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Ⅳ. 研究成果の刊行物・別刷

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Enhanced antitumor effects of suicide gene therapy combined with adenovirally delivered monocyte chemoattractant protein-1

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Summary

Suicide gene therapy using the herpes simplex virus thymidine kinase / ganciclovir (HSV-tk/GCV) system is a well-characterized tool for cancer gene therapy. The HSV-tk/GCV system demonstrates tumor cell killing activity and the bystander effects by which nearby unmodified tumor cells are also killed. However, it does not yet exhibit sufficient efficacy to cure patients of malignancies. Gene therapy aimed at

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enhancing antitumor immune responses may be a promising approach to eradicate tumor cells and prevent tumor recurrence. Recent studies indicate that co-expression of HSV-tk and chemokines including monocyte chemoattractant protein (MCP)-1 increases tumor immunity in mouse models. Furthermore, a bicistronic recombinant adenovirus vector harboring both suicide and chemokine genes in sequence exerts enhanced antitumor effects. The mechanisms underlying these effects were examined by evaluating the activation status of macrophages and T helper 1 (Th1) cytokine gene expression. In addition, codelivery of HSV-tk and MCP-1 genes using the bicistronic adenovirus vector has been reported to display prolonged NK cell-mediated antitumor effects. These findings suggest an immunomodulatory effect of MCP-1 in the context of suicide gene therapy of solid tumors via orchestration of rapid development and prolonged maintenance of innate immune responses.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies with a poor prognosis throughout the world population since it frequently recurs shortly after surgical or non-surgical treatments, including transcatheter arterial chemoembolization, percutaneous ethanol injection therapy, radiofrequency ablation, and chemotherapy [1-3]. This is because of insufficient therapeutic effects, multicentric development of HCC in cirrhotic liver, and distant metastasis. Although these treatments can induce apoptosis of HCC cells, they 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 to prevent tumor recurrence.

Gene therapy strategies for cancer are divided into three major categories: enzyme/prodrug systems such as suicide gene therapy [4-6], immune-gene therapy [7, 8], and tumor suppressor gene replacement therapy [9-11]. Tumor cell-targeted gene therapy with a suicide gene under the transcriptional control of a tumor-specific promoter, such as the herpes simplex virus thymidine kinase (HSV-tk) gene driven by an HCC-specific α-fetoprotein (AFP) promoter, has been reported to have limited effects in some experimental models [4-6]. This is due to incomplete gene transfer and cell killing, even when a highly tumoricidal suicide gene is delivered with a highly transducible recombinant adenovirus (rAd) vector [12].

The suicide gene HSV-tk exhibits tumor cell killing activity in the presence of the prodrug ganciclovir (GCV) (HSV-tk/GCV system) [13] not only in the infected cells, but also in neighboring uninfected cells via bystander effect [14] *in vitro* and *in vivo*. The bystander killing of neighboring uninfected

tumor cells is thought to be not only due to the efflux of toxic phosphorylated GCV metabolites from HSV-tk-expressing cells to uninfected tumor cells through gap junctions [15], but also due to immune-mediated anti-tumor effects via macrophages, T lymphocytes or natural killer (NK) cells involving HSV-tk expressing tumor cells [16, 17]. If so, the enhancement of host immune responses by a cytokine gene combined with the HSV-tk/GCV system may exert synergistic effects. Although several types of immunotherapy using cytokines have been used to enhance the bystander effect of HSV-tk xenogeneic cells [16, 18, 19], satisfactory results have not yet been obtained for many types of tumors, including HCC.

Monocyte chemoattractant protein (MCP)-1 is a chemokine [20] that regulates the recruitment and activation of monocytes/macrophages to inflammatory sites and tumor tissues. Activation includes lysosomal enzyme release and tumoricidal activity in both mice and humans [21]. Since MCP-1 has been shown to regulate the chemotaxis and tumoricidal effects of blood monocytes, it may be an important mediator of tumor regression. Previous studies indicated that the transfectant-derived MCP-1 could recruit monocytes to tumor tissues and eventually cause tumor regression [22-24]. However, transiently expressed MCP-1 could not achieve anti-tumor effects in glioma cells [25, 26]. Some tumors express MCP-1 endogenously [27, 28], and recently it was suggested that endogenous expression of MCP-1 in breast cancer cells leads to angiogenesis and tumor progression [29].

Adenovirally delivered MCP-1 potentiates the antitumor effects of the HSV-tk/GCV suicide gene system

In the initial project, we investigated whether adenovirally delivered MCP-1 potentiates the anti-tumor effects of the HSV-tk/GCV system in HCC cells *in vivo*. Recombinant replication-defective adenovirus vector Ad-MCP-1 (Fig.1) harboring the human MCP-1 gene [20] driven by the CAG promoter [30] was prepared. To estimate whether induction of apoptosis by HSV-tk/GCV concomitant with MCP-1 treatment enhance anti-tumor effect *in vivo*, subcutaneous tumor foci of the HCC cell line HuH7 established in athymic nude mice were transduced with the recombinant adenovirus (rAd) harboring HSV-tk gene, Ad-tk, followed by GCV administration. Two days after the start of GCV treatment, Ad-MCP-1 was injected into tumor foci and tumor development was monitored. The growth of HuH7 tumor was markedly suppressed when treated with both Ad-tk/GCV and Ad-MCP-1.

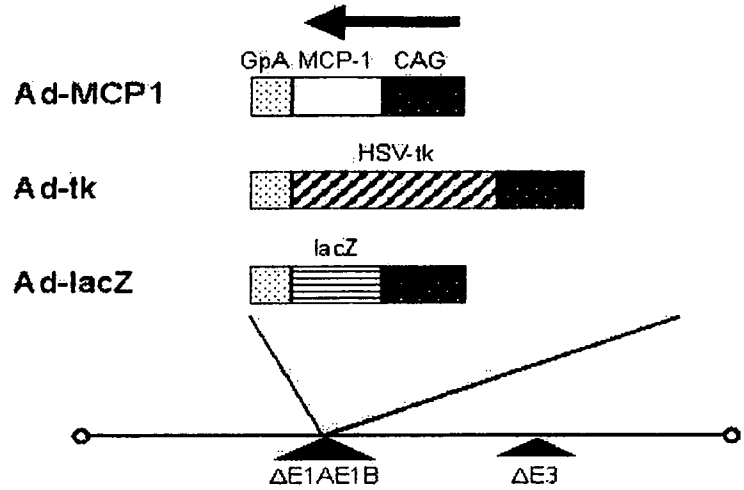

Figure 1. Schematic representation of recombinant adenoviruses (rAds). rAd expressing each gene under the control of the CAG promoter. Ad-MCP-1, Ad-tk and Ad-lacZ expressing monocyte chemoattractant protein (MCP)-1, herpes simplex virus thymidine kinase gene (HSV-tk) and beta-galactosidase gene (LacZ). Solid lines indicate the rAd genome. A closed triangle below the rAd genome represents a deletion of adenovirus early regions. The arrow shows the orientation of transcriptions. GpA, rabbit beta-globin poly (A) site. CAG, CAG promoter.

Histopathological analysis showed that incidences of apoptotic and necrotic tumor cells and mononuclear cell infiltration were induced to a greater extent in the tumor foci treated with both Ad-tk/GCV and Ad-MCP-1. In immunohistochemical analysis, most of the infiltrating mononuclear cells observed in the tumor foci were immunohistochemically stained with antibodies against a macrophage marker Mac-1. On the other hand, these stained cells were not detected when tumors were treated with control Ad-lacZ instead of Ad-MCP-1. Furthermore, the synergistic anti-tumor effects induced by the HSV-tk/GCV system and transduced MCP-1 disappeared in animals when carrageenan, a compound known to inactivate macrophage *in vivo*, was administered. Collectively, these results indicate that adenovirally delivered MCP-1 enhanced the anti-tumor effects of the HSV-tk/GCV system synergistically by recruitment/activation of macrophages in tumor tissues.

To understand the cellular basis of the possible synergism of the HSV-tk/GCV system and MCP-1 in tumoricidal effects *in vivo*, the killing activity and TNF-α production of mouse peritoneal macrophages exposed to apoptotic tumor cells were evaluated *in vitro*. When macrophages were exposed to apoptotic tumor cells, their production of TNF-α, a tumoricidal cytokine [31], was markedly increased. Based on this observation, it is likely that increased TNF-α production of macrophages exposed to apoptotic HuH7 cells might account for the synergistic anti-tumor effects induced by codelivery of HSV-tk/GCV system and MCP-1 *in vivo*.

However, the co-expression of two different genes, i.e., the HSV-tk and MCP-1 genes, in the tumor tissues is achieved by using two different promoters of the distinct vectors. Thus, their expression may be uncoupled by interfering with the activity of their promoters and the vectors at the single cell level, or by the insufficient simultaneous transduction of the two vectors in the cell [32-34]. This notion is supported by our data showing an insufficient HCC tumor killing response when gene therapy was carried out using two recombinant adenovirus vector (rAd)s [32].

A bicistronic recombinant adenovirus vector harboring both suicide and MCP-1 genes exerts enhanced, macrophage-dependent, antitumor effects

In the next experiments, we set out to determine if rAds expressing both the HSV-tk and MCP-1 genes under the transcriptional control of a single promoter within a bicistronic unit can potentiate their anti-tumor effectiveness. The bicistronic Ad-tk-MCP1, harboring the HSV-tk gene and the human MCP-1 gene in sequence and driven by the CAG promoter was prepared [32, 35, 36] (Fig. 2). Briefly, using the internal ribosomal entry site (IRES) fragment of 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 EcoT22I digested adenovirus 5-dIX DNA-terminal protein complex. The rAds were purified on cesium gradients and their titers were determined by the 50% tissue culture infectious dose ($TCID_{50}$) method [37].

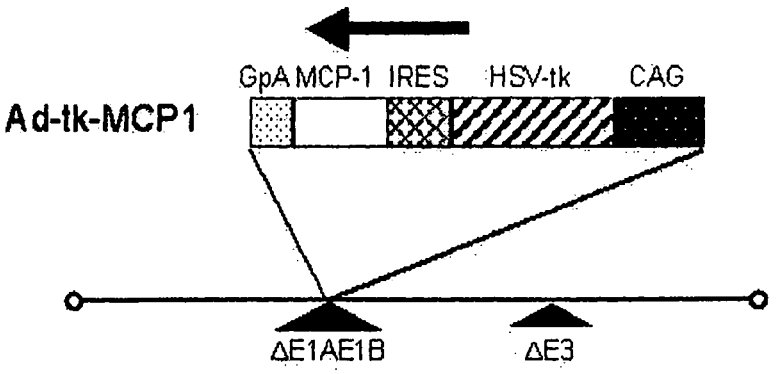

Figure 2. Construction of recombinant adenovirus Ad-tk-MCP1. Bicistronic Ad-tk-MCP1 harboring the HSV-tk gene and the human MCP-1 gene in sequence under the universal CAG promoter within a bicistronic unit including the internal ribosomal entry site (IRES) fragment of encephalomyocarditis virus that allows two cistrons to be translated from a single transcript.

Production of MCP-1 in HuH7 cells infected with Ad-tk-MCP1, Ad-MCP1 and Ad-MCP1 plus Ad-tk was measured by enzyme-linked immunosorbent assay (ELISA) after infection (Fig. 3). MCP-1 production reached a peak level 48 hours after infection with rAds. The amounts of MCP-1 produced by Ad-tk-MCP1 were increased as the multiplicities of infection (MOIs) were increased and reached a peak level at an MOI of 100, although the levels were 1/50 of those seen with Ad-MCP1. In contrast, the cells co-infected with Ad-tk and Ad-MCP1 produced a large amount of MCP-1 comparable to that obtained by a single infection with Ad-MCP1. These data indicate that MCP-1 produced by bicistronic rAd under the control of CAG promoter was less than that produced by Ad-MCP1 alone.

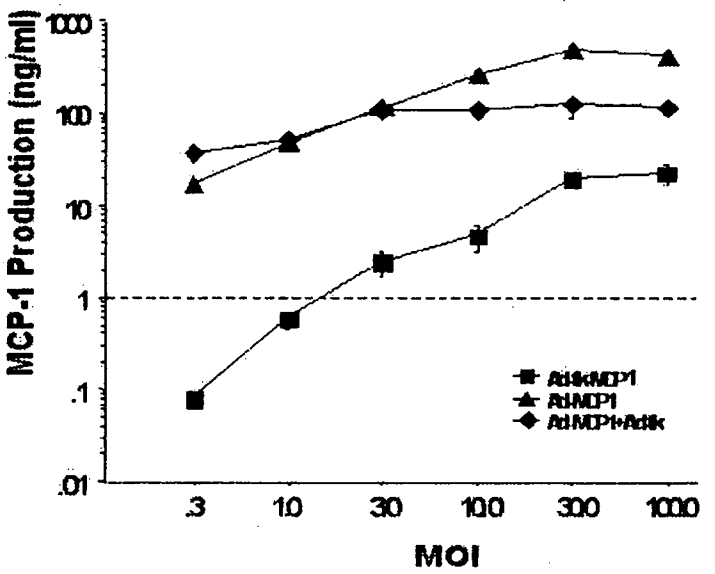

Figure 3. Production of MCP-1 by HuH7 cells infected with rAds. 1×10^5 HuH7 cells were seeded in a well of 12 well plates with 1.0 ml culture medium. Twenty-four hours later, the indicated rAds were infected at various multiplicities of infection (MOIs). Forty-eight hours later, culture medium of each well was collected, the concentrations of MCP-1 were determined by ELISA and the amounts of MCP-1 produced per well were calculated. The data are the means with standard error bars. (Modified from reference[36].)

Production of HSV-tk in HuH7 cells infected with Ad-tk-MCP1, Ad-tk and Ad-MCP1 plus Ad-tk was quantified (Fig. 4). HSV-tk production reached a peak level 72 hours after infection. When compared at varying MOIs, the amounts of HSV-tk reached a peak level at an MOI of 33.3 and remained elevated when higher MOIs were used. Importantly, these rAds resulted in the comparable, marked production of HSV-tk. The results demonstrate that Ad-tk-MCP1, Ad-tk and Ad-MCP1 plus Ad-tk produced similar amounts of HSV-tk when comparable MOIs were used for infection.

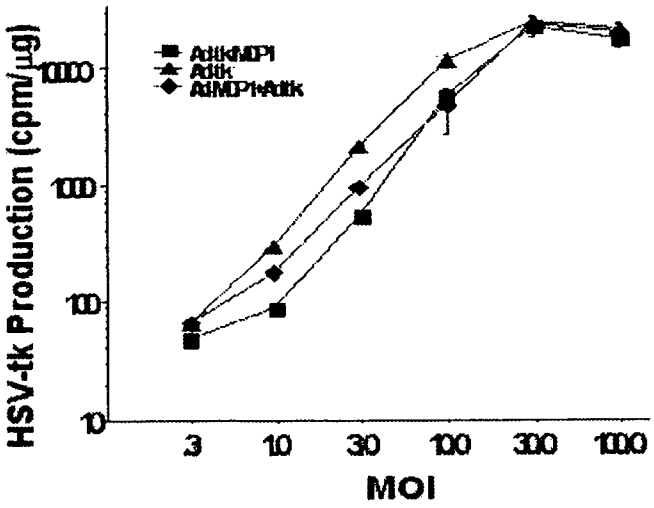

Figure 4. Production of HSV-tk by HuH7 cells infected with rAds. 1×10^6 HuH7 cells were seeded in a well of 6 well plates with 5.0 ml culture medium. Twenty-four hours later, the indicated rAds were infected at various MOIs. Seventy-two hours later, culture medium and cells of each well were collected, the amounts of HSV-tk production per well were calculated. The data are the means with standard error bars. (Modified from reference [36].)

The *in vivo* anti-tumor effects of Ad-tk-MCP1 were analyzed using athymic nude mice (Fig. 5). Despite the reduced production of MCP-1 *in vitro*, the growth of subcutaneous tumors was markedly suppressed in mice treated with Ad-tk-MCP1 compared to those treated with Ad-tk ($P<0.05$), Ad-MCP1 ($P<0.01$), or Ad-lacZ ($P<0.01$). Unexpectedly, the administration of Ad-tk-MCP1 was significantly more effective than the combined administration of Ad-MCP1 plus Ad-tk ($P<0.05$). These data suggest that the anti-tumor killing may be greatly enhanced when MCP-1 is introduced into a cancer cell together with HSV-tk.

MCP-1 is known to recruit and activate macrophage *in vivo*. Therefore, in order to more closely examine the role of MCP1 in our model system, the recruitment of macrophages by rAds infection followed by GCV treatment was analyzed immunohistochemically using an anti-macrophage specific mAb. Most of the infiltrating mononuclear cells in animals treated with Ad-tk-MCP1 or Ad-MCP1 plus Ad-tk stained positively for the macrophage marker Mac-1, but not with control IgG. The number of accumulated Mac-1 positive cells was much greater in the tumors treated with Ad-tk-MCP1 or Ad-MCP1 plus Ad-tk than in those treated with Ad-MCP1 or Ad-tk. In contrast, the Mac-1 positive cells were not detectable when tumors were treated with Ad-lacZ. Furthermore, histopathological analysis showed that the number of necrotic tumor cells was increased to a greater extent in tumors treated with Ad-tk-MCP1.

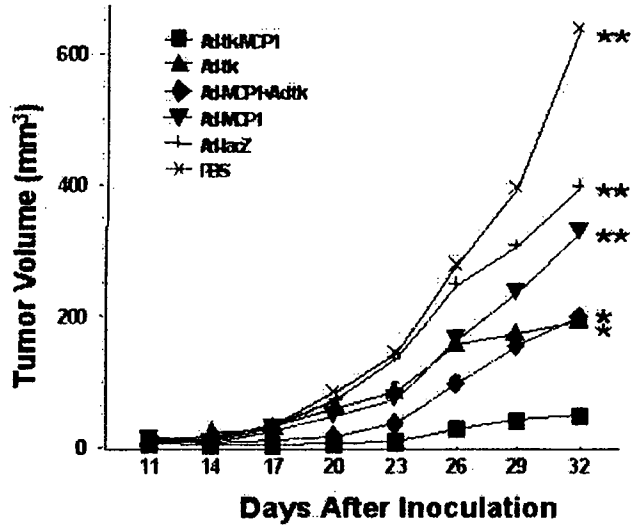

Figure 5. Anti-tumor effects of rAds *in vivo*. Athymic mice were subcutaneously injected with 1×10^7 HuH7 cells on day 0. On days 3 and 4, 1×10^7 $TCID_{50}$ of Ad-tk-MCP1, Ad-tk, Ad-MCP1, Ad-MCP1+Ad-tk, Ad-lacZ or PBS was injected into the tumor foci. Subsequently, 75 mg/kg of GCV was administered for five consecutive days (day 5 to 9). Tumor sizes were monitored. $*P<0.05$ and $**P<0.01$ when compared to Ad-tk-MCP1. (Modified from reference [36].)

The activation state of macrophages recruited into tumor tissues following infection with rAds was analyzed by measuring of TNF-alpha expression, a known macrophage secretagogue [38]. When the levels of TNF-alpha mRNA were semi-quantitatively measured using RT-PCR, TNF-alpha mRNA became detectable in the tumor treated with Ad-tk-MCP1 after 25 cycles of PCR. In addition, tumor development was monitored following the administration of carrageenan, a compound known to inactivate macrophages *in vivo*. The anti-tumor effects of Ad-tk-MCP1 were abolished when carrageenan was administered on days 3 to 5, and on days 12, 19 and 26 ($P<0.05$). Collectively, these data demonstrate that a bicistronic recombinant adenovirus vector Ad-tk-MCP1 expressing both HSV-tk and MCP-1 in sequence under the transcriptional control of a single promoter resulted in the recruitment and activation of macrophages in the tumor, and that these cells may play an important role in tumoricidal activity *in vivo*.

Bicistronic vectors using IRES sequences are widely used to coexpress heterologous genes in a single vector, so that promoter interference, which occurs when using heterologous promoters, does not occur [32-34,39]. Previous studies demonstrated that the level of IRES-dependent gene expression varied from 6 to 100% of that of the promoter-dependent gene in vitro (HeLa, L, and CHO cells) and in vivo (mouse liver), suggesting that the gene needed for higher expression may be positioned as the promoter-dependent gene [40]. In some studies on cancer gene therapy, the cytokine and

suicide genes were positioned as the promoter-dependent and IRES-dependent genes, respectively [41-43], and in other studies, they were positioned in the opposite sequence [44]. Although results from these studies showed that tumor regression was enhanced when suicide and cytokine gene therapies were combined, no comparisons were made between the vectors harboring the two genes in different sequences.

In a series of experiments [36], we evaluated the relative capacities of IRES to mediate the expression of the downstream genes, and found about 6~12% in the case of MCP-1 and 1~4% in the case of HSV-tk when compared with products translated in a promoter-dependent manner in the two bicistronic rAds, Ad-tk-MCP1 and another vector Ad-MCP1-tk that expresses MCP-1 and HSV-tk in sequence, *in vitro*. These results were consistent with a previous report that showed that IRES-dependent gene expression was less efficient than the promoter-dependent expression and that their efficiencies varied depending on the nature of the cell type and reporter genes [40]. Interestingly, our results demonstrated that the administration of Ad-tk-MCP1 was significantly more effective than that of Ad-MCP1-tk *in vivo*. We propose that the HSV-tk/GCV system should be mainly delivered and MCP-1 should be supported when the gene therapy is carried out using the HSV-tk and MCP-1 genes.

Under the experimental conditions, infection with Ad-tk-MCP1 was more effective in terms of tumor suppression than was co-delivery of Ad-tk and Ad-MCP1, although MCP-1 production was much less with Ad-tk-MCP1 than co-delivery of Ad-tk and Ad-MCP1. Ad-tk-MCP1 infection may have resulted in efficient expression of MCP-1 in HuH7 cells that had become apoptotic due to expression of HSV-tk in the presence of GCV. These findings are consistent with our observation that TNF-alpha production was significantly augmented when macrophages were exposed to apoptotic HuH7 cells induced by HSV-tk / GCV *in vitro* [45]. Thus, MCP-1 secreted by apoptotic HuH7 cells may have recruited macrophages more efficiently to these apoptotic cells, thereby resulting in more deleterious effects on tumor formation.

MCP-1 was reported to be destructive in some tumor models [21, 23, 46] but protective in others [47, 48]. Thus, murine colon carcinoma cells expressing MCP-1 failed to metastasize when injected into mice [23], whereas other carcinoma cells showed enhanced metastasis [47]. A recent report demonstrated that monocyte recruitment depended on the level of MCP-1 secreted by the tumor cells and that the effect of monocyte infiltration on tumor growth was dependent on their levels of infiltration. Lower MCP-1 production had little effect on monocyte infiltration, whereas higher production levels appeared to lead to massive infiltration of monocytes / macrophages and eventually tumor destruction [49]. Our results demonstrated that the growth of subcutaneous tumors was markedly suppressed and a strong infiltration of

macrophages was observed when cells were treated with Ad-tk-MCP1, despite the relatively reduced MCP-1 production compared to Ad-MCP1. Several investigators reported that dying HSV-tk-modified cells released soluble factors including cytokines. These factors, in turn, could affect the tumor microenvironment, leading to necrosis and inflammation, infiltration of immune cells, upregulation of costimulatory molecules and the generation of an anti-tumorigenic immune response [16, 50]. In this immunotherapeutically favorable setting, even a minute amount of MCP-1 may stimulate tumor-specific immune-mediate cell killing and enhance local antitumorigenic efficacy, thereby adding to the overall therapeutic effect.

Suicide Gene therapy combined with MCP-1 demonstrates prolonged, NK cell-mediated antitumor effects

Transfection of the MCP-1 into human lung adenocarcinoma cells was found to inhibit the formation of metastases, presumably via the activation of NK cells [46]. Recently, NK cells were reported to mediate long-lived, antigen-specific adaptive recall responses independent of B cells and T cells [51]. These observations suggest that MCP-1 may induce specific tumor immunity by enhancing NK cell functions in our experimental system. To evaluate the long-term systemic immunomodulatory effects of an adenovirus vector expressing MCP-1 together with HSV-tk, the primary subcutaneous HCC tumors were eradicated by using Ad-tk-MCP1 in the murine model, and subsequently the same HCC cells were injected into an another site of the same mice [52]. Moreover, we explored whether innate immune responses induced by NK cells were involved in these procedures.

HCC cell line HuH7 was subcutaneously transplanted into athymic nude mice (day 0) and eradicated with rAds harboring HSV-tk, with or without MCP-1, and the mice were re-challenged with the tumor cells (day 14) (Fig. 6). We found that tumor re-growth was significantly suppressed when the primary tumor cells had been eradicated with Ad-tk-MCP1 compared with Ad-tk. No growth inhibition was observed when Ad-tk-MCP1 or Ad-MCP1 was administered in the absence of HuH7 cell transplantation, or when Ad-lacZ was administered along with mitomycin C (MMC)-treated HuH7 cells. The results demonstrate that, when the primary tumors were eradicated with the HSV-tk/GCV system plus MCP-1, the antitumor effects were maintained.

To evaluate the immunomodulatory effects of rAds expressing HSV-tk, with or without MCP-1, we measured IL-12 production by monocytes which had been co-cultured with HCC cells that had been infected with rAds (Fig.7). Peritoneal exudate cells, which included mostly macrophages, co-cultured with HCC cells infected with rAds expressing MCP-1, produced greater amounts of IL-12 than did macrophages co-cultured with HCC cells infected with rAds expressing HSV-tk. Furthermore, these peritoneal exudate cells secreted larger

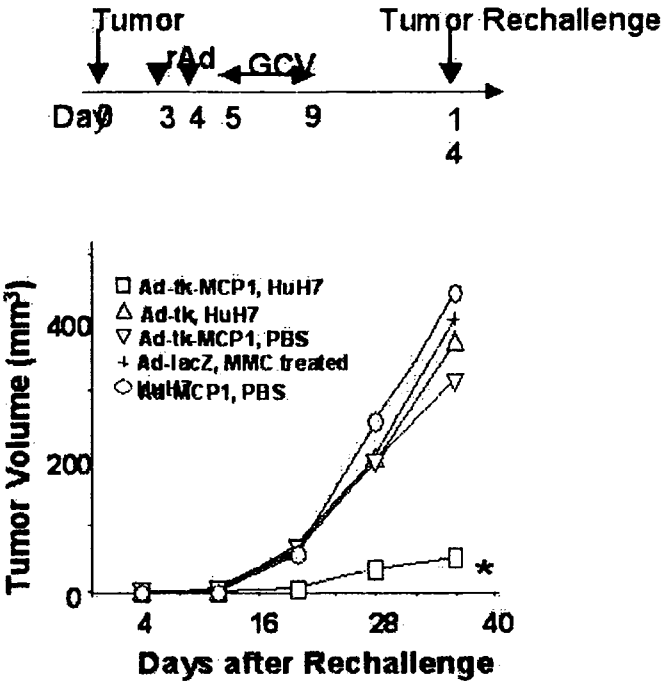

Figure 6. Prolonged antitumor effects of rAds expressing HSV-tk, with or without MCP-1. Mice were subcutaneously injected with 5 x 10^6 HuH7 cells on day 0. On days 3 and 4, 5 x 10^7 TCID50 of Ad-tk-MCP1, Ad-tk, Ad-lacZ or Ad-MCP1 were injected into the tumors, and the mice were intraperitoneally injected with 75 mg/kg GCV every day for the next five days (day 5 to 9). Following complete eradication of the primary tumors, the mice were subcutaneously re-challenged with 3 x 10^6 HuH7 cells at other sites on day 14. Tumor sizes were monitored. *P <0.001 when compared to Ad-tk, HuH7. (Modified from reference [52].)

amounts of IL-12 when HCC apoptosis was induced by the HSV-tk/GCV system.

Antigen-presenting cells (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 [53, 54]. APCs were found to be activated upon recognizing antigens from apoptotic target cells [55], and both macrophages and DCs secrete large amounts of IL-12 when treated with MCP-1 [56-58]. These findings suggest that recognition of apoptotic tumor cells together with MCP-1, may greatly activate macrophages, thereby enhancing IL-12 secretion.

In addition to IL-12, IL-18 is a proinflammatory cytokine produced by activated macrophages that has been shown to augment both innate and acquired immunity [59], and, in combination with IL-12, to induce T helper 1 cell development and NK cell activation [60]. We therefore assayed IL-12 and IL-18 production after tumor re-challenge. Serum concentrations of IL-12 and IL-18 were significantly higher after tumor re-challenge in mice whose

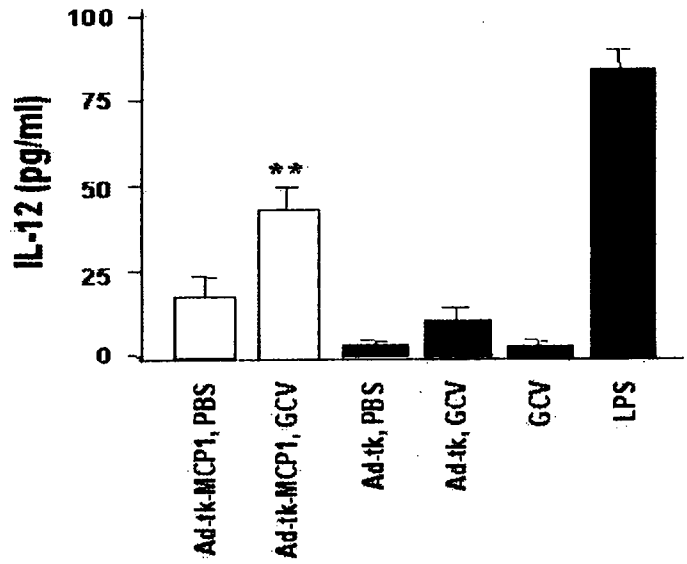

Figure 7. IL-12 production by monocytes co-cultured with apoptotic or non- apoptotic HCC cells infected with rAds *in vitro*. HuH7 cells were infected with Ad-tk-MCP1 and Ad-tk at an MOI of 5 for 24 hours. Aliquots of 1 x 10^5 mouse peritoneal exudate cells, which included most macrophages, were co-cultured with 1 x 10^5 rAd-treated HuH7 cells, and treated with or without GCV for two days, and the concentrations of IL-12 in the media were evaluated using an immunoassay. Each value is the mean ± SE. $^{**}P < 0.05$ when compared to Ad-tk, GCV. (Modified from reference [52].)

primary tumors had been eradicated with Ad-tk-MCP1 compared with mice whose tumors had been eradicated with Ad-tk. Moreover, serum concentrations of IL-12 peaked after primary tumors were eradicated (day 9) and were sustained thereafter. Furthermore, the administration of anti-IL-12 significantly inhibited the antitumor effects conferred by Ad-tk-MCP1 and reduced the serum concentrations of IL-12 to an undetectable level. The combined treatment of anti-IL-12 and anti-IL-18 Ab further diminished anti-tumor effects and reduced both serum IL-12 and IL-18 levels to undetectable levels. The results suggest the critical involvement of IL-12 and IL-18 in the antitumor effects induced by Ad-tk-MCP1 on tumor re-growth.

Since 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-positive NK cells was significantly higher upon tumor re-challenge 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. Similarly, the numbers of F4/80 or Mac-1 positive cells [49, 58] tended to be higher upon tumor re-challenge in mice whose primary tumors had been eradicated with Ad-tk-MCP1. Moreover, the mRNA of IFN-γ, an NK cell secretague [61], became detectable after 30 PCR cycles in the re-challenged tumors of animals whose primary tumors had been eradicated with Ad-tk-MCP1, and was greatly

amplified after 40 PCR cycles. These results demonstrate that NK cells were recruited and activated into re-challenged 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 re-challenge using anti-AGM1, F4/80, Mac-1, CD11c and CD45R Abs. The numbers of F4/80- and Mac-1-positive cells were significantly increased in the spleens of mice treated with Ad-tk-MCP1 compared with mice treated with Ad-tk. In contrast, the numbers of AGM1- and CD45R-positive 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 positive cells. A flow cytometrical analysis of splenocyte single cell suspensions demonstrated that the numbers of DX5- and F4/80-positive cells tended to be higher in the spleens of mice treated with Ad-tk-MCP1. In contrast, treatment with carrageenan decreased the numbers of macrophages in the spleen and at re-challenge sites, and slightly increased the numbers 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 eradication of primary tumors by HSV-tk/GCV plus MCP-1. Finally, anti-AGM1 Ab [62, 63] significantly inhibited the antitumor immunity conferred by Ad-tk-MCP1, and carrageenan partially inhibited the antitumor immunity of Ad-tk-MCP1 (Fig. 8). The results indicate that anti-tumor effects were mainly mediated by NK cells.

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 gene alone was not sufficient to prolong immunity. MCP-1 secreted by apoptotic HuH7 cells was observed to 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 [36, 45]. Moreover, we showed that the numbers of Mac-1- and F4/80-positive cells were increased in the spleens of mice after tumor re-challenge. Indeed, MCP-1 has been shown to activate murine peritoneal macrophages and to enhance the expression of CD11b (Mac-1) in BALB/c mice [49, 58]. 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, which can activate NK cells, thus exerting antitumor effects (Fig. 9).

IL-12-stimulated NK cells are known to exhibit potent cytotoxic activity against various tumor cells [64-66]. NK cells are a part of the innate immune system, a first-line defense against tumor cells, and exert anti-tumor effects rapidly without any prior sensitization [67]. Depletion of NK cells has been

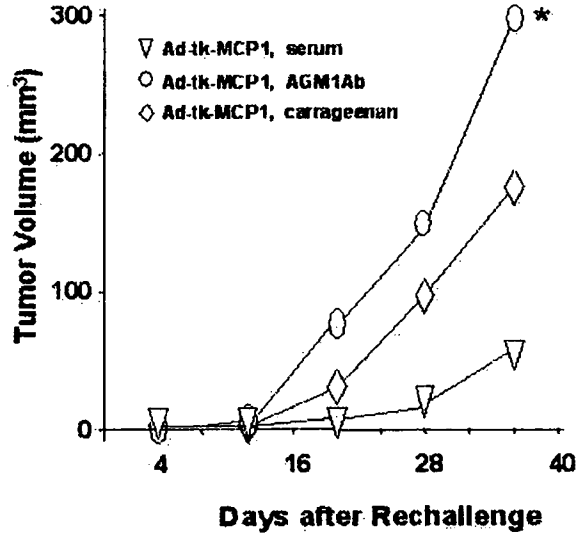

Figure 8. Suppression of prolonged antitumor effects of rAds using anti-asialo GM1 (AGM1) Ab or carageenan. At the rechallenge with HuH7 cells, Ad-tk-MCP1-treated animals were intraperitoneally administered with anti-AGM1 Ab (Ad-tk-MCP1, AGM1Ab), rabbit serum (Ad-tk-MCP1, serum) or carrageenan (Ad-tk-MCP1, carrageenan). Tumor sizes were monitored. $*P < 0.05$ when compared to Ad-tk-MCP1, serum. (Modified from reference [52].)

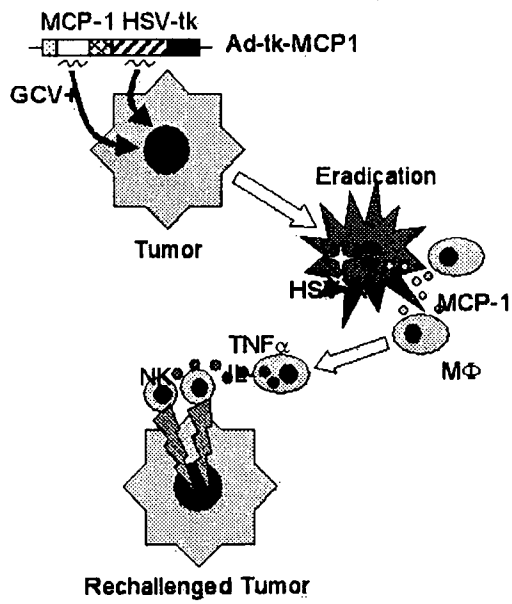

Figure 9. Schematic representation of prolonged antitumor effects induced by rAd harboring both HSV-tk and MCP-1 genes. Ad-tk-MCP1 exerts macrophage-dependent antitumor effects synergistically induced by the HSV-tk/GCV system and MCP-1. During eradication of the primary tumors, activated macrophages in the tumor tissues and the peripheral lymphoid organs induce the secretion of cytokines, including TNFα, IL-12 and IL-18, which can activate NK cells, thus exerting antitumor effects against the re-challenged tumors.