

as mean number of spots per 3×10^5 cells. The number of specific spots was determined by subtracting the number of spots in the absence of antigen. Responses were considered positive if more than 10 specific spots were detected and if the number of spots in the presence of antigen was at least twofold greater than the number of spots in the absence of antigen. The negative controls consisted of incubation of PBMCs with a peptide representing an HLA-A24 restricted epitope derived from HIV envelope protein (HIVenv₅₈₄) [33] and were always < five spots per 3×10^5 cells. The positive controls consisted of 10 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO, USA) and 500 ng/ml ionomycin (Sigma) or a cytomegalovirus (CMV) pp65-derived peptide (CMVpp65₃₂₈) [34].

HLA-A24 restricted peptide epitopes used in this study, AFP₄₀₃ (KYIQESQAL), AFP₄₂₄ (EYYLQNAFL), AFP₄₃₄ (AYTKKAPQL), AFP₃₅₇ (EYSRRHPQL) [27], hTERT₁₀₈₈ (TYVPLLGSL), hTERT₈₄₅ (CYGDMENKL), hTERT₁₆₇ (AYQVCGPPL) (unpublished), hTERT₄₆₁ (VYGFVRAFL), hTERT₃₂₄ (VYAETKHFL) [35], Her-2/neu₈ (RWGLLLALL) [36], MRP3₇₆₅ (VYSDADIFL) and MRP3₆₉₂ (AYVPQQAWI) [37], were synthesized at Mimotope (Melbourne, Australia) and Sumitomo Pharmaceuticals (Osaka, Japan). They were identified using mass spectrometry, and their purities were determined to be > 80% by analytical high performance liquid chromatography (HPLC). Regarding the immunological properties of the epitopes in HCC patients, we found that AFP- and hTERT-derived epitopes were recognized by cytotoxic T lymphocytes in 7.9–21.1% and 6.9–12.5% of 38 and 72 patients, respectively [27,38]. On the other hand, immune responses to Her-2/neu- and MRP3-derived epitopes have not been reported; however, overexpression of Her-2/neu protein was reported to be 2.42% in HCC patients [39], and in our unpublished data the immune responses to Her-2/neu-derived epitope were observed in 5.1% HCC patients (tested 38 patients). In addition, expression of MRP3 protein was reported to be 100% in HCC patients [40]. In our unpublished data, the immune

responses to MRP3-derived epitope were observed in 20.5–25.6% HCC patients (38 tested patients).

Statistical analysis

Results are expressed as mean \pm s.d. Differences between groups were analysed for statistical significance by the Mann–Whitney *U*-test. The estimated probability of tumour recurrence-free survival was determined using the Kaplan–Meier method. The Mantel–Cox log-rank test was used to compare curves between groups. Any *P*-values less than 0.05 were considered statistically significant.

Results

Isolation and characterization of DCs

Adherent cells isolated from PBMCs were differentiated into DCs in the presence of IL-4 and granulocyte–macrophage colony-stimulating factor (GM-CSF). DCs from each study patient were shown to develop high levels of MHC class II (HLA-DR) and co-stimulatory molecule B7-2 (CD86) and showed the absence of markers for mature monocytes (CD14). In addition, DCs obtained were phenotypically immature (CD80^{low}CD83^{low}) and classified to myeloid (CD11c⁺CD123⁻) and plasmacytoid (CD11c⁻CD123⁺) subsets (Table 2). Furthermore, sufficient numbers (at least 1×10^7) of functional DCs were isolated from 200 ml of peripheral blood in all patients in this clinical trial.

Safety of autologous DC administration

DC administration was performed during TAE therapy, in which DCs were mixed together with Gelfoam and infused through an arterial catheter following Lipiodol injection. Adverse events were monitored clinically and biochemically after DC infusion (Table 3). There were no grades III or IV National Cancer Institute common toxicity criteria adverse

Table 2. Properties of infused dendritic cells.

Patient no.	%CD14 ⁻ HLA-DR ⁺	lin ⁻ HLA-DR ⁺				
		%CD11c ⁺ CD123 ⁻	%CD11c ⁻ CD123 ⁺	%CD80 ⁺	%CD83 ⁺	%CD86 ⁺
1	34.5	5.8	8.4	1.6	1.9	11.5
2	56.2	49.7	39.3	4.1	3.2	92.4
3	35.6	63.6	6.1	0	5.7	96.0
4	32.7	22.6	56.5	1.9	1.2	61.6
5	40.4	24.3	57.3	34.9	27.7	94.4
6	60.4	35.8	47.0	11.4	1.5	84.4
7	46.4	54.7	10.7	12.9	12.5	83.2
8	62.8	7.6	32.3	19.0	20.5	49.1
9	55.2	35.2	35.5	28.8	18.3	73.0
10	34.0	9.5	7.0	12.1	8.4	31.8
Mean	45.8	30.9	30.0	12.7	10.1	67.7
s.d.	11.9	20.5	20.5	11.9	9.3	28.9

Table 3. General clinical outcome.

Patient no.	Adverse events					DTH to KLH	Tumour recurrence	Survival w/o HCC (months)	Survival (months)	Death/alive
	Fever (days)	Vomiting	Abdominal pain	Encephalopathy	Others					
1	1	No	No	No	No	n.d.	Yes	6	17	Death
2	2	No	No	No	No	n.d.	Yes	13	17	Alive
3	No	No	No	No	No	n.d.	Yes	8	30	Alive
4	4	Yes	No	No	No	n.d.	Yes	11	36	Alive
5	No	No	No	No	No	n.d.	n.d.	2	2	Death
6	No	No	No	No	No	n.d.	Yes	5	34	Alive
7	8	No	No	No	No	n.d.	Yes	14	22	Death
8	No	No	No	No	No	Positive	No	9	9	Death
9	5	Yes	No	No	No	n.d.	Yes	6	30	Alive
10	No	No	No	No	No	Positive	Yes	22	24	Alive
Mean								9.6	22.1	
s.d.								5.7	11.0	
11	No	No	No	No	No	n.d.	Yes	4	6	Death
12	No	No	No	No	No	n.d.	Yes	9	24	Death
13	No	No	Yes	No	No	n.d.	Yes	2	8	Alive
14	No	Yes	Yes	No	No	n.d.	Yes	3	9	Death
15	3	No	No	No	No	n.d.	Yes	18	20	Death
16	1	No	No	No	No	n.d.	Yes	8	36	Alive
17	No	No	No	No	No	n.d.	Yes	6	12	Death
18	No	No	No	No	No	n.d.	Yes	11	36	Alive
19	5	No	Yes	Yes	No	n.d.	Yes	7	30	Alive
20	No	No	No	No	No	n.d.	Yes	5	11	Death
21	No	No	No	No	No	n.d.	n.d.	4	4	Alive
Mean								7.0	17.8	
s.d.								4.5	12.0	

Adverse events/others: myalgia, gastrointestinal disorder, bleeding, hepatic abscess and autoimmune diseases; DTH: delayed-type hypersensitivity skin test; KLH: keyhole limpet haemocyanin; n.d.: not determined.

events associated with DC infusion and TAE in this study. Furthermore, the adverse events of the patients treated with DC infusion and TAE were compared with the control patients treated with TAE alone in terms of fever, vomiting, abdominal pain, encephalopathy, myalgia, ascites, gastrointestinal disorders, bleeding, hepatic abscess and autoimmune diseases. Although the clinical courses of five of the patients infused with DCs were complicated with high fever, there were no significant differences in the frequency or severity of adverse events associated with DC infusion. There was also no clinical or serological evidence of hepatic failure or autoimmune response in any patients. Thus, the current treatment of DC infusion was performed safely at the same time as TAE in patients with cirrhosis and HCC.

Kinetics and *in situ* effects of DCs following infusion into HCC tissues

DCs were labelled with ^{111}In -oxine and infused into tumour tissues through an arterial catheter following TAE in two patients (numbers 9 and 10). The kinetics of ^{111}In -labelled DCs were monitored by a gamma camera after the infusion (Fig. 1a). Radioactivity at the tumour site decreased after the infusion, but was still detectable up to 14 days after the

infusion, indicating that the DCs stayed alive for more than 2 weeks. However, tracking of labelled DCs to regional lymph nodes was not seen in the current imaging, due possibly to insufficient numbers of migrated DCs, or otherwise due to DC paralysis in tumour tissues [41].

One of the patients (number 10) proceeded unexpectedly to curative surgery 17 days after the DC infusion. The tissue radioactivity was investigated using autoradiography and analysed on an image analyser (Fig. 1b,c,d). Surprisingly, radioactivity was still detectable in the tumour tissue and surrounding liver parenchyma. In addition, immunohistochemistry of the liver tissue was performed using MoAbs specific for cell surface markers CD8, CD14 (monocyte), CD20 (B cell), CD1a (DC) HLA-DR (antigen-presenting cell) and CD83 (DC maturation/activation) (Fig. 2). Immunohistochemical analysis revealed many CD1a-positive DCs in the area surrounding HCC nodules treated with autologous DCs and TAE 17 days previously. In addition, CD83-positive cells and HLA-DR-positive antigen-presenting cells were seen in the same areas of liver tissue. Interestingly, CD83-positive cells were rarely detected in liver tissues of HCC patients, as reported previously [42]. Many CD8⁺ T cells, CD14⁺ monocytes and CD20⁺ B cells were recruited in the same area. In conjunction with the detection of

Fig. 1. Kinetics of autologous dendritic cells (DCs) following infusion into tumour tissues in patients with hepatocellular carcinoma (HCC). DCs were labelled with ^{111}In -oxine and infused into tumour tissues through an arterial catheter following transcatheter hepatic arterial embolization (TAE). (a) Scintigraphic images of the depot were acquired with a gamma camera 7 days after injection. (b) In one case, the treated HCC nodule was accidentally resected surgically 17 days after DC infusion. Bar represents 1 cm. Resected tissues were stained with haematoxylin and eosin (c) and, using the tissue comparable to the inset in (c), autoradiography was conducted and analysed on an image analyser (d).

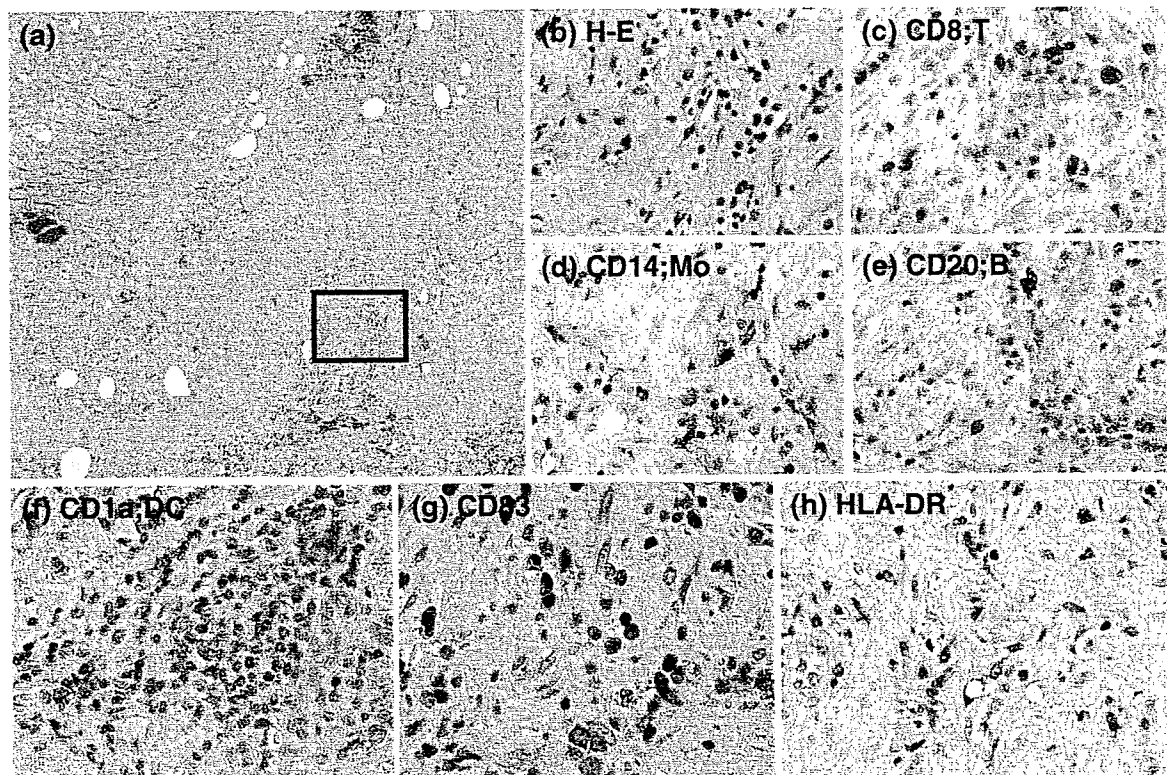
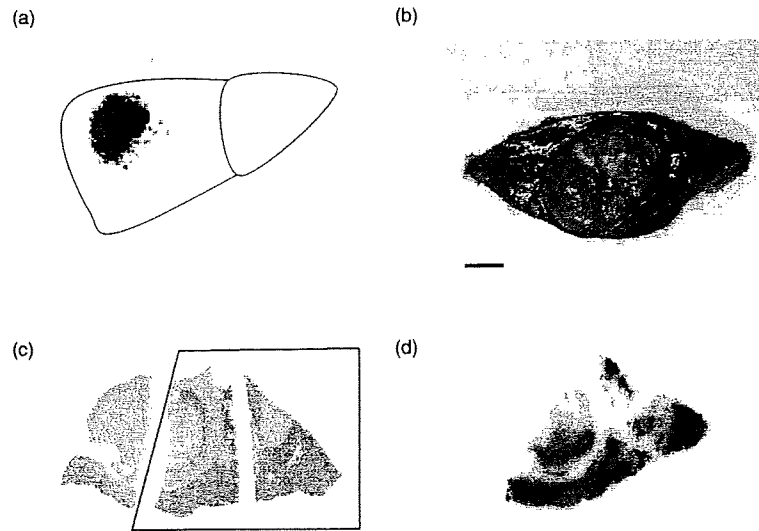


Fig. 2. Immunohistochemical analysis of a surgically resected liver tissue containing a nodule of hepatocellular carcinoma in a patient infused with autologous dendritic cells (DCs) 17 days previously, described in the legend to Fig. 1. The liver tissue was stained with haematoxylin and eosin (a and b) and for CD8 (c), CD14 (d), CD20 (e), CD1a (f), CD83 (g) and human leucocyte antigen D-related (HLA-DR) (h). (b) Close-up of the inset in (a). Areas comparable to (b) are indicated in (c–h). Cells were stained positively in brown. Original magnifications, $\times 40$ (a) and $\times 400$ (b–h).

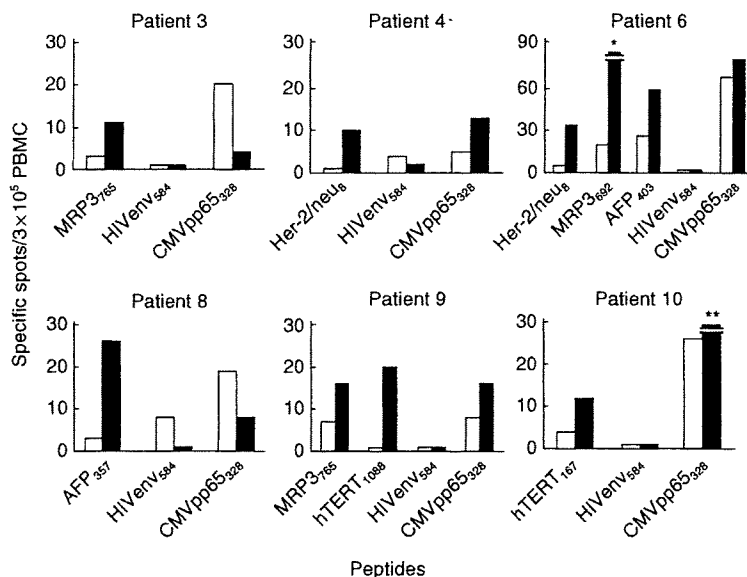


Fig. 3. Immune responses to human leucocyte antigen (HLA)-A24 restricted peptide epitopes derived from tumour antigens in patients infused with dendritic cells (DCs). Peripheral blood mononuclear cells (PBMCs) were obtained before (open bars) and 4 weeks after the infusion (solid bars), pulsed with the peptides derived from AFP, hTERT, Her-2/neu and MRP3, and interferon (IFN)-̑ production was quantified by enzyme-linked immunospot assay (ELISPOT). Negative controls consisted of incubation of PBMC with a peptide representing an HLA-A24 restricted epitope derived from HIV envelope protein (HIVenv₅₈₄). Positive controls consisted of 10 ng/ml phorbol 12-myristate 13-acetate (PMA) and 500 ng/ml ionomycin or a cytomegalovirus (CMV) pp65-derived peptide (CMVpp65₃₂₈). Responses were considered positive if more than 10 specific spots were detected and if the number of spots in the presence of peptide was at least twofold greater than the number of spots in the absence of peptide. *230 specific spots; **32 specific spots.

radioactivity, these data indicate that DCs were viable for more than 17 days following the infusion and seemed to contribute to the recruitment and activation of immune cells.

Immune responses induced following DC infusion

To evaluate the immunomodulatory effects of DCs infused into HCC tissues, PBMCs were obtained 4 weeks after the infusion, pulsed with the peptides derived from AFP, hTERT, Her-2/neu and MRP3 and IFN-̑ production was quantified by ELISPOT. Interestingly, frequencies of IFN-̑-producing cells in response to stimulation with HLA-A24-restricted peptide epitopes derived from tumour antigens, Her-2/neu, MRP3, hTERT and AFP, were increased 2–20-fold in six of the eight HLA-A24-positive patients after the treatments (Fig. 3). In addition, in two of the cases DCs were stimulated with KLH before the infusion, and the intradermal test was performed 2 weeks later. Both cases displayed a positive reaction (Table 3), indicating that DCs induced cellular immunity successfully following the treatments [43]. Collectively, these results demonstrated that DC infusion induced immune responses to unprimed tumour antigens, suggesting that antigen-non-specific immature DCs in the tumour tissues enhanced tumour immunity *in vivo*.

Recurrence-free survival following DC infusion

A further objective of this study was to determine the clinical response to DC infusion. Therefore, we compared recurrence-free survival between patients treated with TAE with ($n = 10$) and without ($n = 11$) DC administration (Table 3). While there was a trend for the patients infused with DCs to display longer recurrence-free survival, these differences did not reach statistical significance [mean recurrence-free survival (\pm s.d.) of patients with and without DC administration: 9.6 ± 5.7 and 7.0 ± 4.5 months, respectively; $P = 0.13$]. In addition, there was no correlation between infused DC phenotypes characterized by the surface markers (Table 2) and recurrence-free survival after the infusion. Although the strong CD8⁺ response against MRP3₆₉₂ was induced in patient 6 (Fig. 3) the clinical outcomes, including recurrence-free survival, were not distinct from the other DC-treated patients.

Discussion

Tumour recurrence rates after curative treatments for HCC are extremely high in patients with active hepatitis and cirrhosis related to HBV and HCV infections [4–6]. Although current treatments, including surgical resection and RFA,

displayed almost complete prevention of local recurrence in the cirrhotic liver, the surrounding non-tumour liver tissues exhibited high carcinogenic potentials to develop *de novo* HCC. Significant risk factors for recurrence have been reported to be cirrhosis and a higher grade of hepatitis activity [44–46]. IFN therapy and anti-inflammatory drugs have been implicated in the reduction of HCC recurrence [47,48]; however, they did not prevent recurrence satisfactorily. To develop novel therapies targeting HCC recurrence, many immune-based trials have been performed [8].

The current study indicates that immature DC infusion following TAE therapy did not cause additional adverse events in patients with cirrhosis and HCC, and that infused DCs remained alive around the tumour tissues for more than 17 days, as indicated by monitoring the kinetics of ¹¹¹In-DC, and contributed to the recruitment and activation of immune cells *in situ*. Immune responses were induced to the tumour antigen peptides with HLA-A24 binding motif. Furthermore, intradermal tests were positive when stimulated with KLH. Ultimately, the patients treated with this DC-based immunotherapy did not display statistically longer recurrence-free survival. This study demonstrated the feasibility, safety and bioactivity of transcatheter arterial DC infusion into tumour tissues for patients with cirrhosis and HCC. The data suggest the ability of an active immunotherapeutic strategy to generate antigen-specific cytotoxicity in HCC patients.

Transcatheter arterial DC infusion into tumour tissues following TAE treatment was feasible and safe for patients with cirrhosis and HCC whose hepatic reserve was decreased, similar to reports from other DC immunotherapy trials [20–22]. Mild toxicity included more patients with fever compared to TAE treatment alone, while their durations were comparable. Two patients complained of infrequent vomiting. To date, no clinical or radiological features of autoimmune diseases have been detected in the current study, nor have other DC immunotherapy trials reported any significant autoimmune events [20–22].

¹¹¹In-labelled DCs were detectable inside and around the HCC nodules after the infusion, suggesting that they were infused precisely to the targeted nodules and stayed alive longer than expected. If DCs died after the infusion, the radioactive materials should have been released from the labelled DCs. Furthermore, they seemed to contribute to the recruitment and activation of immune cells by immunohistochemical analysis. In this procedure, immature DCs that did not express CD83 molecule were infused to liver tissues, and only a few infiltrating inflammatory cells were reported to express CD83 in liver tissues [42,49]. However, many immune cells were shown to express CD83 in the tissues surrounding the treated HCC nodules. As CD83 expression is known to be limited on mature DCs and activated B lymphocytes [50,51], the infused immature DCs were suggested to become mature after the infusion, and to recruit activated lymphocytes in the liver tissues.

Interestingly, the results demonstrate that the antigen-non-specific, immature DC infusion may induce the immune responses to unprimed tumour antigens. Because immature DCs are known to display high endocytic and phagocytic activity [52,53], they can take up tumour antigens in the apoptotic tissues treated with TAE therapy. Following the endocytosis and phagocytosis of tumour antigens, DCs may move to secondary lymphoid organs, including regional lymph nodes, become activated and mature and induce antigen-specific cell-mediated and humoral immune responses. Even uncharacterized antigens can be processed and presented to the host immune systems by DCs in this study, and the immune responses to unprimed antigens may be induced. The current *in vivo* immune induction may omit the identification of tumour antigenic epitopes from the development of tumour immunotherapy.

The recurrence-free survival rates were not increased significantly by this strategy. This therapeutic effect was probably limited due to the insufficient anti-tumour immune responses. To enhance the tumour antigen presentation to T lymphocytes, DCs could be transduced with MHC class I and class II genes [54,55] and co-stimulatory molecules, e.g. CD40, CD80 and CD86 [56,57], and loaded with tumour-associated antigens including tumour lysates, peptides and RNA transfection [58]. To induced natural killer (NK) and NK T cell activation, DCs could be stimulated and modified to produce larger amounts of cytokines, e.g. IL-12, IL-18 and type I IFN [57,59]. Furthermore, DC migration in secondary lymphoid organs could be induced by expression and transduction of chemokine genes, e.g. CCR7 [60–62], by maturation using inflammatory cytokines [63], matrix metalloproteinases and Toll-like receptor (TLR) ligands [64], and by combination of different administration routes including *i.v.* and *i.d.* injections [65]. Importantly, the current study suggests an initial strategy to develop a novel immunotherapy with DCs for the prevention of HCC recurrence.

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Prolonged, NK Cell-Mediated Antitumor Effects of Suicide Gene Therapy Combined with Monocyte Chemoattractant Protein-1 against Hepatocellular Carcinoma

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Tumor recurrence rates remain high after curative treatments for hepatocellular carcinoma (HCC). Immunomodulatory agents, including chemokines, are believed to enhance the antitumor effects of tumor cell apoptosis induced by suicide gene therapy. We therefore evaluated the immunomodulatory effects of a bicistronic recombinant adenovirus vector (rAd) expressing both HSV thymidine kinase and MCP-1 on HCC cells. Using an athymic nude mouse model (BALB/c-*nu/nu*), primary s.c. tumors (HuH7; human HCC cells) were completely eradicated by rAd followed by treatment with ganciclovir. The same animals were subsequently rechallenged with HCC cells, tumor development was monitored, and the recruitment or activation of NK cells was analyzed immunohistochemically or by measuring IFN- γ mRNA expression. Tumor growth was markedly suppressed as compared with that in mice treated with a rAd expressing the HSV thymidine kinase gene alone ($p < 0.001$). Suppression of tumor growth was associated with the elevation of serum IL-12 and IL-18. During suppression, NK cells were recruited exclusively, and Th1 cytokine gene expression was enhanced in tumor tissues. The antitumor activity, however, was abolished either when the NK cells were inactivated with anti-asialo GM1 Ab or when anti-IL-12 and anti-IL-18 Abs were administered. These results indicate that suicide gene therapy, together with delivery of MCP-1, eradicates HCC cells and exerts prolonged NK cell-mediated antitumor effects in a model of HCC, suggesting a plausible strategy to prevent tumor recurrence. *The Journal of Immunology*, 2007, 178: 574–583.

Despite curative treatments including surgical resection and liver transplantation for hepatocellular carcinoma (HCC),² tumor recurrence rates remain high, probably because of insufficient therapeutic effects and the multicentric development of HCC in cirrhotic liver (1–3). Although nonsurgical treatments of HCC such as radiofrequency ablation, transcatheter arterial embolizations, and transcatheter arterial chemotherapy induce apoptosis of HCC cells, these treatments do not enhance antitumoral immunity sufficiently. Therefore, gene therapy aimed at enhancing antitumor immune responses may be a promising approach to induce sufficient inhibitory effects for the prevention of tumor recurrence.

Although killing tumor cells with cytotoxic genes such as suicide gene/prodrug systems consisting of HSV thymidine kinase (HSV-tk) and ganciclovir (GCV) may lead to the genera-

tion of effective immunity (4, 5), cell killing alone is insufficient to increase many antitumor responses (6–8). Recently, however, coexpression of HSV-tk and chemokines was found to increase tumor immunity in animal models in which neither HSV-tk nor chemokine expression alone was sufficient (9). In addition, we previously demonstrated that, at the local treatment site, the antitumor effects of the HSV-tk/GCV system were enhanced by codelivery of MCP-1, a member of the CC chemokine family (8, 10). MCP-1 has been shown to stimulate the cytotoxic activity of monocytes, enhance the expression of adhesion molecules such as CD11b and CD11c, and induce the cytotoxic and migratory activities of NK cells (11–14). Moreover, transfection of the MCP-1 into human lung adenocarcinoma cells inhibited the formation of metastases, presumably via the activation of NK cells (15). It was recently reported that NK cells can mediate long-lived, Ag-specific adaptive recall responses independently of B cells and T cells (16). These observations suggest that MCP-1 can induce specific tumor immunity by enhancing NK cell functions even in this system.

Thus, we evaluated the long-term systemic immunomodulatory effects of a bicistronic recombinant adenovirus vector (rAd) expressing both HSV-tk and MCP-1 (Ad-tk-MCP1). After the primary s.c. HCC tumors in athymic nude mice were eradicated by using Ad-tk-MCP1, the same HCC cells were injected into another site of the same mice to prove the presence of NK cell-mediated, long-term immunity. Moreover, we explored whether innate immune responses induced by NK cells were involved in these procedures. In this study, we provide definitive evidence to indicate that codelivery of a suicide gene and MCP-1 exerts prolonged NK cell-mediated antitumor effects in this model, suggesting a plausible strategy to prevent HCC recurrence.

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² Abbreviations used in this paper: HCC, hepatocellular carcinoma; AGM1, asialo GM1; BNL, BNL 1ME A.7R.1 HCC cell line; DC, dendritic cell; GCV, ganciclovir; HSV-tk, HSV thymidine kinase; MMC, mitomycin C; MOI, multiplicity of infection; rAd, recombinant adenovirus vector; Ad-tk, rAd expressing HSV-tk; Ad-tk-MCP1, rAd expressing both HSV-tk and MCP-1; Ad-MCP1, rAd expressing MCP-1; Ad-lacZ, rAd expressing lacZ; TCD₅₀, 50% tissue culture infectious dose.

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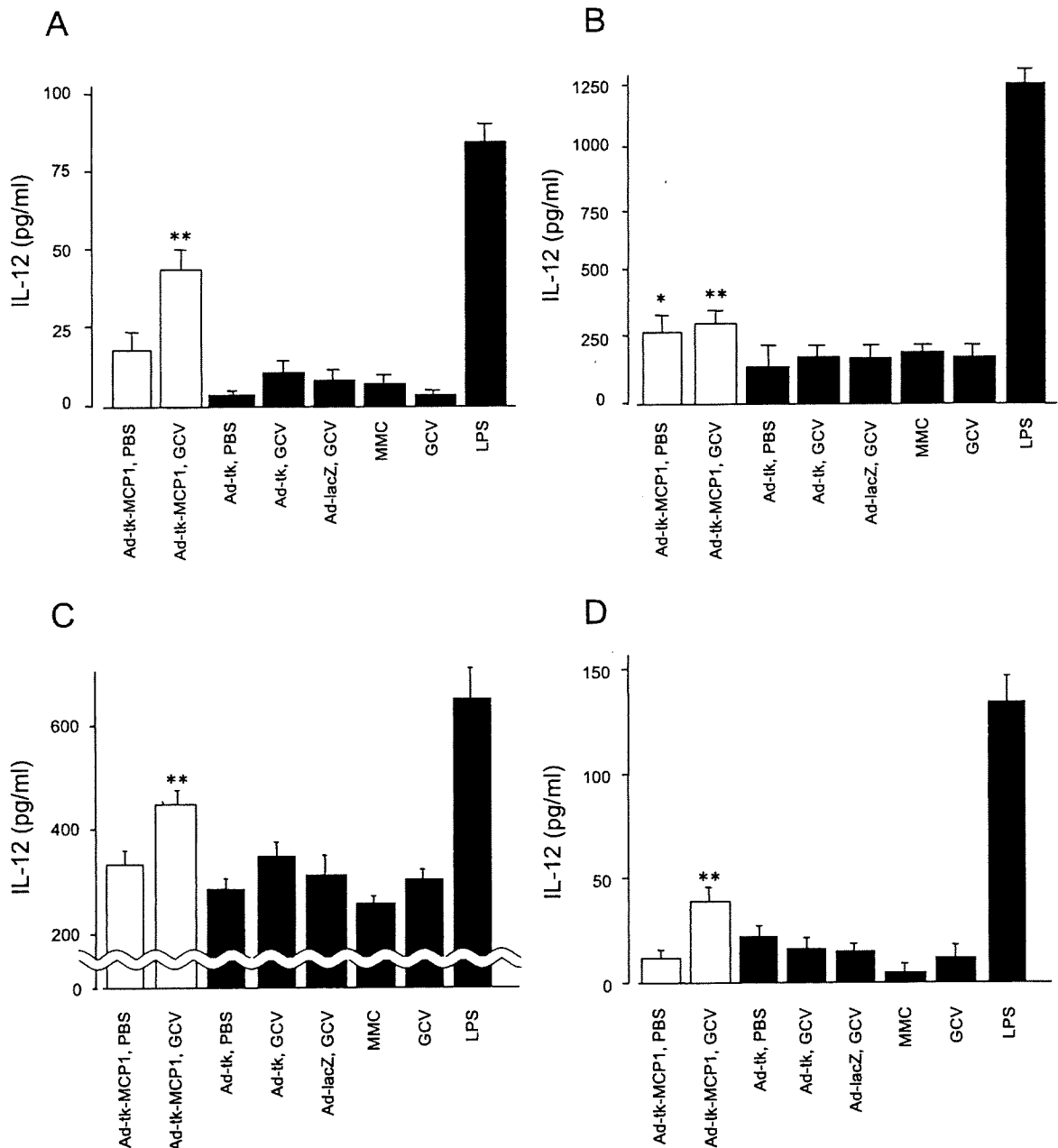


FIGURE 1. IL-12 production by monocytes and DCs cocultured with apoptotic or nonapoptotic HCC cells infected with rAds in vitro. HuH7 cells were infected with Ad-tk-MCP1, Ad-tk, and Ad-lacZ at an MOI of 5 for 24 h. Aliquots of 10^5 mouse (A) and human (C) monocytes or 10^5 mouse (B) and human (D) DCs were cocultured with 10^5 rAd- or MMC-treated HuH7 cells and treated with or without GCV for two days, and the concentrations of IL-12 in the medium were evaluated using an immunoassay. Each value is the mean \pm SE of triplicate experiments. *, $p < 0.05$ compared to Ad-tk with PBS (Ad-tk, PBS); **, $p < 0.05$ compared to Ad-tk with GCV (Ad-tk, GCV) by the Mann-Whitney U test.

Materials and Methods

Recombinant adenoviruses

The bicistronic Ad-tk-MCP1 (10), which harbors the HSV-tk gene and the human MCP-1 gene in sequence and is driven by a CAG promoter constructed from a cytomegalovirus enhancer, a chicken β -actin promoter and part of rabbit β -globin, was prepared, purified, and titrated according to the protocols supplied by the manufacturer (Takara Bio) as described (17, 18). Briefly, using the internal ribosomal entry site (IRES) fragment of the encephalomyocarditis virus, the plasmid ptk-IRES-MCP1 (tk-MCP1) was constructed and the fragment was inserted into the cosmid vector (pAd-

tk-MCP1). Ad-tk-MCP1 was subsequently generated by transfecting 293 cells with pAd-tk-MCP1 and *Eco*T22I-digested adenovirus 5-dIX DNA-terminal protein complex. The rAd expressing HSV-tk (Ad-tk), lacZ (Ad-lacZ) and MCP-1 (Ad-MCP1) were constructed in the same way (8). The rAds were purified on cesium gradients and their titers were determined by the 50% tissue culture infectious dose (TCID₅₀) method (19).

Cell lines and culture

The human HCC cell line HuH7 (20) and the mouse HCC cell line BNL 1ME A.7R.1 (BNL) were cultured in DMEM (Invitrogen Life Technologies)

supplemented with 10% heat-inactivated FBS (Invitrogen Life Technologies). When infected with Ad-tk-MCP1 or Ad-MCP1, BNL cells produced MCP-1 protein at similar levels as HuH7 cells (data not shown), suggesting that human MCP-1 protein was efficiently expressed in the infected human and mouse HCC cell lines.

Preparation of dendritic cells (DCs) and monocytes

Murine DCs were generated using the method of Lutz et al. (21). Briefly, bone marrow cells were harvested from 6-wk-old male BALB/c-*nu/nu* mice (CLEA Japan). Erythrocytes were lysed with ammonium chloride potassium buffer (BioWhittaker), and the nucleated cells were plated in plastic bacteriologic dishes in 10 ml of RPMI 1640 supplemented with 10% heat-inactivated FBS and 20 ng/ml murine GM-CSF (PeproTec), with the culture medium refreshed every 3 days. On day 8, the nonadherent DCs were collected. Purity was routinely >95% CD11c⁺ DC as determined by FACS analysis.

Thioglycollate-elicited murine peritoneal exudate cells were collected as described (22). Briefly, nude mice were i.p. injected with 2 ml of 3% fluid thioglycollate medium (Wako Pure Chemical) and sacrificed 4 days later, followed by peritoneal lavage with 10 ml of cold PBS. Approximately 90% of the collected peritoneal cells were positive for both Mac-1 (CD11b) and I-A^d MHC class II when stained with PE-conjugated anti-Mac-1 Ab (clone M1/70; BD Pharmingen) and FITC-conjugated I-A^d MHC class II (clone AMS-32.1; BD Pharmingen).

Human monocytes and DCs were isolated from healthy blood donors (23). Briefly, PBMCs were isolated by centrifugation in Lymphoprep tubes (Nycomed). PBLs were then incubated in 6-well cell culture plates and the resultant adherent cells were collected as a monocyte population consisting of ~70% CD14⁺ (clone MφP9; BD Pharmingen) cells, as determined by flow cytometric analysis. The monocyte population was further grown into differentiated DCs by culturing them for 1 wk in CellGro DC medium (Good Manufacturing Practice grade; Cell Genix) supplemented with 100 ng/ml GM-CSF (Cell Genix) and 50 ng/ml IL-4 (Cell Genix). The cells were collected with viability of >80%, and >60% of cells were identified as CD14⁺ HLA-DR⁺ (clone L243; BD Pharmingen) DCs.

Assays for IL-12 production *in vitro*

HuH7 cells were infected with Ad-tk-MCP1, Ad-tk, or Ad-lacZ at a multiplicity of infection (MOI) of 5 for 24 h. Aliquots of 10⁵ DCs or monocytes were cocultured with 10⁵ rAd- or mitomycin C (MMC)-treated HuH7 cells in 1.0 ml of culture medium in a 24-well tissue culture plate and treated with or without GCV for two days at 37°C. The concentrations of IL-12 in the medium were quantitated using an immunoassay kit (BioSource International).

Animal studies

The following investigations were conducted in accordance with the Institutional Animal Care and Use Committee guidelines of Kanazawa University. Six-week-old male athymic nude mice were s.c. injected with 5 × 10⁶ HuH7 cells on day 0. On days 3 and 4, 5 × 10⁷ TCID₅₀ (100 μl) of Ad-tk-MCP1, Ad-tk, or Ad-MCP1 was injected into the s.c. tumors, and the mice were treated with 75 mg/kg GCV injected into the peritoneal cavity every day for the next 5 days (days 5–9). Following complete eradication of the primary tumors, the mice were s.c. rechallenged on day 14 with 3 × 10⁶ HuH7 cells or injected with 1 × 10⁵ BNL cells at a distance of >3 cm from the primary challenge site. Nine of 80 (11.3%) mice treated with Ad-tk-MCP1 and 9 of 44 (20.4%) treated with Ad-tk did not show a complete eradication of the primary tumor by the final measurement and therefore were excluded from a rechallenge experiment. In some experiments, Ad-tk-MCP1-treated animals were i.p. administered 200 μl of 1 mg/ml polyclonal rabbit anti-asialo GM1 (AGM1) Ab (Wako Pure Chemical), an Ab against NK cells (24, 25), or 200 μl of rabbit serum (Sigma-Aldrich), 1 ml of 2 mg/ml carrageenan (Sigma-Aldrich), which inactivates macrophages *in vivo* (26–28), or 1 ml of PBS on days 11, 12, 13, 20, 27, 34, 41, and 48. In another series of experiments, Ad-tk-MCP1-treated animals were i.p. administered 250 μg of neutralizing goat anti-mouse IL-12 Ab (Sigma-Aldrich), 225 μg of anti-IL-12 Ab plus 25 μg of anti-mouse IL-18 Ab (93-10C; Medical & Biological Laboratories), or 250 μg of control IgG Ab (goat and/or rat; Sigma-Aldrich) on days 14 and 17. Tumor sizes were measured every 4 days after the second tumor injection, and tumor volumes were calculated according to the formula (longest diameter) × (shortest diameter)²/2.

In another series of experiments, immunocompetent BALB/c-*cl* mice (CLEA Japan) were s.c. injected with 1 × 10⁵ BNL cells infected with each rAd at an *in vitro* MOI of 100 on day 0, GCV was administered i.p. for the next 5 days (days 1–5), and the primary tumors were completely eradicated.

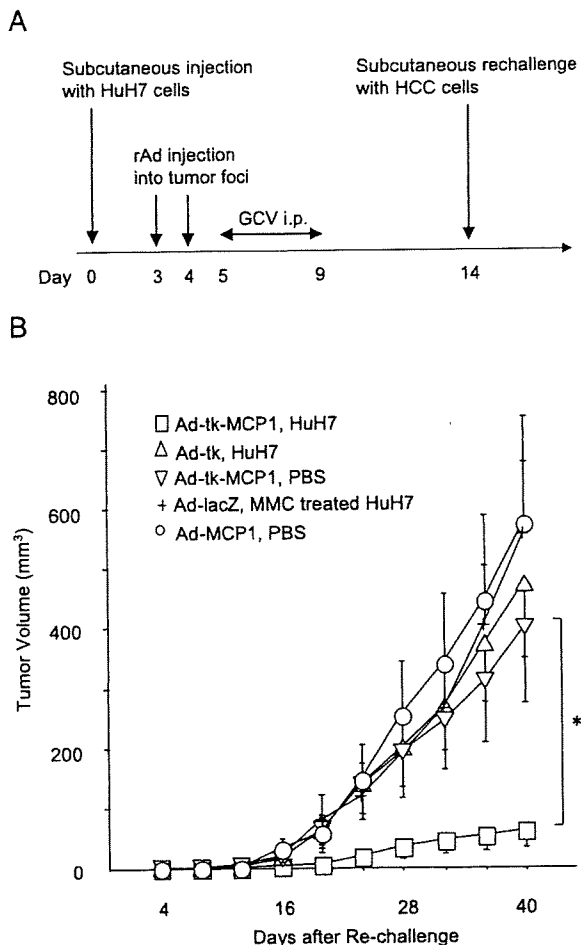


FIGURE 2. Prolonged antitumor effects of rAds expressing HSV-tk with or without MCP-1 in an athymic nude mouse model of HCC. **A**, Mice were s.c. injected with 5 × 10⁶ HuH7 cells on day 0. On days 3 and 4, 5 × 10⁷ TCID₅₀ of Ad-tk-MCP1, Ad-tk, Ad-lacZ, or Ad-MCP1 were injected into the tumors, and the mice were i.p. injected with 75 mg/kg GCV every day for the next 5 days (days 5–9). Following complete eradication of the primary tumors, the mice were s.c. rechallenged with 3 × 10⁶ HuH7 cells at other sites on day 14. **B**, Tumor sizes were measured every 4 days. The results are the means of three independent experiments. *, *p* < 0.001 compared to Ad-tk with HuH7 (Ad-tk, HuH7) by the Mann-Whitney's *U* test.

These mice were s.c. injected with 1 × 10⁴ BNL cells in other sites on day 14, and the tumor sizes were measured every 7 days.

ELISA for serum IL-12 and IL-18

Mouse sera were collected before the injection of s.c. primary tumors and after the rechallenge with tumors, and IL-12 and IL-18 concentrations were measured using immunoassay kits (IL-12 from BioSource International and IL-18 from Medical & Biological Laboratories).

Immunohistochemical analysis

Tumor tissues and spleens were resected on day 16 (2 days after tumor rechallenge). The tissue samples, except those used for F4/80 (A3-1; Serotec) staining, were embedded in OCT compound (Sakura Finetek) and snap frozen in liquid nitrogen. Cryostat sections of frozen tissues were fixed in cold acetone for 10 min, followed by rinsing three times in PBS. The tissue samples used for F4/80 staining were fixed in 10% phosphate-buffered formalin and embedded in paraffin. To avoid nonspecific staining,

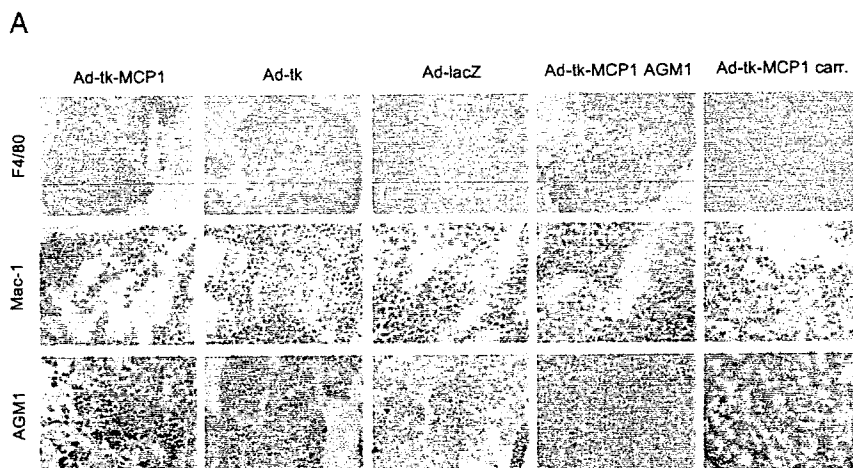
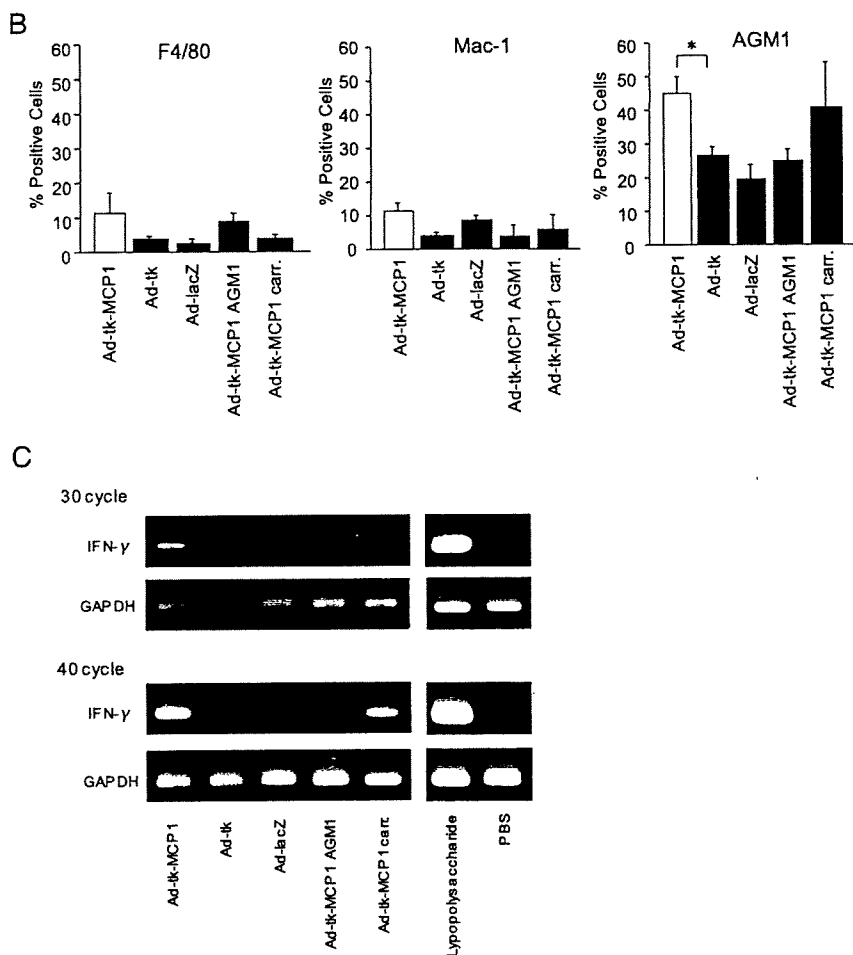


FIGURE 3. Expression of AGM1, F4/80, and Mac-1 Ags and IFN- γ mRNA in rechallenged tumor tissues. In the experiment described in the legend to Fig. 2, tumor tissues were resected 2 days after tumor rechallenge and analyzed immunohistochemically and estimated for IFN- γ mRNA expression by RT-PCR. *A*, Tumor tissues obtained from mice whose primary tumors were treated with Ad-tk-MCP1, Ad-tk, and Ad-LacZ were stained with anti-AGM1, F4/80, and Mac-1 Abs. Original magnification, $\times 100$. *B*, Quantitative morphometric analysis showing the proportions of positive cells in areas of 100 tumor cells. Values are the means \pm SE of triplicate experiments. *, $p < 0.05$ compared to Ad-tk by the Mann-Whitney's *U* test. *C*, RT-PCR were conducted in accordance with the manufacture's protocol as described in *Materials and Methods*. Bands corresponding to IFN- γ (384 bp) and GAPDH (265 bp) were detected. Splenocytes treated with 1 $\mu\text{g}/\text{ml}$ LPS were used as a positive marker and tumor tissues treated with PBS were used as a negative control. carr., Carrageenan.



avidin and biotin in the tissues were blocked using a blocking kit (Vector Laboratories). The slides were subsequently incubated with Abs against AGM1, F4/80, Mac-1, CD11c (HL3; BD Pharmingen), or CD45R (RA3-6B2; BD Pharmingen) for 30 min at room temperature. Negative controls included staining with the corresponding isotype for each Ab and subsequent staining with the secondary Ab. The reactions were visualized using a VECTASTAIN ABC Standard kit (Vector Laboratories), followed by counterstaining with hematoxylin.

RT-PCR for IFN- γ gene expression

Total RNA was extracted from tumor tissues resected on day 10 using a total cellular RNA isolation kit (Ambion) according to the manufacturer's protocol. Each RT-PCR was performed using 1 μg of total RNA and an oligo(dT) adaptor primer and an RNA PCR kit (avian myeloblastosis virus), version.2.1 (Takara Bio). The amplification protocol consisted of an initial denaturation at 94°C for 2 min followed by 30 or 40 cycles of

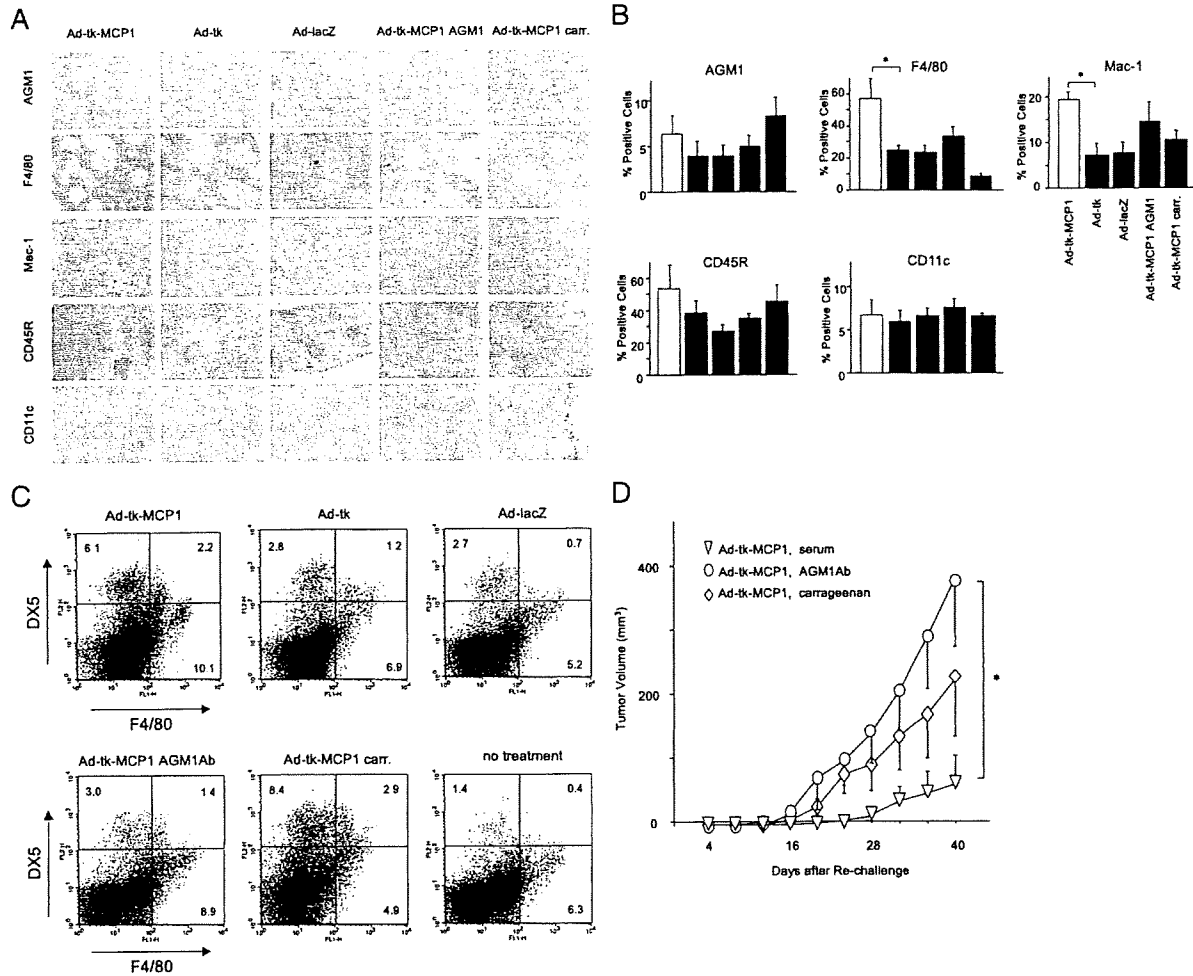


FIGURE 4. A–C, Immunohistochemical detection of AGM1, F4/80, Mac-1, CD11c, and CD45R in spleens. In the experiment described in the legend to Fig. 2, spleens were resected 2 days after the tumor rechallenge. A, The numbers of immune cells in the spleens were analyzed immunohistochemically using Abs against AGM1, F4/80, Mac-1, CD11c, and CD45R. Original magnification, $\times 100$. B, Quantitative morphometric analysis showing the percentage of positive cells in 50×400 power fields. Each value is the mean \pm SE of triplicate experiments. *, $p < 0.05$ compared with Ad-tk by the Mann-Whitney *U* test. C, Surface expression of DX5 and F4/80 in cell populations obtained from spleens was assessed by FACS. The results are representative of two independent experiments. carr., Carrageenan. D, The effects of anti-AGM1 Ab or carrageenan on the growth of rechallenged tumors. At the rechallenge with HuH7 cells, Ad-tk-MCP1-treated animals were i.p. administered with 200 μ l of 1 mg/ml anti-AGM1 Ab (Ad-tk-MCP1, AGM1 Ab), 200 μ l of rabbit serum (Ad-tk-MCP1, serum) or 1 ml of 2 mg/ml carrageenan (Ad-tk-MCP1, carrageenan) as described in *Materials and Methods*. Tumor sizes were measured every 4 days. The results are the means of two independent experiments. *, $p < 0.05$ compared to Ad-tk-MCP1 with PBS or serum (Ad-tk-MCP1, serum) by the Mann-Whitney *U* test.

denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and an extension at 72°C for 1.5 min. The PCR primers for the mouse IFN- γ and GAPDH genes were purchased from R&D Systems.

Flow cytometry

Single cell suspensions of splenocytes were resuspended in PBS containing 1% BSA and 0.1% sodium azide and incubated for 30 min on ice with FITC-conjugated rat anti-mouse-F4/80 and PE-conjugated rat anti-mouse pan NK cells (DX5; BD Pharmingen) or with FITC-conjugated rat anti-mouse-CD4 (BD Pharmingen) and PE-conjugated rat anti-mouse CD8 (BD Pharmingen). The cells were washed, resuspended in PBS, and analyzed in a FACScan with CellQuest software.

Statistical analysis

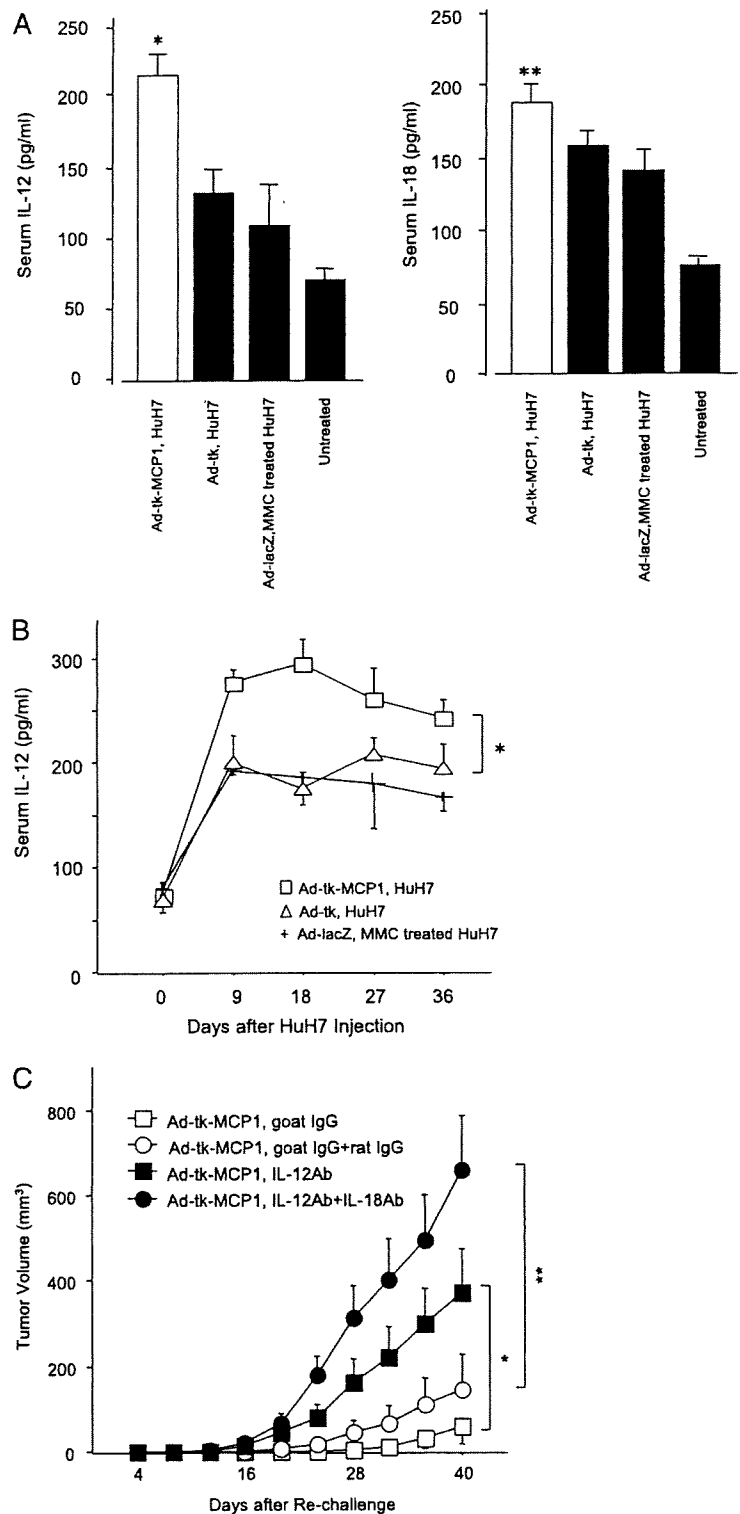
All results were expressed as means \pm SE. The statistical significance of differences between groups was evaluated by repeated measures ANOVA for the duration of the serum levels of IL-12 or the Mann-Whitney *U* test for the other results.

Results

Apoptotic HCC cells expressing MCP-1 augment IL-12 production by monocytes and DCs in vitro

IL-12, which was originally identified as an NK-stimulatory factor and a cytotoxic lymphocyte maturation factor, is one of the most promising cytokines in cancer treatment because of its multiple effects. IL-12 is produced by APCs such as macrophages, DCs, and B cells following the appropriate stimuli (29–31). To evaluate the immunomodulatory effects of rAds expressing HSV-tk with or without MCP-1 (Fig. 1), we measured IL-12 production by monocytes and DCs, both of which had been cocultured with HCC cells that had been infected with rAds (Fig. 1). Murine peritoneal exudate cells, consisting mostly of macrophages, and human monocytes cocultured with apoptotic HCC cells induced by the HSV-tk/GCV system plus MCP-1 produced greater amounts of IL-12

FIGURE 5. Roles of IL-12 and IL-18 in growth suppression of rechallenged HuH7 cells. **A**, Mouse sera were collected before s.c. injection of primary tumor cells (untreated) and 2 days after rechallenge with HuH7 cells, and IL-12 and IL-18 concentrations were measured using immunoassay kits. Each value is the mean \pm SE of triplicate experiments. *, $p < 0.01$; **, $p < 0.05$ compared to Ad-tk by the Mann-Whitney *U* test. **B**, Serum concentrations of IL-12 were monitored every 9 days after the injection of primary tumors. Each value is the mean \pm SE of triplicate experiments. *, $p < 0.05$ compared to Ad-tk with HuH7 (Ad-tk, HuH7) by repeated measures ANOVA. **C**, At rechallenge with HuH7 cells, Ad-tk-MCP1-treated animals were i.p. administered with 250 μ g of anti-IL-12 Ab (Ad-tk-MCP1, IL-12Ab), 225 μ g of anti-IL-12 Ab plus 25 μ g of anti-IL-18 Ab (Ad-tk-MCP1, IL-12Ab+IL-18Ab), or 250 μ g of control IgG Ab (Ad-tk-MCP1, goat IgG or Ad-tk MCP1, goat IgG+rat IgG). Tumor sizes were measured every 4 days. The results are representative of two independent experiments. *, $p < 0.05$ compared to Ad-tk-MCP1 with goat IgG (Ad-tk-MCP1, goat IgG); **, $p < 0.01$ compared to Ad-tk-MCP1 with goat IgG plus rat IgG (Ad-tk-MCP1, goat IgG+rat IgG) by the Mann-Whitney *U* test.



than did those cocultured with apoptotic HCC cells induced by the HSV-tk/GCV system alone (Fig. 1, A and C). Murine bone marrow DCs tended to produce IL-12 when cocultured with HCC cells infected with rAds expressing MCP-1 without regard to HSV-tk-induced apoptosis (Fig. 1B). Human DCs produced

large amounts of IL-12 when cocultured with HSV-tk/GCV-induced apoptotic tumor cells, which expressed MCP-1, as did human monocytes (Fig. 1D). Thus, the phenomena observed in this xenograft model may also be observed under human allogeneic conditions.

When we measured DC maturation markers we found that their expression levels did not change when these cells were cocultured with tk/MCP-1 transduced HCC cells, whereas CD86 expression was elevated when the DCs were incubated with apoptotic HCC cells (data not shown).

Prolongation of the antitumor effects of the HSV-tk/GCV system by codelivery of the MCP-1 gene in an athymic nude mouse model of HCC

To determine the effects of HSV-tk/GCV plus MCP-1 in a murine model of HCC, HuH7 cells were s.c. transplanted into athymic nude mice and eradicated with rAds harboring HSV-tk with or without MCP-1, and the mice were rechallenged with HuH7 cells (Fig. 2A). We found that tumor regrowth was significantly lower when the primary tumor cells had been eradicated with Ad-tk-MCP1 as compared with Ad-tk (tumor volume 40 days after rechallenge, $59.2 \pm 24.9 \text{ mm}^3$ ($n = 22$) vs $471.2 \pm 118.6 \text{ mm}^3$ ($n = 20$), $p < 0.01$) (Fig. 2B). No growth inhibition was observed when Ad-tk-MCP1 or Ad-MCP1 was administered in the absence of HuH7 cell transplantation (tumor volume, $339.6 \pm 124.3 \text{ mm}^3$, $n = 18$, and $575.3 \pm 179.1 \text{ mm}^3$, $n = 12$, respectively) or when Ad-lacZ was administered along with MMC-treated HuH7 cells (tumor volume, $554.8 \pm 125.6 \text{ mm}^3$, $n = 18$). The results demonstrate that, when the primary tumors were eradicated with the HSV-tk/GCV system plus MCP-1, the antitumor effects were maintained.

Recruitment and activation of NK cells in rechallenged tumors

Serum MCP-1 concentration was below the detection limit of the ELISA used when the s.c. tumors were injected with rAds, whereas the tumor produced MCP-1 in vitro upon infection with Ad-tk-MCP-1 (data not shown). Moreover, we could not detect adenovirus DNA in these rechallenged tumors by using PCR (data not shown), negating the possibility that adenovirus infection contributed to the rejection of the rechallenged tumor. These results indicate that the injected human MCP-1 gene functioned locally in the primary s.c. tumors, thereby modulating the subsequent response to the rechallenged tumor. Because athymic nude mice possess NK cells and macrophages but not T lymphocytes, we determined the migration of these cells by an immunohistochemical analysis. The number of AGM1⁺ NK cells was significantly higher upon tumor rechallenge in mice whose primary tumors had been eradicated with Ad-tk-MCP1 plus GCV than in those whose primary tumors had been eradicated with Ad-tk plus GCV ($p < 0.05$) (Fig. 3, A and B). Similarly, the numbers of F4/80 or Mac-1 positive cells (32, 33) tended to be higher upon tumor rechallenge in mice whose primary tumors had been eradicated with Ad-tk-MCP1. Moreover, the mRNA of IFN- γ secreted by NK cells (34) became detectable after 30 PCR cycles in the rechallenged tumors of animals whose primary tumors had been eradicated with Ad-tk-MCP1 and was greatly amplified after 40 PCR cycles (Fig. 3C). These results demonstrate that NK cells were recruited and activated into rechallenged tumor tissues, presumably inhibiting tumor cell growth in mice whose primary tumors had been eradicated with HSV-tk/GCV plus MCP-1.

To monitor the activation state of innate immunity in extrahepatic lymphoid organs, we determined immunohistochemically the numbers of immune cells in the spleen after tumor rechallenge using anti-AGM1, F4/80, Mac-1, CD11c, and CD45R Abs (Fig. 4, A and B). The numbers of F4/80⁺ and Mac-1⁺ cells were significantly increased in the spleens of mice treated with Ad-tk-MCP1 compared with mice treated with Ad-tk ($p < 0.05$). In contrast, the numbers of AGM1⁺ and CD45R⁺ cells tended to be higher in the spleens of mice treated with Ad-tk-MCP1, but there was little dif-

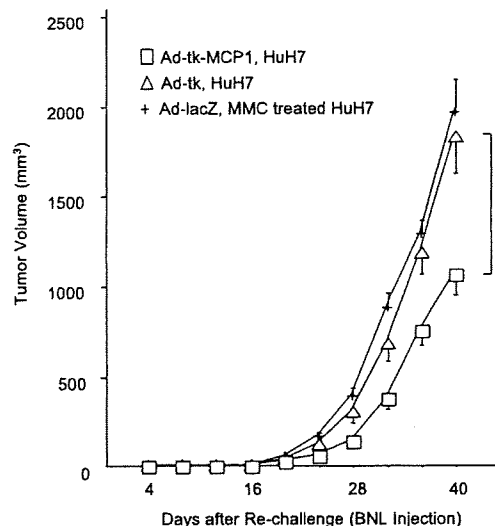


FIGURE 6. Antitumor effects of rAds expressing HSV-tk with or without MCP-1 against a second unprimed cell line (BNL) in an athymic nude mouse model of HCC. As described in the legend to Fig. 3, following complete eradication of the primary tumors the mice were s.c. injected with 1×10^3 BNL cells at other sites on day 14. Tumor sizes were measured every four days. The results are the means of two independent experiments. *, $p < 0.01$ compared to Ad-tk with HuH7 (Ad-tk, HuH7) by the Mann-Whitney's U test.

ference in the numbers of CD11c⁺ cells. A flow cytometrical analysis of splenocyte single cell suspensions demonstrated that the numbers of DX5⁺ and F4/80⁺ cells tended to be higher in the spleens of mice treated with Ad-tk-MCP1 (Fig. 4C). In contrast, treatment with carrageenan decreased the number of macrophages in the spleen and at rechallenge sites and slightly increased the number of NK cells in the spleen. Collectively, these results suggest that alterations in the proportions of cell subsets in splenocytes may reflect the activation status of the innate immune system following the eradication of primary tumors by HSV-tk/GCV plus MCP-1. Finally, an anti-AGM1 Ab (35, 36) significantly inhibited the antitumor immunity conferred by Ad-tk-MCP1 (tumor volume 40 days after rechallenge, $385.4 \pm 106.3 \text{ mm}^3$ ($n = 22$) vs $64.2 \pm 43.6 \text{ mm}^3$ ($n = 16$), $p < 0.05$), and carrageenan partially inhibited the antitumor immunity of Ad-tk-MCP1 (tumor volume, $242.6 \pm 100.8 \text{ mm}^3$ ($n = 14$) vs $53.8 \pm 22.9 \text{ mm}^3$ ($n = 22$), $p = 0.22$) (Fig. 4D). The results indicate that antitumor effects were mainly mediated by NK cells.

Involvement of IL-12 and IL-18 in sustained antitumor effects

IL-18 is a proinflammatory cytokine produced by activated macrophages that has been shown to augment both innate and acquired immunity (37) and, in combination with IL-12, induce Th 1 cell development and NK cell activation (38). We therefore assayed IL-12 and IL-18 production after tumor rechallenge. Serum concentrations of IL-12 and IL-18 were significantly higher after tumor rechallenge in mice whose primary tumors had been eradicated with Ad-tk-MCP1 compared with mice whose tumors had been eradicated with Ad-tk ($p < 0.05$) (Fig. 5A). Moreover, serum concentrations of IL-12 peaked after primary tumors were eradicated (day 9) and were sustained thereafter ($p < 0.05$) (Fig. 5B). Furthermore, the administration of anti-IL-12 significantly inhibited the antitumor effects conferred by Ad-tk-MCP1 (Fig. 5C) and reduced the serum concentrations of IL-12 to an undetectable level

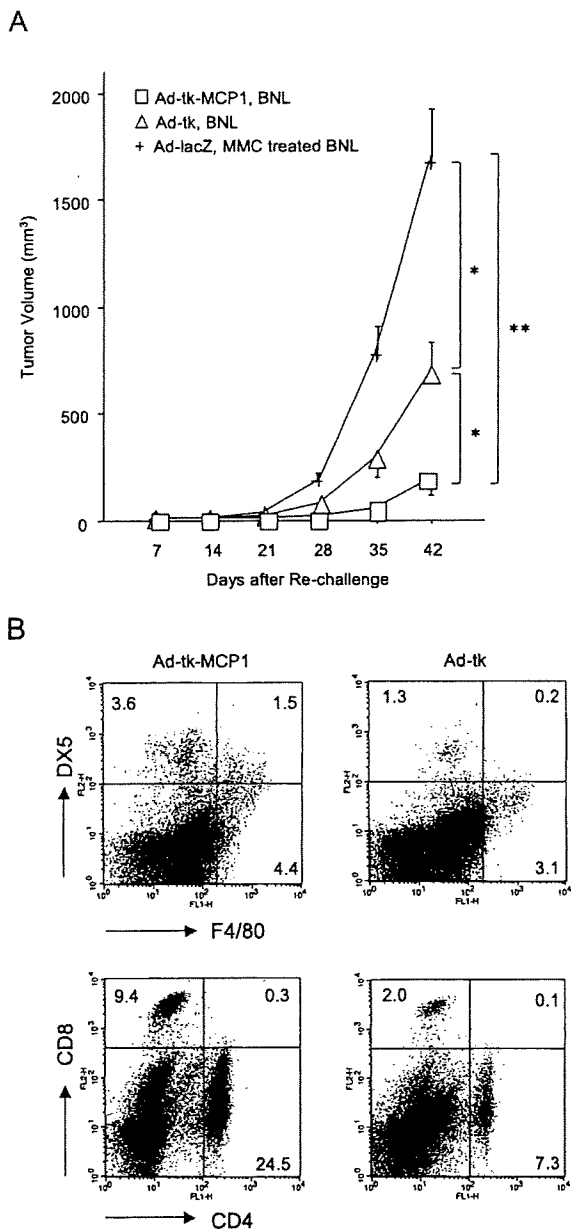


FIGURE 7. Prolonged antitumor effects of rAds expressing HSV-tk with or without MCP-1 in an immunocompetent mouse model of HCC. *A*, On day 0, mice were s.c. injected with 1×10^5 BNL cells infected with Ad-tk-MCP1, Ad-tk, or Ad-lacZ at an in vitro MOI of 100. The mice were i.p. injected with 75 mg/kg GCV per day for the next 5 days (days 1–5). Following complete eradication of the primary tumors, the mice were s.c. rechallenged with 1×10^4 BNL cells at other sites on day 14. Tumor sizes were measured every 7 days. The results are the means of three independent experiments. **, $p < 0.001$ compared to Ad-lacZ with MMC-treated BNL (Ad-lacZ, MMC treated BNL); *, $p < 0.01$ compared to Ad-tk with BNL (Ad-tk, BNL) or Ad-lacZ with MMC-treated BNL (Ad-lacZ, MMC-treated BNL) by the Mann-Whitney *U* test. *B*, Spleens were resected 70 days after the injection of primary tumor cells, and surface expression of DX5, F4/80, CD4, and CD8 in cell populations obtained from spleens was assessed by FACS. The results are representative of two independent experiments.

(data not shown). The combined treatment of anti-IL-12 and anti-IL-18 Ab further diminished antitumor effects (Fig. 5C) and reduced both serum IL-12 and IL-18 levels to undetectable levels

(data not shown). The results suggest the critical involvement of IL-12 and IL-18 in the antitumor effects induced by Ad-tk-MCP1 on tumor regrowth.

Innate immune responses to heterologous tumor injection in an athymic nude mouse

To estimate the involvement of innate immune responses in the antitumor effects observed with HSV-tk/GCV plus MCP-1, we re-challenged mice with heterologous tumor administration. The growth of a second unprimed cell line (BNL; transformed liver cells derived from BALB/c mice) was significantly suppressed when HuH7 cells had been eradicated with Ad-tk-MCP1 as compared with Ad-tk (tumor volume, 1059.5 ± 110.6 mm³ ($n = 12$) vs 1825.4 ± 197.9 mm³ ($n = 12$), $p < 0.01$) when Ad-lacZ was administered with MMC-treated HuH7 cells (tumor volume, 1960.8 ± 183.8 mm³, $n = 12$) (Fig. 6). These results indicate that the innate immune responses contributed to the prolonged antitumor effects of HSV-tk/GCV plus MCP-1 gene transfer.

Prolonged antitumor effects against mouse HCC of rAd expressing HSV-tk and MCP-1 in an immunocompetent mouse

Finally, we evaluated the antitumor responses in immune-competent mice using the same experimental procedures (Fig. 7A). The growth of rechallenged tumors was significantly lower when the primary tumor cells had been eradicated with Ad-tk-MCP1 as compared with Ad-tk (tumor volume 42 days after rechallenge, 170.3 ± 54.2 mm³ ($n = 22$) vs 488.9 ± 120.1 mm³ ($n = 22$), $p < 0.01$), similarly observed on athymic nude mice injected with human HCC. In addition, the growth of rechallenged tumors was significantly suppressed in mice whose primary tumors had been eradicated with Ad-tk as compared with those treated with Ad-lacZ and MMC (488.9 ± 120.1 mm³ ($n = 22$) vs 1666.4 ± 259.2 mm³ ($n = 22$), $p < 0.01$). Furthermore, when we isolated splenocytes 70 days after the injection of primary tumor cells we found that the numbers of CD4⁺ and CD8⁺ cells were increased in mice treated with Ad-tk-MCP1 (Fig. 7B). Collectively, these results confirm that antitumor effects may be dependent not only on innate immunity but on acquired immune responses.

Discussion

In the current study, we observed that when monocytes were cocultured with apoptotic HCC cells infected with Ad-tk-MCP1, these immune cells produced large amounts of IL-12. Interestingly, in both nude and immunocompetent mice the growth of rechallenged HCC cells was markedly suppressed after the primary tumor cells had been eradicated with Ad-tk-MCP1 followed by GCV administration. Furthermore, these prolonged in vivo antitumor effects were associated with the production of IL-12 and IL-18 and mediated by NK cells.

Monocytes produced large amounts of IL-12 when cocultured with apoptotic HCC cells induced by the HSV-tk/GCV system plus MCP-1. APCs, such as macrophages, DCs, and B cells produce IL-12, which was originally identified as an NK-stimulatory factor and shown to exhibit considerable antineoplastic activity (39, 40). APCs were found to be activated upon the recognition of Ags from apoptotic target cells (41), and both macrophages and DCs secrete large amounts of IL-12 when treated with MCP-1 in vitro (33, 42, 43). These findings suggest that the recognition of apoptotic tumor cells together with MCP-1 may activate macrophages and DCs, thereby enhancing IL-12 secretion.

We demonstrated that the antitumor effects were maintained when the tumor cells had been eradicated with Ad-tk-MCP1, a vector that expresses both a suicide gene and a chemokine, but that either alone was not sufficient to prolong immunity in our models.

We previously demonstrated that MCP-1 secreted by apoptotic HuH7 cells may recruit and activate macrophages efficiently, although these effects did not occur when the tumor cells were treated with the rAd expressing either HSV-tk or MCP-1 (8, 10). Moreover, we observed that the numbers of Mac-1⁺ and F4/80⁺ cells were increased in the spleens of mice after tumor rechallenge. Indeed, MCP-1 has been shown to activate murine peritoneal macrophages and enhance the expression of CD11b (Mac-1) in BALB/c mice (32, 33). Collectively, these results suggest that during eradication of the primary tumors, activated macrophages in the tumor tissues and the peripheral lymphoid organs can induce the secretion of cytokines, including IL-12 and IL-18, that can activate NK cells, thus exerting antitumor effects.

IL-12-stimulated NK cells exhibit potent cytotoxic activity against various tumor cells (31, 44, 45). NK cells are a part of the innate immune system, a first-line defense against tumor cells, and exert antitumor effects of NK cells rapidly without any prior sensitization (46). The depletion of NK cells has been shown to promote metastases or tumor growth after rechallenge with primary tumor cells (15, 44, 47). We demonstrated here that the growth of rechallenged parental tumor cells or newly challenged heterologous tumor cells was suppressed after eradication of the primary tumors. Therefore, augmentation of NK-mediated innate immune responses may be an attractive strategy for preventing HCC recurrence, including the growth of differentially transformed tumor cells.

We observed that NK cell-mediated antitumor effects were prolonged after primary tumor cells had been eradicated with Ad-tk-MCP1. Several lines of evidence indicate that the inhibitory effects of NK cells on tumor growth were maintained and were detectable at the site of the primary tumor even after treatment discontinuation (36, 48). Although the mechanisms involved in these responses are not yet known, a number of tumor model systems have demonstrated the important roles of NK cells in early tumor clearance, leading to the establishment of adaptive immunity. It was recently reported that NK cell-mediated immune responses featured hallmarks of adaptive immunity such as acquired immunity, long-lived memory, and Ag specificity (16). DCs expressing IL-12 have been shown to confer NK-mediated tumor protection in which NK activation is dependent on both DC-NK interaction and IL-12 secretion (49). Moreover, NK cell-derived IFN- γ may provide early immune regulation that alters the outcome and quality of adaptive immunity (50). Furthermore, MCP-1 has been shown to induce DC migration to lesions where NK cytolytic responses are activated (51). Consistent with these observations, we demonstrated that the antitumor responses were abolished when NK cells were inactivated by treatment with the AGM1 Ab and that NK cells were recruited and IFN- γ production enhanced in the rechallenged tumors.

We observed that the growth of rechallenged heterologous tumors was suppressed to a lesser extent than that of homologous tumors in our nude mice model. Athymic nude mice lack T lymphocyte-mediated immune responses, but the numbers and functions of macrophages and NK cells are preserved. Moreover, nude mice have limited populations of extrathymically matured T lymphocytes, including $\gamma\delta$ T cells (52), and these may be reduced slightly by treatment with AGM1 Ab (53). Both NK cells and V δ 1 γ δ T lymphocytes have been reported to prevent the growth of s.c. melanoma cells, with both cell types detected at the sites of the s.c. tumors (47). Therefore, we cannot exclude the possibility that the memory subset of $\gamma\delta$ T cells affects antitumor immunity against homologous and heterologous cells, thus leading to differences in the magnitude of tumor suppression.

Although the results presented here are promising, a number of problems remain to be solved before this approach can be used clinically. First, s.c. tumor models using an HCC cell line may not be fully comparable to HCCs in patients. Second, problems using rAds need to be resolved before they can be applied clinically. However, in patients treated with nonsurgical procedures such as percutaneous radiofrequency ablation therapy and transcatheter arterial chemotherapy, the administration of rAd vectors may ensure tumor cell killing, thus enhancing the antitumor effects on residual tumor cells and recurrent HCC.

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Disclosures

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Different Signaling Pathways in the Livers of Patients With Chronic Hepatitis B or Chronic Hepatitis C

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The clinical manifestations of chronic hepatitis B (CH-B) and chronic hepatitis C (CH-C) are different. We previously reported differences in the gene expression profiles of liver tissue infected with CH-B or CH-C; however, the signaling pathways underlying each condition have yet to be clarified. Using a newly constructed cDNA microarray consisting of 9614 clones selected from 256,550 tags of hepatic serial analysis of gene expression (SAGE) libraries, we compared the gene expression profiles of liver tissue from 24 CH-B patients with those of 23 CH-C patients. Laser capture microdissection was used to isolate hepatocytes from liver lobules and infiltrating lymphoid cells from the portal area, from 16 patients, for gene expression analysis. Furthermore, the comprehensive gene network was analyzed using SAGE libraries of CH-B and CH-C. Supervised and unsupervised learning methods revealed that gene expression was correlated more with the infecting virus than any other clinical parameters such as histological stage and disease activity. Pro-apoptotic and DNA repair responses were predominant in CH-B with p53 and 14-3-3 interacting genes having an important role. In contrast, inflammatory and anti-apoptotic phenotypes were predominant in CH-C. These differences would evoke different oncogenic factors in CH-B and CH-C. **In conclusion**, we describe the different signaling pathways induced in the livers of patients with CH-B or CH-C. The results might be useful in guiding therapeutic strategies to prevent the development of hepatocellular carcinoma in cases of CH-B and CH-C. *Supplementary material for this article can be found on the HEPATOLOGY website (<http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html>). (HEPATOLOGY 2006;44:1122-1138.)*

The human liver infected with hepatitis B virus (HBV) and hepatitis C virus (HCV) develops chronic hepatitis, cirrhosis, and in some instances, hepatocellular carcinoma (HCC).¹⁻³ The virological features of these 2 viruses are completely different. HBV is a DNA virus that integrates into the host genome.^{4,5} HBV proteins, which have been reported to have transcriptional transactivator activity, may be related to

the occurrence of HCC.⁶⁻⁹ By contrast, HCV is a positive stranded RNA virus that replicates in the cytoplasm.² There are some reports that HCV proteins localize to the nucleus or interact with nuclear proteins.^{10,11} Nevertheless, both viruses infect the liver and cause chronic hepatitis, which is not distinguishable by histological examination or clinical manifestations.¹² In chronic viral hepatitis, increased numbers of immunoregulatory cells infiltrate the liver, but the functional relevance of these cells to the pathogenesis of chronic hepatitis is not known.

We previously reported that the gene expression profiles in the livers of patients with chronic hepatitis B (CH-B) or chronic hepatitis C (CH-C) are different, and revealed some characteristic features of each disease.¹³ However, the independent expression profiles of infiltrated lymphocytes and hepatocytes have yet to be clarified, as do the detailed signaling pathways underlying these 2 conditions.

In this study, we investigated the signaling pathways underlying CH-B and CH-C using cDNA microarray and serial analysis of gene expression (SAGE) techniques. Using laser capture microdissection (LCM), we selectively isolated hepatocytes from liver lobules and infiltrat-

Abbreviations: CH-B, chronic hepatitis B; CH-C, chronic hepatitis C; SAGE, serial analysis of gene expression; HBV, hepatitis B virus; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; GO, gene ontology; LCM, laser capture microdissection; ALT, alanine aminotransferase; aRNA, antisense RNA; CTL, cytotoxic lymphocyte; Cy, cyanine; EGFR, epidermal growth factor receptor; cDNA, complementary DNA; IFN, interferon; NF- κ B, nuclear factor- κ B; NK cells, natural killer cells.

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ing lymphoid cells from the portal area, from biopsy specimens, and analyzed their gene expression profiles.

Patients and Methods

Patients. The subjects were 27 patients with CH-B and 26 with CH-C at the Graduate School of Medicine, Kanazawa University Hospital, Japan, between 1999 and 2003 (Table 1). Informed consent was obtained from all patients and ethics approval for the study was obtained from the ethics committee for human genome/gene analysis research at Kanazawa University Graduate School of Medicine. Liver biopsy samples were taken from 24 CH-B patients and 23 CH-C patients, and were divided into 3 portions: one was immersed in formalin for histological assessment, another was immediately frozen in liquid nitrogen for further RNA isolation, and the final portion was frozen in OCT compound for LCM analysis and stored at -80°C until use. Tissue samples from the remaining 6 patients with HCC were surgically obtained from the noncancerous parts of the liver and immediately frozen in liquid nitrogen for SAGE analysis. For normal liver, surgically obtained tissue samples of 6 patients who showed no clinical signs of hepatitis were used, as described.¹³

The grading and staging of chronic hepatitis were histologically assessed according to the method described by Desmet et al.¹⁴ (Table 1). There were no significant differences in the degree of histological activity or staging, nor in the sex or age of patients with CH-B or CH-C (Table 1).

Treatment of Cultured Cells With Interferon- α . Huh-7 cells were treated with recombinant interferon- α (IFN- α) (Schering-Plough Corp., Osaka, Japan) at a concentration of 1000 IU/mL for 6 hours, and were harvested for analysis of induced gene expression by cDNA microarray.

Preparation of cDNA Microarray Slides. In addition to the in-house cDNA microarray slides consisting of 1080 cDNA clones as described,^{13,15-19} we made a new cDNA microarray slide for a detailed analysis of the signaling pathways involved in metabolism and enzyme function in liver disease. Besides cDNA microarray analysis, a total of 256,550 tags were obtained from hepatic SAGE libraries (derived from normal liver, CH-C, CH-C related HCC, CH-B, and CH-B related HCC), including 52,149 unique tags. Among these, 16,916 tags with more than 2 hits were selected to avoid the effect of sequencing errors in the libraries. From these candidate genes, 9614 nonredundant clones were obtained from Incyte Genomics (Incyte Corp., Beverly, MA), Clontech (Nippon Becton Dickinson, Tokyo, Japan), and Invitro-

gen (Invitrogen Japan K.K., Tokyo, Japan). Each clone was sequence validated and PCR amplified by Dragon Genomics (Takara Bio, Otsu, Japan), and the cDNA microarray slides (Liver chip 10k) were constructed using SPBIO 2000 (Hitachi Software, Fukuoka, Japan) as previously described.^{13,15-19}

Laser Capture Microdissection. Hepatocytes in liver lobules and infiltrated lymphoid cells in the portal area were isolated by LCM using a CRI-337 LCM system (Cell Robotics, Albuquerque, NM)¹⁸ (Fig. 1). Frozen liver biopsy specimens in OCT compound were sliced into sections 8 μm thick, immediately fixed in methanol for 5 minutes, and kept on dry ice. Tissue samples were quickly stained with toluidine blue and dissected. Around 500 lymphoid cells and a similar number of hepatocytes were excised from 3 slides and immersed in a denaturing solution. Dissection was completed within 5 minutes for each slide.

RNA Isolation and Antisense RNA Amplification. Total RNA was isolated from liver biopsy samples using an RNA extraction kit (Micro RNA Extraction Kit, Stratagene, La Jolla, CA). Aliquots of total RNA (5 μg) were subjected to amplification with antisense RNA (aRNA) using a Message Amp aRNA kit (Ambion, Austin, TX) as recommended by the manufacturer. About 25 μg of aRNA was amplified from 5 μg of total RNA, assuming that 500-fold amplification of mRNA was obtained. Total RNA from LCM samples was isolated with a carrier nucleic acid (20 ng poly C) using RNAqueous-Micro (Ambion). The quality and degradation of the isolated RNA were estimated after electrophoresis using an Agilent 2001 bioanalyzer (Agilent Technologies, Palo Alto, CA) (Fig. 1B). RNA isolation typically yielded 20-40 ng total RNA from 500 cells. Half of the obtained RNA was amplified twice as described above to yield 20-40 μg aRNA. Antisense RNA (20 μg) was used for further labeling procedures. The optimum conditions of LCM and reproducibility of data were assessed repeatedly.

Hybridization on cDNA Microarray Slides and Image Analysis. As a reference for each microarray analysis, aRNA samples prepared from the normal liver tissue from 1 of the patients were used. Test RNA samples fluorescently labeled with cyanine 5 (Cy5) and reference RNA labeled with Cy3 were used for microarray hybridization as described.^{13,15-19} Quantitative assessment of the signals on the slides was carried out by scanning on a ScanArray 5000 (General Scanning, Watertown, MA) followed by image analysis using GenePix Pro 4.1 (Axon Instruments, Union City, CA) as described.

Processing of cDNA Microarray Data. Hierarchical clustering of gene expression was performed by BRB-Ar-

Table 1. Characteristics of Patients, as Used for Analyses of Whole Liver Biopsy, LCM, and SAGE Samples

Patient No.	Virus	Age	Sex	ALT	A	F	Viral load (LEG/mL, KIU/mL)	HCV serotype	HBeAg	LCM Hep	LCM Ly
Whole liver biopsy samples											
1	HBV	34	F	45	1	1	8.2	na.	+		
2	HBV	64	F	119	1	1	>8.7	na.	+		
3	HBV	49	M	21	1	1	<3.7	na.	-		
4	HBV	29	M	194	2	1	7.5	na.	+		
5	HBV	47	M	10	1	2	<3.7	na.	-		
6	HBV	53	F	43	1	2	7.3	na.	+		
7	HBV	24	M	42	2	2	7.1	na.	+		
8	HBV	18	M	400	2	2	8.0	na.	+		
9	HBV	20	M	188	2	2	6.2	na.	+		
10	HBV	59	M	68	2	3	4.4	na.	+		
11	HBV	36	F	29	2	3	4.2	na.	-		
12	HBV	60	M	33	2	3	7.4	na.	+		
13	HBV	60	F	28	2	3	<3.7	na.	-		
14	HBV	35	F	145	3	3	7.6	na.	+		
15	HBV	64	M	48	1	4	7.1	na.	-		
16	HBV	55	M	30	1	4	7.4	na.	-		
17	HBV	34	F	45	2	4	8.5	na.	+		
18	HBV	54	M	159	2	4	5.5	na.	+		
19	HBV	60	M	121	3	4	4.6	na.	+		
20	HCV	24	M	34	1	1	>850	I	-		
21	HCV	68	F	43	1	1	720	II	-		
22	HCV	64	F	117	1	2	590	na.	-		
23	HCV	69	M	6	1	2	300	II	-		
24	HCV	42	M	59	1	2	410	II	-		
25	HCV	73	M	19	1	2	140	I	-		
26	HCV	43	M	98	2	2	60	I	-		
27	HCV	70	M	56	2	2	600	I	-		
28	HCV	70	F	26	2	3	350	I	-		
29	HCV	65	M	21	2	3	290	I	-		
30	HCV	47	M	225	2	3	120	I	-		
31	HCV	58	M	200	2	3	410	I	-		
32	HCV	57	F	116	2	3	490	I	-		
33	HCV	63	F	39	2	4	290	I	-		
34	HCV	76	M	54	2	4	660	I	-		
35	HCV	67	M	67	2	4	240	I	-		
36	HCV	46	M	111	2	4	>850	I	-		
37	HCV	63	M	64	2	4	60	na.	-		
LCM samples											
38(2)	HBV	64	F	119	1	1	>8.7	na.	+	+	+
39	HBV	31	F	114	1	1	8.5	na.	+	+	+
40	HBV	68	F	41	2	2	5.5	na.	+		+
41	HBV	29	M	140	2	2	>8.7	na.	+		+
42	HBV	40	M	80	2	2	<3.7	na.	-		+
43	HBV	45	M	83	2	3	6.1	na.	+		+
44(10)	HBV	59	M	68	2	3	4.4	na.	+	+	+
45(14)	HBV	35	F	145	3	3	7.6	na.	+	+	+
46(21)	HCV	68	F	43	1	1	720	II	-	+	+
47	HCV	47	M	33	1	1	50	I	-	+	+
48	HCV	67	M	80	2	2	114	II	-		+
49	HCV	73	M	71	2	2	>850	II	-		+
50	HCV	67	M	70	2	2	>851	I	-		+
51	HCV	59	F	43	2	3	>852	I	-		+
52(31)	HCV	58	M	200	2	3	410	I	-	+	+
53(32)	HCV	57	F	116	2	3	490	I	-	+	+
SAGE samples											
54	HBV	55	M	34	1	1	5.9	na.	-		
55	HBV	70	F	31	2	2	7.7	na.	-		
56	HBV	72	M	22	1	4	6.3	na.	+		
57	HCV	71	M	128	1	4	440	I	-		
58	HCV	69	F	84	2	4	212	I	-		
59	HCV	49	M	150	2	4	60	I	-		

Abbreviations: na., not applicable; LCM, laser capture microdissection; ALT, alanine aminotransferase; SAGE, serial analysis of gene expression; A, activity; Hep., hepatocyte obtained by LCM; Ly., lymphocyte obtained by LCM; F, fibrosis; No., if the sample was obtained from the same patient, the new sample number is shown with the old one; HCV RNA was assayed by Amplicor Monitor Test (KIU/mL); HBV DNA was assayed by transcription-mediated amplification (LEG/mL).