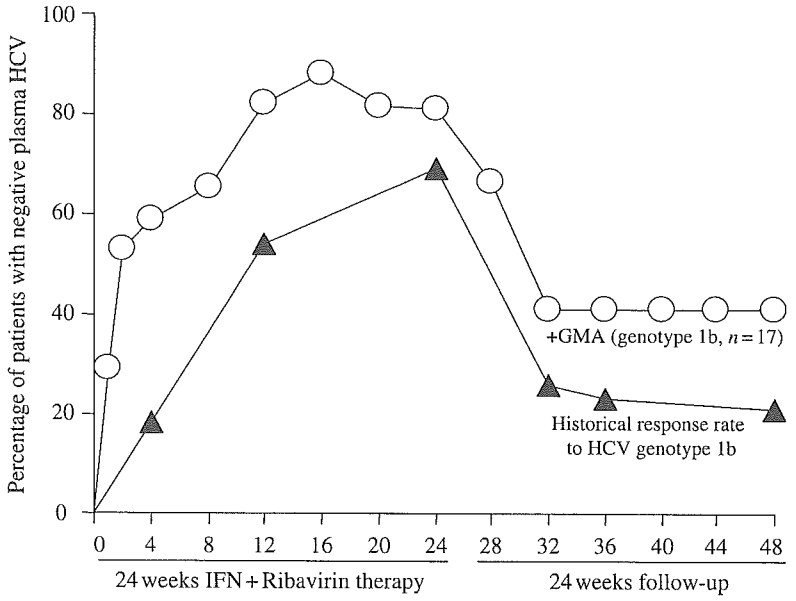

Fig. 1 The percentage of patients who became HCV (HCV-RNA) negative with time during IFN-α + ribavirin therapy and the follow-up for two groups of patients. The solid triangles represent the historical response to IFN-α2b + ribavirin in patients with HCV genotype 1b while the open circles represent the response to the same regimen in 17 patients with HCV genotype 1b who received five GMA sessions prior to initiation of IFN + ribavirin therapy. For clarity, SD values are not shown. It can be seen that in the group receiving GMA, the response was more rapid with a greater fraction of patients in remission throughout the study time. The historical data are according to Iino S. Future outlook for treatment of chronic hepatitis C: IFN + ribavirin combination therapy (Rinsho Byori, 2001; 49: 747–54 with permission of the publisher).

therapy and the follow-up period. Eleven of 24 patients were IFN naïve and 13 had relapsed following a short-period response to IFN. Plasma HCV-RNA was 612.9 ± 312.9 kU/mL (range 100–850 kIU/mL). As shown, at the end of the 24-week IFN/RBV therapy, 20 of 24 patients (83%) were HCV negative, and by the end of the follow-up period, 14 of 24 patients (58%) had maintained their remission. With respect to HCV genotypes, all seven patients with genotype 2a or 2b maintained their remission throughout the follow-up period (100% sustained response). To say that this small group of seven patients with 2a or 2b responded to the therapy more rapidly and fully compared with patients who harboured HCV genotype 1b. Additionally, in Fig. 1, the historical response to IFN/RBV in patients with HCV genotype 1b is presented for comparison with the results of this study on 17 patients who received GMA prior to IFN/RBV. In the GMA group, the response is more rapid with a greater fraction of patients achieving sustained remission.

Changes in serum ALT and AST levels during the study

At entry, the group ALT was 108.1 ± 80.4 IU/L (range 41–373 IU/L). The corresponding values for AST were 73.42 ± 45.09 IU/L and 31–226 IU/L. The mean ALT fell following the start of IFN/RBV therapy and was within normal range after 12 weeks ($P < 0.001$) and remained at this level during the 24-week follow-up period ($P < 0.0001$). The changes in serum AST were very similar to ALT (results not presented).

Changes in peripheral blood leucocyte counts

Figure 2 shows total peripheral blood WBC and granulocyte (neutrophil) counts *vs* time during GMA, IFN/RBV therapy and follow-up. There was no marked fall in either WBC or granulocyte counts during the week in which patients received five GMA sessions. At entry, WBC count ($\times 10^3/\mu L$) was 4.826 ± 0.745 (range 3.92–6.61). The corresponding values after five GMA sessions were 4.602 ± 0.908 (range 6.40–3.11). Similarly, for neutrophils (%), entry values were 55.65 ± 8.64 (range 35–70.9). The values after five GMA sessions were 50.73 ± 12.04 (range 34–68). However, both WBC and granulocyte counts fell significantly during IFN/RBV therapy which were reversed during the follow-up.

Effects of the treatment on peripheral blood lymphocyte counts

Figure 3 shows lymphocyte counts at entry, at the end of the last GMA session and up to the end of the observation period. At entry, lymphocyte count (%) was 39.96 ± 8.87 (range 21.9–52.6). Just prior to the start of IFN/RBV therapy, it had

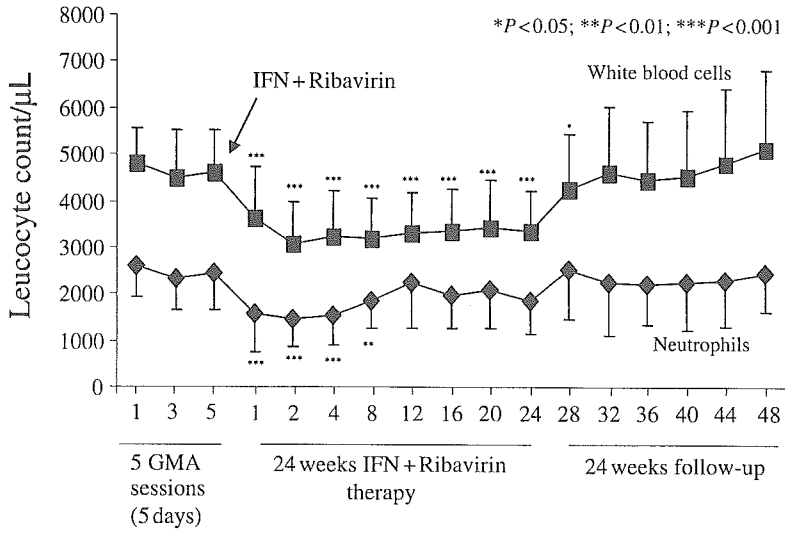

Fig. 2 Total white blood cell (WBC) and granulocyte counts with time during 5 days of GMA and IFN + ribavirin therapy together with the follow-up time. As shown, no marked fall in peripheral blood leucocytes was seen during GMA, but both WBC and neutrophil counts fell during IFN + ribavirin therapy with recovery during the follow-up. For additional comments see Discussion. The *P*-values for the time points shown are relative to the entry levels.

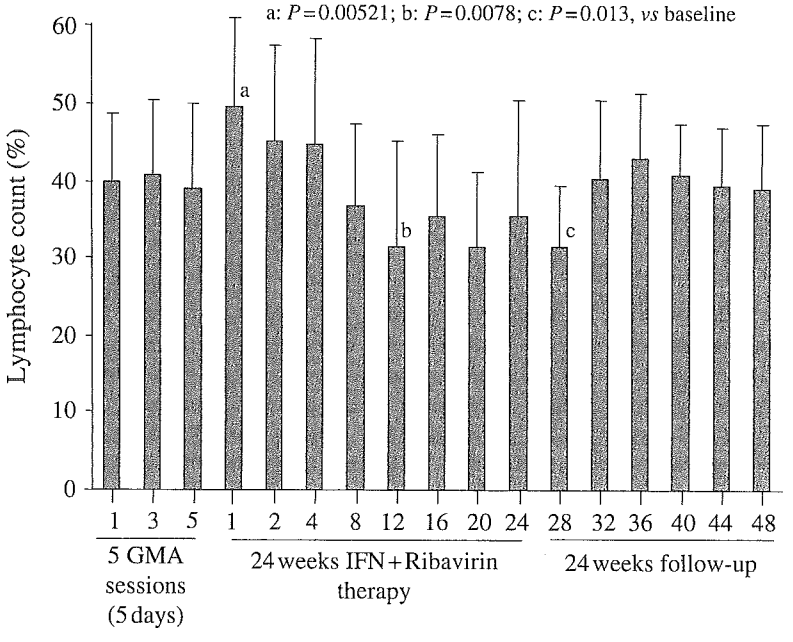

Fig. 3 Changes in lymphocyte counts at entry, at the end of the last GMA session (start of IFN/ribavirin therapy) and up to the end of the observation period. At entry, the lymphocyte count (%) was 39.96 ± 8.87 (range 21.9–52.6). At the start of IFN/ribavirin therapy, it had increased to 49.51 ± 11.55 (range 28–69) (P = 0.0052). Similar to WBC and granulocyte counts (Fig. 4), lymphocyte counts began to fall during IFN/ribavirin therapy and by week 12 were 31.47 ± 13.74 (range 9.0–54.4) (P < 0.0078). After cessation of IFN/ribavirin therapy (fallow-up) blood leucocytes began to rise again.

increased to 49.51 ± 11.55 (range 28–69) (P = 0.0052). Obviously, the upper limit is higher than normal laboratory values. The anomaly might be associated with HCV infection and/or IFN/RBV therapy. However, it is not attributable to removal of granulocytes by GMA because earlier work has shown that at the time of the rise in lymphocyte count, the peripheral blood granulocyte count returns to baseline level, within a few hours [12]; the lost granulocytes are replaced by CD10 negative (naive) neutrophils [13]. Indeed, Fig. 2 does not show any marked fall in WBC or neutrophils during the 5-day GMA course. Similar to WBC and granulocyte counts, lymphocyte counts began to fall during IFN/RBV therapy and by week 12 was 31.47 ± 13.74 (range 9.0–54.4) (P < 0.0078). After cessation of IFN/RBV therapy, blood lymphocyte counts began to rise again (Fig. 3).

Generation of the complement C3a during GMA

Figure 4 shows generation of complement activation fragment, C3a in the peripheral blood (Adacolumn inflow) and in the blood that emerged from the column (blood return to patients). The data show a dramatic increase in this major complement activation product at the column outflow, by more than 10-fold higher relative to inflow.

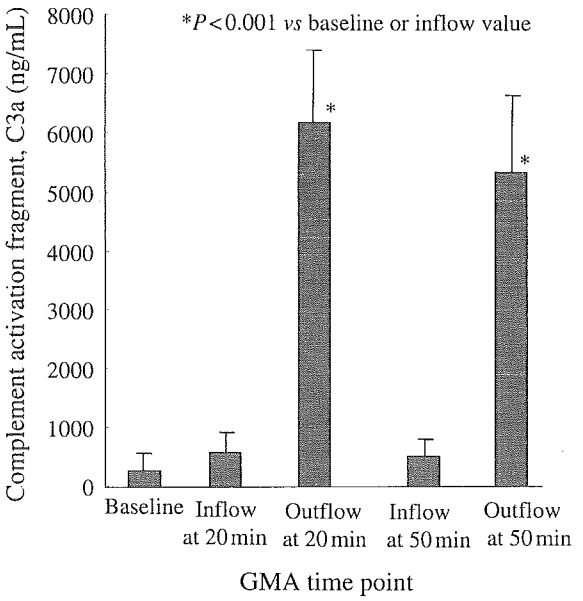

Fig. 4 Generation of complement activation fragment, C3a in the peripheral blood (Adacolumn inflow) and in the blood that emerged from the column (blood return to patients). The data show a dramatic increase in blood levels of the major active complement, C3a at the column outflow, by more than 10-fold higher relative to the column inflow.

Effects of GMA on TNF-α and IL-8

Figure 5 shows the effects of GMA on TNF-α and IL-8 produced by leucocytes. As indicated, blood samples were taken from the Adacolumn inflow at the start of GMA (peripheral blood) and outflow at 50 min. This was performed on two separate occasions, the first GMA session and the fifth (last) session. The amounts of TNF-α and IL-8 produced by leucocytes were significantly reduced not only by leucocytes that had passed through the column, but also by peripheral blood leucocytes after five sessions.

Treatment safety and patient compliance

All 24 patients completed their five GMA sessions according to the protocol and no severe adverse events related to GMA were seen. Further, during GMA, no marked fall in haematological or biochemical parameters were seen in spite of the fact that column removes up to 65% of neutrophils and 55% of monocytes from the blood in the column [12]. Only two incidences of transient mild headache were reported during GMA. In both cases, the symptoms receded within 3 h without medication. Two patients did not complete the follow-up; one had accidental broken leg at week 12 and the other received intermittent IFN/RBV during the follow-up according to the physician's discretion (see Table 1). As shown in Figs 2 and 3, during IFN/RBV therapy, both WBC, neutrophil and lymphocyte counts fell significantly and were reversed during the follow-up. Similarly, Hb (g/dL) fell from an entry value of 14.16 ± 1.19 (range 11.7–16.3) to 11.73 ± 1.67 (range 7.6–15.2) at week 12. The fall in Hb reversed during the follow-up.

DISCUSSION

The only novelty in this study is addition of GMA to the widely adopted [5,6] IFN/RBV therapy. GMA with Adacolumn in patients with rheumatoid arthritis and ulcerative colitis reported unexpected suppression of TNF-α, IL-1β, IL-6 and IL-8 produced by leucocytes [12,13,24–26], an increase in CD4+/CD8+ T cells [12], a rise in CD10 negative (naive) neutrophils [13] and generation of active complement including SC5b-9 complex and the opsonin C3bi by the column carriers [20]. In view of the evidence that granulocytes, monocytes/macrophages are extrahepatic replication sites for HCV [17–19] and an increase in CD4+/CD8+ T cells subsequent to a GMA course [12], we had the impression that GMA might enhance the efficacy of IFN/RBV. Earlier in a patient with ulcerative colitis–HCV co-morbidity, genotype 1b who was intolerant to IFN, we achieved sustained remission after GMA [27]. Following this case, we undertook a preliminary study in six patients with high plasma HCV-RNA. GMA was performed at one session/week for 5 weeks. Despite a fall of over 50% in HCV-RNA during the GMA procedure, HCV-RNA increased again during the waiting time for the next session [28]. It appeared that one session/week for five consecutive weeks was an inappropriate GMA frequency. Accordingly, in this study, we tried one GMA/day.

In Japan, the standard treatment for HCV is 6–10 million units of IFN for 24 weeks (daily for 2–4 weeks, then three times a week for 20–22 weeks) plus RBV; this was followed in this study. This regimen normalizes plasma AST and ALT, but plasma HCV-RNA becomes negative in <50% of patients after the treatment [29]. Patients who do not respond to this regimen are considered to harbour IFN-resistant HCV. However, the majority of patients included in this study had high plasma viraemia and were harbouring HCV genotype 1b which very poorly responds to IFN/RBV [5–7,30]. In spite of this, 20 of 24 patients (83%) became HCV negative and of these, 14 (58%) achieved sustained remission (40% genotype 1b and 100% of genotype 2a or 2b). An 83% initial response rate in patients with high plasma HCV load and a 58% sustained remission rate are the highest reported in the literature for genotype 1b [5–7,30]. In our experience, patients who maintain their remission up to 24 weeks following the end of treatment are unlikely to have a viral relapse. Our impression that an increase in circulating lymphocyte level was one factor for good initial response was overshadowed by the fall in lymphocytes during IFN/RBV therapy. Hence, it is likely that one or more course of GMA during drug therapy and follow-up can increase the number

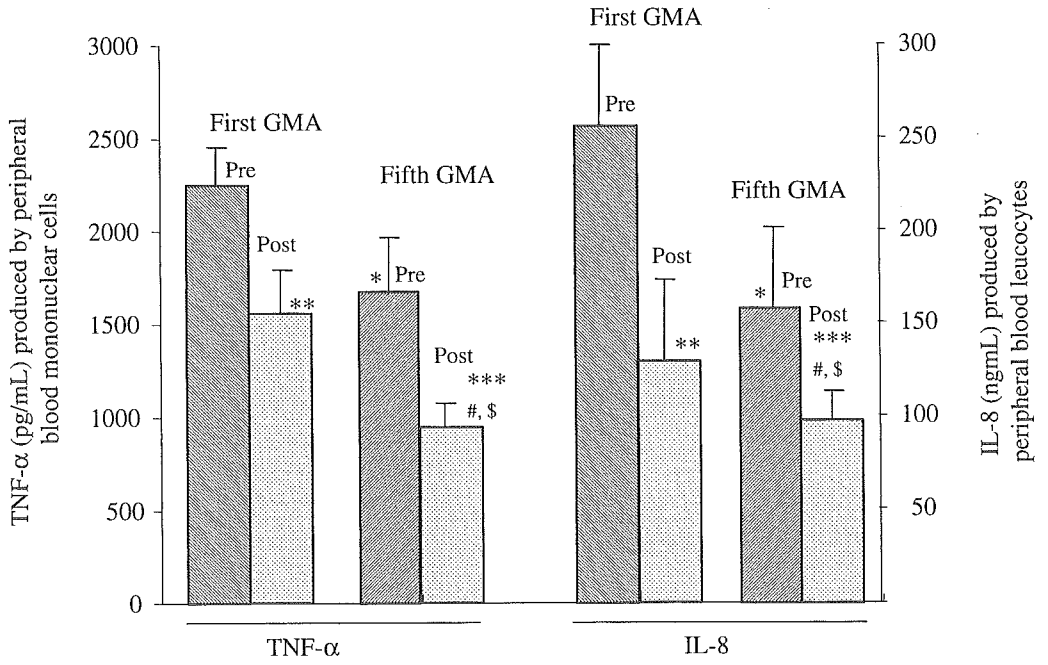

Fig. 5 Effects of GMA on TNF-α and IL-8 produced by lipopolysaccharide stimulated blood leucocytes. As indicated, blood samples were taken from the Adacolumn inflow at the start of GMA (peripheral blood) and outflow at 50 min. This was performed on two separate occasions, the first GMA session and the fifth (last) session. The amount of cytokine produced by leukocytes was significantly reduced not only by passing through the column but also by peripheral blood after five sessions. The results show that GMA is associated with reduced cytokine production by blood leucocytes.

patients with sustained remission. We also observed GMA-related suppression of TNF-α and IL-8 produced by blood leucocytes, a significant rise in lymphocyte counts and generation of the active complement, C3a.

Poor HCV response to IFN is associated with defective CD4+/CD8+ T-cell function [8–11]. Hence, a decrease in lymphocytes during IFN/RBV therapy is likely to be a negative factor in HCV response to INF/RBV. The mechanism(s) by which IFN/RBV causes a fall in blood leucocytes is not clear to us. A study by Zella et al. [31] reports that IFN-α2b increases the expression of apoptosis receptor CD95 and chemokine receptors CCR1 and CCR3 in monocytoid cells. Similarly, Kaser et al. [32] report that IFN-α promotes activation-induced T-cell death by upregulation of Fas (CD95/APO-1) and Fas ligand expression, while a study by Manna et al. [33] reports that IFN-α potentiates TNF-induced apoptosis.

For GMA-related enhanced efficacy of IFN/RBV, the following speculations are in line with the observed actions of GMA. First, as reported above, GMA generates complement activation products, C3a together with SC5b-9 complex and the opsonin C3bi [12,20]. Complement is an integral component of body's defence [34] and generation of C3a, SC5b-9 and C3bi theoretically should contribute to HCV killing both in the plasma and within the liver via opsonization of the viral particles and enhanced cell-mediated immunity. Secondly, GMA has been associated with a sustained fall in TNF-α, IL-1β, IL-6 and IL-8 released by peripheral blood leucocytes [12,13,24–26]. It is known [35–37] that down-regulation of pro-inflammatory cytokines compromises the activities of the so-called 'suppressor of cytokine signalling' (SOCS). These are several intracellular proteins induced by inflammatory cytokines [35–37]. SOCS are well known to have a strong inhibitory role on the activities of interferons [38–40] including the anti-HCV action of IFN-α [40]. This may lead us to assume that an enhanced anti-HCV efficacy of IFN/ribavirin following GMA reflects absence of a strong inhibitory effect on IFN-α by SOCS. Thirdly, GMA depletes infected leucocytes and increases lymphocytes which should provide a healthier immune function at the start of IFN/RBV therapy. Certainly, these speculations need to be further supported in future controlled studies and if true, they can serve as the basis for GMA to become an adjunct to IFN/RBV, a very safe, feasible and natural procedure. Additional issues which might affect the efficacy of GMA and need to be evaluated in future controlled trials include: (i) frequency of

GMA (once a day, three times a week etceteras); (ii) duration of one GMA session; and (iii) duration of one treatment course (1 week, 2 weeks or longer?).

In conclusion, this study has shown that adsorptive depletion of infected granulocytes and monocytes/macrophages enhances the anti-HCV efficacy of IFN/RBV therapy. Generation of active complement opsonins during GMA is thought to contribute to HCV killing, and additional mechanism(s) including increased lymphocytes and suppression of specific cytokines are likely to be involved. Clearly further controlled studies are necessary to determine the full efficacy of GMA in the treatment of HCV.

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Short Communication

Full-Length Sequence of Hepatitis B Virus Belonging to Genotype H Identified in a Japanese Patient with Chronic Hepatitis

Akira Nakajima[1,2,3], Mitsugu Usui[2], Tran Thien Tuan Huy[1], Naomi Khaing Than Hlaing[1], Naohiko Masaki[4], Tetsutaro Sata[1] and Kenji Abe[1]*

[1]*Department of Pathology, National Institute of Infectious Diseases, Tokyo 162-8640,*
[2]*PALMA Bee'Z Research Institute Co., Ltd., Kanagawa 210-0855,* [3]*Sanko Junyaku Co., Ltd., Tokyo 101-0032 and*
[4]*Division of Gastroenterology, International Medical Center of Japan, Tokyo 162-8655, Japan*

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SUMMARY: We have isolated and cloned the full-length nucleotide sequence of the hepatitis B virus (HBV) genome (denoted HBV-IM806-2) recovered from a Japanese patient with chronic hepatitis. This patient had a history of travel to Bangkok, Thailand, and then suffered the onset of acute hepatitis B 3 months after his return to Japan. The HBV-IM806-2 isolate was composed of 3,215 nucleotides and showed the highest similarity to genotype H of HBV. Interestingly, 24 amino acid residues specific for genotype H were identified throughout the full genome sequence. Furthermore, phylogenetic analysis based on the full genome sequence confirmed that IM806-2 belonged to genotype H and was more closely related to the prototype of the Los Angeles strain than to the Nicaragua strain.

More than 350 million people worldwide are chronically infected with hepatitis B virus (HBV) and are at risk of dying as a result of the occurrence of hepatocellular carcinoma accompanying HBV infection. By characterization of the viral genome, HBV has been classified into genotypes A through G with an inter-genotypic diversity of at least 8% in the full genome sequence (1,2). In addition to this classification, a newly described genotype H has been found in Nicaragua and the U. S. (3). It seems that the distribution of genotype H is restricted to the northern part of Latin America, however, the exact distribution of this genotype remains unclear due to the lack of a rapid and simple method of identification such as genotyping by PCR or PCR-RFLP. Among the sequence records deposited in the database, only 8 isolates of genotype H have been sequenced in the full genome to date. Here we report the identification and entire nucleotide sequence of the HBV belonging to genotype H isolated from a Japanese patient with chronic hepatitis.

A 61-year-old man underwent a medical examination at the International Medical Center of Japan, Tokyo and was diagnosed with chronic hepatitis. Serological findings were positive for HBsAg, HBeAg and anti-HBc, but negative for anti-HCV and HCV RNA. The patient had visited Bangkok, Thailand, 30 years previously and had had sexual contact with a woman there. Three months after his return to Japan, he suffered the onset of acute hepatitis with HBV infection which developed into chronic hepatitis B. We conducted a sequencing analysis of the HBV obtained from this patient. To obtain a full-length sequence, we amplified HBV DNA by PCR using a primer combination of HBV4 (sense; 5′-CCG GAA AGC TTA TGC TCT TCT TTT TCA CCT CTG CCT AAT CAT C-3′; the HindIII site is underlined) and HBV4R (antisense; 5′-CCG GAG AGC TCA TGC TCT TCA AAA AGT TGC ATG GTG CTG GTG-3′; the SacI site is underlined) as reported previously (4). Viral DNA was extracted from 100 μl of serum using a DNA/RNA extraction Kit (SepaGene RV-R, Sanko Junyaku Co., Ltd., Tokyo, Japan). The resulting pellet was resuspended in 50 μl of RNase-free water and maintained at −20°C until use. The PCR conditions included pre-incubation at 94°C, 2-min activation of Blend Taq-Plus DNA polymerase (Toyobo Co., Ltd., Tokyo, Japan) followed by 40 cycles of PCR (94°C for 15 sec, 55°C for 45 sec and 72°C for 3 min 20 sec with a final extension for 7 min at 72°C). The PCR products were separated by 1% agarose gel electrophoresis and purified using a QIA quick gel extraction kit (Qiagen, Inc., Chatsworth, Calif., USA) in preparation for sequence analysis. Purified PCR products were cloned into the HindIII/SacI sites of pUC19 vector. Cloned HBV DNA was subjected to sequencing using an ABI PRISM™ Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, Calif., USA). Sequences of cloned HBV DNA were determined using the automated DNA sequencer, ABI 3100-Avant Genetic Analyzer (Applied Biosystems).

The sequence reported in this paper have been deposited in the DDBJ/GenBank/EMBL under the accession number AB205010.

The HBV genome recovered in this study was compared with the 35 isolates of HBV with a full-length sequence in the database. Nucleotide sequences were multiple aligned using GENETYX for Windows version 7 software (Genetyx, Tokyo, Japan) and were calculated using the Kimura two-parameter method; phylogenetic trees were constructed by the neighbor-joining method (5). To confirm the reliability of the pairwise comparison and phylogenetic tree analysis, bootstrap resampling and reconstruction were carried out 1,000 times.

The full genome sequence of the HBV was obtained from our Japanese patient with chronic hepatitis and was named HBV-IM806-2. IM806-2 was composed of 3,215 bases. When compared with other previously reported HBV isolates with a full genome sequence, IM806-2 showed high overall identity (98.9%) with the prototype of the Los Angeles strain (AY090460) and 97.4% identity with the Nicaragua

*Corresponding author: Mailing address: Department of Pathology, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan. Tel: +81-3-5285-1111 ext. 2624, Fax: +81-3-5285-1189, E-mail: kenjiabe@nih.go.jp

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	Amino acid	P gene															C gene		X gene				S gene		
		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
	M57663 Philippine	D	N	C	P	S	S	A	S	S	A	L	D	N	K	S	R	S	R	V	A	I	P	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
	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
	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
	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
	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
	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
	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
	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
	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
	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
	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
	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
	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
	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
	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
	X75657 France	E	N	S	P	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
	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
	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
	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
	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
	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
	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
	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
	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.

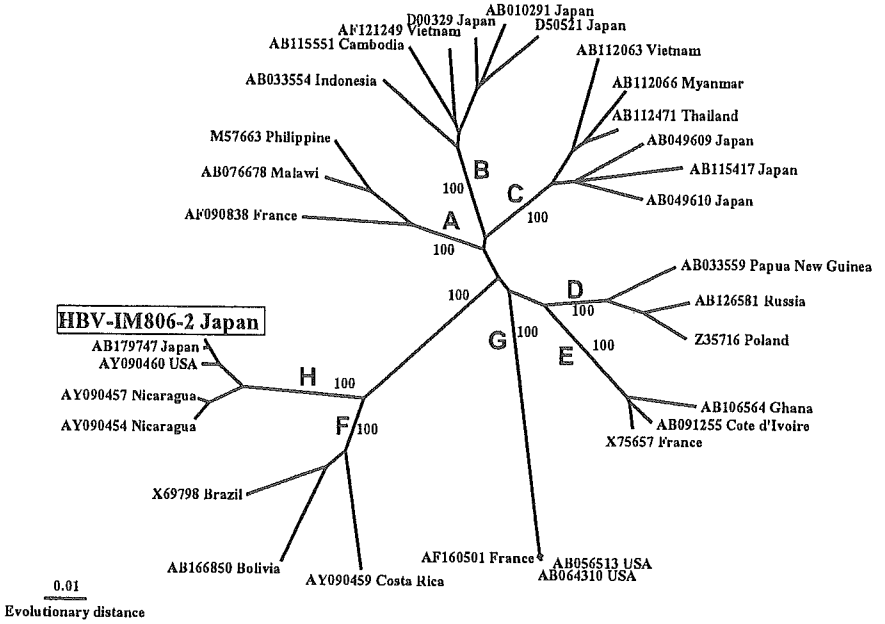

Fig. 2. Phylogram generated by neighbor-joining analysis of genetic distances in the full-length sequence of HBV.

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

NAOTA TAURA[1], KEISUKE HAMASAKI[1], KAZUHIKO NAKAO[2], TATSUKI ICHIKAWA[1], DAISUKE NISHIMURA[1], TAKASHI GOTO[1], MARIKO FUKUTA[1], HIROSHI KAWASHIMO[1], YASUHIDE MOTOYOSHI[1], HIDETAKA SHIBATA[1] and KATSUMI EGUCHI[1]

[1]The First Department of Internal Medicine and [2]Health Research Center, Nagasaki University School of Medicine, Sakamoto 1-7-1, Nagasaki 852-8501, Japan

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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.

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

Correspondence to: Dr Naota Taura, The First Department of Internal Medicine, Nagasaki University School of Medical, Sakamoto 1-7-1, Nagasaki 852-8501, Japan
E-mail: ntaura-gi@umin.ac.jp

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

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.

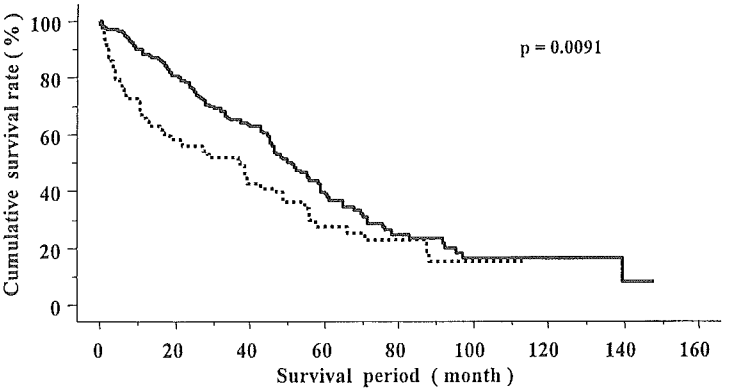

Figure 1. The cumulative survival rate in patients with subclinical HCC (group 1, thick line) and in patients with symptomatic HCC (group 2, thin line).

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

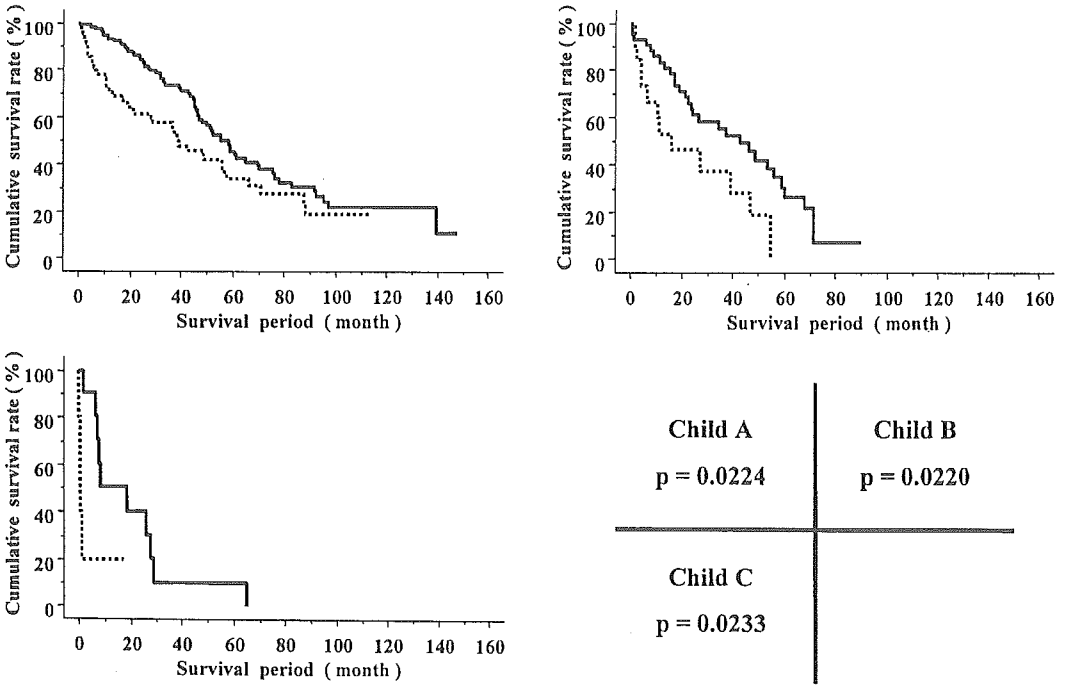

Figure 2. The cumulative survival rate in patients with subclinical HCC (group 1, thick line) and in patients with symptomatic HCC (group 2, thin line) according to Child-Pugh staging.

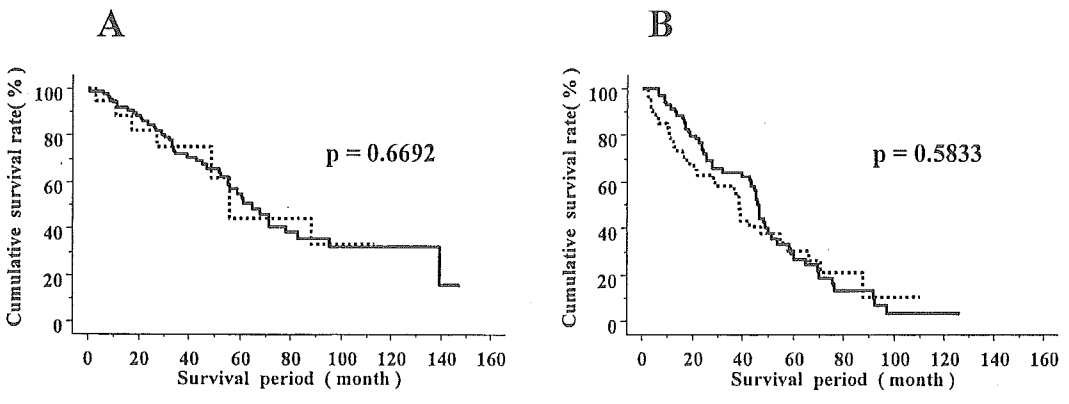

Figure 3. The cumulative survival rate in patients with subclinical HCC (group 1, thick line) and in patients with symptomatic HCC (group 2, thin line) who received treatment. A, Patients with RFA and/or PEI; B, Patients with TAC.

(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

TOSHINOBU HAYASHI[1], KAZUHIKO NAKAO[1,2], YUJI NAGAYAMA[4], OHKI SAITOH[4], TATSUKI ICHIKAWA[3], HIROKI ISHIKAWA[3], KEISUKE HAMASAKI[3], KATSUMI EGUCHI[3] and NOBUKO ISHII[1,2]

[1]Division of Clinical Pharmaceutics and [2]Health Research Center and [3]First Department of Internal Medicine, Graduate School of Biomedical Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8501; [4]Deparment of Medical Gene Technology, Atomic Bomb Disease Institute, Graduate School of Biomedical Sciences, Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan

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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.

Correspondence to: Dr Kazuhiko Nakao, Health Research Center, Graduate School of Biomedical Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan
E-mail: kazuhiko@net.nagasaki-u.ac.jp

Key words: hepatocellular carcinoma, irradiation, sulindac, poly (I:C)

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 $1x10^6$ 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 ($1x10^6$ cells/mouse), MH134 cells ($5x10^5$ cells/mouse) and BNL1ME.A.7R.1 cells ($1x10^6$ 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 ($3x10^6$ 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.

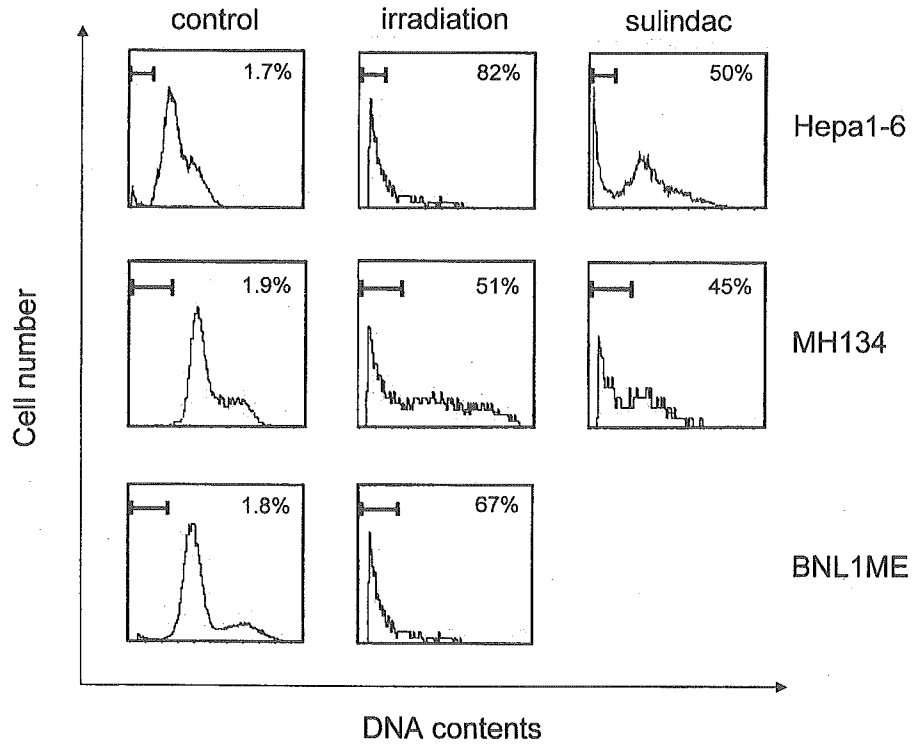

Figure 1. Apoptosis induced by irradiation and sulindac in murine hepatoma cells. Hepa1-6, MH134 and BNL1ME.A.7R.1 cells were irradiated by 40 Gy, or Hepa1-6 and MH134 cells were treated with 2 mM sulindac. Cells were stained with propidium iodide and subjected to DNA content analysis by a flow cytometer.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

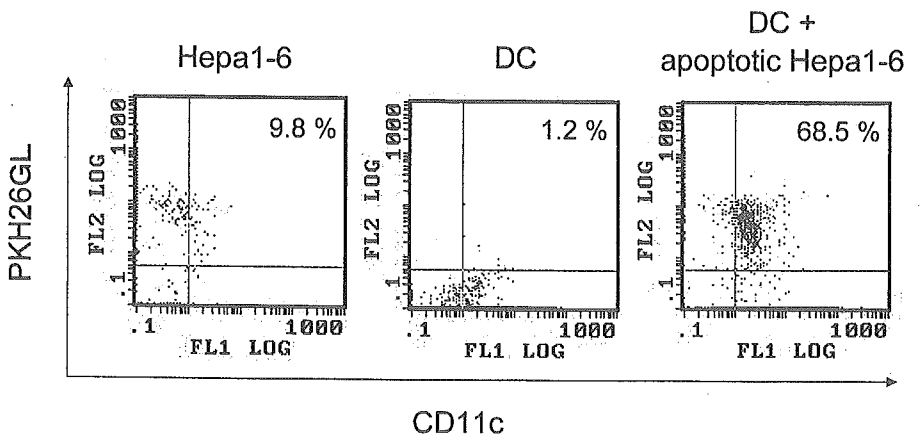

Figure 2. Uptake of apoptotic hepatoma cells by DCs. Hepa1-6 cells were dyed red using PKH26GL, induced apoptosis by 40 Gy irradiation, and cocultured with DCs at a ratio of 1:1. CD11c positive and PKH26GL positive cells were analyzed by a flow cytometer. The percentages of cells with double positive ($CD11c^+$ 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.

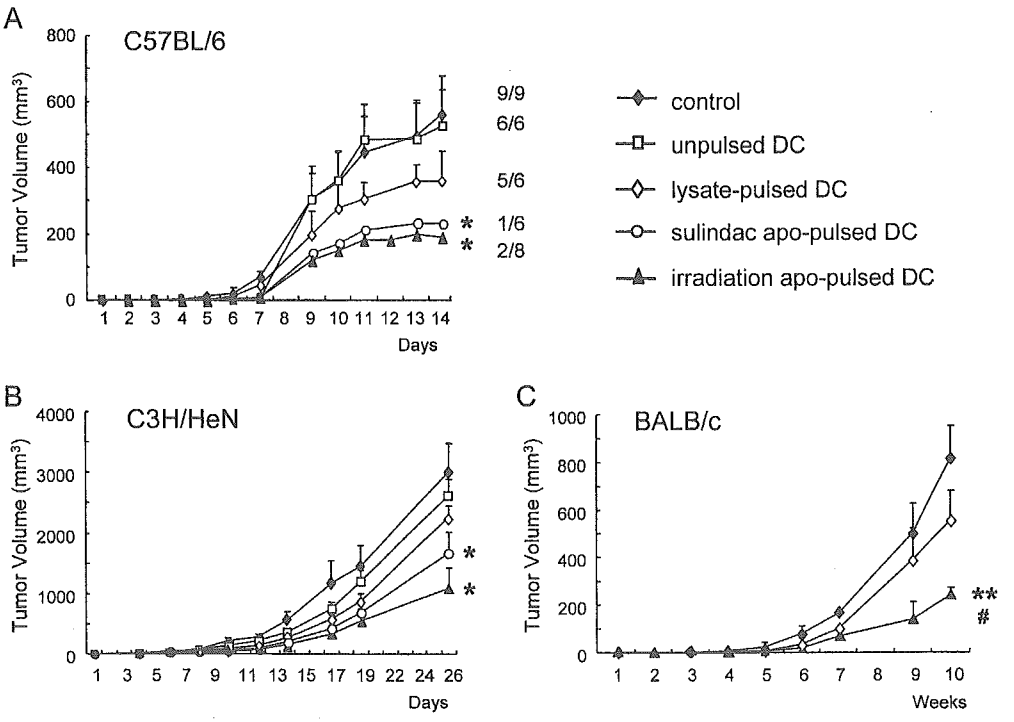

Figure 3. Protective antitumor effect of vaccination with DCs pulsed with apoptotic hepatoma cells *in vivo*. Mice were vaccinated twice with a one week interval with the injection of PBS alone (control), unpulsed DCs, DCs pulsed with hepatoma cell lysates, and DCs pulsed with sulindac- or irradiation-induced 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.