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Administration of Multiple Cytokine Genes with Anti-tumor Activity Inhibits both Tumor Incidence and Tumor Growth

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Abstract

The finding of reporter gene expression in muscle cells after intramuscular injection of a reporter gene containing DNA has suggested that injection of a certain gene in its naked form could induce an expression of the injected gene. The result proposed the concept, namely DNA or genetic vaccine technology, that injection of an antigen gene could induce a specific immune response against the antigen. Although the concept was initially applied to vaccination technology, the result also means that administration of cytokine genes with anti-tumor activity could exert their functions when they are applied as a naked form of DNA. To test the possibility, plasmid vector containing granulocyte macrophage-colony stimulation factor (GM-CSF) and interleukin-12 (IL-12) genes, which are known as one of the most potent anti-tumor cytokines, were constructed and injected into mice together with syngeneic tumor cells. When the cytokine gene containing plasmid was injected on the same day of tumor cell injection, a tumor mass developed in 4 out of 5 mice tested. Even among the 4 mice, the tumor mass of a mouse disappeared 2 weeks after tumor development. In addition, tumor generation was significantly delayed in cytokine gene injected mice and the average tumor size was about 51.5% that of vector control injected mice. These results suggested that tumor treatment through the injection of multiple cytokine genes with potent anti-tumor activity significantly inhibits tumor development and growth, and that the method could be considered as one of the tools for efficient tumor treatment.

Key Words: Tumor, cytokine, DNA

INTRODUCTION

Currently, surgical operation, chemotherapy and/or radiation therapy are the most frequently applied methods of tumor treatment. The fact that most tumor cells have been known to express tumor-specific and/or tumor-associated antigens suggests that the induction of specific immune responses against the antigen could be one of the most efficient methods of inhibiting tumor cell growth. However, most progressive tumors have been known to be able to evade the immune system even in the presence of antigens, possibly through the low-level expression of immune-related cell surface molecules such as the major histocompatibility complex (MHC) antigen,[1] the deficiency in accessory signals required for proper immune stimulation,[2-5] and/or the absence or low-level of T cell functions, such as cytokine secretion.[6,7] Consequently, numerous efforts have been concentrated on the enhancement of specific immune responses against tumor cells in tumor-bearing hosts for the efficient inhibition of tumor growth.

Cytokine treatment has been regarded as one of the most efficient methods of enhancing immune responses against tumor cells, since lower secretion of cytokines and/or secretion of the immune response inhibitor have been detected in tumor patients.[8-11] In order to treat tumors with cytokines, cytokines with anti-tumor activity have been systematically applied to inhibit tumor growth, and effective eradication of immunogenic tumors in a rodent tumor model system has been reported.[12] However, application of the results from animal studies to human clinical trials does not seem to be promising in some cases due to the systemic toxicity and high cost of treatment due

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to the necessity of multiple high-dose cytokine injections.[13-16] In this regard, Tepper et al.[17] launched a pioneering work on immune-mediated cancer therapy and suggested the potential for the transfection of tumor cells with cytokine genes as a tumor treatment strategy by engineering tumor cells to produce cytokines to inhibit tumor growth *in vivo* and to induce systemic immunity against the untreated parental tumor cells.[18-24] However, the effect of cytokines administered through cytokine gene transfected tumor cells did not turn out to be as effective as direct cytokine treatment. In addition, clinical application of the method of tumor treatment was not plausible because the specific tumor cells from a tumor patient had to be manipulated to express anti-tumor cytokines to cope with the parental tumor cells.

A report on gene expression after intramuscular injection of a reporter gene containing plasmid vector without any delivery system suggested that the specific gene could be expressed in host muscle cells by injecting the DNA for the gene into host muscle tissue.[25] This result meant that anti-tumor cytokines could be applied into a tumor-bearing host through the injection of cytokine genes to inhibit tumor growth. Also, the report of long-lasting expression of injected genes in muscle tissue suggested that an anti-tumor cytokine could be expressed for a long period with an injection.[25,26] Since the drawbacks of cytokine-mediated tumor treatment include systemic toxicity and the high cost of treatment caused by multiple high-dose anti-tumor cytokine injection, the injection of cytokine genes with anti-tumor activity could be a method of treating tumors by using cytokines without systemic toxicity at low cost. Previously, other investigators have tested whether cytokine gene-mediated tumor treatment could be applied to inhibit tumor growth by using anti-tumor cytokines, such as granulocyte macrophage-colony stimulation factor (GM-CSF), interleukin-2 (IL-2) and interleukin-12 (IL-12), and they found that the method was effective in inhibiting tumor growth.[23,24,27-31] These results meant that injection of multiple cytokine genes could be more effective than single cytokine gene injection in tumor treatment. To test this possibility, we constructed a plasmid vector containing both GM-CSF and IL-12 genes,[32-39] which have been known as one of the most effective anti-tumor cytokines, and tested whether the injection of multiple cytokine genes could induce anti-tumor activity in an animal tumor model.

MATERIALS AND METHODS

Chemicals, plastics, cell lines and mice

Unless otherwise specified, the general chemicals and plastics used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and Falcon Labware (Becton-Dickinson, San Jose, CA, U.S.A.), respectively. Restriction enzymes and nucleic acid modifying enzymes were purchased from POSCO Chemical Co. (Sungnam, Korea).

DAP.3 mouse fibroblast cells and murine GM-CSF (mGM-CSF) dependent FDC-P1 cells were kindly provided by Dr. Ronald N. Germain (National Institutes of Health, Bethesda, MD, U.S.A.) and Dr. Donald Metcalf (Walter and Eliza Hall Institute of Medical Research, Victoria, Australia), respectively.[40,41] The cells were cultured in DMEM medium supplemented with 10% FBS (HyClone, Logan, Utah, U.S.A.). Murine IL-3, which shares its receptor with mGM-CSF was additionally supplemented for mGM-CSF dependent FDC-P1 cell growth.

Four to six-week-old inbred BALB/c mice were purchased from Korea Research Institute of Bioscience and Biotechnology (Yoosung, Korea).

Construction of plasmid vectors

Murine GM-CSF gene containing pUC19-GM-CSF vector was provided by Dr. D. S. Heo (Seoul National University Medical School, Seoul, Korea). Clones msp35 and msp40, cDNAs encoding murine IL-12 (mIL-12) subunits p35 and p40, respectively, were provided by Dr. J. H. Park (Changwon National University, Changwon, Korea).[42] As an initial step in constructing the plasmid vector containing mGM-CSF and both subunits of mIL-12 gene, mGM-CSF gene and the genes for both subunits of mIL-12 were separately cloned into pcDNA3.1 (+) plasmid vector (Invitrogen, Netherlands). Briefly, mGM-CSF gene containing *Hin*d III-*Eco*R I fragment of pUC19-GM-CSF was subcloned into the polycloning site of pcDNA3.1 (+) to generate pcDNA3.1-GM-CSF (Fig. 1A). To construct the plasmid vector containing both subunits of mIL-12, *Hin*d III-*Eco*R I fragment of both msp35 and msp40 genes were separately subcloned into pcDNA3.1 (+)

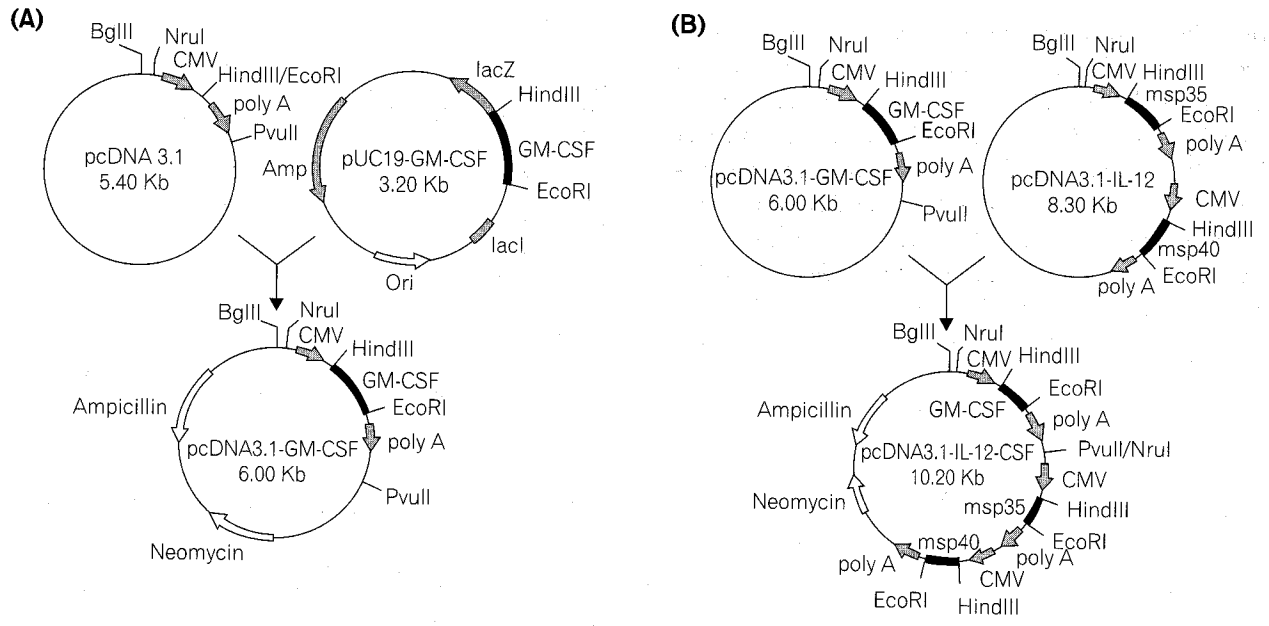

Fig. 1. Construction of plasmid vector containing mGM-CSF and mIL-12 genes. (A) Cloning of mGM-CSF gene into pcDNA3.1 (+). (B) Cloning of mGM-CSF gene from pcDNA3.1 (+) into mIL-12 gene containing pcDNA3.1-IL-12 to generate pcDNA3.1-IL-12-GM-CSF, which contains the genes for mGM-CSF and both subunits of mIL-12.

to generate pcDNA3.1-msp35 and pcDNA3.1-msp40, respectively. Then, *Bgl* II-*Pvu* II fragment containing CMV promoter, msp35 gene and polyadenylation signal of pcDNA3.1 (+) was subcloned into *Bgl* II-*Pvu* II/*Nru* I site of pcDNA3.1-msp40 to generate pcDNA-3.1-IL-12 which contains the genes for both subunits of mIL-12. Finally, *Bgl* II/*Pvu* II fragment containing mGM-CSF gene with CMV promoter and polyadenylation signal of pcDNA3.1 (+) was subcloned into *Bgl* II-*Pvu* II/*Nru* I site of pcDNA3.1-IL-12 to generate the pcDNA3.1-IL-12-GM-CSF which contains the genes for mGM-CSF and both subunits of mIL-12 (Fig. 1B). Commercially-available pcDNA3.1 (+) vector, which is the most frequently used plasmid vector for DNA vaccine technology, was used as a control vector.

Transfection of the plasmid construct

In order to transfect the constructed plasmid vector into DAP.3 mouse fibroblast cells, plasmid DNA was initially purified through a CsCl/EtBr preparation. Then, 10^5-10^6 of DAP.3 cells were transfected with $15-20$ μg of the plasmid by using the $CaPO_4$-mediated transfection method. Plasmid transfected cells were then selected for 3 weeks by growing the cells in the medium containing 450 μg/ml of G418. The level and integrity of the expressed cytokines were determined from culture supernatant, which was obtained by culturing 2×10^6 transfected cells in 10 ml medium for 7 days, through the biological assays as described below.

Measuring the cytokine activity

In order to measure the biological activity of mGM-CSF in culture supernatant, the level of support for the growth of mGM-CSF dependent FDC-P1 cells was measured.[41] Briefly, growth-factor-starved 10^4 FDC-P1 cells were suspended in 100 μl of DMEM medium supplemented with 10% FBS (HyClone Laboratories Inc., Logan, UT, U.S.A.) and added into each well of microtitre plates which contained each test sample. After 24 hr incubation, 1 μCi of [*methyl*-^{3}H] Thymidine (Amersham Lifescience, IL, U.S.A.) was added to each well and incubated for another $12-16$ hr. The cells were then harvested on glass-fiber filter with a cell harvester (Inotech, Switzerland) and the tritium content was measured with a liquid scintillation counter (Packard, IL, U.S.A.). In this experiment, rmGM-CSF purchased from R&D System (Minneapolis, MN, U.S.A.) was used as a positive control.

In order to measure the biological activity of produced mIL-12, the level of support for proliferation of PHA-activated T lymphoblasts were measured.[39] Briefly, 5×10^5 cells/ml of peripheral blood mononuclear cells (PBMC), which had been isolated from the peripheral blood of healthy volunteers by Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation, were cultured for 3 days in RPMI 1640 medium containing 5 μg/ml of PHA. The cultures were then split 1 : 1 with fresh medium and cultured for 1 additional day in the presence of rhIL-2 (50 U/ml). Then, the cells were washed and resuspended in the medium at a density of 4×10^4 cells/well in the presence of anti-human IL-2 antibody and 100 μl of serially diluted samples. After 24 hr incubation, the same procedure of proliferation assay was conducted as in mGM-CSF assay, except rhIL-12 (Pharmingen, San Diego, CA, U.S.A.) was used as a positive control for the experiment.

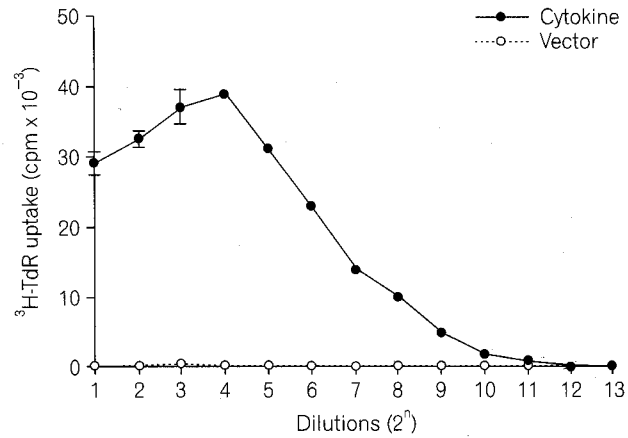

Fig. 2. Measuring the biological activity of mGM-CSF produced from pcDNA3.1-IL-12-GM-CSF plasmid transfected DAP.3 mouse fibroblast cells to confirm the integrity of the vector. The results represent the means and standard errors of triplicates. Cytokine and vector represent the results from pcDNA3.1-IL-12-GM-CSF and control vector transfected cell culture supernatants, respectively.

Injection of tumor cells and plasmid vectors and measuring tumor growth

In order to generate tumor-bearing mice, 1×10^5 of CT-26 mouse colon carcinoma cells[43] were injected subcutaneously into syngeneic BALB/c mice. One hundred micrograms of pcDNA3.1 (+) and pcDNA-3.1-IL-12-GM-CSF plasmid DNA were injected subcutaneously into each mouse for negative control and experiment, respectively, on the same day into the same site of tumor cell injection. Five mice were used as a group in each experiment.

Tumor growth was initially measured 7 days after the injection of both the tumor cells and cytokine gene containing plasmid vector, and every 2 days after that. Tumor diameter was determined by measuring the widest and narrowest diameter of the tumor mass and expressed as an average. Mice with tumors less than 2 mm in diameter were scored negative on tumor incidence.

Statistical significance of the data has been analyzed through unpaired two-tailed t test by using GraphPad Instat® software.

RESULTS AND DISCUSSION

In order to prepare the plasmid vector containing both mGM-CSF and mIL-12 genes, genes for mGM-CSF and both subunits of mIL-12 were cloned into pcDNA3.1 (+) vector, which is the most frequently used plasmid vector for DNA vaccine technology (Fig. 1). Since confirmation of the integrity of the construct was prerequisite for testing the effect of multiple cytokine gene treatment on tumor growth inhibition, culture supernatant from pcDNA3.1-IL-12-GM-CSF vector-transfected DAP.3 cells were tested to determine whether there was any biological activity of mGM-CSF and mIL-12. When serially diluted samples of the culture supernatant were used to support the growth of mGM-CSF dependent FDC-P1 cells, the culture supernatant efficiently supported the growth of FDC-P1 cells, although the culture supernatant from control vector transfected cells could not support the growth of T lymphoblast cells (Fig. 2). This result suggested that biologically active mGM-CSF was produced from the pcDNA3.1-IL-12-GM-CSF vector construct. When a recombinant mGM-CSF was used as a control to determine the concentration of mGM-CSF within the culture supernatant, mGM-CSF activity in the culture supernatant was determined as 2.9 $\times 10^6$ U/ml (data not shown). Similarly, serially diluted samples of culture supernatant were also tested to support the proliferation of PHA-stimulated T lymphoblast cells, which is a standard method of measuring IL-12 activity. The culture supernatant from pcDNA3.1-IL-12-GM-CSF transfected cells supported the growth of T lymphoblast cells very efficiently (Fig.

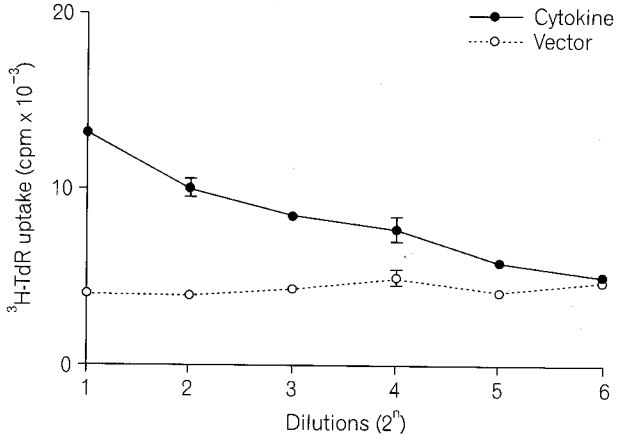

Fig. 3. Measuring the biological activity of mIL-12 produced from pcDNA3.1-IL-12-GM-CSF plasmid transfected DAP.3 mouse fibroblast cells to confirm the integrity of the vector. The results represent the means and standard errors of triplicates. Cytokine and vector represent the results from pcDNA3.1-IL-12-GM-CSF and control vector transfected cell culture supernatants, respectively.

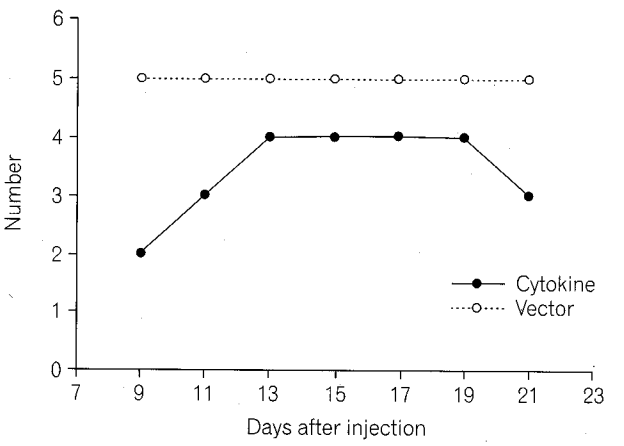

Fig. 5. Tumor incidence after the injection of tumor cells and plasmid vectors. Cytokine and vector represent the results from pcDNA3.1-IL-12-GM-CSF- and control vector-treated mice, respectively. Mice with tumors less than 2 mm in diameter were scored negative on tumor incidence.

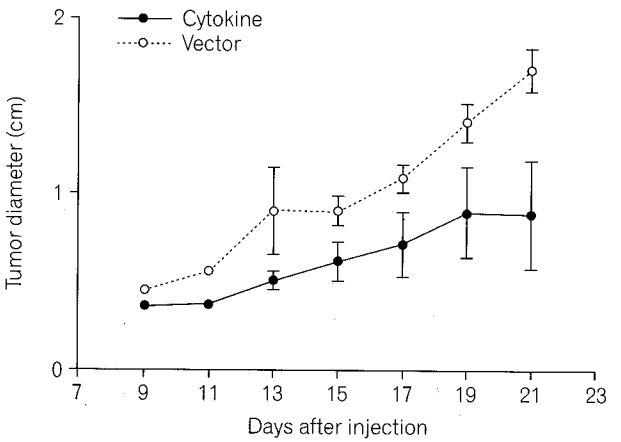

Fig. 4. Mean diameter of the tumor mass after the injection of tumor cells and plasmid vectors. Cytokine and vector represent the results from pcDNA3.1-IL-12-GM-CSF- and control vector-treated mice, respectively. The results of multiple cytokine gene-treated mice were calculated only from the mice with tumors at the time of measurement.

3). As in the experiment for the mGM-CSF assay, the culture supernatant from control vector transfected cells was unable to support the efficient growth of T lymphoblast cells. This result also suggested that biologically active mIL-12 was produced from pcDNA3.1-GM-CSF-IL-12 plasmid vector. When a recombinant mIL-12 was used as a control to determine the activity of mIL-12 within the culture supernatant, the activity was determined as 500 U/ml (data not shown). Taken together, these results suggested that the mGM-CSF and mIL-12 gene containing pcDNA3.1-IL-12-GM-CSF plasmid vector was constructed correctly and that the vector could be used to test whether multiple cytokine gene treatment could inhibit tumor cell growth in tumor-bearing mice.

To test whether the injection of multiple cytokine genes could exert anti-tumor activity, mGM-CSF and mIL-12 gene-containing pcDNA3.1-IL-12-GM-CSF vector was injected into BALB/c mice together with syngeneic CT-26 tumor cells as described in Materials and Methods, and the tumor diameter and tumor incidence were monitored (Fig. 4 and 5). Regarding tumor growth, the tumor diameter of cytokine gene-treated mice was smaller than that of control vector-treated mice even at 7 days after injection, which was the first time of measurement. Differences in tumor diameter increased as time passed and the average tumor diameter of control vector-treated mice was eventually about twice that of cytokine gene-treated mice (Fig. 4). The result suggested that multiple cytokine gene treatment could inhibit tumor growth significantly ($p<0.05$) when the genes were applied in a naked DNA form. This phenomenon does not seem to be restricted to specific tumor cells because the same treatment efficiently inhibited the growth of M-MSV transformed fibroblast-like M-MSV-BALB/3T3 cells (data not shown).[44]

Since multiple cytokine gene treatment showed an inhibitory effect on tumor growth, we tested whether

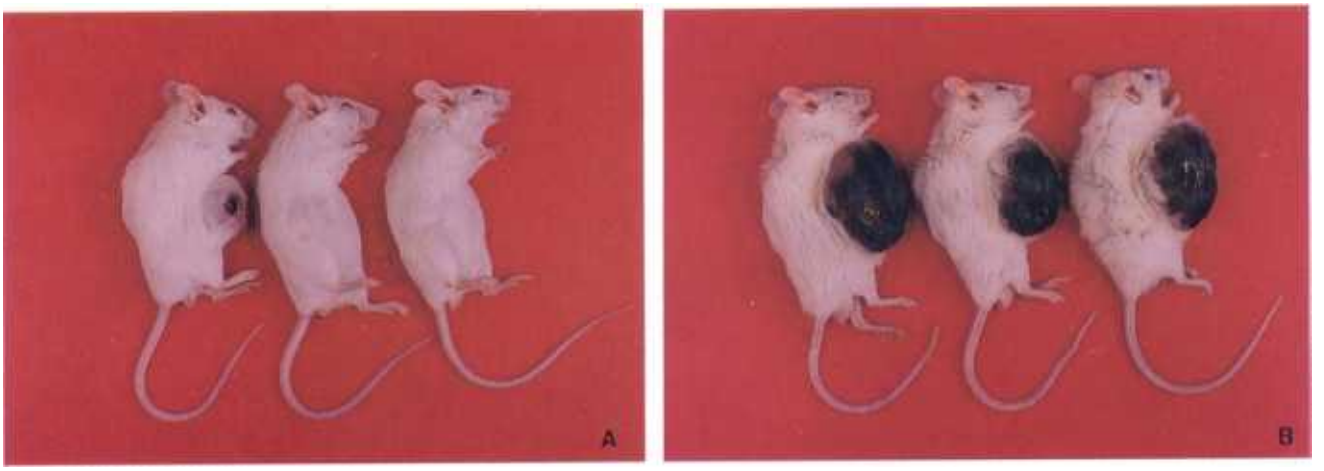

Fig. 6. Representative pictures of the mice which were taken 21 days after injection. (A) Multiple cytokine gene-treated mice. Middle and right mice are without tumor development after tumor cells and cytokine gene injection. Left mouse is the one with tumor development after the same injection. (B) Control vector injected mice.

the same treatment had any effect on tumor incidence, as well (Fig. 5). A palpable tumor mass developed in all 5 control vector-treated mice even at 7 days after injection, which was the first time of tumor measurement, and it was maintained during the whole period of monitoring. However, tumor mass developed in only 2 of 5 multiple cytokine gene-treated mice during the same period. Although a tumor mass developed in 2 more mice 9 and 11 days after injection, respectively, the mass of one mouse disappeared 21 days after injection. These results indicated that multiple cytokine gene treatment has a significant inhibitory effect ($p < 0.05$) on tumor incidence. As a reference, pictures of the experimental mice were taken and they show that the size of the tumor mass in multiple cytokine gene-treated mice (Fig. 6A) was much smaller than in control vector-treated mice (Fig. 6B).

Overall, treatment of multiple cytokines with anti-tumor activity in their naked DNA form could efficiently inhibit both tumor development and tumor incidence. Since the drawbacks of cytokine treatment, which has been known as one of the most efficient methods of tumor treatment by enhancing the immune responses against tumor antigens, are systemic toxicity and the high cost caused by multiple high-dose cytokine injections, then cytokine gene treatment could be the best alternative method to take advantage of cytokine treatment without systemic toxicity at low cost. In addition, this method could be applied to a broad range of tumor cell treatments because there is no need to manipulate each type of tumor cell which is required by the cytokine gene transfection method. Consequently, this protocol could be considered as one of the methods of immune-mediated tumor treatment. Characterization of the immune responses induced by multiple cytokine gene treatment is continuing. In addition, the effects of multiple cytokine gene treatment including interleukin-2, which has also been known as one of the most potent cytokines with anti-tumor activity, on tumor growth is being tested.

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