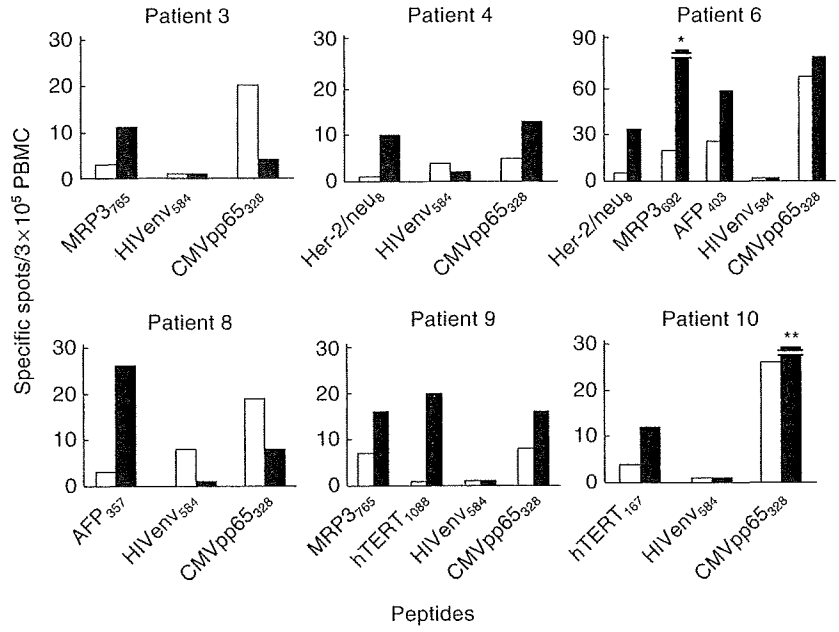

Fig. 3. Immune responses to human leucocyte antigen (HLA)-A24 restricted peptide epitopes derived from tumour antigens in patients infused with dendritic cells (DCs). Peripheral blood mononuclear cells (PBMCs) were obtained before (open bars) and 4 weeks after the infusion (solid bars), pulsed with the peptides derived from AFP, hTERT, Her-2/neu and MRP3, and interferon (IFN)-ã 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 ($HIVenv_{584}$). 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 ($CMVpp65_{328}$). 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 lease 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-ã production was quantified by ELISPOT. Interestingly, frequencies of IFN-ã-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 (± s.d.) of patients with and without DC administration: 9·6 ± 5·7 and 7·0 ± 4·5 months, respectively; $P = 0{\cdot}13$]. 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 $MRP3_{692}$ 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 carcinogenetic 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 ^{111}In-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].

^{111}In-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 generation 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 eradiated by using Ad-tk-MCP1, the same HCC cells were injected into an 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; $TCID_{50}$, 50% tissue culture infectious dose.

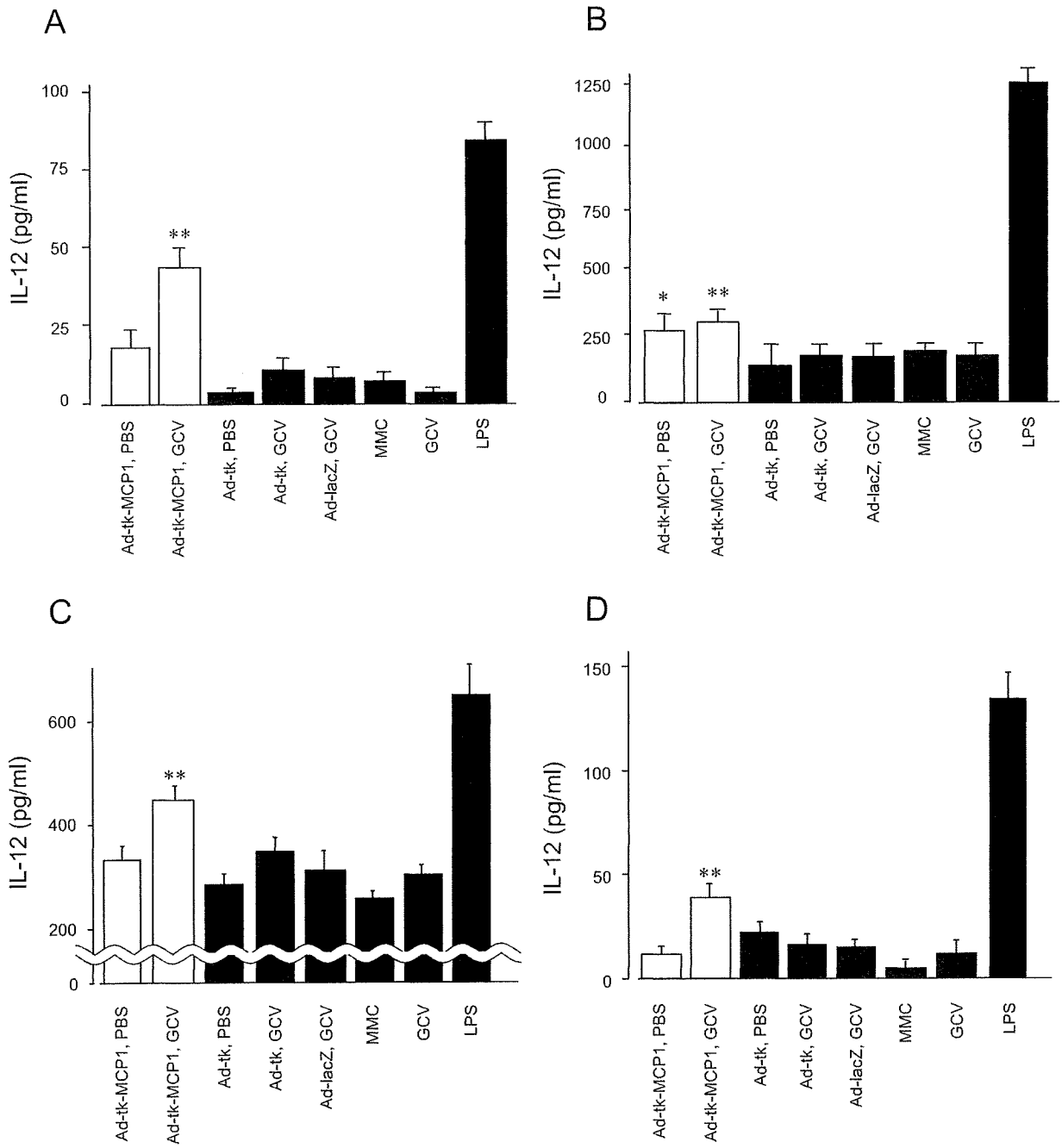

FIGURE 1. IL-12 production by monocytes and DCs cocultured with apoptotic or nonapoptotic HCC cells infected with rAds in vitro. HuH7 cells were infected with Ad-tk-MCP1, Ad-tk, and Ad-lacZ at an MOI of 5 for 24 h. Aliquots of 10^5 mouse (*A*) and human (*C*) monocytes or 10^5 mouse (*B*) and human (*D*) 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 ± 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 ($TCID_{50}$) 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 $I\text{-}A^d$ MHC class II when stained with PE-conjugated anti-Mac-1 Ab (clone M1/70; BD Pharmingen) and FITC-conjugated $I\text{-}A^d$ 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 $CD14^-HLA\text{-}DR^+$ (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 10^5 DCs or monocytes were cocultured with 10^5 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 5×10^6 HuH7 cells on day 0. On days 3 and 4, 5×10^7 $TCID_{50}$ (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 3×10^6 HuH7 cells or injected with 1×10^3 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-jcl mice (CLEA Japan) were s.c. injected with 1×10^5 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. These mice were s.c. injected with 1×10^4 BNL cells in other sites on day 14, and the tumor sizes were measured every 7 days.

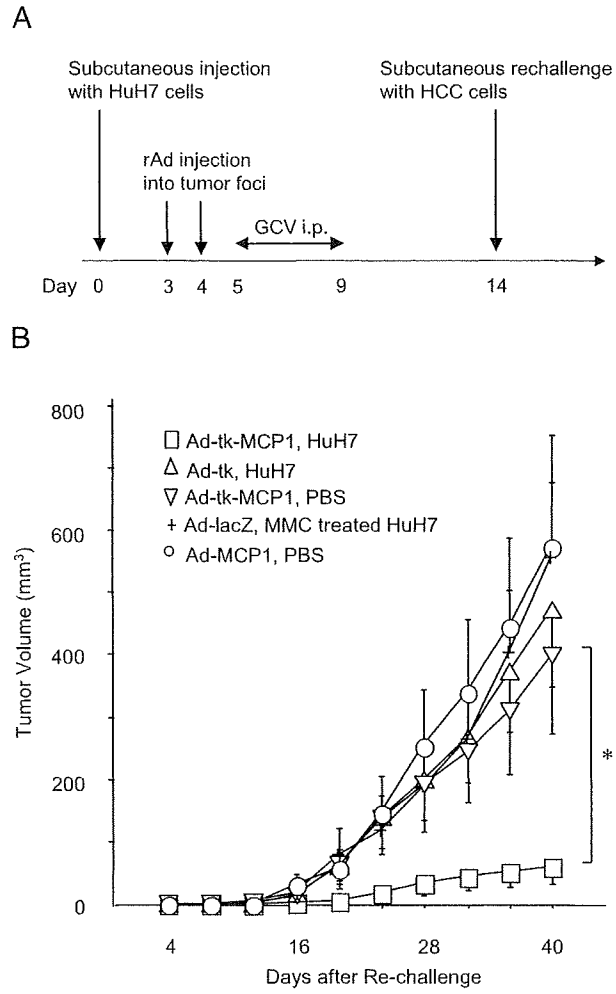

FIGURE 2. Prolonged antitumor effects of rAds expressing HSV-tk with or without MCP-1 in an athymic nude mouse model of HCC. *A*, Mice were s.c. injected with 5×10^6 HuH7 cells on day 0. On days 3 and 4, 5×10^7 $TCID_{50}$ of Ad-tk-MCP1, Ad-tk, Ad-lacZ, or Ad-MCP1 were injected into the tumors, and the 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 3×10^6 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.

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,

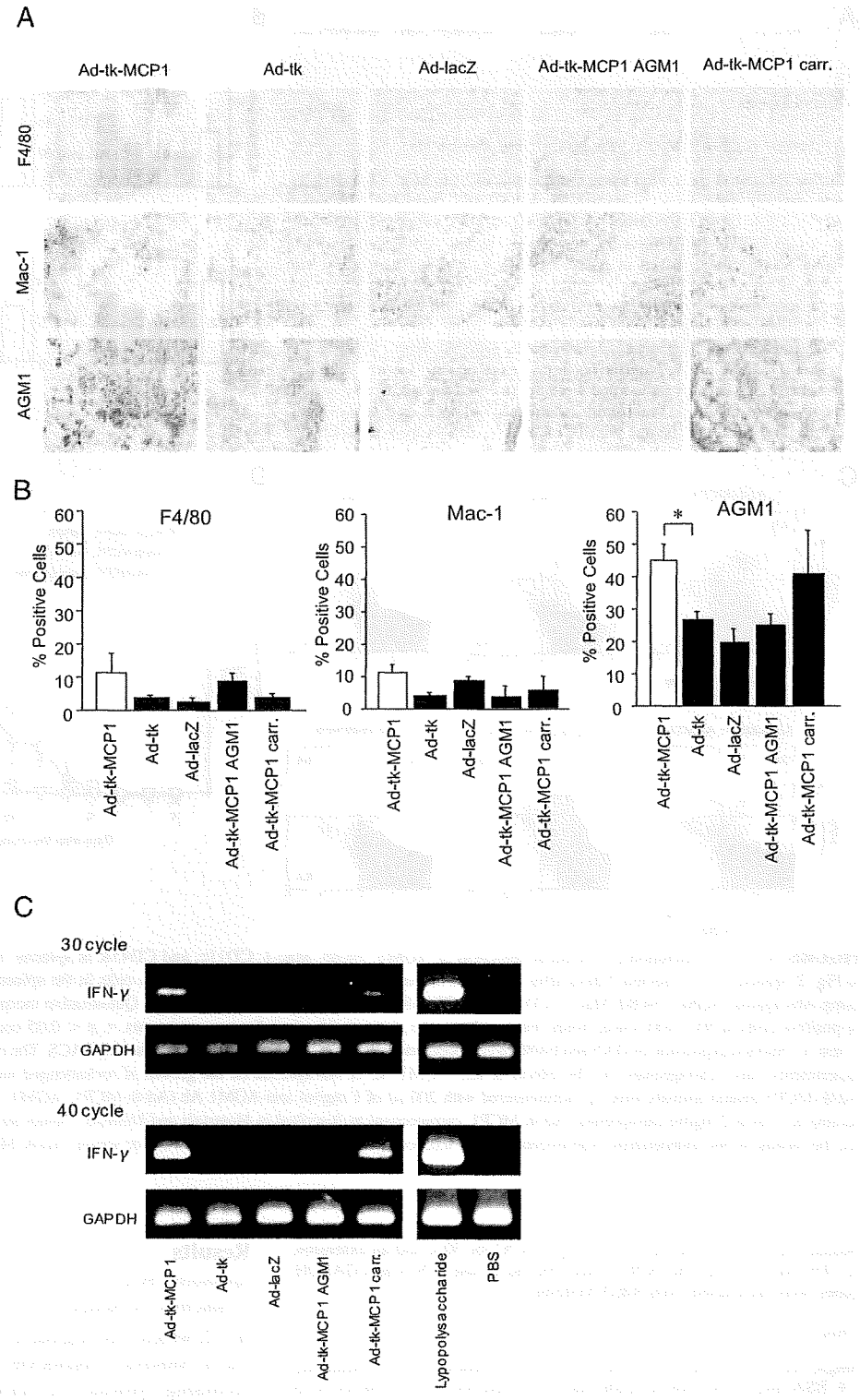

FIGURE 3. Expression of AGM1, F4/80, and Mac-1 Ags and IFN-γ mRNA in rechallenged tumor tissues. In the experiment described in the legend to Fig. 2, tumor tissues were resected 2 days after tumor rechallenge and analyzed immunohistochemically and estimated for IFN-γ mRNA expression by RT-PCR. *A*, Tumor 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, ×100. *B*, Quantitative morphometric analysis showing the proportions of positive cells in areas of 100 tumor cells. Values are the means ± 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 μg/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

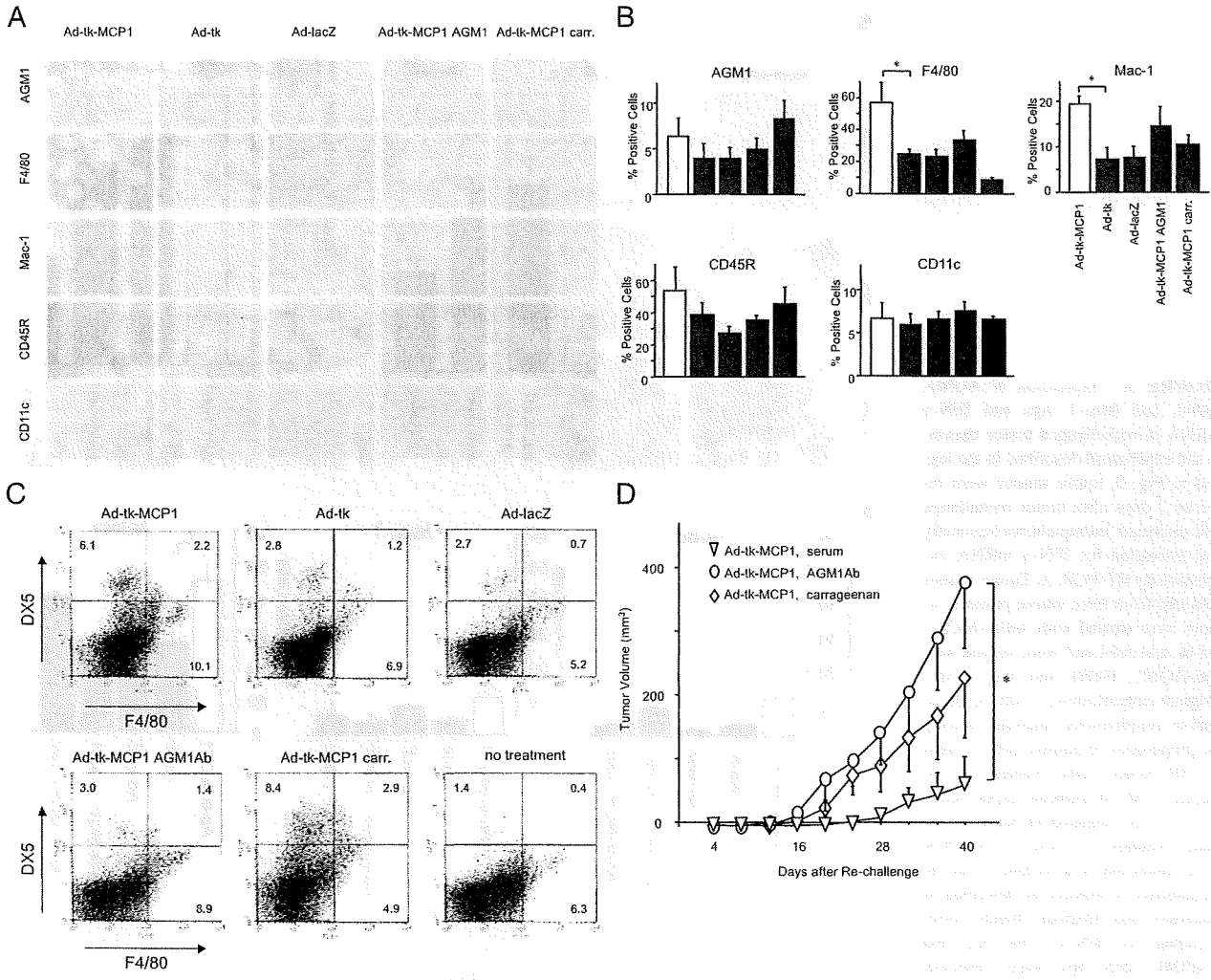

FIGURE 4. *A–C*, Immunohistochemical detection of AGM1, F4/80, Mac-1, CD11c, and CD45R in spleens. In the experiment described in the legend to Fig. 2, spleens were resected 2 days after the tumor rechallenge. *A*, The numbers of immune cells in the spleens were analyzed immunohistochemically using Abs against AGM1, F4/80, Mac-1, CD11c, and CD45R. Original magnification, ×100. *B*, Quantitative morphometric analysis showing the percentage of positive cells in 50 ×400 power fields. Each value is the mean ± 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 μl of 1 mg/ml anti-AGM1 Ab (Ad-tk-MCP1, AGM1 Ab), 200 μl 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 ± 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

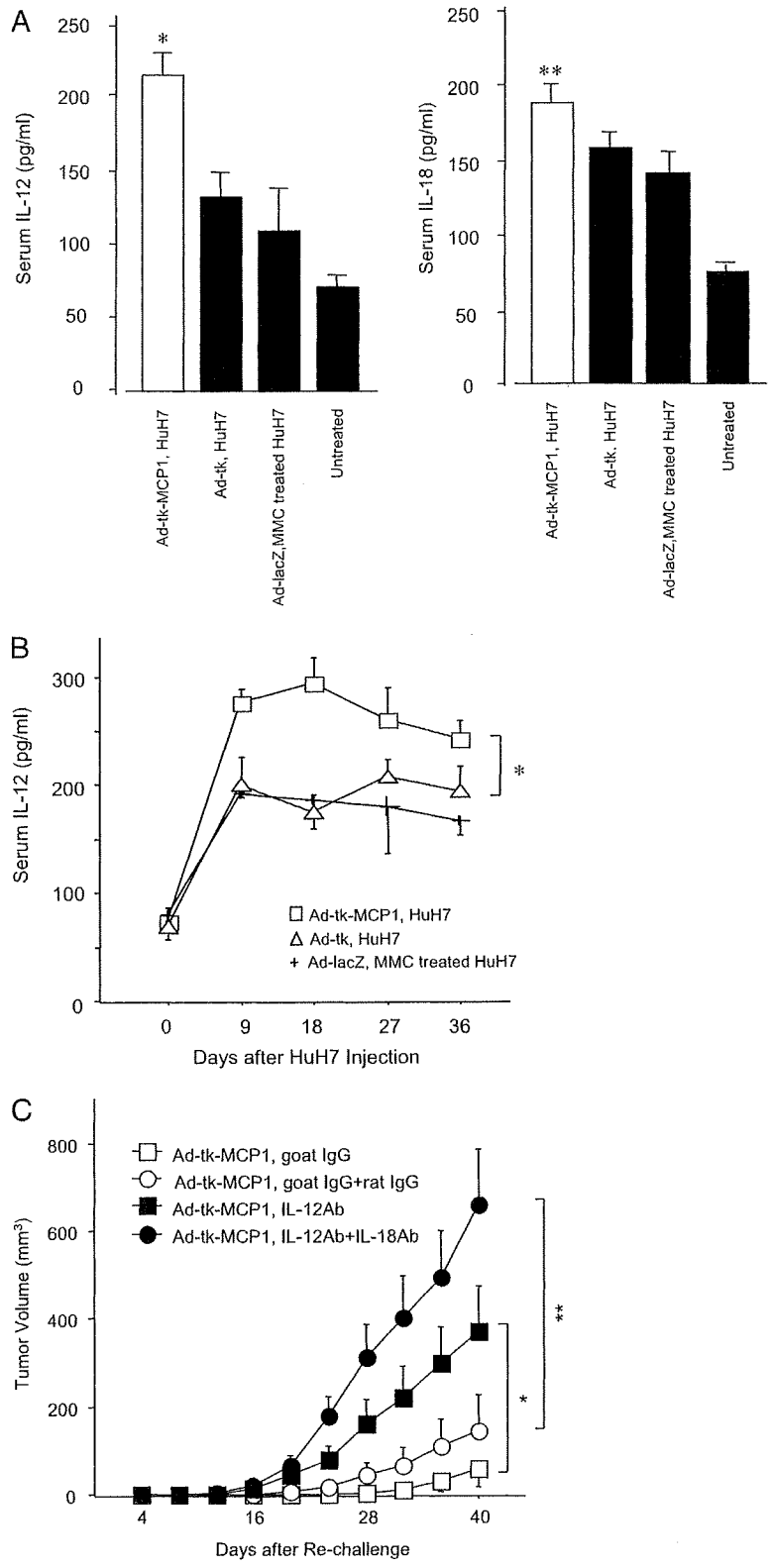

FIGURE 5. Roles of IL-12 and IL-18 in growth suppression of rechallenged HuH7 cells. *A*, Mouse sera were collected before s.c. injection of primary tumor cells (untreated) and 2 days after rechallenge with HuH7 cells, and IL-12 and IL-18 concentrations were measured using immunoassay kits. Each value is the mean ± 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 ± 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 μg of anti-IL-12 Ab (Ad-tk-MCP1, IL-12Ab), 225 μg of anti-IL-12 Ab plus 25 μg of anti-IL-18 Ab (Ad-tk-MCP1, IL-12Ab+IL-18Ab), or 250 μg 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-MCP, goat IgG); **, $p < 0.01$ compared to Ad-tk-MCP1 with goat IgG plus rat IgG (Ad-tk-MCP, 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. 1*B*). 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. 1*D*). 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. 2*A*). 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 ± 24.9 mm^3 (n = 22) vs 471.2 ± 118.6 mm^3 (n = 20), $p < 0.01$) (Fig. 2*B*). 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 ± 124.3 mm^3, n = 18, and 575.3 ± 179.1 mm^3, n = 12, respectively) or when Ad-lacZ was administered along with MMC-treated HuH7 cells (tumor volume, 554.8 ± 125.6 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. 3*C*). 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 $Mac\text{-}1^+$ 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 difference 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. 4*C*). 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 ± 106.3 mm^3 (n = 22) vs 64.2 ± 43.6 mm^3 (n = 16), $p < 0.05$), and carrageenan partially inhibited the antitumor immunity of Ad-tk-MCP1 (tumor volume, 242.6 ± 100.8 mm^3 (n = 14) vs 53.8 ± 22.9 mm^3 (n = 22), $p = 0.22$) (Fig. 4*D*). The results indicate that antitumor effects were mainly mediated by NK cells.

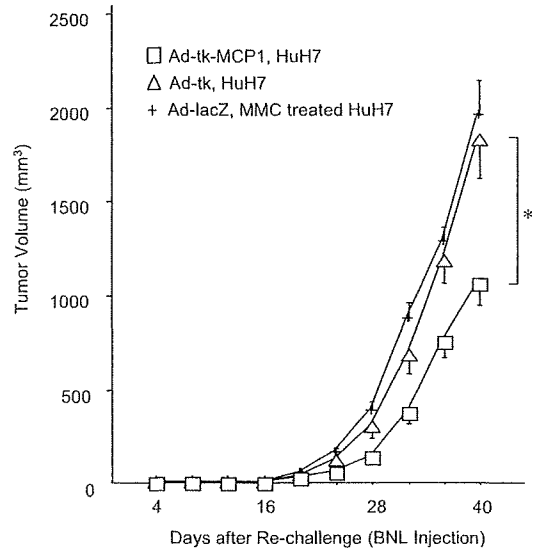

FIGURE 6. Antitumor effects of rAds expressing HSV-tk with or without MCP-1 against a second unprimed cell line (BNL) in an athymic nude mouse model of HCC. As described in the legend to Fig. 3, following complete eradication of the primary tumors the mice were s.c. injected with 1×10^3 BNL cells at 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.

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. 5*A*). Moreover, serum concentrations of IL-12 peaked after primary tumors were eradicated (day 9) and were sustained thereafter ($p < 0.05$) (Fig. 5*B*). Furthermore, the administration of anti-IL-12 significantly inhibited the antitumor effects conferred by Ad-tk-MCP1 (Fig. 5*C*) and reduced the serum concentrations of IL-12 to an undetectable level

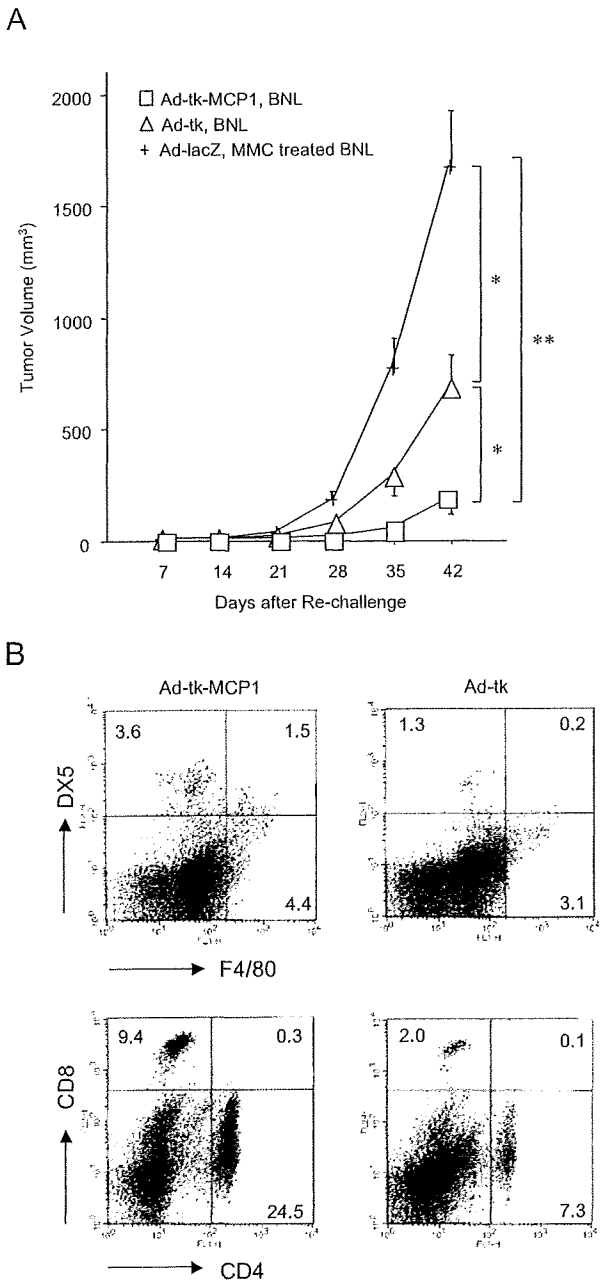

FIGURE 7. Prolonged antitumor effects of rAds expressing HSV-tk with or without MCP-1 in an immunocompetent mouse model of HCC. *A*, On day 0, mice were s.c. injected with 1×10^5 BNL cells infected with Ad-tk-MCP1, Ad-tk, or Ad-lacZ at an in vitro MOI of 100. The mice were i.p. injected with 75 mg/kg 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. 5*C*) 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 rechallenged 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 mm^3 ($n = 12$) vs 1825.4 ± 197.9 mm^3 ($n = 12$), $p < 0.01$) when Ad-lacZ was administered with MMC-treated HuH7 cells (tumor volume, 1960.8 ± 183.8 mm^3, $n = 12$) (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. 7*A*). 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 mm^3 ($n = 22$) vs 488.9 ± 120.1 mm^3 ($n = 22$), $p < 0.01$), 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 mm^3 ($n = 22$) vs 1666.4 ± 259.2 mm^3 ($n = 22$), $p < 0.01$). 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. 7*B*). 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 Vδ1γ δT 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 γ δT 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

The authors have no financial conflict of interest.

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Hepatitis B virus X protein overcomes oncogenic RAS-induced senescence in human immortalized cells

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Chronic infection with hepatitis B virus (HBV) is a major risk factor for hepatocellular carcinoma. The HBV X protein (HBx) is thought to have oncogenic potential, although the molecular mechanism remains obscure. Pathological roles of HBx in the carcinogenic process have been examined using rodent systems and no report is available on the oncogenic roles of HBx in human cells *in vitro*. We therefore examined the effect of HBx on immortalization and transformation in human primary cells. We found that HBx could overcome active RAS-induced senescence in human immortalized cells and that these cells could form colonies in soft agar and tumors in nude mice. HBx alone, however, could contribute to neither immortalization nor transformation of these cells. In a population doubling analysis, an N-terminal truncated mutant of HBx, HBx-D1 (amino acids 51–154), which harbors the coactivation domain, could overcome active RAS-induced cellular senescence, but these cells failed to exhibit colonigenic and tumorigenic abilities, probably due to the low expression level of the protein. By scanning a HBx expression library of the clustered-alanine substitution mutants, the N-terminal domain was found to be critical for overcoming active RAS-induced senescence by stabilizing full-length HBx. These results strongly suggest that HBx can contribute to carcinogenesis by overcoming active oncogene-induced senescence. (*Cancer Sci* 2007; 98: 1540–1548)

Chronic infection with HBV is a major risk factor for HCC worldwide. HBV belongs to the Hepadnavirus family. Its genome is a 3.2-kb, circular, partially double-stranded DNA molecule with four overlapping open reading frames: PC-C, PS-S, P and X.[1] The HBV genome, which is converted to covalently closed circular DNA in the nucleus after infection, serves as the template for transcription, generating the four viral transcripts that encode the HBV core and polymerase polypeptides, the large surface antigen polypeptide, the middle and major surface antigen polypeptides, and the HBx polypeptide. HBV replicates by reverse transcription of viral pregenomic 3.5-kb RNA using the HBV polymerase that catalyzes RNA-dependent DNA synthesis and DNA-dependent DNA synthesis.[1,2] It is converted into the 3.2-kb partially double-stranded genomic DNA inside the viral capsid.

The critical role of HBV chronic infection in HCC has been well established etiologically, whereas the mechanism by which HBV causes transformation of hepatocytes remains unclear.[3–5] HBx has long been suspected of playing a positive role in hepatocarcinogenesis, as avian hepadnaviruses missing the X open reading frame seem not to be associated with HCC. HBx consists of 154 aa and is a multifunctional regulator that modulates many host cell functions through its interactions with a variety of host factors.[5] HBx consists of both a negative regulatory domain[6] and a coactivation domain that is required for the augmentation of virus and host genes.[7,8] HBx was reported to transform rodent immortal cells *in vitro*,[9,10] and a high incidence of HCC has been reported in transgenic mice overexpressing HBx.[11,12] However, the functional role of HBx in the transformation is still controversial. Some independent groups proposed collaborating roles of HBx in the hepatocarcinogenic process.[13–15] Although these reports are informative, all were experimentally assessed in rodent systems. Because mouse and human primary cells have different telomere biology,[16] DNA damage check point control mechanisms and cell cycle progression,[17,18] developing a human system to address the functional role of HBx is critically important. Here we report that we established human fibroblast cells stably expressing HBx protein and analyzed the effects of HBx expression on the ability to confer an immortal phenotype and tumorigenic potential.

Materials and Methods

Retroviral vectors. All constructs for the expression of HBx (subtype adr) proteins, pNKF-HBx (aa 1–154), pNKF-HBx-D1 (aa 1–50) and pNKF-HBx-D5 (aa 51–154) have been described previously.[8] The retrovirus vectors pBabe-puro, hygro, puro-H-RAS^{V12} hygro-hTERT and pWZL-blast were kindly provided by W. C. Hahn (Dana-Farber Cancer Institute, Harvard).[19,20] To construct pBabe-blast, the blasticidin S cDNA of pWZL-blast was used as a template to amplify the PCR products of blasticidin S with the primer set of AAGCTTACCATGGCCAAGCCTTTGT and ATCGATTTAGCCCTCCCACACATAA, generating an artificial *Hind*III site at the 5-end and a *Cla*I site at the 3′-end, respectively. The HBx cDNA of pNKF-HBx was used as a template to amplify the PCR products of HBx with a primer set of TGATCAATGGACTACAAAGACGAT and CTCGAGAGAT-CTTTAATTAATTAA, generating an artificial *Fba*I site at the 5-end and an *Xho*I site at the 3′-end, respectively. The PCR products were digested and inserted into the *Bam*HI and *Sal*I sites of the pBabe-blast vector. The *Eco*RI and *Bgl*II fragments of HBx-D1 and HBx-D5 from pNKF-HBx-D1 and pNKF-HBx-D5 were, respectively, inserted into the *Eco*RI and *Bgl*II sites of the pBabe-blast-HBx vectors. An alanine scanning method was applied to construct a series of HBx clustered alanine substitution mutants (designated 'cm') by site-directed mutagenesis. The mutagenesis was carried out using a splicing PCR method with all of the mutated oligonucleotide primer sets. The target sequence of seven aa residues was changed to AAASAAA, and all of the HBx-encoding DNA fragments bearing the clustered mutations were introduced into the *Eco*RI and *Bam*HI sites of pNKFLAG, generating the pNKF-Xcm1 to pNKF-Xcm21 constructs. The

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Abbreviations: aa, amino acid; DMEM, Dulbecco's modified Eagle's medium; HBV, hepatitis B virus; HBx, hepatitis B virus X protein; HCC, hepatocellular carcinoma; hTERT, human telomerase reverse transcriptase; OIS, oncogene-induced senescence; PCR, polymerase chain reaction; PD, population doubling; SA-β-gal, senescence-associated β-galactosidase; SDS-PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis.

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*Eco*RI and *Bgl*II fragments of HBx-cm1 to HBx-cm21 from pNKF-Xcm1 to pNKF-Xcm21 were, respectively, inserted into the *Eco*RI and *Bgl*II sites of the pBabe-blast-HBx vectors. All of the constructs were sequenced by the dideoxy method using the *Taq* sequencing primer kit and a DNA sequencer (370A; Applied Biosystems).

Virus production and cell lines. Amphotropic retroviruses were produced by transfection of the 293T producer cell line with a retroviral vector and a vector encoding replication-defective helper viruses, pCL-Ampho (Imgenex), using FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer's recommendations. Two days after the transfection, culture supernatants were collected, filtered, supplemented with 4 μg/mL polybrene, and used for infection. Two days after the infection, drug selection of infected cells was started, and the selected populations were used in all of the experiments. Infected cell populations were selected in puromycin (1.0 μg/mL), blasticidin S (4 μg/mL) and hygromycin (80 μg/mL) for up to 2 weeks.

Cell culture. Human lung fibroblasts (TIG3) from the Japanese Collection of Research Bioresources were maintained in DMEM with 10% heat-inactivated fetal bovine serum (JRH Biosciences). Human foreskin fibroblasts, BJ and BJ-hTERT-LT-ST-H-RAS^{V12} cells were maintained as described previously.[19] These human fibroblasts were not clonal and were maintained as populations. BJ cells and TIG3 cells have a finite lifespan, and were used at PD between 25 and 35. PD were determined using the formula:

$$PD = Log(Nf/Ni)/Log2,$$

where Nf = the number of cells counted and Ni = the number of cells seeded. Comparisons of means and standard deviations were carried out using the unpaired *t*-test.

Western blot analysis. Cells were harvested, washed with phosphate-buffered saline (–), and sonicated in a lysis buffer (50 mM Tris-HCl [pH 7.4], 200 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 10 μg/mL aprotinin and 10 μg/mL dithiothreitol). Total lysates were fractionated by SDS-PAGE, transferred onto nitrocellulose membranes and subjected to western blot analysis with antibodies. Anti-FLAG M2 antibody and anti-β-actin antibody were from Sigma. Anti-RAS antibody F-235 (sc-29), anti-p53 antibody DO-1 (sc-126) and anti-p21 antibody F-5 (sc-6246) were from Santa Cruz. Anti-p16 antibody was from BD PharMingen. The proteins were visualized by enhanced chemiluminescence according to the manufacturer's instructions (Amersham).

Analysis of senescence. SA-β-Gal staining was carried out using the Senescence Detection Kit (Oncogene) as instructed by the manufacturer. For each sample, at least 200 cells were counted in randomly chosen fields.

Telomerase activity assays. Total lysates of cells were subjected to the telomerase repeat amplification protocol using a TRAPEZE kit (Intergen) according to the manufacturer's instructions.

Soft-agar colony formation assays. Soft-agar growth assays were carried out as described previously.[19] At the time of plating in soft agar, cultures were trypsinized and counted, and 5×10^3 or 5×10^4 total cells were mixed with 1.5 mL of 0.35% Noble agar-DMEM (top layer) and then poured on top of 5 mL of solidified 0.7% Noble agar-DMEM (bottom layer) in 6-cm-diameter dishes. After 3 weeks, colonies were counted, and pictures were taken.

Tumorigenicity assays. A total of 1×10^6 cells were resuspended in 50 μL Matrigel solution (BD Matrigel Basement Membrane Matrix HC; BD Biosciences) and immediately injected subcutaneously into 8-week-old female nude mice (BALB/cAnNCrl-nu BR). 2-D tumor sizes were measured once a week. The tumor volume (mm^3) was calculated using the formula (length × width^2)/2.[21]

Results

Effect of HBx on cellular senescence of human primary cells. During immortalization, human cells differ from rodent cells in the regulation of telomere length[22,23] and cell cycle checkpoints.[24,25] Human cells must bypass two barriers to become immortalized: replicative senescence and crisis. Replicative senescence is characterized by an irreversible growth arrest but continued metabolic activity.[26] Crisis is characterized by widespread cell death.[26,27] By the introduction of hTERT, human primary cells avoid these two barriers and can become immortalized.[28–30]

It is possible that HBx contributes to the immortalization process of human primary cells, but not to the cellular transformation process. If so, it may facilitate cellular transformation indirectly by overcoming two crises, M1 and M2. To study whether this does facilitate cellular transformation, it is best to use human primary hepatocytes as HBV is a hepatotropic virus. However, human primary hepatocytes are almost impossible to obtain for such an experimental approach. HBx exhibits its transactivation function not only in hepatoma cell lines but also in various carcinoma and sarcoma cell lines. Under these situations, we addressed whether HBx contributes to the immortalization of human primary fibroblasts, BJ cells and TIG3 cells that have been well studied for cellular senescence and immortalization. We used hTERT-introduced BJ and TIG3 cells for positive controls of immortal cells.

The human primary fibroblasts, BJ cells and TIG3 cells were infected with the HBx-expression retroviruses and cultured in the presence of the selection drug, blastcidin S. The drug-resistant polyclonal cells were selected and characterized. Three different constructs of HBx were used to map the responsible domain: full-length HBx (HBx-wt), HBx-D1, which lacks the N-terminal negative regulatory domain, and HBx-D5, which lacks the coactivation domain (Fig. 1a). First we examined HBx expression in the primary human fibroblasts. We found that full-length HBx and HBx-D5 were highly but equally expressed, whereas expression of HBx-D1 was very weak in the blastcidin S-selected clones (Fig. 1b). We hypothesized that HBx expression may confer an immortal phenotype, which could contribute to cellular transformation and tumorigenesis, but we observed that the BJ cells expressing HBx proteins stopped dividing at PD 69.6 ± 0.9 (errors ± SD) (HBx-wt), PD 66.6 ± 1.6 (HBx-D5), PD 66.1 ± 1.4 (HBx-D1) and PD 60.5 ± 0.6 (control cells) (Fig. 1c). TIG3 cells, another human fibroblast, expressing HBx proteins stopped dividing at PD 77.2 ± 1.1 (HBx-wt), PD 75.1 ± 0.8 (HBx-D5), PD 75.1 ± 0.1 (HBx-D1) and PD 75.4 ± 0.2 (control cells) (Fig. 1d). Although a very minor extended lifespan (2–4 PD) was observed with HBx-wt-expressing primary human fibroblasts, the HBx protein could not elicit immortalization. We examined whether the effect of HBx on delay of cellular senescence was correlated with putative augmentation of telomerase activity in HBx-introduced BJ and TIG3 cells (Fig. 1e) as activation of the hTERT promoter was observed in hepatoma cell lines that were transiently cotransfected with the HBx expression vector and luciferase reporter vector of the hTERT promoter (S. Murakami *et al.* unpublished data, 2005). Telomerase activity in the extracts of cells expressing HBx-wt or HBx-D1 was slightly higher than that of cells expressing empty vector or HBx-D5 in both kinds of cells (Fig. 1e), but we failed to detect an increase in hTERT protein expression (data not shown). Therefore, the relevance of the weak augmentation of telomerase activity in the HBx-expressing primary cells remains unclear.

Effect of HBx on immortalized BJ-hTERT cells. Next, we addressed whether HBx facilitates the cellular transformation process

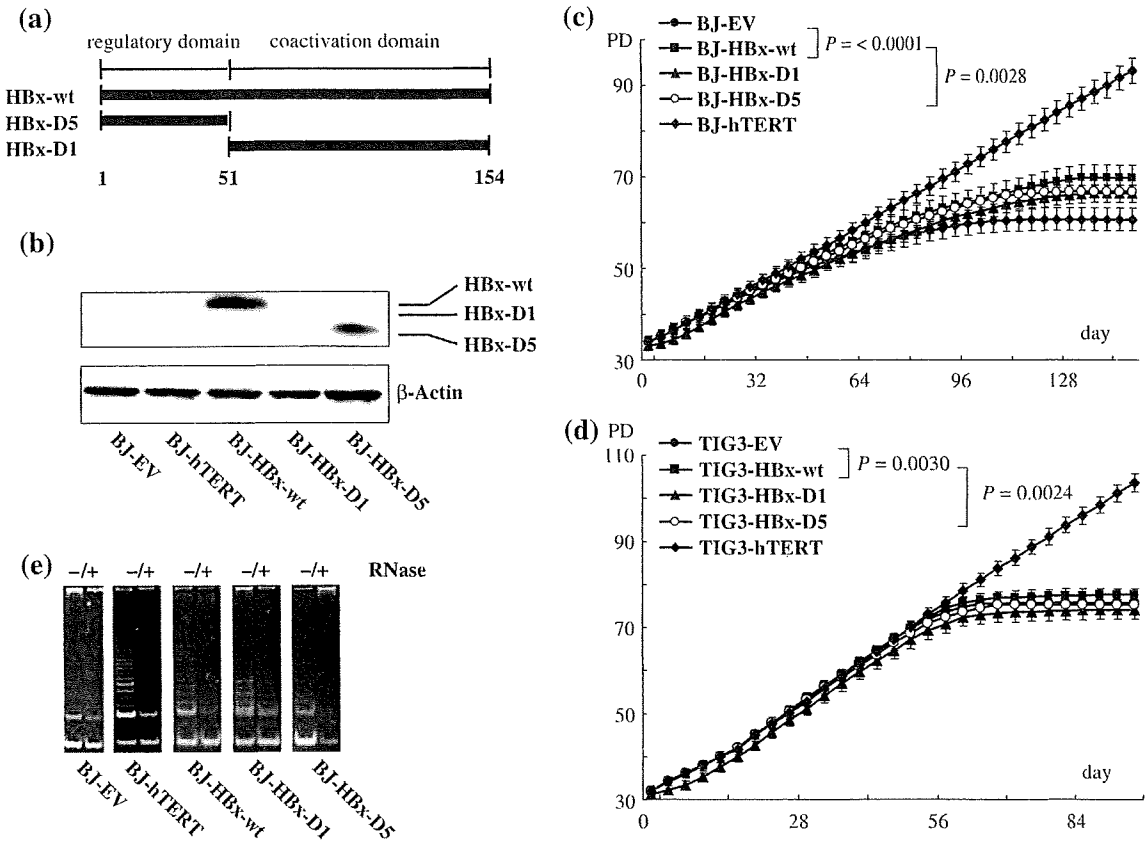

Fig. 1. Hepatitis B virus protein X (HBx) can not immortalize human primary cells, but weakly affects cellular senescence and telomerase activity. (a) Schematic representation of the HBx proteins.[5,8] The amino acids (aa) of full-length HBx (154 aa residues) and truncated HBx are shown. HBxD1 harbors the carboxy-terminal coactivation domain, spanning aa residues 51–154, whereas, HBxD5 harbors the amino-terminal negative regulatory domain, spanning aa residues 1–50. (b) Expression of HBx, HBx-D1 and HBx-D5 proteins in infected BJ cells. Total cell lysates of BJ cells infected with the empty vector (EV), human telomerase reverse transcriptase (hTERT), HBx, HBx-D1 and HBx-D5 expression retroviruses were fractionated by sodium dodecylsulfate–polyacrylamide gel electrophoresis and subjected to western blot analysis with anti-FLAG M2 antibody. (c) Effect of HBx on replicative senescence in BJ cells. BJ cells were infected with a control vector (filled circles) or hTERT (filled diamonds) and with a retrovirus encoding wild-type HBx (filled squares), HBx-D1 (filled triangles) or HBx-D5 (open circles). Cells infected with pBabe-puro- and pBabe-blast were selected with 1 μg/mL puromycin and 4 μg/mL blasticidin S, respectively. After 8 days of drug selection, triplicate samples of 1×10^5 cells were plated and grown under normal conditions (day 0). (d) Effect of HBx mutants on replicative senescence in TIG3 cells. Symbols are the same as in (c). (e) Telomerase activity in BJ cells as demonstrated by telomerase activity assay (TRAP). Total cell lysates (200 ng) prepared from BJ cells infected with control vector, hTERT, HBx, HBx-D1, and HBx-D5 were subjected to TRAP assay using a TRAPEZE kit (Intergen).

using human immortal cells. For this purpose, we used BJ-hTERT cells – these were BJ-derived cells immortalized by the introduction of hTERT, as characterized previously.[19] HBx-wt as well as its truncated mutants had no effect on cell proliferation, telomerase activity or cell transformation. Using the newly established TIG3-hTERT cells, we confirmed that the stable expression of HBx, XD1 or XD5 did not affect cell proliferation or cell transformation (data not shown). These results indicate the inability of HBx alone to transform these human immortalized cells.

Ability of HBx to overcome H-RAS^{V12}-induced senescence in BJ cells immortalized by hTERT Seeing as HBx did not exhibit the ability to immortalize primary human fibroblasts or to elicit transformation into hTERT-induced immortal primary human fibroblasts, we considered whether HBx functioned together with an oncogene and induced cell transformation. Senescence induced by active oncogene expression (OIS), such as oncogenic RAS, is one of the anticancer processes in which tumor suppressors and their related networks are involved, as demonstrated *in vitro* and recently also *in vivo*.[31,32] Overcoming OIS is critical for cellular transformation *in vitro* and cancerous cell proliferation *in vivo*.[31] Therefore, we addressed whether HBx has a collaborating role in transforming cells in the presence of oncogenic RAS or in overcoming RAS-induced senescence.

To examine the effect of HBx on RAS-induced senescence-like growth arrest, we introduced H-RAS^{V12} into BJ-hTERT, BJ-hTERT-HBx-wt, BJ-hTERT-HBx-D1 and BJ-hTERT-HBx-D5 cells using a retrovirus (Fig. 2d). BJ-hTERT cells expressing H-RAS^{V12} stopped proliferating within several days of RAS introduction. In contrast, BJ-hTERT cells expressing both H-RAS^{V12} and HBx-wt (BJ-hTERT + H-RAS^{V12} + HBx-wt) continued to proliferate to more than 80 PD (Fig. 2a). Although HBx-D1 also demonstrated the ability to overcome active RAS-induced senescence, HBx-D5 failed to overcome OIS (Fig. 2a). We also found that the growth rate of BJ-hTERT + H-RAS^{V12} + HBx-wt cells was much higher than that of BJ-hTERT + H-RAS^{V12} + HBx-D1 cells, probably reflecting the fact that some portion of the latter cells were positive for SA-β-gal (Fig. 2b,c). Consistent with this result, cells staining positive for SA-β-gal were significantly fewer in BJ-hTERT +

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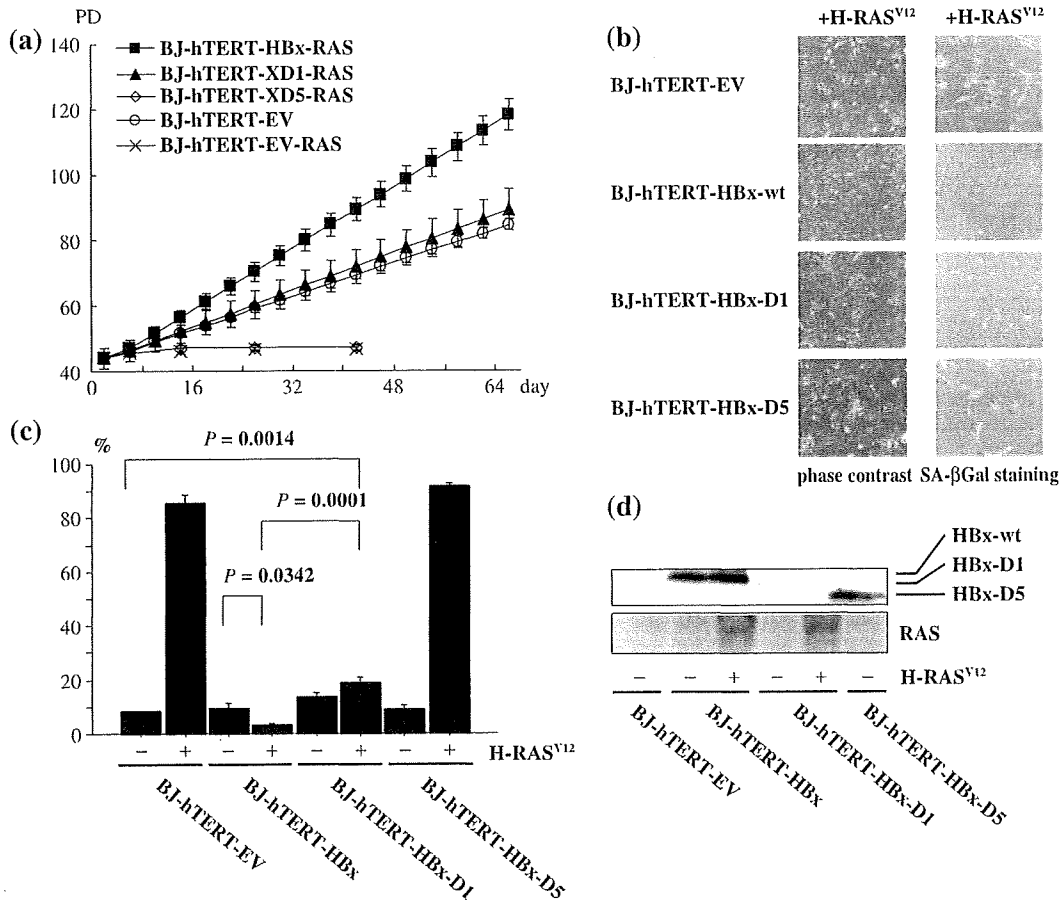

Fig. 2. Hepatitis B virus protein X (HBx) can overcome H-RAS^{V12}-induced cellular senescence of human immortalized cells. (a) Effect of HBx on H-RAS^{V12} induced senescence. BJ-human telomerase reverse transcriptase (hTERT) cells (open circles) and H-RAS^{V12}-induced BJ-hTERT-HBx-wt (filled squares), BJ-hTERT-HBx-D1 (filled triangles), BJ-hTERT-HBx-D5 (filled diamonds) cells and BJ-hTERT-empty vector (EV) (cross) are shown. After 10 days of drug selection at population doubling (PD) 42, triplicate samples of 1×10^5 cells were plated and grown under normal conditions (day 0). (b) HBx overcomes H-RAS^{V12}-induced senescence of human immortalized cells. H-RAS^{V12} and EV, full-length or truncated forms of HBx were introduced into BJ-hTERT cells. Left panel shows photographs 10 days after infection of the H-RAS^{V12}-expression retrovirus. Right panels show senescence-associated β-galactosidase (SA-β-Gal) staining 10 days after infection. (c) The percentage of cells positive for SA-β-Gal was determined in BJ cells stably expressing HBx-wt, HBx-D1, HBx-D5 or empty vector, with or without H-RAS^{V12} on day 9 after infection. Bars = mean ± SD. (d) Western blot analysis of RAS-induced cells. Total cell lysates from BJ-hTERT cells stably expressing HBx-wt, HBx-D1, HBx-D5 or EV together with or without H-RAS^{V12} were prepared and fractionated by sodium dodecylsulfate–polyacrylamide gel electrophoresis, then subjected to western blot analysis. HBx-wt, HBx-D1 and HBx-D5 were detected with anti-FLAG M2 antibody. RAS protein was detected with anti-RAS antibody.

H-RAS^{V12} + HBx-wt than in BJ-hTERT + H-RAS^{V12} + HBx-D1 (Fig. 2c). These results indicate that HBx-wt has the ability to overcome RAS-induced senescence. HBx-D1, the coactivator domain of HBx, seems to be indispensable and sufficient for overcoming RAS-induced senescence analyzed by the PD analysis, although HBx-D1 did not show the same ability as HBx-wt. The incomplete ability of HBx-D1 may be due to the low expression of HBx-D1 in the blasticidin S-selected clones in BJ-hTERT cells, as observed with the BJ cells (see Discussion).

HBx protein is required for anchorage-independent growth and tumor formation in nude mouse in response to H-RAS^{V12}. HBx can overcome RAS-induced senescence (examined by the PD analysis) and can indicate that HBx and RAS can induce cell transformation. Therefore, we examined whether BJ-hTERT + H-RAS^{V12} + HBx-wt and BJ-hTERT + H-RAS^{V12} + HBx-D1 cells can form colonies in soft agar. We found that BJ-hTERT + H-RAS^{V12} + HBx-wt cells showed cell number-dependent formation of colonies, which were much smaller size than those of control cells, BJ-hTERT + H-RAS^{V12} + SV40 LT + ST[20,33] (Fig. 3a,b). In contrast, BJ-hTERT + H-RAS^{V12} + HBx-D1 cells could not form colonies in soft agar (Fig. 3a), although these cells overcame RAS-induced senescence. This result strongly suggests that HBx-D1 is not equivalent to HBx-wt in its ability to make colonies in soft agar.

Next we tested the tumor-forming ability of BJ-hTERT + H-RAS^{V12} + HBx-wt or HBx-D1 cells in nude mice. BJ-hTERT + H-RAS^{V12} + HBx-wt cells were found to form tumors in four of eight mice, although these tumors grew much more slowly and were much smaller than those formed by BJ-hTERT + H-RAS^{V12} + SV40 LT + ST cells (eight of eight animals) (Fig. 3c). In contrast, BJ-hTERT + H-RAS^{V12} + HBx-D1 cells did not generate tumors in nude mice (Fig. 3c), consistent with the results of the soft-agar assay. These results indicate that HBx contributes to cellular transformation by collaborating with active RAS in human immortalized cells. To our knowledge, this is the first report showing that HBx plays a critical role in

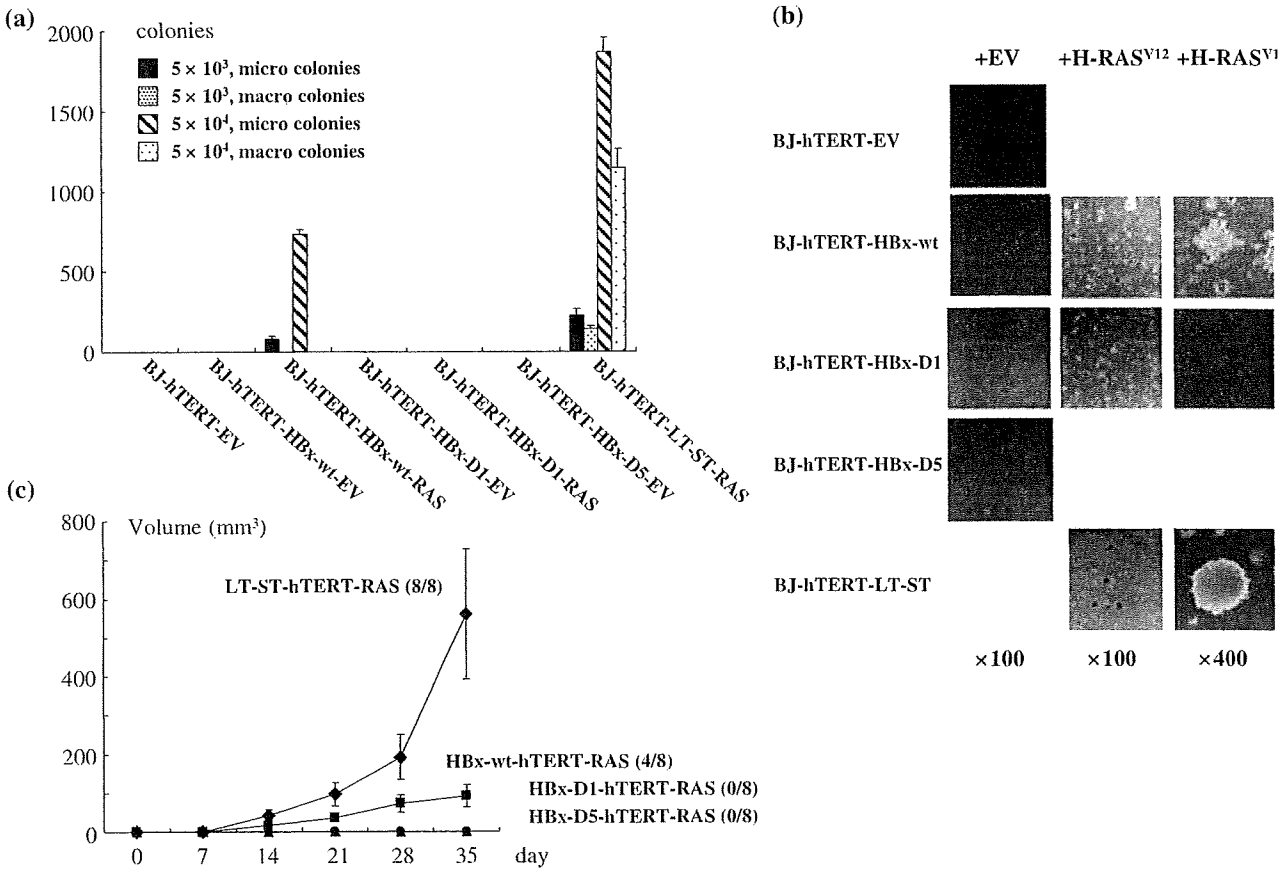

Fig. 3 (a,b) Anchorage-independent growth in soft agar and (c) tumorigenicity and tumor-forming ability in nude mice of cells expressing hepatitis B virus X protein (HBx) and H-RAS^{V12}. (a) Soft-agar assays were carried out as described in Materials and Methods.[19] After 3 weeks, colonies were counted and pictures were taken. The colony-forming ability of BJ-human telomerase reverse transcriptase (hTERT) cells stably expressing wild-type or truncated HBx with or without H-RAS^{V12} is indicated at the bottom. H-RAS^{V12}-introduced BJ-hTERT-LT-ST cells were the positive control. (b) Morphology of colonies in the soft-agar assay. Colonies were photographed 21 days after seeding. (c) Tumor formation in nude mice was carried out as described previously in Materials and Methods.[19,21] Tumor sizes were measured once a week. Each point on the graph represents the average volume of tumors. BJ-hTERT-LT-ST-RAS (filled diamonds), BJ-hTERT-HBx-RAS (filled squares), BJ-hTERT-HBx-D1 (filled circles), and BJ-hTERT (filled triangles) cells are shown. Error bars indicate the mean ± SD for each time point.

cellular transformation, collaborating with active RAS in human immortalized cells.

Effects of HBx on p16 and p21 expression and the ability of HBx to overcome RAS-induced senescence. Overexpression of RAS causes oncogene-induced premature senescence in normal human fibroblasts (Fig. 4c) and hTERT-immortalized human fibroblasts (Fig. 2a), but RAS failed to induce premature senescence in HBx-wt- or HBx-D1-introduced BJ-hTERT cells (Fig. 2a). We next examined the effect of stable expression of HBx in BJ cells with or without expression of hTERT, as interference with both the p53 and pRb pathways is necessary to avoid RAS-induced cellular senescence, in which p16 and p21 are the critical downstream effectors of pRb and p53, respectively. Expression of p16 and p21 was upregulated in HBx-wt- or HBx-D1-introduced BJ-hTERT cells; however, HBx-D5 has no ability to induce the expression of these genes. The presence of H-RAS^{V12} resulted in downregulation of the augmented expression of p16 and p21 in HBx-wt- or HBx-D1-introduced BJ cells and BJ-hTERT cells (Fig. 4a,b). These results suggest that HBx can suppress expression of p53, p16 and p21 in H-RAS^{V12}-introduced cells, contributing to overcoming RAS-induced senescence. Next we examined whether HBx-wt and H-RAS^{V12} not immortalized by hTERT were sufficient for cellular transformation. We introduced H-RAS^{V12} into BJ-HBx-wt, BJ-HBx-D1 and BJ-HBx-D5 cells and analyzed them by PD analysis and soft-agar colony assay. In the PD analysis, H-RAS^{V12}-introduced BJ-HBx-wt and BJ-HBx-D1 cells did overcome RAS-induced cellular senescence but stopped cell division at PD 62, which is approximately the cellular senescence of BJ cells (Figs 1c,4c), whereas H-RAS^{V12}-introduced BJ-HBx-D5 did not overcome senescence and stopped cell division. These results suggest that HBx can overcome RAS-induced senescence but can not immortalize the cells (Fig. 4c). In the soft-agar colony formation assay, BJ-HBx-wt-H-RAS^{V12} and BJ-HBx-D1-H-RAS^{V12} could but BJ-HBx-D5-H-RAS^{V12} could not form very tiny colonies, suggesting that HBx-wt and H-RAS^{V12} in the absence of hTERT may enable the cells to proliferate in an anchorage-independent manner (data not shown).

As HBx-D1, which was very weakly expressed, exhibited almost the same ability as HBx-wt to upregulate the tumor suppressor genes and to overcome RAS-induced senescence in these cells, we wondered whether HBx-D1 missing the N-terminal domain may have some negative effect on cell proliferation. Because the transient expression level of HBx-D1 in BJ cells was similar to those in HepG2 cells, as reported previously

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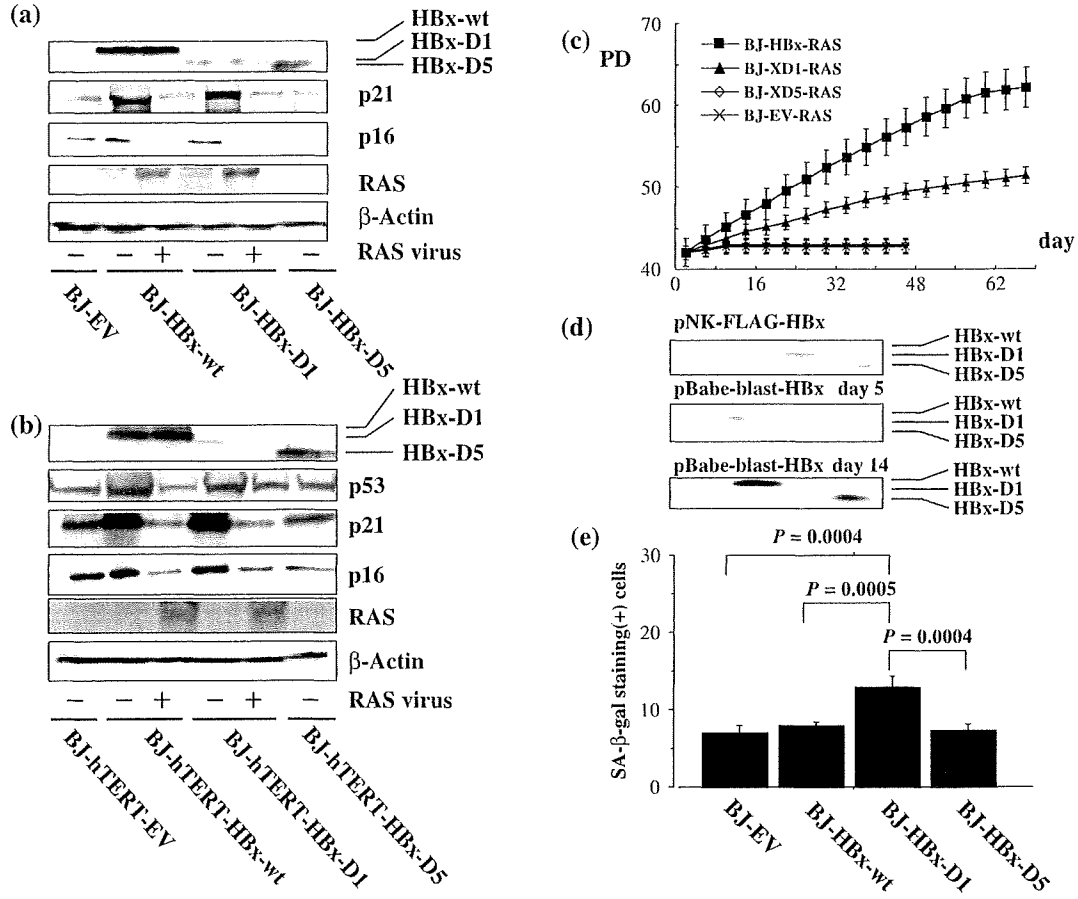

Fig. 4. Effect of hepatitis B virus X protein (HBx) on p16 and p21 expression and the ability of HBx to overcome H-RAS^{V12}-induced cellular senescence of human normal cells. Total cell lysates from BJ-human telomerase reverse transcriptase (hTERT) cells stably expressing HBx-wt, HBx-D1, HBx-D5 or empty vector together with or without H-RAS^{V12} were prepared, and fractionated by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE), then subjected to western blot analysis. Expression of (a) p16 and p21 proteins and (b) p53, p16 and p21 proteins. (c) Effect of HBx on H-RAS^{V12}-induced senescence. Population doublings (PD) of H-RAS^{V12}-induced BJ-HBx-wt (filled squares), BJ-HBx-D1 (filled triangles), BJ-HBx-D5 (open diamonds) and BJ-EV (cross) cells are shown. After 10 days of drug selection, at PD 44, triplicate samples of 1×10^5 cells were plated and grown under normal conditions (day 0). (d) Expression of HBx, HBx-D1 and HBx-D5 proteins in infected BJ cells. Total cell lysates of BJ cells transfected with mammalian expression plasmids of FLAG-HBx-wt, FLAG-HBx-D1 and FLAG-HBx-D5 were fractionated by SDS-PAGE and subjected to western blot analysis with anti-FLAG M2 antibody (upper panel). Total cell lysates of BJ cells infected with the empty vector (EV), HBx-wt, HBx-D1 and HBx-D5 expression retroviruses were fractionated by SDS-PAGE and subjected to western blot analysis with anti-FLAG M2 antibody (middle and bottom panel). (e) The percentage of cells positive for senescence-associated β-galactosidase (SA-β-Gal) was determined in BJ cells stably expressing HBx-wt, HBx-D1, HBx-D5 or empty vector (EV) on day 40 after infection. Bars = mean ± SD.

(Fig. 4d),[8] it was not due to the construct design of the vector. The expression of HBx-D1 was slightly lower than those of HBx-wt and HBx-D5 on day 5 after selection, much lower on day 10 after selection (data not shown). On day 14 after selection, the expression of HBx-D1 reached the lowest level, and after day 14 that expression level was kept (Figs 1b,4d). HBx-D1-introduced BJ cells grew slower than HBx-wt- or HBx-D5-introduced BJ cells (data not shown) and contained more SA-β-Gal-positive cells during proliferation (Fig. 4e). These results suggest that cells expressing lower levels of HBx-D1 proliferated more than cells expressing higher levels of HBx-D1, due to some toxic or antiproliferative effect of the coactivation domain of HBx in the human primary cells (see Discussion).

Important region of HBx for overcoming cellular senescence and anchorage-independent growth. As HBx exhibited the ability to overcome active RAS-induced senescence, we next tried to identify the critical regions of HBx for overcoming cellular senescence. BJ-hTERT cells were infected with retroviruses expressing one of the clustered alanine-substituted mutants covering all parts of HBx,[34] and a series of cell clones stably expressing these HBx-cm mutants, BJ-hTERT-HBx-cm, was established (Fig. 5). H-RAS^{V12} was then introduced into BJ-hTERT-HBx-cm1 to BJ-hTERT-HBx-cm21 cells and cell proliferation was examined. The regions covering HBx-cm8 to HBx-cm10, and those covering HBx-cm19 to HBx-cm21 were found to be not critical for overcoming active RAS-induced senescence and anchorage-independent growth as the BJ-hTERT-RAS clones expressing these HBx-cm mutants proliferated and formed colonies in soft agar, similar to BJ-hTERT-HBx-wt-H-RAS^{V12} cells. The BJ-hTERT-RAS clones expressing HBx-cm1 to HBx-cm7, and those expressing HBx-cm14 to HBx-cm16, were like BJ-hTERT-HBx-D1-RAS, which can grow but at a much reduced rate compared with BJ-hTERT-HBx-RAS cells. The HBx regions covering HBx-cm11 to HBx-cm13, HBx-cm17