# Diverse efficacy of vaccination therapy using the α-fetoprotein gene against mouse hepatocellular carcinoma

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**Abstract.** Antitumor vaccination therapy approaches using naked plasmid DNA or recombinant viruses encoding tumor-associated antigens are currently in development. In the present study, we examined the therapeutic efficacy of vaccination using the mouse α-fetoprotein (AFP) gene in mouse hepatocellular carcinoma (HCC) cells. C57L/J or C3H/HeN mice were primed with an injection of naked plasmid DNA expressing mouse AFP followed by a booster of replication-defective adenovirus expressing mouse AFP (plasmid-AFP prime/adenovirus-AFP booster vaccination). The mice were then challenged with high AFP-producing Hepa1-6 cells or low AFP-producing MH134 cells, respectively, and the tumor growth rate was monitored. Plasmid-AFP prime/adenovirus-AFP booster vaccination promoted protective immunity against Hepa1-6 cells, and significantly increased the number of interferon-γ-producing splenic cells in C57L/J mice. In addition, this vaccination protocol repressed the growth of pre-established Hepa1-6 tumors in C57L/J mice. However, plasmid-AFP prime/adenovirus-AFP booster vaccination did not induce protective immunity against MH134 cells in C3H/HeN mice. These results suggest that vaccination with the AFP gene is a promising strategy to treat HCC, but its outcome may be affected by the level of AFP expression in HCC or by the immunological response of the host.

## Introduction

Hepatocellular carcinoma (HCC) is one of the most common fatal malignancies worldwide, and is especially common in several parts of Asia and Africa (1). Although advances in medical technology have permitted the early recognition and treatment of HCC (2,3), the 5-year survival rate for HCC patients has barely reached 40% and the annual death rate from HCC exceeds 30,000 in Japan (3,4). Therefore, there is an urgent need to develop new approaches to treat HCC.

In recent years, increasing efforts have been made to use antitumor vaccination strategies, including genetically modified tumor cells (5,6), dendritic cells (DC) that are either pulsed or transduced with tumor-associated antigens (7-9), synthetic peptides containing tumor-specific epitopes (10,11), and naked plasmid DNA or recombinant viruses encoding tumor-associated antigens (12,13). All of these antitumor vaccination approaches aim to induce specific immunological responses to tumor-associated antigens, resulting in the destruction of tumor cells and the protection of patients from relapses.

α-fetoprotein (AFP) is an oncofetal protein that is expressed in fetal liver and down-regulated after birth, but it is frequently re-expressed in HCC (14). Because of its specific expression in HCC, the enhancer/promoter region of the AFP gene has been used in gene therapy approaches to HCC (15-18). Recently, it has been reported that AFP-specific immune responses can be detected in patients with AFP-expressing HCC (19). This finding suggests that AFP is a possible target for HCC-specific vaccination therapy. Vollmer *et al* first reported that vaccination in mice with dendritic cells (DC) transduced with the AFP gene generated AFP-specific and protective immunity against lymphoma cells expressing an exogenous AFP gene (20). In addition, using a similar model, the same group reported that vaccination with an AFP-expressing naked plasmid followed by a booster vaccination with an AFP-expressing adenovirus also promoted AFP-specific and protective immunity, but that vaccination with a naked plasmid alone did not (21). In contrast, Grimm *et al* (22) reported that vaccination with AFP-expressing naked plasmid DNA together with plasmids expressing interleukin-12 (IL-12) and granulocyte-macrophage colony stimulating factor (GM-CSF) significantly repressed the growth of pre-established Hepa1-6 hepatoma, which produces

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large amounts of AFP endogenously. Taken together, these results suggest that immunotherapy with the AFP gene could be a possible approach to the treatment of HCC.

Clinically, the amount of AFP production in each HCC is different, and the immunological background in each patient is not the same. Therefore, in the present study, we examined whether combined vaccination with naked DNA and adenovirus expressing AFP, which is reported to promote antitumor immunity against AFP-expressing lymphoma cells, can induce effective antitumor immunity in two different mouse hepatoma models. One of these used C57L/J mice inoculated with highly AFP-producing Hepa1-6 cells, while the other used C3H/HeN mice inoculated with low AFP-producing MH134 cells.

## Materials and methods

*Mice and cell lines.* C57L/J mice and C3H/HeN mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and Charles River Japan (Osaka, Japan), respectively. All mice were kept in the animal facility of the Nagasaki University and handled in accordance with guidelines for animal experimentation. Mice aged 6-8 weeks old were used for experiments. The mouse hepatoma cells, Hepa1-6 and MH134, and the human HeLa cervical cancer cell line were maintained in DMEM (Life Technologies Inc., San Diego, CA) supplemented with 10% fetal bovine serum (FBS).

*Plasmid and adenoviral vectors.* The mouse AFP (mAFP) cDNA clone, MAFP (B557), was a generous gift from Professor Shinzo Nishi (Hokkaido University, Sapporo, Japan). To construct an mAFP expression plasmid vector, pmAFP, the mAFP cDNA was inserted into an expression vehicle, pBudCE4, that contained the cytomegalovirus (CMV) promoter (Invitrogen, San Diego, CA). Plasmid vectors expressing mouse GM-CSF, pRx-mGHCSF-bsr, and macrophage colony stimulating factor (M-CSF), pCAhMCSF, were purchased from the RIKEN GeneBank (Tsukuba, Japan). All plasmids were prepared using an EndoFree Mega Prep Kit (Qiagen, Tokyo, Japan) to eliminate endotoxin contamination. The mAFP expression adenoviral vector, AdmAFP, was constructed using an *in vitro* ligation method that was recently established (23). In brief, mAFP cDNA was inserted into a shuttle plasmid vector, pHMCMV6, which contains the CMV promoter, and digested with I-CeuI and PI-SceI. The resulting fragment was inserted into an I-CeuI/PI-SceI-digested adenoviral plasmid vector, pAdHM4, which contains a complete E1/E3-deleted adenovirus type 5 genome. The resultant AFP-expressing adenoviral plasmid vector was digested with PacI, and transfected into 293 human embryonal kidney (HEK) cells to yield AdmAFP. The pHMCMV6 and pAdHM4 plasmids were kindly provided by Dr H. Mizuguchi (National Institute of Health Sciences, Tokyo, Japan). Adenovirus was propagated in HEK293 cells and purified using two rounds of CsCl gradient centrifugation.

*AFP detection.* HeLa cells were transfected with pmAFP by the lipofection method using Superfect (Qiagen, Tokyo, Japan) or infected with AdmAFP. These cells were lysed and subjected to Western blotting using a goat polyclonal anti-mAFP antibody (also provided by Professor Shinzo Nishi) and a horseradish peroxidase-labelled anti-goat antibody (Chemicon International Inc., Temecula, CA). The lysates from Hepa1-6 and MH134 cells were also analyzed for the presence of AFP.

The total RNA from HeLa cells transfected with pmAFP or infected with AdmAFP and that from MH134 cells was extracted using the guanidium isothiocyanate method and subjected to reverse transcription PCR (RT-PCR). The primers used for the PCR were 5'-TCCAGGCAACAACCAT TATTA-3' and 5'-TTTCCTCGTGTAACCAATAAG-3'.

*Immunization protocol.* pmAFP, pCAhMCSF (pM-CSF) and pRx-mGHCSF-bsr (pGM-CSF) were diluted in PBS/25% sucrose to a concentration of 100 μg/100 μl and injected into the right posterior tibialis muscle of the mice. Three days before injection of the plasmids, 12.5% bupivacaine (100 μl) was injected to induce muscle inflammation for efficient gene expression (24). Two weeks after injection of the plasmids, AdmAFP was diluted in PBS to a concentration of $1x10^9$ pfu/100 μl and injected into the same location as a booster vaccination.

*In vivo studies.* Tumor challenge was performed 2 weeks after the last immunization. Hepa1-6 cells ($1x10^7$ cells/mouse) and MH134 cells ($5x10^5$ cells/mouse) were suspended in 100 μl of serum-free medium and were subcutaneously (s.c.) injected into the right flank of C57L/J mice and C3H/HeN mice, respectively, and the tumor growth rate was monitored. The tumor was measured in two dimensions, and its size was calculated using the formula; (width$^2$ x length)/2. Alternatively, Hepa1-6 cells ($1x10^7$ cells/mouse) were s.c. injected into the right flank of control mice. Seven days after tumor inoculation, the immunization protocol was started.

*ELISPOT assay.* An ELISPOT assay was performed to measure the number of splenic cells secreting IFN-γ, as described previously (25). Briefly, 1 week after the last immunization, the C57L/J or C3H/HeN mice were sacrificed, and their splenic cells were harvested. The isolated splenic cells were incubated for 2 days with RPMI-1640 medium (Life Technologies Inc., San Diego, CA) containing10% FBS, 10 IU/ml of recombinant mouse IL-2 (R&D systems, Minneapolis, MN) and 50 μM of 2-mercaptoethanol. These splenic cells were then co-cultured with 20 Gy irradiated Hepa1-6 cells or MH134 cells. Twenty-four hours later, an ELISPOT assay was performed with the mouse IFN-γ ELISPOT assay kit (R&D systems) according to the manufacturer's instructions.

*Statistical analysis.* The statistical significance in all the experiments was calculated using an unpaired Student's t-test.

## Results

*Detection of AFP.* A Western blot analysis was performed to determine the levels of AFP expression in HeLa cells transfected with pmAFP or infected with AdmAFP, as well as the endogenous levels of AFP expression in Hepa1-6 and MH134 cells. As shown in Fig. 1A, HeLa cells infected

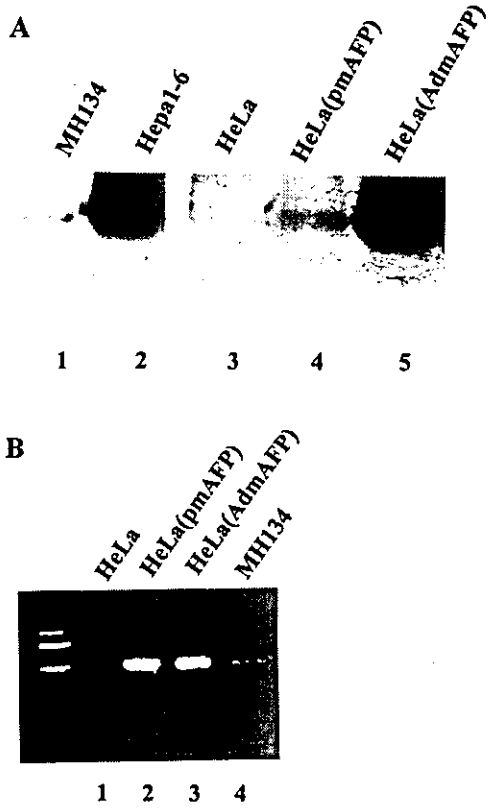


Figure 1. AFP expression in HeLa cells transduced with the mAFP gene and in mouse hepatoma cells. (A), HeLa cells were transfected with (lane 4) or without (lane 3) pmAFP or infected with AdmAFP at a multiplicity of infection (MOI) of 100 (lane 5), and a Western blot analysis was performed using anti-mAFP antibody. Cell lysates from Hepa1-6 cells (lane 1) and MH134 cells (lane 2) were also analyzed. (B), HeLa cells were transfected with (lane 2) or without (lane 1) pmAFP or infected with AdmAFP at a MOI of 100 (lane 3), and RT-PCR was performed to detect AFP mRNA expression. Total RNA from MH134 cells (lane 4) was also subjected to RT-PCR.

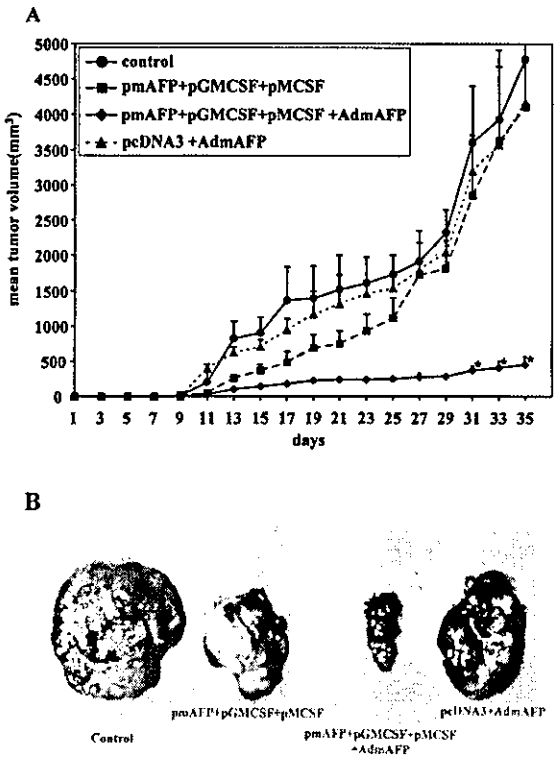


Figure 2. Effect of plasmid-AFP prime/adenovirus-AFP booster immunization on the growth of Hepa1-6 cells in C57L/J mice. (A), pmAFP (100 μg/mouse), pGM-CSF (50 μg/mouse) and pM-CSF (50 μg/mouse) were injected into two groups of C57L/J mice. pcDNA3, a vehicle plasmid, was injected into one group of mice. Two weeks later, AdmAFP ($1x10^9$ pfu/mouse) was injected into two groups of mice as a booster immunization. Control mice were not given any immunization. Two weeks after the last immunization, Hepa1-6 cells ($1x10^7$ cells/mouse) were s.c. inoculated into the right flank of the mice. Tumor volume was determined as described in Materials and methods. Data are expressed as mean ± SE (n=6). *$p<0.05$ versus other groups of mice. (B), A representative Hepa1-6 tumor in each group that was removed at the end of study (day 35).

with AdmAFP produced large amounts of AFP, but those transfected with pmAFP produced lesser amounts of AFP. Hepa1-6 cells produced more abundant amounts of AFP than MH134 cells, as described previously (22). In addition, the expression of AFP mRNA in HeLa cells transduced with the mAFP gene and in MH134 cells was confirmed by RT-PCR (Fig. 1B).

*Plasmid-AFP prime/adenovirus-AFP booster vaccination generates protective immunity against Hepa1-6 cells in C57L/J mice, but not against MH134 cells in C3H/HeN mice.* We determined whether AFP plasmid vaccination with or without an AFP adenovirus booster could promote protective immunity against Hepa1-6 and MH134 cells in C57L/J and C3H/HeN mice, respectively (Figs. 2 and 3). These mice were primed with an injection of pmAFP, pM-CSF and pGM-CSF, or with pcDNA3 (vehicle) alone, followed 2 weeks later by a booster of AdmAFP, or the lack thereof. Another 2 weeks later, the C57L/J and C3H/HeN mice were challenged with Hepa1-6 and MH134 cells, respectively, and the tumor growth rate was monitored. Two of the six C57L/J mice immunized with a pmAFP prime/AdmAFP booster were completely protected from the challenge of Hepa1-6. In the remaining four mice, the growth of Hepa1-6 was significantly delayed, compared with the other groups ($p<0.05$). However, vaccination with pmAFP, pM-CSF and pGM-CSF or AdmAFP alone did not induce such protective immunity against Hepa1-6 cells in C57L/J mice (Fig. 2A). A representative Hepa1-6 tumor in each group, which was removed at the end of study, is shown in Fig. 2B. In contrast, no protective immunity against MH134 cells was observed in C3H/HeN mice using any vaccination protocol, including pmAFP prime/AdmAFP booster vaccination (Fig. 3).

*Plasmid-AFP prime/adenovirus-AFP booster vaccination increases the frequency of IFN-γ-producing splenic cells in C57L/J mice.* The frequency of IFN-γ-producing splenic cells from immunized C57L/J mice was determined using an ELISPOT assay (Fig. 4). Similarly to the *in vivo* study (Fig. 2A), the frequency of IFN-γ-producing splenic cells

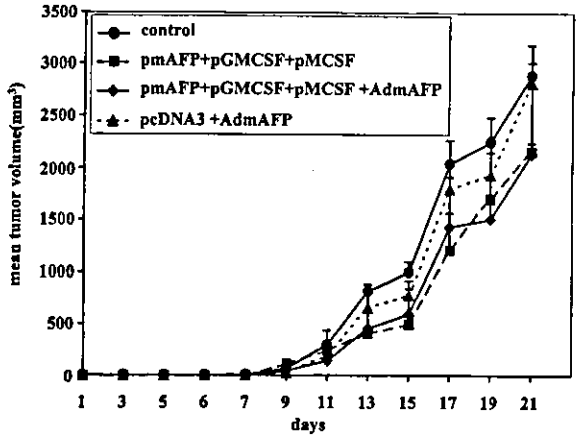


Figure 3. Effect of plasmid-AFP prime/adenovirus-AFP booster immunization on the growth of MH134 cells in C3H/HeN mice. C3H/HeN mice were immunized as described in the Fig. 2 legend. Two weeks after the last immunization, MH134 cells ($5x10^5$ cells/mouse) were s.c. inoculated into the right flank of C3H/HeN mice. Tumor volume was determined as described in Materials and methods. Data are expressed as mean ± SE (n=6).

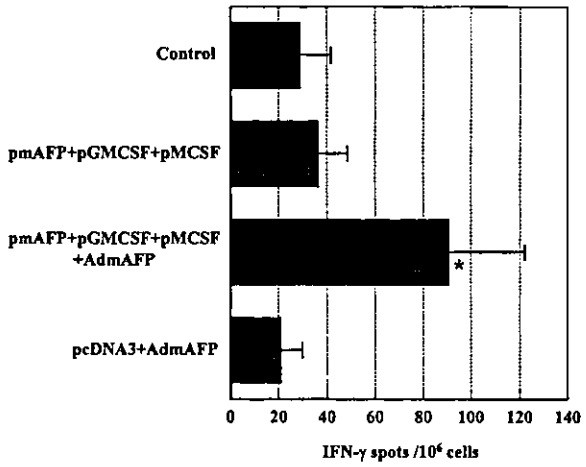


Figure 4. IFN-γ ELISPOT assay. Splenic cells derived from immunized C57L/J mice or control mice were cultured with irradiated Hepa1-6 cells for 24 h, and a mouse IFN-γ ELISPOT assay was performed. The spots in each well were counted under a microscope. Values are expressed as the number of spot-forming cells relative to the number of spleen cells added to each well at the start of the culture. Data are expressed as mean ± SD (n=4).

from C57L/J mice immunized with a pmAFP prime/AdmAFP booster was significantly higher than that using other protocols ($p<0.05$). These results suggested that immunization of C57L/J mice using the pmAFP prime/AdmAFP booster vaccination might elicit T-cell responses to Hepa1-6 cells. We also performed similar experiments using splenic cells from immunized C3H/HeN mice, but could not detect similar responses (data not shown).

*Plasmid-AFP prime/adenovirus-AFP booster vaccination shows an antitumor effect against pre-established Hepa1-6 cells in C57L/J mice.* Finally, we elucidated the antitumor effect of immunization with a pmAFP prime/AdmAFP booster against pre-established Hepa1-6 tumors. As shown in Fig. 5, the growth of Hepa1-6 tumors was significantly retarded by immunization with a pmAFP prime/AdmAFP booster, although rejection of the Hepa1-6 tumor was not observed.

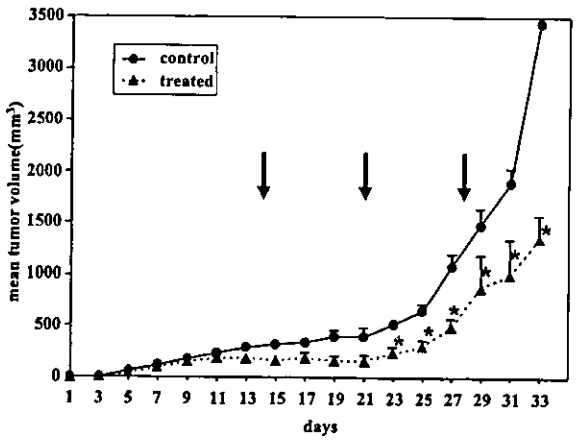


Figure 5. Antitumor effect of pmAFP prime/AdmAFP booster immunization on pre-established Hepa1-6 tumors in C57L/J mice. Hepa1-6 cells ($1x10^7$ cells/mouse) were s.c. inoculated into the right flank of C57L/J mice. Seven days after tumor inoculation, the mice were immunized with pmAFP (100 μg/mouse), pGM-CSF (50 μg/mouse) and pM-CSF (50 μg/mouse). At this point, the tumors had reached an average volume of 100 $mm^3$. At days 14, 21 and 28, AdmAFP ($1x10^9$ pfu/mouse) was injected into the mice as a booster immunization (arrow). Control mice were not given any immunization. Tumor volume was determined as described in Materials and methods. Data are expressed as mean ± SE (n=6). *$p<0.05$ versus control.

## Discussion

In the present study, we injected naked plasmids expressing M-CSF and GM-CSF together with an AFP-expressing plasmid as the primary vaccination protocol. This was based on the observations that co-immunization of M-CSF and GM-CSF genes with the HIV-1 genome enhanced the CTL response against HIV-1 (26), and that Hepa1-6 cells transduced with the membrane form of the M-CSF gene were rejected and this led to CTL immunity in C57L/J mice (27). However, in our experiments, single (Fig. 2) and even repeated vaccination (data not shown) with plasmids expressing AFP, M-CSF and GM-CSF failed to induce protective immunity against Hepa1-6 tumors in C57L/J mice. Similar results suggesting that vaccination using naked plasmids expressing AFP and GM-CSF cannot induce protective immunity against lymphoma cells expressing exogenous AFP in C57BL/6 mice have been reported (20). In contrast to these observations, Grimm *et al* (22) have reported that vaccination with a plasmid encoding AFP and plasmids expressing IL-12 and GM-CSF led to rejection of pre-established Hepa1-6 tumors in C57L/J mice. IL-12 is known to stimulate NK cells, promote maturation of CTL, differentiate the Th1-type immune response and induce antiangiogenic effects (28,29). Since we used M-CSF, rather than IL-12, this may in part explain the difference between our results and those of

Grimm *et al*. Taken together, the kinds of cytokine or growth-factor genes that are chosen for co-immunization with the tumor-associated antigen gene could be crucial in achieving a favorable outcome for antitumor vaccination therapy.

A booster vaccination with adenovirus-AFP, in addition to the primary plasmid-AFP vaccination, generated protective immunity against Hepa1-6 tumors in C57L/J mice, as shown in a previous report using an AFP-expressing lymphoma model (21), but adenovirus-AFP vaccination alone did not promote such immunity. In addition, the adenovirus-AFP booster vaccination in C57L/J mice significantly increased the frequency of IFN-γ-producing splenic cells, probably including T-cells, but an adenovirus-AFP vaccination alone did not achieve a similar result. These results suggest that a booster immunization with adenovirus-AFP greatly enhances the immunological responses against Hepa1-6 cells in C57L/J mice and that the plasmid-AFP primary vaccination is needed prior to the adenovirus booster. Furthermore, this vaccination protocol significantly repressed the growth of pre-established Hepa1-6 tumors, which are commonly used as a model in clinical vaccination therapy. Recently, Nagayama *et al* reported a similar observation whereby the repeated injection of adenovirus expressing the thyrotropin receptor, but not that of plasmid DNA encoding the same receptor, successfully induced Graves' hyperthyroidism in mice (30). At present, it is not clear why an adenovirus booster is superior to a plasmid DNA booster. However, Ambriovic *et al* have suggested that the injection of adenovirus rather than plasmid DNA induces a local inflammation and stimulates cytokine production, which provides a benefit for immunization efficacy (31). In addition, as shown in Fig. 1A, the level of AFP expression was much greater in the adenovirus infected cells than in the plasmid DNA transfected cells. Therefore, the higher level of AFP expression with adenovirus injection, compared to injection of naked plasmid DNA, may account for the advantages shown by the adenovirus booster.

In contrast, plasmid-AFP prime/adenovirus-AFP booster vaccination could not induce protective immunity against MH134 tumors in C3H/HeN mice. This vaccination protocol also failed to increase the frequency of IFN-γ-producing splenic cells in C3H/HeN mice (data not shown). Since MH134 cells produce a much lower level of AFP than Hepa1-6 cells (Fig. 1A), the AFP epitope may not be presented adequately to stimulate a T-cell response, although MH134 cells express class 1 MHC molecules (32) to the same extent as Hepa1-6 cells (22,33). In addition, Ribas *et al* recently reported that C3H/HeN mice, but not C57BL/6 mice, receiving multiple vaccinations with DCs expressing the MART-1 tumor antigen show decreased protection against melanoma, which is associated with a change from a type 1 to a type 2 cytokine response in C3H/HeN mice (34). Taken together, these observations and our results suggest that it may be possible that the plasmid-AFP prime/adenovirus-AFP booster vaccination induces a similar phenomenon in C3H/HeN mice, through which the protective immunity against MH134 cells was diminished.

In conclusion, we have shown that immunotherapy using a plasmid/adenovirus expressing AFP induced an effective antitumor immunity against highly AFP-producing Hepa1-6 cells in C57L/J mice, but not against low AFP-producing MH134 cells in C3H/HeN mice. These results suggest that although AFP-mediated vaccination therapy is a promising approach to treat HCC, its efficacy is probably dependent on the level of AFP expression in HCC or on the immunological response of patients.

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## References

1. Di Bisceglie A, Carithers RJ and Gores GJ: Hepatocellular carcinoma. Hepatology 28: 1161-1165, 1998.
2. Sato Y, Nakata K, Kato Y, Shima M, Ishii N, Koji T, Taketa K, Endo Y and Nagataki S: Early recognition of hepatocellular carcinoma based on altered profiles of α-fetoprotein. N Engl J Med 328: 1802-1806, 1993.
3. Predictive factors for long term prognosis after partial hepatectomy for patients with hepatocellular carcinoma in Japan. Cancer 74: 2772-2780, 1994.
4. Annual report of disease-related death in Japan. J Health Welfare Stat 41: 47-60, 1994.
5. Wang Q, Yu H, Zhang L, Ju D, Pan J, Xia D, He L, Wang J and Cao X: Vaccination with IL-18 gene-modified, superantigen-coated tumor cells elicits potent antitumor immune response. J Cancer Res Clin Oncol 127: 718-726, 2001.
6. Kinoshita Y, Kono T, Yasumoto R, Kishimoto T, Wang CY, Haas GP and Nishisaka N: Antitumor effect on murine renal cell carcinoma by autologous tumor vaccines genetically modified with granulocyte-macrophage colony-stimulating factor and interleukin-6 cells. J Immunother 24: 205-211, 2001.
7. Nakamura M, Iwahashi M, Nakamori M, Ueda K, Matsuura I, Noguchi K and Yamaue H: Dendritic cells genetically engineered to simultaneously express endogenous tumor antigen and granulocyte macrophage colony-stimulating factor elicit potent therapeutic antitumor immunity. Clin Cancer Res 8: 2742-2749, 2002.
8. Wu J, Wang XH, Yang TC, Xian J and Zheng WL: Dendritic cells transfected with carcinoembryonic antigen-vaccinia recombinant virus induces CEA-specific immunity mediated by cytotoxic T lymphocytes *in vitro*. Di Yi Jun Yi Da Xue Xue Bao 22: 256-258, 2002.
9. Heiser A, Coleman D, Dannull J, Yancey D, Maurice MA, Lallas CD, Dahm P, Niedzwiecki D, Gilboa E and Vieweg J: Autologous dendritic cells transfected with prostate-specific antigen RNA stimulate CTL responses against metastatic prostate tumors. J Clin Invest 109: 409-417, 2002.
10. Fisk B, Blevins TL, Wharton JT and Ioannides CG: Identification of an immunodominant peptide of HER-2/neu protooncogene recognized by ovarian tumor-specific cytotoxic T lymphocyte lines. J Exp Med 181: 2109-2117, 1995.
11. Salgaller ML, Weber JS, Koenig S, Yannelli JR and Rosenberg SA: Generation of specific anti-melanoma reactivity by stimulation of human tumor-infiltrating lymphocytes with MAGE-1 synthetic peptide. Cancer Immunol Immunother 39: 105-116, 1994.
12. Mendiratta SK, Thai G, Eslahi NK, Thull NM, Matar M, Bronte V and Pericle F: Therapeutic tumor immunity induced by polyimmunization with melanoma antigens gp100 and TRP-2. Cancer Res 61: 859-863, 2001.
13. Schreurs MW, De Boer AJ, Figdor CG and Adema GJ: Genetic vaccination against the melanocyte lineage-specific antigen gp100 induces cytotoxic T lymphocyte-mediated tumor protection. Cancer Res 58: 2509-2514, 1998.
14. Chen H, Egan JO and Chiu JF: Regulation and activities of alpha-fetoprotein. Crit Rev Eukaryot Gene Expr 7: 11-41, 1997.

15. Ishikawa H, Nakata K, Mawatari F, Ueki T, Tsuruta S, Ido A, Nakao K, Kato Y, Ishii N and Eguchi K: Utilization of variant-type of human alpha-fetoprotein promoter in gene therapy targeting for hepatocellular carcinoma. Gene Ther 6: 465-470, 1999.
16. Ueki T, Nakata K, Mawatari F, Tsuruta S, Ido A, Ishikawa H, Nakao K, Kato Y, Ishii N and Eguchi K: Retrovirus-mediated gene therapy for human hepatocellular carcinoma transplanted in athymic mice. Int J Mol Med 1: 671-675, 1998.
17. Mawatari F, Tsuruta S, Ido A, Ueki T, Nakao K, Kato Y, Tamaoki T, Ishii N and Nakata K: Retrovirus-mediated gene therapy for hepatocellular carcinoma: selective and enhanced suicide gene expression regulated by human alpha-fetoprotein enhancer directly linked to its promoter. Cancer Gene Ther 5: 301-306, 1998.
18. Ido A, Nakata K, Kato Y, Nakao K, Murata K, Fujita M, Ishii N, Tamaoki T, Shiku H and Nagataki S: Gene therapy for hepatoma cells using a retrovirus vector carrying herpes simplex virus thymidine kinase gene under the control of human alpha-fetoprotein gene promoter. Cancer Res 55: 3105-3109, 1995.
19. Bei R, Budillon A, Reale MG, Capuano G, Pomponi D, Budillon G, Frati L and Muraro R: Cryptic epitopes on alpha-fetoprotein induce spontaneous immune responses in hepatocellular carcinoma, liver cirrhosis, and chronic hepatitis patients. Cancer Res 59: 5471-5474, 1999.
20. Vollmer CM, Eilber FC, Butterfield LH, Ribas A, Dissette VB, Koh A, Montejo LD, Lee MC, Andrews KJ, McBride WH, Glaspy JA and Economou JS: Alpha-fetoprotein-specific genetic immunotherapy for hepatocellular carcinoma. Cancer Res 59: 3064-3067, 1999.
21. Meng WS, Butterfield LH, Ribas A, Dissette VB, Heller JB, Miranda GA, Glaspy JA, McBride WH and Economou JS: alpha-fetoprotein-specific tumor immunity induced by plasmid prime-adenovirus boost genetic vaccination. Cancer Res 61: 8782-8786, 2001.
22. Grimm CF, Ortmann D, Mohr L, Michalak S, Krohne TU, Meckel S, Eisele S, Encke J, Blum HE and Geissler M: Mouse alpha-fetoprotein-specific DNA-based immunotherapy of hepatocellular carcinoma leads to tumor regression in mice. Gastroenterology 119: 1104-1112, 2000.
23. Mizuguchi H and Kay MA: Efficient construction of a recombinant adenovirus vector by an improved *in vitro* ligation method. Hum Gene Ther 9: 2577-2583, 1998.
24. Vitadello M, Schiaffino MV, Picard A, Scarpa M and Schiaffino S: Gene transfer in regenerating muscle. Hum Gene Ther 5: 11-18, 1994.
25. Carvalho LH, Hafalla JC and Zavala F: ELISPOT assay to measure antigen-specific murine CD8(+) T cell responses. J Immunol Methods 252: 207-218, 2001.
26. Kim JJ, Yang JS, Lee DJ, Wilson DM, Nottingham LK, Morrison L, Tsai A, Oh J, Dang K, Dentchev T, Agadjanyan MG, Sin JI, Chalian AA and Weiner DB: Macrophage colony-stimulating factor can modulate immune responses and attract dendritic cells *in vivo*. Hum Gene Ther 11: 305-321, 2000.
27. Dan Q, Sanchez R, Delgado C, Wepsic HT, Morgan K, Chen Y, Jeffes EW, Lowell CA, Morgan TR and Jadus MR: Non-immunogenic murine hepatocellular carcinoma Hepa1-6 cells expressing the membrane form of macrophage colony stimulating factor are rejected *in vivo* and lead to $CD8^+$ T-cell immunity against the parental tumor. Mol Ther 4: 427-437, 2001.
28. Lamont AG and Adorini L: IL-12: a key cytokine in immune regulation. Immunol Today 17: 214-217, 1996.
29. Melero I, Mazzolini G, Narvaiza I, Qian C, Chen L and Prieto J: IL-12 gene therapy for cancer: in synergy with other immunotherapies. Trends Immunol 22: 113-115, 2001.
30. Nagayama Y, Kita-Furuyama M, Ando T, Nakao K, Mizuguchi H, Hayakawa T, Eguchi K and Niwa M: A novel murine model of Graves' hyperthyroidism with intramuscular injection of adenovirus expressing the thyrotropin receptor. J Immunol 168: 2789-2794, 2002.
31. Ambriovic A, Adam M, Monteil M, Paulin D and Eloit M: Efficacy of replication-defective adenovirus-vectored vaccines: protection following intramuscular injection is linked to promoter efficiency in muscle representative cells. Virology 238: 327-335, 1997.
32. Atarashi Y, Yasumura S, Nambu S, Yoshio Y, Murakami J, Takahara T, Higuchi K, Watanabe A, Miyata K and Kato M: A novel human tumor necrosis factor alfa mutein, F4614, inhibits *in vitro* and *in vivo* growth of murine and human hepatoma: implication for immunotherapy of human hepatocellular carcinoma. Hepatology 28: 57-67, 1998.
33. Nakatsuka K, Sugiyama H, Nakagawa Y and Takahashi H: Purification of antigenic peptide from murine hepatoma cells recognized by Class-I major histocompatibility complex molecule-restricted cytotoxic T-lymphocytes induced with B7-1-gene-transfected hepatoma cells. J Hepatol 30: 1119-1129, 1999.
34. Ribas A, Butterfield LH, McBride WH, Dissette VB, Koh A, Vollmer CM, Hu B, Chen AY, Glaspy JA and Economou JS: Characterization of antitumor immunization to a defined melanoma antigen using genetically engineered murine dendritic cells. Cancer Gene Ther 6: 523-536, 1999.