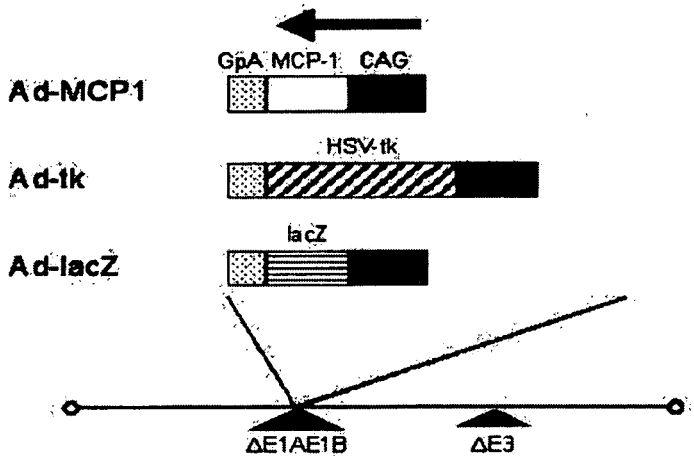

Figure 1. Schematic representation of recombinant adenoviruses (rAds). rAd expressing each gene under the control of the CAG promoter. Ad-MCP-1, Ad-tk and Ad-lacZ expressing monocyte chemoattractant protein (MCP)-1, herpes simplex virus thymidine kinase gene (HSV-tk) and beta-galactosidase gene (LacZ). 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].

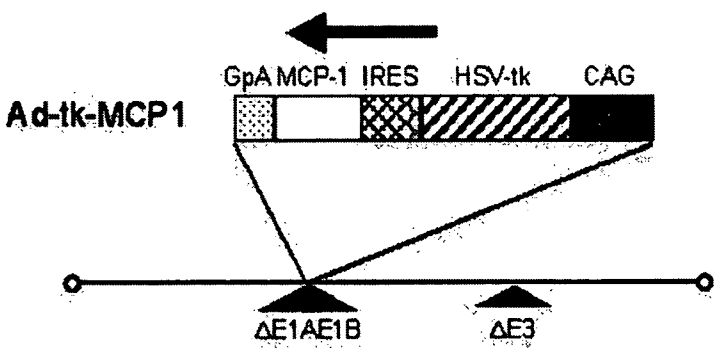

Figure 2. Construction of recombinant adenovirus Ad-tk-MCP1. Bicistronic Ad-tk-MCP1 harboring the HSV-tk gene and the human MCP-1 gene in sequence under the universal CAG promoter within a bicistronic unit including the internal ribosomal entry site (IRES) fragment of encephalomyocarditis virus that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

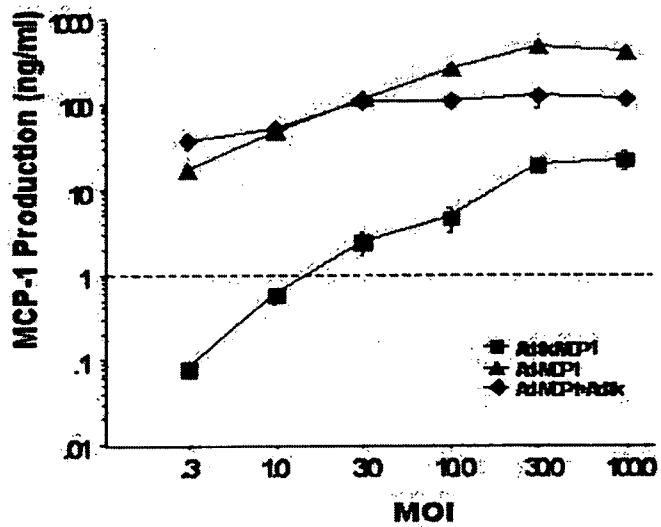

Figure 3. Production of MCP-1 by HuH7 cells infected with rAds. 1×10^5 HuH7 cells were seeded in a well of 12 well plates with 1.0 ml culture medium. Twenty-four hours later, the indicated rAds were infected at various multiplicities of infection (MOIs). Forty-eight hours later, culture medium of each 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.

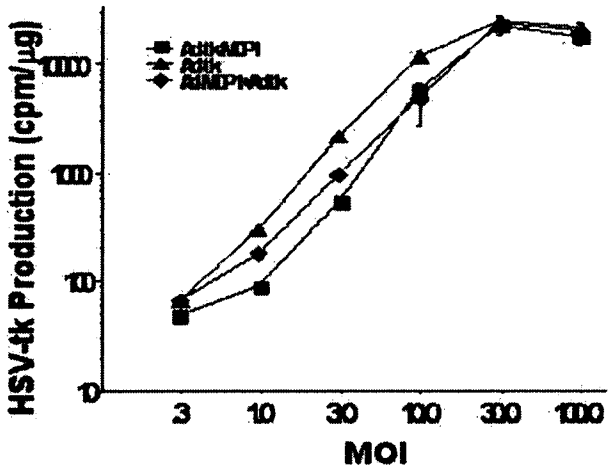

Figure 4. Production of HSV-tk by HuH7 cells infected with rAds. 1×10^6 HuH7 cells were seeded in a well of 6 well plates with 5.0 ml culture medium. Twenty-four hours later, the indicated rAds were infected at various MOIs. Seventy-two hours later, culture medium and cells of each well were collected, 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.

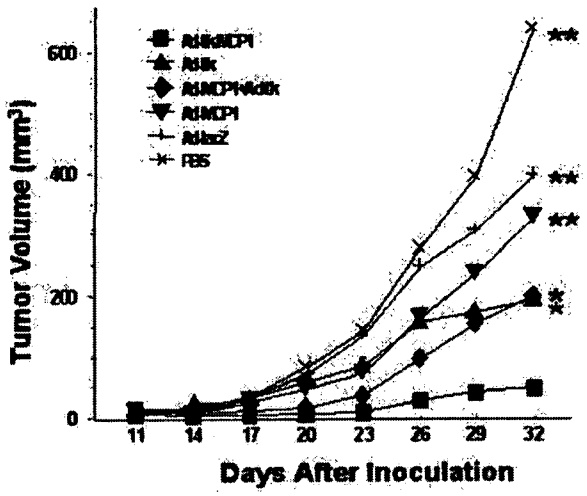

Figure 5. Anti-tumor effects of rAds *in vivo*. Athymic mice were subcutaneously injected with 1×10^7 HuH7 cells on day 0. On days 3 and 4, 1×10^7 $TCID_{50}$ of Ad-tk-MCP1, Ad-tk, Ad-MCP1, Ad-MCP1+Ad-tk, Ad-lacZ or PBS was injected into the tumor foci. Subsequently, 75 mg/kg of GCV was administered 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

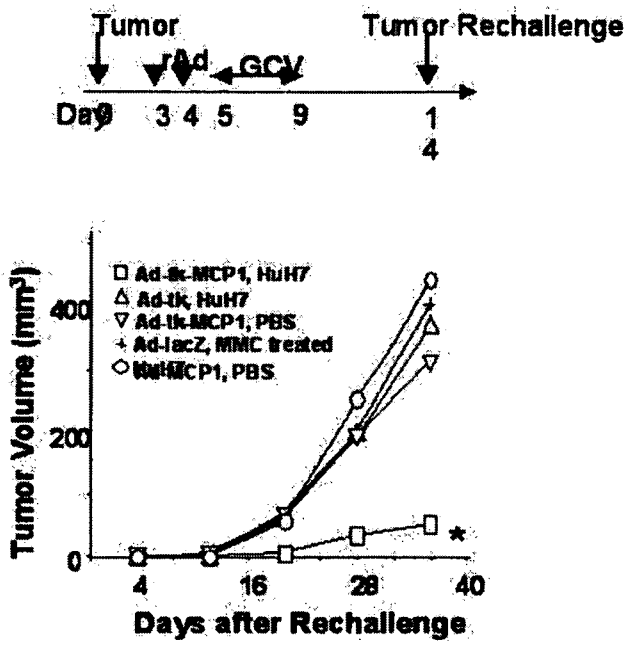

Figure 6. Prolonged antitumor effects of rAds expressing HSV-tk, with or without MCP-1. Mice were subcutaneously injected with 5 x 10^6 HuH7 cells on day 0. On days 3 and 4, 5 x 10^7 TCID50 of Ad-tk-MCP1, Ad-tk, Ad-lacZ or Ad-MCP1 were injected into the tumors, and the mice were intraperitoneally injected 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

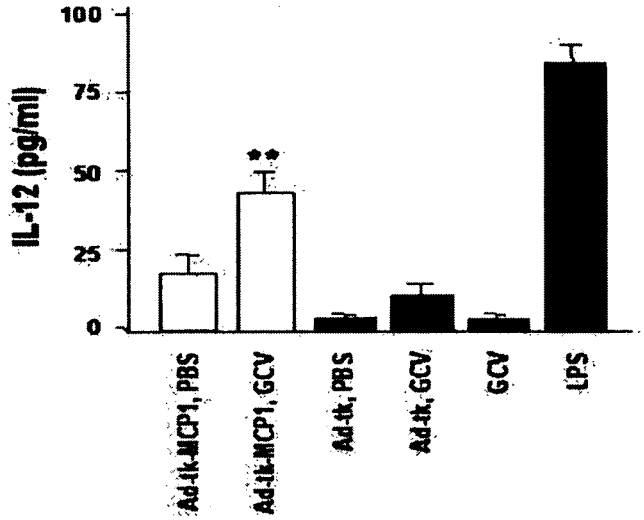

Figure 7. IL-12 production by monocytes co-cultured with apoptotic or non- apoptotic HCC cells infected with rAds *in vitro*. HuH7 cells were infected with Ad-tk-MCP1 and Ad-tk at an MOI of 5 for 24 hours. Aliquots of 1 x 10^5 mouse peritoneal exudate cells, which included most macrophages, were co-cultured 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

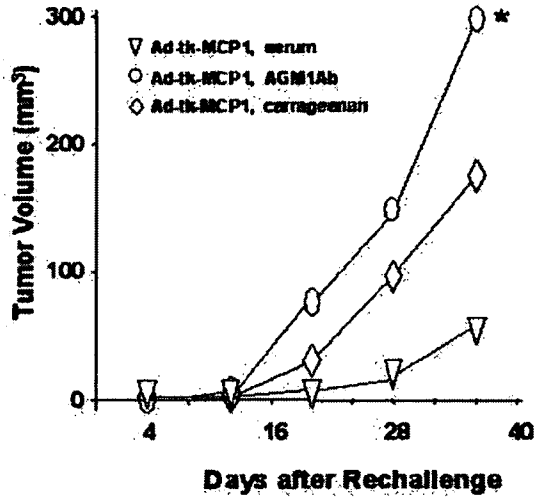

Figure 8. Suppression of prolonged antitumor effects of rAds using anti-asialo GM1 (AGM1) Ab or carageenan. At the rechallenge with HuH7 cells, Ad-tk-MCP1-treated animals were intraperitoneally administered with anti-AGM1 Ab (Ad-tk-MCP1, AGM1Ab), rabbit serum (Ad-tk-MCP1, serum) or carrageenan (Ad-tk-MCP1,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

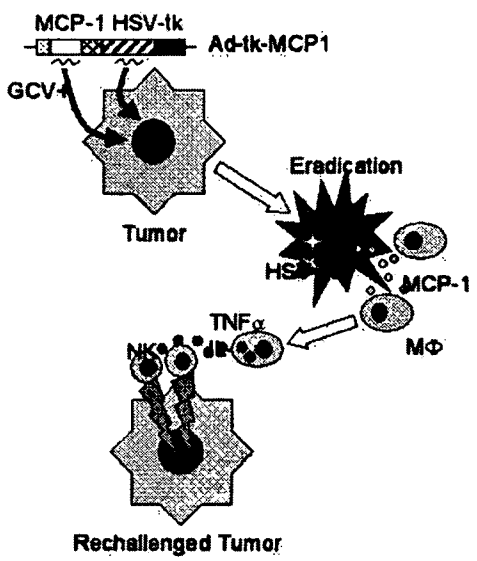

Figure 9. Schematic representation of prolonged antitumor effects induced by rAd harboring both HSV-tk and MCP-1 genes. Ad-tk-MCP1 exerts macrophage-dependent antitumor effects synergistically induced by the HSV-tk/GCV system and MCP-1. During eradication of the primary tumors, activated macrophages 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.

shown to promote metastases or tumor growth after re-challenge with primary tumor cells [46, 65, 68]. Therefore, augmentation of NK-mediated innate immune responses may be an attractive strategy for preventing HCC recurrence.

Recently, not only in nude mice but in immunocompetent mice, we have found that adenovirally-delivered monocyte chemoattractant protein (MCP)-1 highly augmented the antitumor effects of the HSV-tk/GCV system [69]. In an immunocompetent mouse model of colon cancer, subcutaneous tumor foci were directly transduced with both rAds expressing an HSV-tk gene and one of the MCP-1, CD80 and LacZ genes, followed by GCV administration. The growth of tumors was markedly suppressed by codelivery of HSV-tk and MCP-1 genes, which was exclusively associated with the recruitment of monocytes/macrophages, T helper 1 (Th1) cytokine gene expression and cytotoxic activity of the splenocytes. Furthermore, the antitumor effects were more efficient than that obtained by the combination of HSV-tk and CD80 genes. These results suggest an immunomodulatory effect of MCP-1 in the context of suicide gene therapy via orchestration of innate and acquired immune responses.

Perspective

Although the findings presented here are promising, a number of problems remain to be solved before this approach can be used clinically. In general, the presence of rAd vector immunity has impeded clinical use of these vectors [70]. Innate immune responses to the vector particles drastically limit the vector transduction efficiency and the duration of transgene expression. However, in cancer gene therapy, transgene expression for a short duration may be enough for the desired effects. Induction of proinflammatory cytokines and chemokines by rAd vectors might not be a limitation in every situation since rAd vectors can efficiently transduce a broad range of cell types. In terms of the safety of replication-defective adenovirus vectors [71], although the clinical trial designed to correct partial ornithine transcarbamylase deficiency was overshadowed by the death of a patient at the highest dose [72, 73], the first gene therapy treatment for cancer was approved in 2004 [74]. A replication-defective Ad5 vector expressing p53 from a Rous sarcoma virus (RSV) promoter, Gendicine (SiBiono, Shenzen, China), is now approved for use in head and neck squamous cell carcinoma, glioma [75], non-small cell lung cancer [76], bladder carcinoma [77], and ovarian cancer [78]. The use of an E1-deleted adenovirus expressing the HSV-tk shows promise for glioma [79, 80] and prostate cancer [81]. Finally, we look forward toward how these new insights into the development of gene therapy will impact on therapeutic options for cancer in the near future.

Acknowledgements

I am indebted to my many colleagues and collaborators who contributed greatly to the work described in this paper, especially Drs. Tomoya Tsuchiyama, Yoshio Sakai, Takashi Kagaya and Naofumi Mukaida.

References

1. Venook, A.P. 1994, *J Clin Oncol*, 12,1323.
2. Trinchet, J.C., and Beaugrand, M. 1997, *J Hepatol*, 27,756.
3. Bruix, J. 1997, *Hepatology*, 25,259.
4. Kanai, F., Shiratori, Y., Omata, M. 1996, *Hepatology*, 23,1359.
5. Kanai, F., Lan, K.H., Omata, M. 1997, *Cancer Res*, 57,461.
6. Kaneko, S., Hallenbeck, P., Chiang, Y.L. 1995, *Cancer Res*, 55,5283.
7. Cao, G., Kuriyama, S., Qi, Z. 1996, *J Gastroenterol Hepatol*, 11,1053.
8. Qin, X.Q., Tao, N., Barsoum, J. 1998, *Proc Natl Acad Sci U S A*, 95,14411.
9. Fricker, J. 1996, *Mol Med Today*, 2,361.
10. Anderson, S.C., Johnson, D.E., Maneval, D.C. 1998, *Clin Cancer Res*, 4,1649.
11. Bookstein, R., Demers, W., Wills, K. 1996, *Semin Oncol*, 23,66.
12. Anderson, W.F. 1998, *Nature*, 392,25.
13. Culver, K.W., Ram, Z., Blaese, R.M. 1992, *Science*, 256,1550.
14. Wu, J.K., Cano, Cherington, V. 1994, *Neurosurgery*, 35,1094.
15. Elshami, A.A., Saavedra, A., Albelda, S.M. 1996, *Gene Ther*, 3,85.
16. Ramesh, R., Marrogi, A.J., Freeman, S.M. 1996, *Exp Hematol*, 24,829.
17. Freeman, S.M., Ramesh, R., Marrogi, A.J. 1995, *Cancer Lett*, 92,167.
18. Santodonato, L., Ferrantini, M., Belardelli, F. 1996, *Hum Gene Ther*, 7,1.
19. Moriuchi, S., Oligino, T., Glorioso, J.C. 1998, *Cancer Res*, 58,5731.
20. Matsushima, K., Larsen, C.G., Oppenheim, J.J. 1989, *J Exp Med*, 169,1485.
21. Rollins, B.J., Sunday, M.E. 1991, *Mol Cell Biol*, 11,3125.
22. Hirose, K., Hakozaki, M., Matsushima, K. 1995, *Br J Cancer*, 72,708.
23. Huang, S., Singh, R.K., Bar-Eli, M. 1994, *Cancer Immunol Immunother*, 39,231.
24. Huang, S., Xie, K., Bar-Eli, M. 1995, *J Interferon Cytokine Res*, 15,655.
25. Nishi, T., Yoshizato, K., Ushio, Y. 1996, *Cancer Res*, 56,1050.
26. Merighi, M.A., Yoshizato, E. 2002, *Rev Lat Am Enfermagem*, 10,493.
27. Luciani, M.G., Stoppacciaro, A., Ruco, L.P. 1998, *Mol Pathol*, 51,273.
28. Valkovic, T., Lucin, K., Jonjic, N. 1998, *Pathol Res Pract*, 194,335.
29. Salcedo, R., Ponce, M.L., Murphy, W.J. 2000, *Blood*, 96,34.
30. Kanegae, Y., Lee, G., Saito, I. 1995, *Nucleic Acids Res*, 23,3816.
31. Fukumura, D., Salehi, H.A., Jain, R.K. 1995, *Cancer Res*, 55,4824.
32. Sakai, Y., Kaneko, S., Kobayashi, K. 2001, *J Virol Methods*, 92,5.
33. Emerman, M., Temin, H.M. 1984, *Cell*, 39,449.
34. Cullen, B.R., Lomedico, P.T., Ju, G. 1984, *Nature*, 307,241.
35. Miyake, S., Makimura, M., Saito, I. 1996, *Proc Natl Acad Sci U S A*, 93,1320.
36. Tsuchiyama, T., Kaneko, S., Kobayashi, K. 2003, *Cancer Gene Ther*, 10,260.
37. Kanegae, Y., Makimura, M., Saito, I. 1994, *Jpn J Med Sci Biol*, 47,157.
38. Sunderkotter, C., Steinbrink, K., Sorg, C. 1994, *J Leukoc Biol*, 55,410.
39. Emerman, M., Temin, H.M. 1986, *Mol Cell Biol*, 6,792.

40. Mizuguchi, H., Xu, Z., Hayakawa, T. 2000, *Mol Ther*, 1,376.
41. Sharma, S., Miller, P.W., Dubinett, S.M. 1997, *Gene Ther*, 4,1361.
42. Pizzato, M., Franchin, E., Palu, G. 1998, *Gene Ther*, 5,1003.
43. Okada, H., Giezeman-Smits, K.M., Bozik, M.E. 1999, *Gene Ther*, 6,219.
44. Okada, H., Miyamura, K., Yoshida, J. 1996, *Gene Ther*, 3,957.
45. Sakai, Y., Kaneko, S., Kobayashi, K. 2001, *Cancer Gene Ther*, 8,695.
46. Nokihara, H., Yanagawa, H., Sone, S. 2000, *Cancer Res*, 60,7002.
47. Nakashima, E., Mukaida, N., Matsushima, K. 1995, *Pharm Res*, 12,1598.
48. Ueno, T., Toi, M., Matsushima, K. 2000, *Clin Cancer Res*, 6,3282.
49. Nesbit, M., Schaider, H., Herlyn, M. 2001, *J Immunol*, 166,6483.
50. Vile, R.G., Castleden, S., Chong, H. 1997, *Int J Cancer*, 71,267.
51. O'Leary, J.G., Goodarzi, M., von Andrian, U.H. 2006, *Nat Immunol*, 7,507.
52. Tsuchiyama, T., Nakamoto, Y., Sakai Y., Marukawa Y., Kitahara M., Mukaida N., Kaneko S., *J Immunol*, 2007, 178(1):574-83.
53. Brunda, M.J., Luistro, L., Gately, M.K. 1993, *J Exp Med*, 178,1223.
54. Nastala, C.L., Edington, H.D., McKinney, T.G. 1994, *J Immunol*, 153,1697.
55. Albert, M.L., Sauter, B., Bhardwaj, N. 1998, *Nature*, 392,86.
56. Matsukawa, A., Hogaboam, C.M., Kunkel, S.L. 2000, *Exp Mol Pathol*, 68,77.
57. Traynor, T.R., Herring, A.C., Huffnagle, G.B. 2002, *J Immunol*, 168,4659.
58. Biswas, S.K., Sodhi, A. 2002, *J Interferon Cytokine Res*, 22,527.
59. Okamura, H., Kashiwamura, S., Nakanishi, K. 1998, *Curr Opin Immunol*, 10,259.
60. Dinarello, C.A., Novick, D., Rubinstein, M. 1998, *J Leukoc Biol*, 63,658.
61. Carson, W.E., Ross, M.E., Caligiuri, M.A. 1995, *J Clin Invest*, 96,2578.
62. Nagai, M., Masuzawa, T. 2001, *Int Immunopharmacol*, 1,657.
63. van den Broeke, L.T., Daschbach, E., Berzofsky, J.A. 2003, *J Immunol*, 171,5842.
64. Satoh, T., Saika, T., Thompson, T.C. 2003, *Cancer Res*, 63,7853.
65. Lasek, W., Mackiewicz, A., Jakobisiak, M. 2000, *Cancer Gene Ther*, 7,1581.
66. Rakhmilevich, A.L., Janssen, K., Yang, N.S. 2000, *Cancer Gene Ther*, 7,826.
67. Kim, S., Iizuka, K., Yokoyama, W.M. 2000, *Proc Natl Acad Sci U S A*, 97,2731.
68. Orengo, A.M., Di Carlo, E., Ferrini, S. 2003, *J Immunol*, 171,569.
69. Kagaya, T., Nakamoto, Y., Kaneko, S. 2006, *Cancer Gene Ther*, 13,357.
70. Bangari, D.S., Mittal, S.K. 2006, *Curr Gene Ther*, 6,215.
71. Young, L.S., Searle, P.F., Mautner, V. 2006, *J Pathol*, 208,299.
72. Raper, S.E., Chirmule, N., Batshaw, M.L. 2003, *Mol Genet Metab*, 80,148.
73. Raper, S.E., Yudkoff, M., Batshaw, M.L. 2002, *Hum Gene Ther*, 13,163.
74. Peng, Z. 2005, *Hum Gene Ther*, 16,1016.
75. Lang, F.F., Bruner, J.M., Yung, W.K. 2003, *J Clin Oncol*, 21,2508.
76. Swisher, S.G., Roth, J.A., Yin, M. 2003, *Clin Cancer Res*, 9,93.
77. Pagliaro, L.C., Keyhani, A., Dinney, C.P. 2003, *J Clin Oncol*, 21,2247.
78. Wolf, J.K., Bodurka, D.C., Gershenson, D.M. 2004, *Gynecol Oncol*, 94,442.
79. Germano, I.M., Fable, J., Silvers, A. 2003, *J Neurooncol*, 65,279.
80. Immonen, A., Vapalahti, M., Yla-Herttuala, S. 2004, *Mol Ther*, 10,967.
81. Teh, B.S., Ayala, G., Butler, E.B. 2004, *Int J Radiat Oncol Biol Phys*, 58,1520.

Microbiol. Immunol., 51(4), 471–482, 2007

Editor-Communicated Paper

Prediction of Efficient Virological Response to Pegylated Interferon/Ribavirin Combination Therapy by NS5A Sequences of Hepatitis C Virus and Anti-NS5A Antibodies in Pre-Treatment Sera

Ahmed El-Shamy[1], Mikiko Sasayama[1], Motoko Nagano-Fujii[1], Noriko Sasase[2], Susumu Imoto[2], Soo Ryang Kim[2], and Hak Hotta*[,1]

[1]*Division of Microbiology, Kobe University Graduate School of Medicine, Kobe, Hyogo 650–0017, Japan, and* [2]*Division of Gastroenterology, Kobe Asahi Hospital, Kobe, Hyogo 653–0801, Japan*

Communicated by Dr. Yoshiharu Matsuura: Received February 13, 2007. Accepted February 17, 2007

***Abstract*: A considerable number of patients infected with Hepatitis C virus subtype 1b (HCV-1b) do not respond to pegylated interferon/ribavirin combination therapy. In this study we explored a useful factor(s) to predict treatment outcome. A total of 47 HCV-1b-infected patients were treated with pegylated interferon/ribavirin for 48 weeks. Sera of the patients were examined for the entire NS5A sequence of the HCV genome, HCV RNA titers and anti-NS5A antibodies. According to their responses, the patients were divided into two groups, early viral responders who cleared the virus by week 16 (EVR[16w]) and those who did not (Non-EVR[16w]). The mean number of mutations in the V3 region (aa 2356 to 2379) or that in the V3 region *plus* its N-terminally flanking region, which we refer to as interferon/ribavirin resistance-determining region (IRRDR; aa 2334 to 2379), of NS5A obtained from the pretreatment sera was significantly larger for EVR(16w) compared with Non-EVR(16w). Moreover, HCV-1b isolates with ≥5 mutations in V3 or those with ≥6 mutations in IRRDR were almost exclusively found in EVR(16w). Also, the presence of detectable levels of anti-NS5A antibodies in the pretreatment sera was closely associated with EVR(16w). In conclusion, a high degree of sequence variation in V3 (≥5) or IRRDR (≥6) and the presence of detectable levels of anti-NS5A antibodies in the pretreatment sera would be useful factors to predict EVR(16w). On the other hand, a less diverse sequence in V3 (≤4) or IRRDR (≤5) together with the absence of detectable anti-NS5A antibodies could be a predictive factor for Non-EVR(16w).**

***Key words*: Early viral responder (EVR), Non-EVR, Variable (V) 3 region, IFN/RBV resistance-determining region (IRRDR)**

Hepatitis C virus (HCV) is the most common blood-borne pathogen of chronic liver diseases in Japan and U.S.A. (17). It establishes persistent infection that often results in chronic hepatitis followed by liver cirrhosis, from which hepatocellular carcinoma arises (18). HCV is an RNA virus, which belongs to the *Flaviviridae* family. Based on the nucleotide sequence diversity of the viral genome, HCV is classified into six genotypes, HCV-1 to -6, with each genotype being further subdivided into a number of subtypes, such as HCV-1a and -1b (38, 39).

Because of a considerable lag time between the onset of infection and clinical manifestation, the prevalence of HCV-related liver disease is still increasing (17). Thus, effective treatment is essential. There are three independent factors for successful treatment of chronic HCV infection: (i) the virus must be sensitive to treatment, (ii) all infected cells must be eliminated, and (iii) the patients must comply with therapy regimens (7). In this context, global consensus obtains that a combina-

**Address correspondence to* Dr. Hak Hotta, Division of Microbiology, Kobe University Graduate School of Medicine, 7–5–1 Kusunoki-cho, Chuo-ku, Kobe, Hyogo 650–0017, Japan. Fax: +81–78–382–5519. E-mail: hotta@kobe-u.ac.jp

Abbreviations: aa, amino acid; EVR, early viral responder; HCV, hepatitis C virus; IFN, interferon; IRRDR, interferon/ribavirin resistance-determining region; NCR, non coding region; NS, non-structural region; PBS, phosphate buffered saline; PEG, pegylated; RBV, ribavirin; V3, variable region 3.

tion of interferon (IFN) or pegylated IFN (PEG-IFN) *plus* ribavirin (RBV) is the treatment of choice for chronic hepatitis C. Even with this treatment regimen, however, sustained virological response rates for those infected with the most resistant genotypes, HCV-1a and -1b, still hover at ~50% (11, 22). Thus, it is worthy to identify the predictive factors that allow the selection of patients who will achieve the eradication of HCV RNA either before or during treatment, because IFN/RBV combination therapy is more expensive and has several side effects (29).

Predictors of IFN-based therapy can be classified into two categories, pre-treatment and on-treatment factors. Pre-treatment factors comprise of host factors, such as age, gender, obesity, ethanol consumption, hepatic iron overload, fibrosis, immune responses and co-infection with other viruses, and viral factors that mainly include viral genotypes, particular amino acid (aa) sequence variation within a given genotype and viral load. On-treatment factors are mainly related to the viral kinetics within the first few weeks of treatment (7).

As for the particular aa sequence variation within a given HCV genotype, the IFN sensitivity-determining region (ISDR) in the nonstructural protein 5A (NS5A) was first proposed by Enomoto et al. (5, 6). It was further demonstrated that ISDR and its adjacent sequence was able to bind to double-stranded RNA-activated protein kinase (PKR), one of the important antiviral proteins of the host cell, to inhibit its enzymatic activity and, therefore, the combined region is called PKR-binding domain (PKR-BD) (12, 13). A significant correlation between sequence variation in PKR-BD and IFN responsiveness was also reported (31). However, to date, there is a big controversy surrounding the ISDR theory, as some studies supported it (1, 10, 26) and others did not (4, 27, 34). We have demonstrated in clinical and experimental settings that NS5A plays a certain role(s) in determining IFN resistance and HCV RNA titers in both ISDR-dependent and -independent manners (21, 40, 41). Also, there are some reports that showed a correlation between IFN responsiveness and the sequence diversity of the V3 region or its surrounding regions near the carboxy terminus of NS5A (4, 19, 27, 31, 35, 37, 44). Moreover, a significant correlation between IFN responsiveness and the presence of anti-HCV NS5A antibodies in the pre-treatment sera was observed for patients chronically infected with HCV (9, 10); however, this observation is also controversial (36, 44). Thus, despite the uncertainties and still unknown importance of this viral protein in determining the IFN sensitivity, NS5A does warrant investigation to better understand the possible mechanism of virus-mediated IFN resistance and also to find a predictive factor(s) for efficient viral clearance by IFN. In this study we aimed to define molecular markers within different regions of NS5A that can predict efficient viral clearance during PEG-IFN/RBV therapy. We also examined whether anti-NS5A antibodies in the pre-treatment sera could predict efficient viral clearance.

Materials and Methods

Patients. A total of 47 patients chronically infected with HCV-1b, whose diagnosis had been made based on anti-HCV antibody detection, HCV subtype determination according to the method by Okamoto et al. (32) and clinical follow-up, were treated with PEG-IFN α-2b (1.5 μg per kilogram body weight, once weekly, subcutaneously) and RBV (600–800 mg daily, *per os*), according to a standard treatment protocol for Japanese patients established by a hepatitis study group of the Ministry of Health, Labour and Welfare, at Kobe Asahi Hospital, Hyogo Prefecture, Japan. All the patients were confirmed negative for HBsAg using chemiluminescent immunoassay (Abbott Japan Co., Ltd., Tokyo). Before and during the treatment, serum samples were collected from the patients and tested for HCV RNA by reverse transcription (RT)-PCR at intervals of 4 weeks. The quantification of serum HCV RNA titers was performed by RT-PCR with an internal RNA standard derived from the 5′ non-coding region (5′ NCR) of HCV (Amplicor HCV Monitor test, version 2.0, Roche Diagnostics, Tokyo). The thresholds of the low-range and high-range measurements of this assay were 50 and 600 IU/ml, respectively. HCV core antigen in the sera was also quantitatively measured by chemiluminescent immunoassay (Abbott Japan Co., Ltd.).

The study protocol was approved beforehand by the Ethic Committee in Kobe Asahi Hospital, and written informed consent was obtained from each patient prior to the treatment.

NS5A sequence analysis. HCV RNA was extracted from 140 μl of serum using a commercially available kit (QIAmp viral RNA kit; QIAGEN, Tokyo). For amplification of the NS5A region of the HCV genome, the extracted RNA was reverse transcribed and amplified for full-length NS5A using SuperScript One-step RT-PCR for long templates (Invitrogen, Tokyo) and a set of primers, NS5A-F1 (5′-TACTCCCTGCCATCCCTCTC-CTG-3′; sense, nucleotides [nt] 5974 to 5997) and NS5A-F2 (5′-CTCCTTGAGCACGTCCCGGT-3′; antisense, nt 7796 to 7777). The resultant RT-PCR product was subjected to a second-round PCR by using Platinum *Taq* DNA polymerase (Invitrogen) and an inner set of primers, NS5A-F3 (5′-TCTCCAGCCTTACCAT-

CACYCA-3'; sense, nt 6172 to 6193) and NS5A-F4 (5'-CGGTARTGRTCGTCCAGGAC-3'; antisense, nt 7780 to 7761). The samples that were not amplifiable (nos. 3, 23, 47, 61, 65 and 69) using the aforementioned primers were amplified using primer sets reported previously (21). Reverse transcription was performed at 45 C for 30 min and terminated at 94 C for 2 min, followed by the first-round PCR over 35 cycles, with each cycle consisting of denaturation at 94 C for 30 sec, annealing at 55 C for 30 sec and extension at 68 C for 90 sec. The second-round PCR was performed under the same condition. The amplified fragments were purified with QIA quick PCR purification kit (QIAGEN), and visualized by agarose gel electrophoresis and ethidium bromide staining. Nucleotide sequences of the amplified fragments were determined by direct sequencing without subcloning using Big Dye Deoxy Terminator cycle sequencing kit and ABI 337 DNA sequencer (Applied Biosystems, Inc., Japan). The aa sequences were deduced and aligned using GENETYX. Win software version 7.0 (GENETYX Corp., Tokyo).

Detection of anti-NS5A antibodies. Detection of anti-NS5A antibodies in the pre-treated sera was performed using Western blot analysis. Lysates of Huh-7 cells harboring an HCV-1b subgenomic RNA replicon (pFK5B2884Gly; a kind gift from Dr. R. Bartenschlager, University of Heidelberg, Heidelberg, Germany) (20) were prepared in a buffer containing 50 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% glycerol, 100 mM dithiothreitol and 0.1% bromophenol blue, and were subjected to 10% SDS-polyacrylamide gel electrophoresis (1×10^5 cells equivalent per each lane). Lysates of the parental Huh-7 cells served as a control. The samples were then transferred to nitrocellulose membranes. After being blocked with skimmed milk for 1 hr at room temperature followed by washing with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T), the membranes were incubated with each of the patients' sera (1:100 diluted) for 1 hr, washed three times with PBS-T, and incubated with peroxidase-conjugated goat anti-human IgG (1:1,000 diluted; MBL, Nagoya, Japan) at room temperature for another 1 hr. After being washed three times with PBS-T, the positive bands were visualized by using ECL detection system (Amersham Pharmacia Biotech) according to the manufacturer's instruction.

Statistical analysis. The data obtained were statistically analyzed by Student's t test, χ^2 test and the test for the proportion. A P value of <0.05 was considered statistically significant.

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB285035 through AB285081.

Results

Time Course of Viral Clearance and Determination of Early Virological Responders (EVR)

Therapeutic responses of PEG-IFN/RBV-treated patients were monitored at intervals of 4 weeks. The number of patients who cleared HCV from the sera increased with time and reached nearly a plateau level at week 16 after the initiation of the PEG-IFN/RBV therapy (Table 1). Based on this viral clearance kinetics, we provisionally chose week 16 for determination of efficient viral clearance and, accordingly, the patients were classified into early virological responders at week 16 (EVR[16w]) and Non-EVR(16w).

Demographic characteristics of EVR(16w) and Non-EVR(16w) are summarized in Table 2. The mean titer of HCV core antigen was 1.9-times higher for Non-EVR(16w) than that for EVR(16w) although the difference was not statistically significant (P=0.17). HCV RNA titers measured by RT-PCR were almost the same between the two groups. Also, age, gender, body weight, platelet counts, hemoglobin levels, ALT or γ-GTP titers did not significantly differ between EVR(16w) and Non-EVR(16w).

Sequence Analysis of the V3 Region and Its Adjacent Regions of NS5A Obtained from Pre-Treated Sera

The entire NS5A region of the HCV genome was amplified from the pre-treated sera, and the aa sequences deduced. We first determined the consensus sequence by aligning the sequences obtained from all 47 patients and then compared each NS5A sequence to the consensus sequence. A tendency was noted that the number of aa substitutions in the entire NS5A obtained from EVR(16w) was greater than that from Non-EVR(16w) (Table 3). There was no difference in the number of mutations in an N-terminal half of NS5A (aa

Table 1. Time course of the virological response of the patients during PEG-IFN/RBV combination therapy

Week[a]	No. of responders[b] / no. of total (%)
4	6 / 47 (12.8)
8	13 / 47 (27.7)
12	23 / 47 (48.9)
16	31 / 47 (66.0)
20	31 / 47 (66.0)
24	34 / 47 (72.3)

[a] Weeks after initiation of PEG-IFN/RBV combination therapy.
[b] Responders are those who cleared HCV from the sera.

Table 2. Demographic characteristics of EVR(16w) and Non-EVR(16w) patients

Factor	EVR(16w)	Non-EVR(16w)	*P* value
Age	56.0 ± 10.5[a]	59.6 ±9.6	NS[b]
Sex (male/female)	17 / 14	12 / 4	NS
Body weight (kg)	58.3 ± 11.6	63.7 ± 10.9	NS
Platelets ($\times 10^4$/mm^3)	17.3 ± 4.9	14.4 ± 5.4	NS
Hemoglobin (g/dl)	14.1 ± 1.5	14.0 ± 1.6	NS
ALT (IU/liter)	35.3 ± 25.2	44.6 ± 34.2	NS
γ-GTP (IU/liter)	39.0 ± 27.7	53.4 ± 36.1	NS
HCV-RNA (kIU/ml)	1,455 ± 1,195	1,689 ± 1,407	NS
HCV core antigen (fmol/liter)	6,349 ± 6,066	11,879 ± 14,902	NS

[a] Mean ± S.D.
[b] NS, not significant.

Table 3. The number of aa mutations within different regions of NS5A of the HCV genome obtained from pre-treated sera of EVR(16w) and Non-EVR(16w)

NS5A region	EVR (16w)	Non-EVR (16W)	*P* value
Full-NS5A (aa 1972–2419)	22.9 ± 5.4[a]	19.6 ± 4.5	<0.05
N-half (aa 1972–2208)	8.7 ±2.0	9.1 ± 2.6	NS[b]
ISDR (aa 2209–2248)	1.7 ± 2.4	1.3 ± 1.3	NS
PKR-BD (aa 2209–2274)	3.3 ± 3.0	2.8 ± 2.1	NS
Pre-V3 (aa 2334–2355)	1.1 ± 1.2	0.3 ± 0.5	<0.05
V3 (aa 2356–2379)	4.4 ± 1.5	3.0 ± 1.5	<0.01
IRRDR (aa 2334–2379)	5.5 ± 2.0	3.3 ± 1.6	<0.001

[a] Mean ± S.D.
[b] NS, not significant.

Table 4. Correlation between NS5A sequence variation, Anti-NS5A antibodies and EVR(16w)

Criteria	No. of subjects / no. of subtotal [a] (%)		*P* value
	EVR(16w)	Non-EVR(16w)	
V3 mutation ≥5	15 / 31 (48.4)	2 / 16 (12.5)	<0.05
IRRDR mutation ≥6	17 / 31 (54.8)	1 / 16 (6.3)	<0.01
Thr^{2378}	12 / 31 (38.7)	0 / 16 (0.0)	<0.01
Anti-NS5A (+)	22 / 31(71.0)	2 / 16 (12.5)	<0.001

[a] Subtotal no. of EVR(16w) or Non-EVR(16w).

1972 to 2208), the ISDR (aa 2209 to 2248) or the PKR-binding domain (aa 2209 to 2274) between EVR(16w) and Non-EVR(16w). Instead, a significant difference was observed in the V3 region (aa 2356 to 2379), with the mean ± S.D. for EVR(16w) being 4.4 ± 1.5 while that for Non-EVR(16w) 3.0 ± 1.5 ($P<0.01$). We also found a significant difference between the two groups in a V3-upstream region, which we refer to as the pre-V3 region (aa 2334 to 2355). When these two regions were combined, the difference between EVR(16w) and Non-EVR(16w) became more apparent, with the mean ± S.D. being 5.5 ± 2.0 and 3.3 ± 1.6 for EVR(16w) and Non-EVR(16w), respectively ($P<0.001$). We hereafter refer to this combined region as IFN/RBV resistance-determining region (IRRDR).

Only 2 (12.5%) of 16 Non-EVR(16w) had HCV with 5 or more (≥5) mutations in the V3 region whereas 15 (48.4%) of 31 EVR(16w) did (Table 4). The difference between EVR(16w) and Non-EVR(16w) was statistically significant ($P<0.05$). Similarly, only one (6.3%) of 16 Non-EVR(16w) and 17 (54.8%) of 31 EVR(16w) had HCV with ≥6 mutations in IRRDR, with the difference between the two groups being statistically significant ($P<0.01$). It should be noted that, at later time points as well, such as weeks 20 and 24, strong correlation was observed between the virus clearance rate and the sequence variation in the V3 region and IRRDR (data not shown). No correlation was observed between the initial viral load and the number of mutations in the V3 region or IRRDR.

	2334	IRRDR	2379	
	2334	Pre-V3 2355 2356 V3	2379	
Cons	VLTESTVSSALAELATKTFGSSGSSAVDSGTATAPPDQASDDGDKG			
EVR(16w)				**Anti-NS5A**
9	.....E...A.....GLP.....A.			+
10	.....A.....P..A.G..TA.....T.....A.			+
12	I.....A.....EPPG...T.			+
14	.....V.....D.E...A...M.....R.			+
15	..S.....E.....S...P..G..A.			-
18	I.....AT.....S.SK..T.			-
19	..S.....E.....S...P.....A.			+
20	I.....GP.....T.			+
21	.....E...A.....G..P.			+
22	.....P..F..T.			+
23	I.....E.....NE.....A.			-
24	I.....N.....A.....HT.P.....T.			+
25	I..D.....A....D.....A.....HT.P.....T.			-
28	.....P.....T.			+
32	I..D.N..T.....E.....P.....T.			+
35	.....G.....A.....			-
37	.....E..T.....G.L.....N.E			+
38	.....G....P.....T.			+
39	.....E.....L...SL....A.			+
43	.....T.....E.....P.....A.			+
44	I.....A.....H.HS..T..A.			+
45	.....E.....L..V...C...			+
47	.....T.....E			+
57	I.....A...E.....SRP.....T.			-
61	.....E.....YLL....GT.			-
65	...G....TV.....EPP.A.....RP.....A.			+
67	.....E...A.....L.G.....			+
69	.....L.....A...M.....N..AR			-
71	I..D.....V.P.....A.			+
72	I.....R.....A.....GRP.....TE			+
73	.....L.....D.....S.....P.N...A.			-
Non-EVR(16w)				
3	I.....NL.....A.			-
5	.....S.....E...A.....T....GRE			+
6	.....S.....I.....Q.			-
8	.....T.S.G.S..E			-
11	..S.....G.V.....G.....			-
13	I.....P.....A.			+
16	.....S...G.....E			-
17	.....E.....A.			-
27	.....N.L..E..A.			-
30	.....E.....E			-
31	.....E.....			-
33	.....			-
42	.....E.....PP.....A.			-
49	.....A.....P.....A.			-
52	.....E.....S.G...A.			-
59	.....E.....P....GA.			-

Fig. 1. Sequence alignment of IRRDR (pre-V3 and V3 regions) of NS5A of HCV-1b obtained from the pre-treatment sera. The consensus sequence is shown on the top (Cons). The numbers along the sequence indicate aa positions. Dots indicate residues identical to those of the consensus sequence. Thr^{2378} is written in boldface Result of anti-NS5A antibodies detection is also shown on the right.

The IRRDR sequences obtained from all 47 patients were aligned along with the consensus sequence (Fig. 1). We noticed that 12 (38.7%) of 31 EVR(16w) had threonine at position 2378 (Thr^{2378}) whereas none of 16 Non-EVR(16w) did ($P<0.01$) (Table 4).

Sequence Analysis of ISDR and PKR-BD of N55A Obtained from Pre-Treated Sera

As described above, there was no difference in the mean number of mutations in ISDR or PKR-BD between EVR(16w) and Non-EVR(16w) (Table 3). Only 4 patients had HCV with ≥4 mutations in ISDR (Fig. 2), the criteria for IFN-sensitive HCV strains according to the criteria reported by Enomoto et al. (5, 6). Although there appeared to be a trend for patients with HCV having ≥4 mutations in ISDR toward EVR(16w) (3 of 4 patients), the difference was not statistically significant. Also, the prevalence of HCV with ≥4 mutations in ISDR was not significantly different between EVR(16w) (3 of 31 patients; 9.7%) and Non-EVR(16w) (1 of 16 patients; 6.3%). It would be interesting to note, however, that all 3 HCV strains with ≥4 mutations in ISDR obtained from EVR(16w) (nos. 10, 65 and 72) had ≥6 mutations in IRRDR, while the only one strain with ≥4 mutations in ISDR from Non-EVR(16w) (no. 13) had only 3 mutations in IRRDR (Figs. 1 and 2).

Detection of Anti-NS5A Antibodies in the Pre-Treatment Sera

It was previously reported that the presence of anti-NS5A antibodies in the pre-treatment sera was correlated well with IFN responsiveness (9, 10); however, this observation is controversial (36, 44). Therefore, we examined whether or not the patients had detectable levels of anti-NS5A antibodies in the sera before PEG-IFN/RBV treatment. Consistent with the previous observations, 22 (71.0%) of 31 EVR(16w) patients had detectable levels of anti-NS5A antibodies whereas only 2 (12.5%) of 16 Non-EVR(16w) did, with the difference between the two groups being statistically significant ($P<0.001$) (Table 4). Representative results of anti-NS5A-positive and -negative sera are shown in Fig. 3. Thus, there was a strong correlation between the presence of anti-NS5A antibodies and PEG-IFN/RBV responsiveness assessed by EVR(16w).

Proposed Predictive Factors for EVR and Non-EVR

Table 5 summarizes proposed predictive factors for EVR(16w) or Non-EVR(16w). The positive predictive value (PPV) of a factor is the proportion (%) of patients with the factor who have achieved the outcome of interest (either EVR[16w] or Non-EVR[16w]) while the negative predictive value (NPV) is the proportion (%) of patients without the same factor who have not achieved the outcome of interest. High degrees of sequence variation in the V3 region (≥5) and IRRDR (≥6) of HCV NS5A could predict EVR(16w) with PPV of 88.2% ($P<0.01$) and 94.4% ($P<0.0001$), respectively. Similarly, the presence of anti-NS5A antibodies could be a useful predictive factor for EVR(16w), with PPV being 91.7% ($P<0.0001$).

It should be noted that the NPV for the low degree of sequence variation in V3 (≤4) and IRRDR (≤5) alone or the absence of detectable anti-NS5A antibodies alone did not reach a statistically significant level and, therefore, cannot be used as a predictive factor for Non-EVR(16w). On the other hand, the absence of detectable levels of anti-NS5A antibodies together with the low degree of sequence variation in either V3 (≤4) or IRRDR (≤5) could be a useful predictive factor for Non-EVR(16w), with PPV being 81.3% ($P<0.05$) and 87.5% ($P<0.01$), respectively (Table 5).

Viral clearance kinetics at intervals of 4 weeks for HCV-1b isolates with and without the predictive factors is shown in Fig. 4A (≥5 mutations in V3), 4B (≥6 mutations in IRRDR), 4C (the presence of anti-NS5A antibodies) and 4D (the absence of anti-NS5A antibodies plus ≤4 mutations in V3 or ≤5 mutations in IRRDR). High degrees of sequence variation in V3 (≥5) and IRRDR (≥6) were significantly associated with early viral clearance not only at week 16 but also at weeks 12, 20 and 24. Similarly, significant correlation between the presence of anti-NS5A antibodies and early viral clearance at week 16 and thereafter was observed (Fig. 4C). On the other hand, the absence of anti-NS5A antibodies together with lower degrees of mutation in V3 (≤4) or IRRDR (≤5) was significantly associated with persistent HCV viremia at week 16 and thereafter (Fig. 4D).

Discussion

The introduction of IFN/RBV combination therapy was an important breakthrough in the treatment of chronic HCV infection (2, 23). However, the rate of sustained virological response is still unsatisfactory (~50%), particularly in patients infected with HCV genotype 1 (11, 22). Given the significant side effects and high cost associated with the IFN/RBV therapy for a period of 48 weeks (29), it would be beneficial if one could predict ahead of time which patients would, or would not, respond to the therapy. Most of the recent studies have focused on the possible correlation between EVR and viral clearance kinetics during the first few months of the treatment (8, 14, 15, 24). On the