

Dendritic Cells Genetically Engineered to Simultaneously Express Endogenous Tumor Antigen and Granulocyte Macrophage Colony-stimulating Factor Elicit Potent Therapeutic Antitumor Immunity¹

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ABSTRACT

Recently, several studies have shown that vaccine therapy using dendritic cells (DCs) genetically engineered to express a surrogate tumor antigen can effectively induce antitumor immunity. In this study, murine bone marrow DCs were adenovirally transduced with murine endogenous tumor antigen gp70 expressed in CT26 cells and granulocyte macrophage colony-stimulating factor (GM-CSF), and we examined whether antigen-specific CTL responses and therapeutic immunity could be induced in mice immunized with those genetically modified DCs. The cytotoxic activity against CT26 in mice immunized with gp70-transduced DCs was significantly higher than that in control ($P < 0.01$) and was enhanced by GM-CSF-cotransduction ($P < 0.001$). GM-CSF gene transfer into DCs expressing tumor-associated antigen enhances CC chemokine receptor 7 expression on DCs, leading to improved migratory capacity of DCs to draining lymph nodes. Consequently, an effective antitumor immune response would be induced. Vaccination using gp70-transduced DCs provided remarkable therapeutic efficacy in s.c. models. Moreover, it could be sufficiently augmented by GM-CSF-cotransduction of DCs. These results support that vaccination therapy using DCs simultaneously transduced with tumor-associated antigen can elicit potent CTL response, and GM-CSF-cotransduction of DCs could optimize therapeutic response. Further investigation is needed to optimize this vaccine therapy to achieve the obvious benefit in clinical application.

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INTRODUCTION

DCs³ are the most potent antigen-presenting cells capable of initiating T-cell-dependent immune responses (1, 2); therefore, there has been significant interest in immunotherapy using DCs. Several groups have identified TAAs recognized by tumor-specific CD8⁺ CTLs, as well as cloned genes encoding those TAAs (3, 4), and focused on the delivery of TAA-derived proteins/peptides or TAA genes to DCs to elicit specific antitumor responses.

The development of an effective tumor vaccine depends on a system for delivery of the TAA to facilitate the induction of antitumor immune responses. Recently, it has been shown that DCs pulsed with whole tumor antigens, defined tumor antigenic peptides, or total RNA from tumor cells are capable of inducing effective antigen-specific immunity and therapeutic antitumor responses (5–7). We have used a gene-based vaccination strategy using DCs expressing the entire tumor antigen to elicit a potent therapeutic antitumor immunity because this approach has important advantages over peptide/protein- or RNA-based immunization (8, 9). TAA gene expression in DCs causes endogenous processing of multiple and/or undefined antigenic peptides independent of MHC alleles, and the immunization of mice with DCs engineered to express a surrogate tumor antigen induces a tumor-specific CTL response and, moreover, protective and therapeutic immunity (10, 11).

Functions of DCs are affected by several immunostimulatory cytokines within the local tissue environment (12). In particular, GM-CSF is a potent stimulator of DCs (13). Several investigators have reported that GM-CSF gene-transduced tumor vaccine results in efficient tumor suppression and survival benefit in mouse models (14, 15). Recently, it has been reported that genetically modified DCs expressing GM-CSF elicit a specific CTL response and, moreover, therapeutic immunity in murine tumor models (16). Accordingly, GM-CSF seems to be a very attractive cytokine to augment DC immunization therapy. In this study, we transduced DCs simultaneously with TAA gene and GM-CSF gene using Ad vector for the antitumor vaccination therapy.

We used CT26 murine colorectal cancer cells for the tumor-bearing model because CT26 cells are basically poorly immunogenic and endogenously express gp70, an envelope

³ The abbreviations used are: DC, dendritic cell; Ad, adenovirus; GM-CSF, granulocyte macrophage colony-stimulating factor; TAA, tumor-associated antigen; MOI, multiplicity of infection; CCR7, CC chemokine receptor 7; RT-PCR, reverse transcription-PCR; LN, lymph node; FBS, fetal bovine serum; mGM-CSF, murine GM-CSF; mCCR7, murine CCR7; β -gal, β -galactosidase; IL, interleukin.

protein of an endogenous ecotropic murine leukemia virus (17). This model allows us to accurately evaluate the ability of Ad-transduced DCs expressing natural TAA to induce an anti-tumor immune response.

The purpose of this study was to assess whether vaccination using DCs genetically engineered to express natural TAA could induce therapeutic antitumor immunity in an autologous colon cancer model. Furthermore, we investigated the enhancing effect of cotransduction of DCs with the *GM-CSF* gene on this vaccine therapy.

MATERIALS AND METHODS

Mice and Cell Lines. Female 6–8-week-old BALB/c (H-2^d) mice were purchased from Japan SLC Inc. (Hamamatsu, Japan). Syngeneic tumor cell line CT26 (H-2^d), an undifferentiated low immunogenic murine colorectal adenocarcinoma cell line, was kindly provided by Hoffman-La Roche (Kamakura, Japan). Meth-A (H-2^d), a methylcholanthrene-induced sarcoma cell line, was obtained from Riken Gene Bank (Ibaraki, Japan). Human embryonic kidney cell line 293 was purchased from the American Type Culture Collection (Manassas, VA). CT26 and 293 cells were grown in DMEM (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% FBS (Life Technologies, Inc., New York, NY), 2 mM L-glutamine (Life Technologies, Inc.), 100 units/ml penicillin, and 100 µg/ml streptomycin (Life Technologies, Inc.). Meth-A was grown in RPMI 1640 (Nissui Pharmaceutical Co.) supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin.

Generation of DCs *in Vitro* from Bone Marrow. DCs were obtained from murine bone marrow precursors as described by Lutz *et al.* (18). In brief, murine bone marrow cells (2×10^6) were cultured in 100-mm dishes in 10 ml of complete medium containing 200 units/ml recombinant mGM-CSF (kindly provided by Kirin Beer Co., Tokyo, Japan). At day 3 of culture, another 10 ml of fresh medium containing recombinant mGM-CSF were added to the plates. At days 6 and 8, half of the medium was replaced by the fresh complete medium containing mGM-CSF. At day 10, nonadherent cells were collected for flow cytometric analysis and genetic modification.

For phenotypic analysis, nonadherent cells were stained with FITC-conjugated monoclonal antibodies against murine cell surface molecules, including monoclonal antibodies specific for MHC class I (H-2k^d) and II (I-A^d) molecules, CD80 (B7.1), CD86 (B7.2), and CD11c (all from PharMingen, San Diego, CA), and quantified by FACScan (Becton Dickinson, Mountain View, CA). More than 99% of the cells showed high expression of CD11c, CD80, MHC class I antigen, and MHC class II antigen, and around 30% of the cells showed low expression of CD86.

Recombinant Ad Vectors. The cDNA of endogenous murine tumor antigen gp70 was amplified from total RNA of CT26 by RT-PCR using a pair of specific primers (sense, 5'-ATGGAGAGTACAACGCTCTCA; antisense, 5'-GTACCAATCCTGTGT GGTCG). The PCR product was initially cloned for sequencing and then ligated into the *Swa*I site of cosmid vector pAxCawt (Takara, Shiga, Japan) to yield pAxCagp70. Recombinant AxCagp70 was generated by the COS-TPC method as described previously (19). AxCAmGM-CSF

expressing mGM-CSF was generated using the expression cassette of mGM-CSF cDNA (pAdex1CAmGMCSF) as described previously (15), and AxCALacZ expressing a *LacZ* reporter gene was generated using pAxCaiLacZ (Takara) by the COS-TPC method.

Ad Vector-mediated Gene Transfer and Expression in DCs *in Vitro*. To assess the ability of Ad vectors to transfer and express genes of interest in DCs *in vitro*, DCs were infected with AxCALacZ using a centrifugal method (20). DCs (1×10^6 DCs/500 µl) were mixed with 500 µl of AxCALacZ solution at the MOI indicated in a 15-ml polypropylene tube (Falcon) and centrifuged at $2000 \times g$ at 30°C to 35°C for 2 h. Then, DCs were washed twice with PBS and cultured in complete medium containing 200 units/ml mGM-CSF for 48 h, and *LacZ* expression was quantified by flow cytometry using fluorescein di-β-D-galactopyranoside (Molecular Probes, Inc., Eugene, OR). Labeling with propidium iodide (1 µg/ml) was used to facilitate live/dead cell discrimination.

Analysis of mRNA Expression of mCCR7 Using RT-PCR. Total RNA was isolated from genetically modified DCs, and RT-PCR was performed to examine the expression of mCCR7 mRNA using a pair of specific primers (sense, 5'-ACAGCGCCTCCAGAAGAACAGCGG; antisense, 5'-TGACGTCATAGGCAATGTTGAGCTG). CT26 was used as a positive control for gp70 expression. To ensure the quality of the procedure, RT-PCR was performed on the samples using specific primers for β-actin.

Assays for Cytokine Secretion. Genetically modified DCs expressing mGM-CSF were seeded at a concentration of 5×10^5 cells/well and cultured for 48 h, and then supernatants were harvested, and mGM-CSF content was measured in duplicate using an Endogen mGM-CSF ELISA kit (Endogen, Inc., Woburn, MA).

Induction of Antigen-specific CTLs and Cytotoxicity Assay. To determine whether the administration of DCs transfected with AxCagp70 would induce gp70-specific CTLs, DCs were transfected with AxCagp70 (MOI of 100) as described above. Furthermore, to determine whether antitumor activity induced by immunization with DCs expressing gp70 would be enhanced by cotransduction of GM-CSFs, DCs were transfected with AxCagp70 (MOI of 100) and AxCAmGM-CSF (MOI of 5, 10, or 30). BALB/c mice were immunized once by s.c. injection of 5×10^5 genetically modified DCs suspended in 200 µl of PBS. Spleens were removed 14 days after DC inoculation, and then the *in vivo*-primed splenocytes were pooled and cocultured (4×10^6 splenocytes/ml) with irradiated (10,000 rads) CT26 cells (4×10^5 cells/ml) in a 6-well plate (4 ml/well). After 5 days of coculture, the *in vivo*-restimulated splenocytes were assayed in a 4-h ⁵¹Cr release assay as described previously (21, 22). Briefly, CT26 and Meth-A were used as target cells. The target cells, labeled with Na₂⁵¹CrO₄, were incubated in triplicate with effector cells at various E:T ratios at 37°C in a 5% CO₂ atmosphere for 4 h. The supernatant was harvested, and radioactivity was counted using a gamma counter. The maximum amount of ⁵¹Cr incorporated was determined by adding 1 N HCl in target cells. The percentage of cytotoxicity was calculated as follows: percentage of lysis = [(sample cpm - spontaneous cpm)/(maximum cpm - spontaneous cpm)] × 100.

Trafficking Study of s.c. Injected Genetically Modified DCs. We examined whether genetically modified DCs could migrate to regional LNs, following the method described previously (16). DCs were labeled with the fluorescent dye PKH67 (Zynaxis, Malvern, PA) according to the manufacturer's protocol. The cells were washed and incubated with PKH67 staining solution for 5 min. Complete medium containing 10% FBS was added to the cells, followed by removal of unbound PKH67 by extensive washing with PBS. DCs labeled with PKH67 were injected s.c. into the lower abdomen of the mice. After 72 h, the mice were sacrificed, and the draining inguinal LNs were removed. All harvested LNs were crushed gently and suspended in PBS, and then these suspensions were subjected to flow cytometric analysis to detect fluorescence-positive cells within the LN preparation.

Experiment Design of *in Vivo* Tumor Therapy for s.c. Tumor Models. To assess whether preexisting s.c. tumor could be suppressed after immunization with DCs genetically modified to express tumor antigen and mGM-CSF, BALB/c mice (6–8 weeks old) were inoculated s.c. in the right flank with 1×10^6 CT26 cells. Five days later, tumor-bearing mice ($n = 5$ mice/group) were treated with s.c. injection in the opposite flank with 5×10^5 DC-AxCagp70 and DC-AxCagp70/mGM-CSF. Twenty days after treatment, the volume of the s.c. tumor was estimated using the following formula: (short diameter)² \times long diameter \times 0.52. To evaluate whether DC immunization of mice bearing pre-established s.c. tumor resulted in a prolongation of survival in mice models, the same experiment was performed, and the mice were followed for survival.

Statistical Analysis. Quantitative results are expressed as mean \pm SD. Statistical analysis was performed by ANOVA and Fisher's test using Statview 5.0 software (Abacus Concepts, Inc., Berkeley, CA). Analysis of survival was performed using the Kaplan-Meier method, and the generalized log-rank (Mantel-Cox) and Breslow-Gehan-Wilcoxon tests were used to compare the resulting curves of the treatment groups using Statview 5.0 software. $P < 0.05$ was considered to be statistically significant.

RESULTS

Adenoviral Transduction of DCs *in Vitro*. We first evaluated the ability of Ad vectors to transfer and express genes in DCs. Naive DCs showed only background levels of β -gal activity. Expression of β -gal was readily detected using the centrifugal method in the majority (75–83%) of DCs transfected with AxCALacZ at a MOI of 100 and was further increased at a MOI of >100 in a dose-dependent manner. On the other hand, the viability of DCs transfected with Ad vector at various MOIs was examined. At a MOI ≤ 100 , the viability of transduced DCs was similar to that of nontransduced DCs, whereas at a MOI ≥ 200 , the viability of transduced DCs decreased in a dose-dependent manner (data not shown). On the basis of these observations, all subsequent studies were carried out with AxCAgp70 at a MOI of 100. The expression of gp70 mRNA in DCs was confirmed by the RT-PCR when DCs were transfected with AxCAgp70 (data not shown).

In an assay for GM-CSF secretion, DCs transfected with

Table 1 mGM-CSF production of DCs transfected with AxCAmGM-CSF at various MOI (ng/ 5×10^5 cells)

DCs or DC-AxCagp70 were transfected with AxCAmGM-CSF or AxCALacZ at various MOIs and cultured at a concentration of 5×10^5 cells well for 48 h. Then supernatants were harvested and assayed by ELISA.

Vectors	MOI	DC	DC-AxCagp70
AxCAmGM-CSF	0	0	0
	2	66	43
	5	437	483
	10	742	668
	15	1114	1031
	30	1767	1802
AxCALacZ	50	2200	2369
	50	0	NT ^a
	100	2	NT

^a NT, not tested.

AxCAmGM-CSF at various MOIs produced a high concentration of mGM-CSF in a dose-dependent manner. In contrast, DCs transfected with mock virus AxCALacZ did not produce mGM-CSF at all. GM-CSF production from the DCs transfected with AxCAmGM-CSF was not affected by cotransfection of 100 MOI of AxCAgp70 (Table 1).

Tumor-specific CTL Response Induced by Immunization with Genetically Modified DCs. The ability of DCs engineered to express murine TAA gp70 to induce antigen-specific CTL responses was evaluated first. The cytotoxic activity against CT26 in spleen cells of mice immunized with DC-AxCagp70 was significantly higher than that of mice immunized with DC-AxCALacZ or PBS ($P < 0.01$; Fig. 1A). On the other hand, the cytotoxic activity against Meth-A, which does not express gp70, was $<5\%$ in all groups (Fig. 1B). Furthermore, we investigated whether cotransduction with the *mGM-CSF* gene would augment tumor-specific CTL activity induced by immunization with DC-AxCagp70. The cytotoxic activity against CT26 in spleen cells of mice immunized with DCs cotransfected with AxCAgp70 and AxCAmGM-CSF (5 MOI) was remarkably enhanced. Importantly, this enhancing effect of AxCAmGM-CSF was not recognized at a MOI of ≥ 10 (Fig. 2). Accordingly, the optimal dose of AxCAmGM-CSF was thought to be 5 MOI in terms of enhancement of CTL response. We showed that mGM-CSF production from DCs transfected with 5 MOI of AxCAmGM-CSF was 483 ng/ 5×10^5 cells (Table 1).

Enhancement of Migratory Capacity by mGM-CSF Gene Transduction of DCs *in Vivo*. To determine why cotransduction of DCs with the *GM-CSF* gene enhances their capacity to induce tumor-specific CTLs, we investigated the impact of GM-CSF transduction at various MOIs on *in vivo* DC function in terms of migration.

Nontransfected DCs, DC-AxCALacZ, DC-AxCagp70, and DC-AxCagp70/mGM-CSF were stably labeled with a fluorescent dye (PKH67) and s.c. injected into mice. After 3 days, two draining LNs were removed from each mouse. Interestingly, remarkable swelling of LNs in the mice immunized with DC-AxCagp70/mGM-CSF was seen (around 2 mm in diameter), as compared with that in mice immunized with nontransduced DCs (around 0.5 mm). Single-cell suspensions from

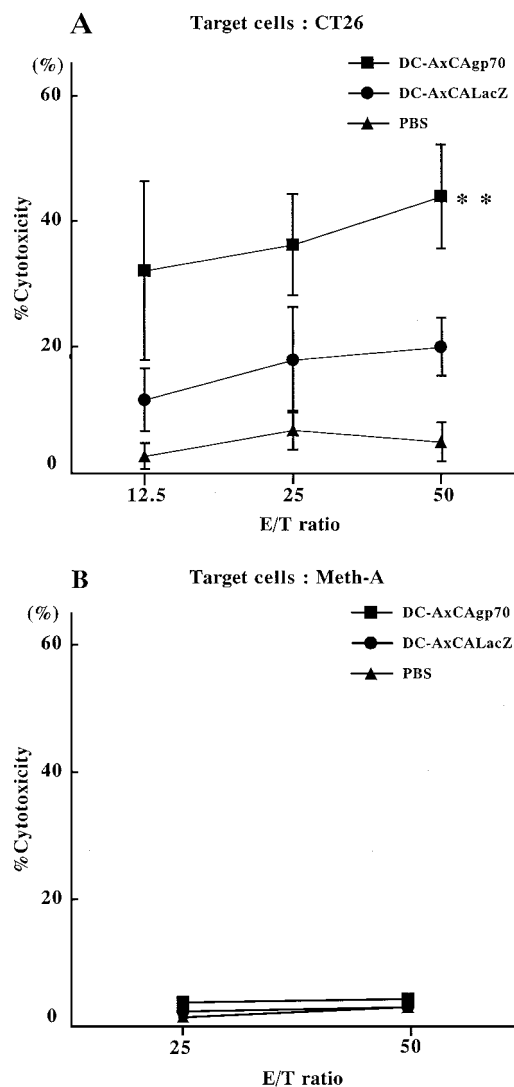


Fig. 1 CTL activity induced by immunization using genetically modified DCs. BALB/c mice were immunized once by s.c. injection of DCs transfected with AxCAg70 (DC-AxCaAgp70, ■), DCs transfected with AxCALacZ (DC-AxCALacZ, ●), or PBS (▲). Spleen cells were isolated 14 days after immunization and restimulated *in vitro* for 5 days with irradiated CT26 cells. CT26 (A) and Meth-A (B) were used as targets to determine the cytotoxic activity of effector cells. Results are shown as the means \pm SD ($n = 6$ for each group). **, significantly different from DC-AxCALacZ and PBS ($P < 0.01$).

draining LNs were analyzed for labeled cells by flow cytometry. A distinct population of dye-labeled cells was found in the LNs of mice immunized with DC-AxCaAgp70, and moreover, the population of labeled cells was significantly increased in the LNs of mice immunized with DC-AxCaAgp70/mGM-CSF. The optimal dose of AxCAmGM-CSF was 5 MOI in terms of enhancing migratory capacity of transduced DCs. In contrast, hardly any fluorescence-positive cells could be found in the LNs of mice injected with nontransduced DCs. (Fig. 3).

mCCR7 mRNA Expression in Genetically Modified DCs. We hypothesized that transduction of the *GM-CSF* gene might affect the expression of CCR7 on DCs and consequently

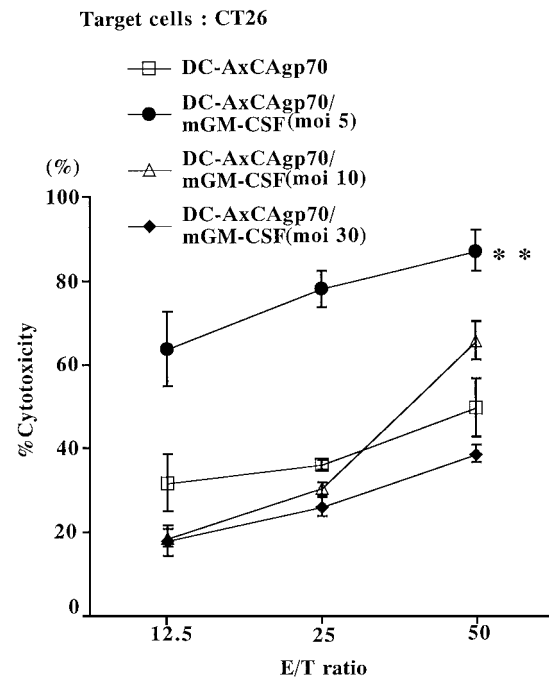


Fig. 2 The enhancement of CTL activity by cotransduction of GM-CSF. DCs were transfected with AxCAg70 alone (DC-AxCaAgp70, □) or AxCAg70 and AxCAmGM-CSF [DC-AxCaAgp70/mGM-CSF (MOI of 5, ●; MOI of 10, △; MOI of 30, ◆)], and BALB/c mice were then immunized once by s.c. injection of these DCs. Spleen cells were isolated 14 days after immunization and restimulated *in vitro* for 5 days with irradiated CT26 cells. CT26 cells were used as targets to assay the cytotoxic activity of effector cells. Results are shown as the means \pm SD ($n = 3$ /group). **, significantly different from DC-AxCaAgp70, DC-AxCaAgp70/mGM-CSF (MOI of 10), and DC-AxCaAgp70/mGM-CSF (MOI of 30; $P < 0.001$).

modify the migratory capacity of DCs. We investigated mRNA expression of *CCR7* on adenoviral gene-transduced DCs by RT-PCR. *CCR7* mRNA expression was not detected in non-transduced DCs. In contrast, *CCR7* mRNA expression on DCs was induced by adenoviral transduction with the *gp70* gene, and importantly, it was markedly up-regulated by cotransduction with the *GM-CSF* gene (Fig. 4).

Therapeutic Efficacy of Genetically Modified DCs in s.c. Tumor Models. We assessed the therapeutic efficacy of vaccination therapy using genetically modified DCs *in vivo*. Vaccination using *gp70* gene-transduced DCs provided remarkable therapeutic efficacy of tumor growth in this preexisting tumor model. Quantitative analysis of the tumor volume on day 26 after tumor implantation, when tumor-bearing mice treated with PBS began to die, confirmed the significant treatment differences between the DC-AxCaAgp70- or DC-AxCaAgp70/mGM-CSF-treated group and the control groups ($P < 0.0001$) and, moreover, between the DC-AxCaAgp70-treated group and the DC-AxCaAgp70/mGM-CSF-treated group ($P < 0.05$; Fig. 5A).

We also evaluated whether the tumor volume reduction in mice treated with DC-AxCaAgp70 or DC-AxCaAgp70/mGM-CSF conferred a survival advantage. Tumor-bearing mice that were immunized with DC-AxCaAgp70 or AxCAg70/mGM-

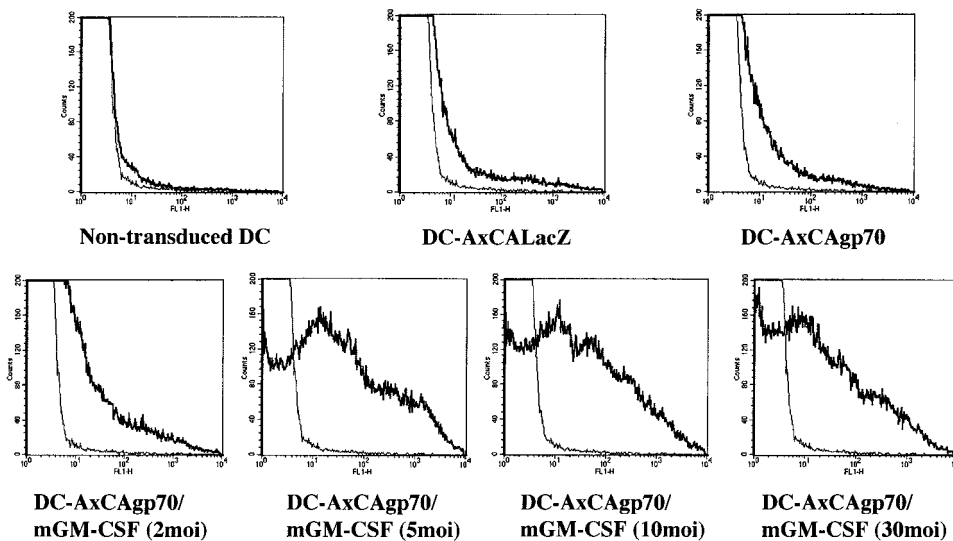


Fig. 3 The enhancement of migratory capacity by GM-CSF gene transduction of DCs *in vivo*. Nontransfected DCs, DC-AxCALacZ, DC-AxCagp70, and DC-AxCagp70/mGM-CSF were labeled with the fluorescent dye PKH67 and injected s.c. into the lower abdomen of mice. After 72 h, single-cell suspensions from the draining LNs were analyzed for labeled cells by flow cytometry.

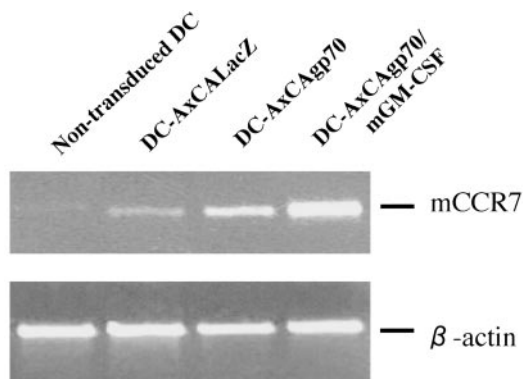


Fig. 4 *mCCR7* mRNA expression in genetically modified DCs. DCs were transfected with AxCALacZ, AxCAg70, or AxCAg70/mGM-CSF; total RNA was extracted from these DCs; and expression of *mCCR7* mRNA was assessed by RT-PCR using a pair of specific primers.

CSF survived significantly longer than mice in the control groups ($P < 0.01$). However, there was no statistically significant difference in survival between the DC-AxCagp70 group and the DC-AxCagp70/mGM-CSF group ($P = 0.3$; Fig. 5B).

DISCUSSION

Recently, as new approaches to achieve efficient DC-based immunotherapy against tumor have been explored, several groups have reported that immunization of mice with DCs adenovirally transduced with surrogate tumor antigen gene, such as ovalbumin, β -gal, or human TAA, elicits specific CTL responses and provides protective and therapeutic immunity against tumor cells, which express the same tumor antigen (10, 11). In this study, we used the murine CT26 colon cancer model. In normal mice, CT26 cells are poorly immunogenic and do not induce detectable tumor-specific CTLs (23). It has been reported that CT26 cells express gp70, an envelope protein of an endog-

enous ecotropic murine leukemia virus. This viral antigen, gp70, which is not expressed in the normal tissue of BALB/c mice, acts as the immunodominant antigen (17). Therefore, the CT26 tumor model is useful to investigate the efficacy of immunotherapy using DCs genetically engineered to express TAA.

Ad vector is a highly efficient and reproducible method of gene transfer. Indeed, several studies have shown that successful adenoviral gene transfer not only into murine DCs (10, 11) but also into human DCs (24) resulted in induction of a T-cell response against tumor. Ad vectors bind to the Coxsackievirus and Ad receptor, which is a high-affinity receptor for the Ad fiber protein, and transduce with the gene into cells (25). However, DCs have a small amount of surface expression of Coxsackievirus and Ad receptor; therefore, high doses of Ad vectors are required for high transduction efficiency, resulting in low viability of transduced DCs (26). To overcome this problem, we used a centrifugal method for adenoviral gene transfer into DCs following the previous report (20). As a result, transgene expression in DCs showed a remarkably high efficiency (75–86%), and DCs maintained high viability at a MOI of 100, which was a fairly lower dose compared with that in the previous studies. It is suggested that adenoviral gene transduction by a centrifugal method is markedly efficient and makes it possible for more than one gene to be transferred simultaneously into DCs, allowing for cotransduction of genes encoding distinct tumor antigens and/or immunostimulatory cytokines.

The present data demonstrated that an Ad vector encoding murine endogenous tumor antigen gp70 could effectively transfer and express the transgene in DCs. *In vivo* administration of these genetically modified DCs elicited a MHC class I-restricted, tumor-specific CD8⁺ CTL response. In s.c. tumor models, we also demonstrated that a single injection with gp70-transduced DCs had a remarkable effect in terms of suppression of tumor growth, and it was translated into a significant survival advantage. These observations underscore the ability of genetically modified DCs expressing endogenous tumor antigen to induce the therapeutic tumor-specific immunity *in vivo*.

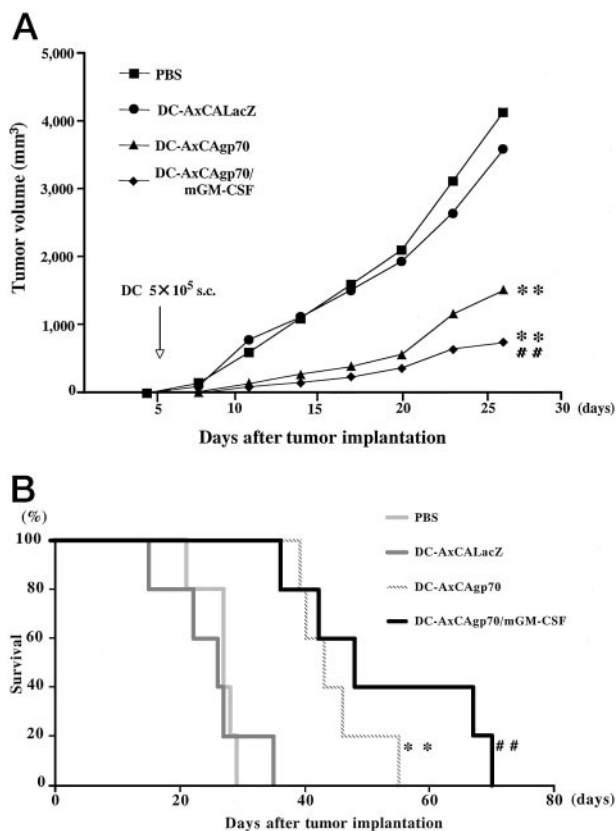


Fig. 5 A, tumor growth suppression in mice immunized with genetically modified DCs in the s.c. tumor model. BALB/c mice were inoculated s.c. in the right flank with 1×10^6 CT26 cells. Five days later, tumor-bearing mice ($n = 5$ mice/group) were treated with s.c. injection in the opposite flank with 5×10^5 DC-AxCALacZ (●), DC-AxCAGp70 (▲), DC-AxCAGp70/mGM-CSF (◆), or PBS (■). Results are presented as mean tumor volumes \pm SD of mice that developed tumors in each group. **, significantly different from DC-AxCALacZ and PBS ($P < 0.0001$). ##, significantly different from DC-AxCAGp70 ($P < 0.05$). B, survival advantage in mice immunized with genetically modified DCs in the s.c. tumor model. BALB/c mice were inoculated s.c. in the right flank with 1×10^6 CT26 cells. Five days later, tumor-bearing mice ($n = 5$ mice/group) were treated with s.c. injection in the opposite flank with 5×10^5 DC-AxCALacZ, DC-AxCAGp70, DC-AxCAGp70/mGM-CSF, or PBS. **, significantly different from DC-AxCALacZ and PBS (log-rank $P < 0.01$, Breslow-Gehan-Wilcoxon $P < 0.01$). ##, significantly different from DC-AxCALacZ and PBS (log-rank $P < 0.005$, Breslow-Gehan-Wilcoxon $P < 0.005$).

The strategy of immunizing mice with TAA-transduced DCs has potential advantages. First, DCs transduced with the entire TAA gene may present multiple epitopes including previously unknown epitopes associated with different MHC class I molecules. Second, TAA gene-transduced DCs may present helper epitopes associated with MHC class II molecules and may induce tumor-specific CD4⁺ T cells (8, 9). Third, DCs are provided with a renewable supply of antigen for presentation by transgene expression, as opposed to a single pulse of peptide. These advantages are also true of vaccine therapy using DCs pulsed with tumor lysate. Considering the clinical application, however, the strategy using tumor lysate requires tumor resection; therefore, its application is quite limited.

Several studies have demonstrated that immunostimulatory cytokines such as GM-CSF and IL-12 augment the efficacy of DC-based vaccines, especially within the local tissue environment. In this regard, GM-CSF seems to have the most profound effects on DC functions (13). GM-CSF gene transduction enhances the capacity of DCs to induce primary immune response (16). Quite recently, it has been reported that GM-CSF gene cotransduction augments the antitumor effect of vaccination with genetically modified DCs expressing surrogate tumor antigen more than IFN- α , IL-12, and CD40 ligand (27). In the present study, DCs were simultaneously transduced with the natural tumor antigen *gp70* gene and the GM-CSF gene, and we assessed the augmenting effect of cotransduction with the GM-CSF gene on therapeutic vaccine therapy using genetically modified DCs in CT26 tumor models. Our findings showed that specific CTL activity against CT26 in the spleen cells from mice immunized with DCs expressing *gp70* was extremely enhanced by cotransduction of DCs with the GM-CSF gene, and, importantly, that the optimal dose of AxCAmGM-CSF that elicited the maximum host immune response against tumor was 5 MOI. The optimal dose was relatively low, and the CTL response was rather suppressed at a higher dose. Indeed, other investigators have also demonstrated that a higher dose of GM-CSF than the optimal one could suppress the function activity of antigen-presenting cells or T lymphocytes, and it could prepare a host environment that accelerates the growth of the subsequent tumor challenge (15).

We focused on the migratory capacity of DCs to investigate the mechanism of this enhancing effect of GM-CSF gene transduction. DCs were labeled with a fluorescent marker and injected s.c. into the lower abdomen of mice. In the inguinal LNs, a significant increase in the number of fluorescence-positive cells was detected after s.c. injection of labeled DCs cotransduced with the *gp70* gene and the GM-CSF gene, compared with that of DCs transduced with the *gp70* gene alone. It is noteworthy that the optimal dose of AxCAmGM-CSF for enhancing the migratory capacity of DCs was 5 MOI, which was exactly consistent with the results of inducing specific CTL activity against CT26. These observations suggest that GM-CSF produced by DCs themselves enhances the migratory capacity and viability of DCs *in vivo*, leading to enhancement of specific CTL activity against tumor.

Recently, it has been demonstrated that some chemokines play a critical role in the migration of DCs (28). Secondary lymphoid tissue chemokine is constitutively expressed in the T-cell area of LNs, high endothelial venules, and mucosal lymphoid tissues and is an agonist for CCR7 (29). CCR7 mRNA expression on DCs increases progressively on their maturation (30). Quite recently, it has been shown that cocultivation of DCs with tumor cells induces CCR7 expression and, furthermore, that CCR7 gene-transduced DCs migrate more efficiently to draining LNs (31). These observations suggest that CCR7 expression in DCs would play an important role in promoting the migration of DCs to draining LNs, which constitutively express the CCR7 ligand, secondary lymphoid tissue chemokine. In the present study, we investigated mRNA expression of CCR7 on genetically modified DCs. Our findings showed that mRNA expression of mCCR7 of DCs adenovirally transduced with *gp70* was obviously up-regulated, whereas nontransduced DCs

did not express CCR7. Importantly, mRNA expression of *CCR7* on DCs transduced with the *gp70* gene was markedly enhanced by *GM-CSF* gene cotransduction. These results are consistent with the present data on the migration capacity of genetically modified DCs. It is proposed that *GM-CSF* gene transfer to DCs expressing TAA enhances the expression of CCR7 on DCs, leading to improved migratory capacity of DCs to draining LNs, in which genetically modified DCs could stimulate T cells. Consequently, a markedly effective antitumor immune response would be induced *in vivo*. These data suggested that GM-CSF produced by DCs themselves could optimize the efficiency of induction of a therapeutic immune response in mice immunized with DCs expressing TAA.

Vaccination using *gp70*-transduced DCs provided remarkable therapeutic efficacy in *s.c.* models, and tumor suppression was also augmented by GM-CSF cotransduction of DCs, and these results were compatible with the CTL activity against CT26 *in vitro*. However, these data were not reflected in the survival advantage. Although the reason for the discrepancy between CTL response and therapeutic efficacy in the tumor model is not clear, it is possible that CTLs generated in regional LNs or spleen would not sufficiently infiltrate the tumor. To achieve a survival advantage, we need to further investigate the timing and interval of DC administration, how many DCs are needed for one injection, and how many times DCs should be injected. Moreover, we need to examine the cotransduction of DCs with other cytokines such as IL-12.

In summary, vaccination therapy using DCs transduced with a gene encoding endogenous TAA resulted in generation of an efficient therapeutic immune response against tumor. Moreover, *GM-CSF* gene cotransduction of DCs enhanced the *in vivo* functions of DCs, especially the migratory capacity with up-regulation of CCR7 expression, and elicited a potent antitumor immune response. This strategy is considered feasible in humans because large numbers of DCs can be easily expanded from peripheral blood, and DCs also can be easily transduced with more than one gene of interest by a centrifugal method using Ad vector. However, further investigation is needed to optimize this vaccine therapy to achieve the obvious benefit in clinical application.

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