Adenovirus-mediated mGM-CSF in vivo Gene Transfer Inhibits Tumor Growth in a Murine Meth A Fibrosarcoma Model

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=Abstract=

The effectiveness of noninfectious recombinant adenovirus encoding murine granulocyte-macrophage colony stimulating factor (mGM-CSF) for the treatment of Meth A fibrosarcoma was investigated in syngeneic BALB/C model. Meth A and HeLa cells transduced with the recombinant adenovirus (Ad.mGM-CSF) produced substantial amounts of mGM-CSF, while WEHI164 cells transduced with the virus did not produce mGM-CSF. Mice inoculated subcutaneously with 1×10^6 Meth A cells, followed by injection of Ad.dE1 as a control, developed large tumors that reached a mean tumor size of 22 mm by day 30. However, tumor development and tumorigenicity were significantly inhibited in mice with a single intratumoral injection of Ad.mGM-CSF at 1×10^8 pfu. Histological examination of the tumors injected with Ad.mGM-CSF revealed dense infiltrates of neutrophils, histiocytes, lymphocytes, and eosinophils associated with apoptotic cell death. The results suggest that the recombinant adenovirus encoding GM-CSF have a potential use for cancer gene therapy.

Key Words: Adenovirus vector, GM-CSF, Cancer gene therapy

INTRODUCTION

Gene therapy of human cancer is under intensive investigation (6,23). Cancer immuno-gene therapy has been a challenging but promising modality among various gene therapeutic approaches for the treatment of cancer, as it may have a great potential abrogating malignant tumor cells under microenvironment by generating tumor-specific immune responses of the host. It appears that any gene transfer system using viral or nonviral vectors currently available can not deliver therapeutic genes into all the tumor cells. However, immune-stimulatory genes do not necessarily have to be delivered into all the tumor cells, as far as a small portion of tumor cells expressing them may be able to trigger systemic, tumor-specific immune responses of the host.

Adenovirus vectors have been used extensively in cancer gene therapy, as they deliver therapeutic genes into cells or tissues efficiently and transduced genes are expressed transiently and

strongly (26). Long-term expression of therapeutic genes may not be absolutely required in cancer gene therapy using immune-potentiating genes. Therefore, adenovirus vectors among other gene transfer systems may be suitable vectors for tumor vaccines or cancer immuno-gene therapy (26).

Vaccination with tumor cells secreting immune-stimulatory proteins such as IL-2 (11,27) and IL-12 (4) have been shown to induce systemic tumor-specific immune response in syngeneic hosts. GM-CSF is one of immunopotentiating agents that can expand the numer of potent antigen-presenting cells such as dendritic cells as well as mature macrophages (10,15). Consequently, GM-CSF can enhance the host immunity to respond to foreign antigens. Since Dranoff et al. (8) showed that vaccination with irradiated murine melanoma cells expressing mGM-CSF stimulated potent, specific, and long-lasting antitumor immunity, a number of studies have been carried out to examine the efficacy and molecular mechanism of tumor vaccines expressing GM-CSF. Ex vivo cancer gene therapy using GM-CSF-encoded retrovirus or adenovirus vectors was performed in human osteosarcoma, Lewis lung carcinoma, colon carcinoma, melanoma, and renal cell carcinoma models (1, 2,14,18,24,25). Adenovirus-mediated mGM-CSF gene therapy combined with suicide genes or other cytokine was carried out in the murine model for melanoma or hepatic metastases of colon carcinoma (3,5). These studies revealed that GM-CSF plays an important role in eliciting potent, long-lasting antitumor immune responses in the hosts. However, molecular mechanism of antitumor effect of GM-CSF is still not clear. Futhermore, adenovirus-mediated mGM-CSF in vivo cancer gene therapy has not been reported in murine Meth A fibrosarcoma model. In this study, we constructed a noninfectious recombinant adenovirus encoding mGM-CSF and investigated antitumor effect of the recombinant adenovirus in the Meth A tumor model.

MATERIALS AND METHODS

Tumor cells and virus

Meth A and WEHI164 sarcoma cells were obtained from Dr. Young Sang Kim (Chungnam National University) and the Korean Type Culture Collection (KTCC), respectively. The tumor cells were grown at 37℃ in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS)-penicillin-streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids. HeLa cells were grown in Dulbecco's modified essential medium containing 10% FBS-penicillin-streptomycin.

An adenovirus mutant (Ad.dE1) with a deletion of E1 region was spontaneously obtained during the construction of replication-defective recombinant adenovirus using pJM17. Ad.dE1 virus can propagate only in 293 cells in which E1A and E1B proteins of adenovirus are constitutively expressed (13), but not in HeLa, Meth A and WEHI164 cells. Recombinant and mutant adenoviruses for animal studies were propagated in 293 cells. Virus purification was done by sequential centrifugation in CsCl step gradient as previously reported (17). Virus titers were determined on 293 cells as plaque-forming unit (pfu).

Experimental animals

BALB/C female mice (6∼7 weeks old) were obtained from Dae Han laboratory animal center, Korea, adjusted to five mice per cage, and kept in isolation under strictly controlled specific-pathogen-free conditions. Animals were exposed to 12-h light/12-h dark cycles, and standard feed and water were provided *ad libitum.*

Construction of an adenovirus recombinant

The mGM-CSF cDNA fragment was isolated by digesting pCRIImGM-CSF with *Nco*I, blunt-ending with Klenow enzyme, and then digesting with *Bam*HI. The cDNA fragment was inserted into the *Eco*RI (blunt-ended by Klenow) and

*Bam*HI sites of adenovirus transfer plasmid, pXC/
CMV encoding HCMV promoter and SV40 poly
(A) sequences, by standard cloning procedures
(21). The resulting pX/CMV/mGM-CSF was
cotransfected with pJM17 into 293 cells by cal-
cium phosphate DNA transfection method (12).
The correct recombinant adenovirus encoding
mGM-CSF, generated by in vivo homologous
recombination, was confirmed by restriction en-
zyme analyses of its viral DNA and plaque-pu-
rified twice.

Expression analyses of mGM-CSF

HeLa monolayer cells growing on 60-mm
dishes were uninfected or infected with Ad.
mGM-CSF at multiplicity of infection (MOI)
of 100 and 300 or with Ad.dE1 at MOI of 100.
Cells were harvested at 48 h post-transduction
and RNAs were obtained by cytoplasmic RNA
extraction method (21). An equal quantity of cy-
toplasmic RNAs was electrophoresed by using
MOPS buffer on 1% agarose gel containing 6%
formaldehyde. The gel was transferred to a ni-
trocellulose membrane. The mGM-CSF and hu-
man β-actin cDNA fragments were labeled with
[α-^{32}P]ATP by nick translation and used as
probes. After hybridization, the membrane was
washed and autoradiographed.

HeLa, Meth A, or WEHI164 cells growing
on 60-mm dishes were infected with Ad.mGM-
CSF or Ad.dE1 as a control virus at various
MOI. Culture media were taken out at 24 h post-
transduction. Amounts of mGM-CSF secreted
into the culture media were measured by mGM-
CSF ELISA-kit (Amersham).

Establishment and treatment of Meth A fibrosarcoma model

Tumors were generated by subcutaneous in-
jection of 10^6 cells of Meth A into each of 15
mice. The mice were then randomly divided
into three groups (n=5 per a group). Ad.mGM-
CSF or Ad.dE1 viruses in 50 μl of 10 mM Tris-
HCl buffer (pH 7.4) containing 1 mM MgCl$_2$,
10% (v/v) glycerol, and polybrene (20 μg/ml)
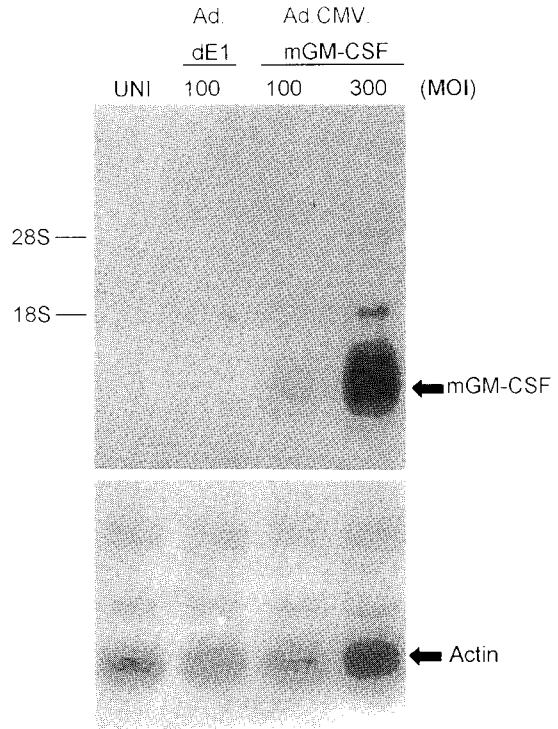
Figure 1. Northern blot analysis of mGM-CSF ex-
pression in HeLa cells. HeLa cells were uninfected
(UNI) and transduced with Ad.dE1 at MOI of 100,
and Ad.mGM-CSF at MOI of 100 and 300. Thirty
micrograms of cytoplasmic RNAs were subjected
to Northern blot by using ^{32}P-labeled mGM-CSF
cDNA fragment as a probe. To confirm the presence
of apporoximately equal amounts of RNA samples,
filter was then stripped and reprobed with human β-
actin cDNA.

were intratumorally injected at a week after in-
jection of tumor cells. One group of mice was
left untreated. Tumor size was measured in in-
tervals of 2~3 days. Linear calipers were used
to measure the longest (a) and shortest (b) di-
ameters. The tumor size was calculated by the
formula: (a+b)/2.

Histology

Tumor samples excised from the mice were
fixed in 10% buffered formalin and embedded
in paraffin for histologic examination. Five-mi-
crometer thick sections from the paraffin blocks
were stained with hematoxylin-eosin (H & E)
and examined under light microscopre.
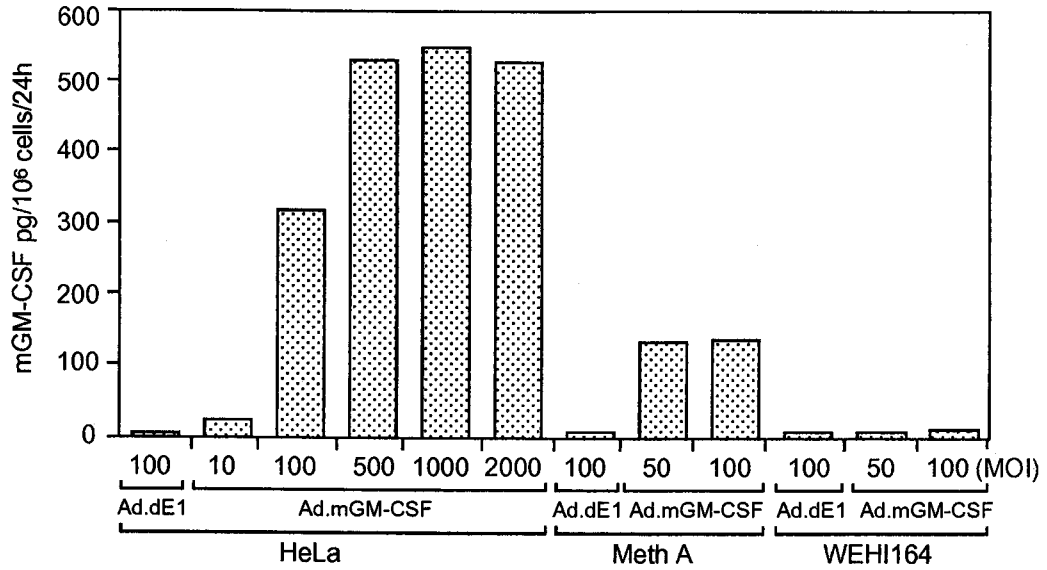
Figure 2. Secretion of mGM-CSF into culture media of HeLa, Meth A, and WEHI164 cells transduced with Ad.mGM-CSF. Tumor cells were transduced with Ad.dE1 and Ad.mGM-CSF at different MOIs. Culture media were taken out at 24 h post-transduction. Amounts of mGM-CSF present in the media were measured by ELISA.
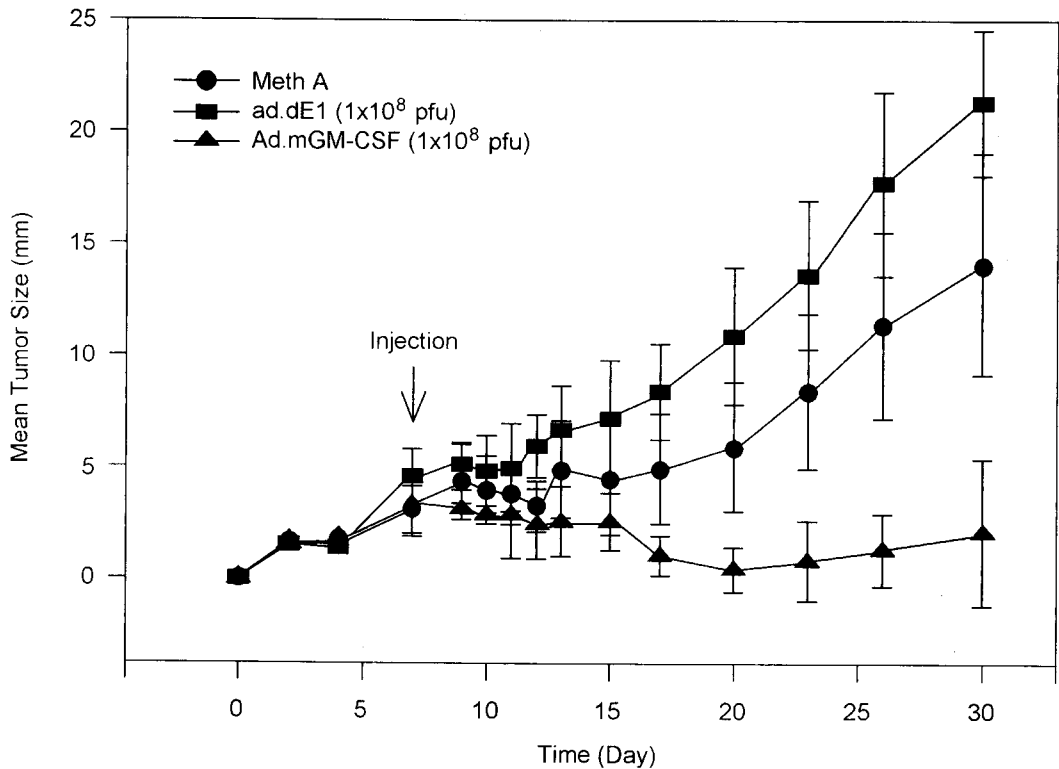
Figure 3. Inhibition of tumor development by Ad.mGM-CSF. Fifteen mice were inoculated with 1×10^6 Meth A cells subcutaneously and divided into three groups (five mice per a group). Ad.mGM-CSF and Ad.dE1 viruses at 1×10^8 pfu were intratumorally injected at 7 days after injection of tumor cells, respectively. One group was left untreated. Average values of tumor size of mice were expressed by sum of longest and shortest diameters of tumor, which were divided by two.

A   B
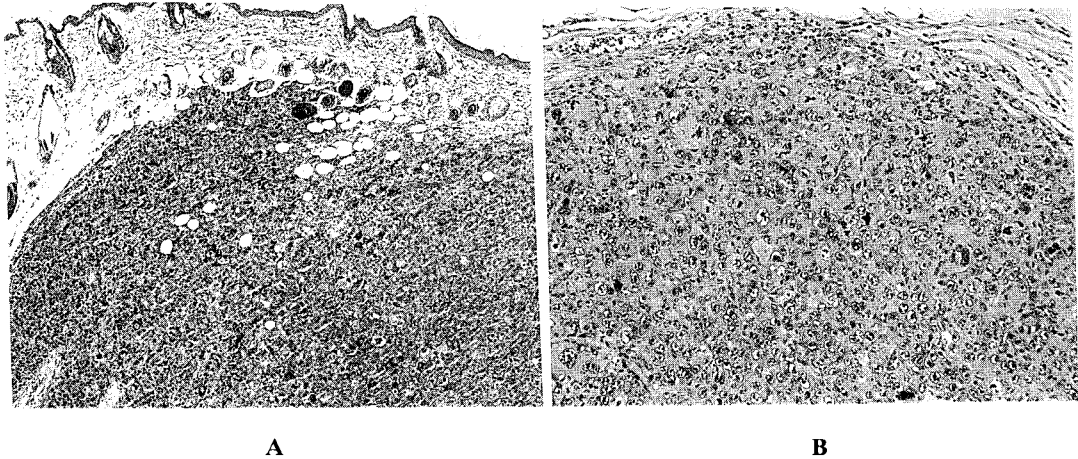
Figure 4. Histological examination of tumor nodules treated with Ad.dE1. **(A)** Nodular proliferation of pleomorphic tumor cells is present in the subcutaneous adipose tissue of mice in control group at 26 days after Ad. dE1 treatment (H & E × 40). **(B)** Sparse infiltrates of lymphoid cells and neutrophils were seen on higher magnification (H & E × 100).

Table 1. Antitumor effect by Ad.mGM-CSF in murine Meth A tumor model

Intratumoral injections	n	Survival (days)	Tumorigenicity (%)
None	5	64, 68, 75, 83, >120	80
Ad.dE1	5	50, 53, 64, 68, >120	80
Ad.mGM-CSF	5	68, 81, >120, >120, >120	40

BALB/C mice (n = five mice per a group) were injected with 1×10^6 Meth A cells subcutaneously. Ad.dE1 and Ad.mGM-CSF viruses at 1×10^8 pfu were once directly injected into palpable tumor nodules at a week after injection of Meth A tumor cells. Tumorigenicity was determined at the end of observation. None stands for tumor-bearing mice left untreated.

RESULTS

Expression of mGM-CSF in tumor cells transduced with the recombinant adenovirus

HeLa cells were transduced with Ad.mGM-CSF at MOI of 100 and 300, and the cytoplasmic RNAs were prepared. A presence of mGM-CSF-specific RNA was analyzed by Northern blot (Figure 1). The mGM-CSF transcript was detected only in HeLa cells transduced with Ad.mGM-CSF. Next, we determined whether tumor cells transduced with Ad.mGM-CSF secreted mGM-CSF into culture media. HeLa, Meth A, and WEHI164 cells were transduced with the recombinant adenovirus at various MOI.

Culture media obtained at 24 h post-transduction were analyzed by ELISA-kit for mGM-CSF (Figure 2). About 317 and 135 pgs of mGM-CSF/ml medium were present in the culture media of the HeLa and Meth A cells transduced with Ad.mGM-CSF at 100 MOI, respectively. However, mGM-CSF was not detected in the culture media of the virus-transduced-WEHI 164 cells.

Antitumor effect of Ad.mGM-CSF

As substantial amounts of mGM-CSF was produced from Ad.mGM-CSF-transduced Meth A cells, but not from the virus-transduced WEHI164 cells, we used Meth A cells to examine whether Ad.mGM-CSF has an antitumor
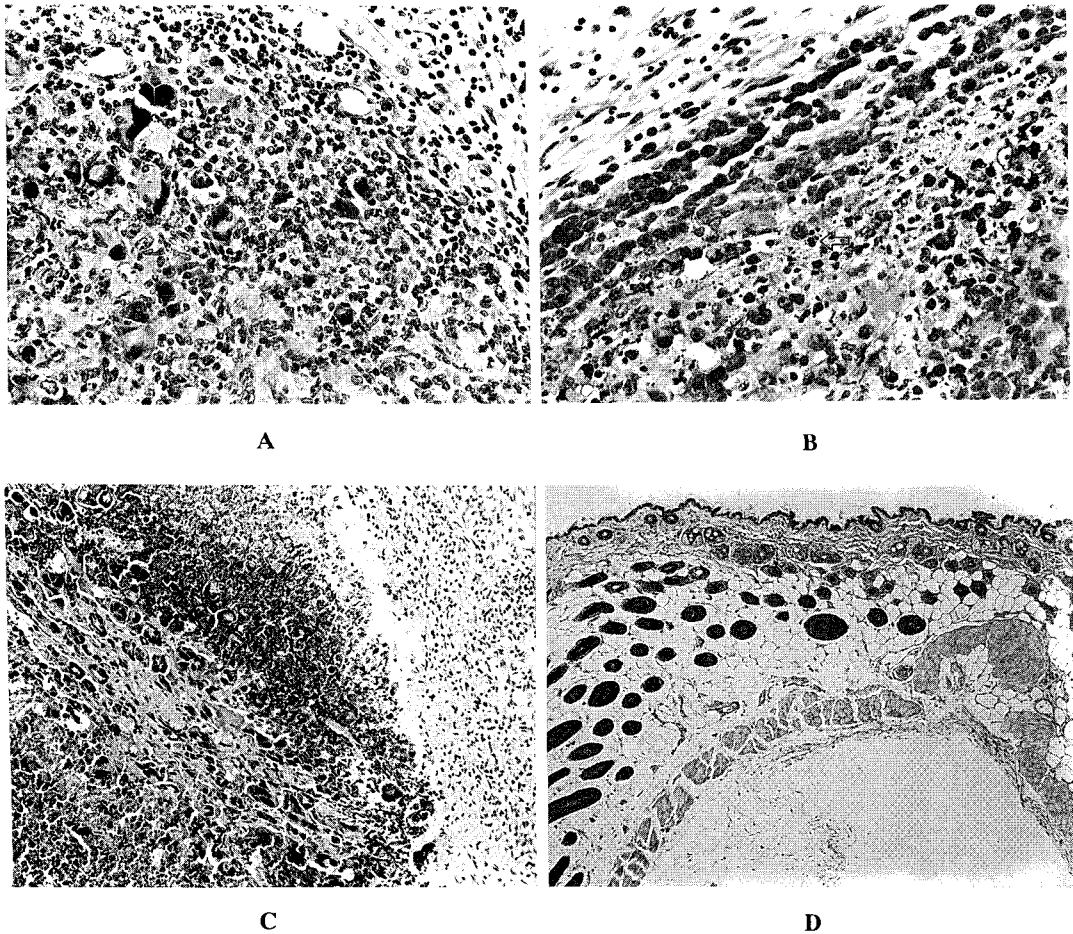
Figure 5. Histological examination of tumor nodules treated with Ad.mGM-CSF. **(A)** Moderately dense cellular infiltrate, consisting of neutrophils, histiocytes, lymphocytes, and a few eosinophils, is present at the periphery of tumor and between tumor cells in mice at 3 days after Ad.mGM-CSF treatment (H & E × 200). **(B)** A confluent focus of apoptotic cell death of tumor cells (indicated by an empty arrow) is seen (H & E × 200). **(C)** Massive necrosis and apoptotic cell death of tumor cells are present in mice at 7 days after Ad.mGM-CSF treatment. Tumor cells are surrounded by a dense infiltrate of lymphoid cells, neutrophils, and histiocytes (H & E × 100). **(D)** No residual tumor is present in mice at 26 days after Ad.mGM-CSF treatment (H & E × 40).

in murine tumor model. Fifteen syngeneic mice were injected with 1×10^6 Meth A cells subcutaneously. Mice with palpable tumors were divided into three groups (five mice per a group) randomly. A single intratumoral injection of 1×10^8 pfu of Ad.mGM-CSF and Ad.dE1 as a control virus was given to mice in the experimental and control groups at a week after injection of tumor cells, respectively. Five mice were left untreated. Tumor size of each mouse was measured almost every other day by day 30

after injection of tumor cells. Average values of tumor size of mice in three groups are shown in Figure 3. Tumor developed to 22 mm in mice injected with control adenovirus, Ad.dE1, and to 13 mm in mice left untreated by day 30. However, tumor development of mice injected with Ad.mGM-CSF was inhibited notably. It is interesting to note that the control mice injected with Ad.dE1 virus developed larger tumor than the untreated. The reason is not clear, but we may speculate that Ad.dE1 contains the

E4orf6 gene, product of which is known to bind and inactivate p53, and the gene product expressed in tumor nodule at low level might have contributed to the tumor development.

To assess treatment outcome, mice with tumors were studied for long-term survival. Eight mice in control groups, namely four of five mice left untreated and four of five mice injected with Ad.dE1 virus, died by 83 days because of large development of tumor (Table 1). Spontaneous tumor regression was observed from day 17 in a mouse in the untreated mice and from day 15 in a mouse in the mice injected with Ad.dE1, and the mice survived more than 120 days. Thus tumorigenicity of mice in the control groups was 80%. Tumor development was initially inhibited in all the five mice treated with Ad.mGM-CSF. However, two of five mice of which tumor development recurred at day 17 and after day 30, and consequently died by 68 and 81 days, respectively. Three out of five mice treated with Ad.mGM-CSF became tumor-free and survived over 120 days, although we can not rule out that one of three tumor-free mice might have occurred spontaneously. Thus, tumorigenicity of the mice treated with Ad. mGM-CSF was 40%. The results suggest that a single intratumoral injection of Ad.mGM-CSF inhibited tumor development upto 50% in the murine Meth A tumor model.

Immune cells are recruited at the tumor site by Ad.mGM-CSF

Histological examination of tumor samples was performed at 3, 7, and 26 days after the teatments of mice with Ad.dE1 and Ad.mGM-CSF. The tumor volume of the control group steadily increased with time. At 26 days after the treatment of Ad.dE1 (Figure 4A and B), nodular proliferations of pleomorphic tumor cells which invaded the adjacent skeletal muscle bundles were observed in the subcutaneous adipose tissue, suggesting that tumor developed subcutaneously. Sparse infiltrates of lymphoid cells and neutrophils were identified in the tu-

mor of the control group. At 3 days after the treatment of Ad.mGM-CSF (Figure 5A and B), a moderately dense cellular infiltrate, consisting of neutrophils, histiocytes, lymphocytes, and a few eosinophils, was identified at the periphery of tumor and between tumor cells. Apoptotic cell death of tumor cells, indicated by an arrow in Figure 5B, was focally present. Macrophages with phagocytic apoptotic remnants were also present. At 7 days after the treatment of Ad. mGM-CSF, confluent foci of apoptotic tumor cell death were prominent (Figure 5C). There was no residual tumor at 26 days after the treatment of Ad.mGM-CSF (Figure 5D). Thus, histological examination of tumors suggests that the antitumor effect by the treatment of tumor-bearing mice with Ad.mGM-CSF might result from a recruitment of immune cells at the tumor site.

DISCUSSION

In search of a therapeutic means using an immune-potentiating gene for the treatment of cancer, it is important which immune-modulatory gene as well as which gene transfer system are being used. In this study human adenovirus vector and a gene for mGM-CSF were used. Human adenovirus vector has been known to transduce broad spectrum of tissues such liver, lung, brain, muscle, and macrophages (16). However, it appears that adenovirus vector-mediated gene transduction or expression-efficiency depends on cell types, as HeLa cells transduced with Ad.mGM-CSF produced about two-fold more mGM-CSF than Meth A cells at the same MOI. WEHI164 cells transduced with the virus almost did not produce mGM-CSF under the same condition (Figure 2). These results suggest that when adenovirus vector is being used in any gene therapy experiment, transduction or expression-efficiency of target cells by adenovirus vector may be a critical factor for positive outcome.

The irradiated GM-CSF-transduced melanoma

with retroviral vector has been shown to stimulate potent, specific, and long-lasting antitumor immunity in murine B16 melanoma model (8). The tumor vaccine induced potent, T-cell mediated antitumor immune responses. Subsequently phase I study was carried out with an autologous GM-CSF gene-transduced tumor vaccine for renal cell carcinoma patients (24). An objective partial response was observed in a patient. Murine colon carcinoma cells producing GM-CSF after retroviral transduction resulted in prolonged survival and reduced tumorigenicity (14). In vitro transduction of Lewis lung carcinoma cells with adenovirus-encoded mGM-CSF suppressed tumor formation in syngeneic mice, and the transduced and irradiated Lewis lung carcinoma cells induced regression of pre-established wild type tumors without in vitro selection for transductants (18). Low levels of specific antitumor cytotoxic T lymphocytes (CTLs) were detected in mice inoculated with adenovirus-encoded mGM-CSF. In hepatic metastases model of colon carcinoma, local expression of GM-CSF in hepatic tumor and prolonged IL-2 expression were necessary to generate persistent antitumor immunity (5). In melanoma-bearing mice, adenovirus-mediated GM-CSF and cytosine deaminase genes transfer followed by 5-fluorocytosine administration generated potent antitumor response (3). These studies indicated that GM-CSF is an effective tool for the treatment of cancer by eliciting potent, long-lasting, tumor-specific immune responses of the host. Our results confirm the antitumor effect of Ad.mGM-CSF in fibrosarcoma model, which was not reported previously. However, the precise molecular mechanism of the antitumor effect of GM-CSF is still not clear. We examined whether tumor-specific CTL is present in the tumor-bearing mice treated with Ad. mGM-CSF. So far we could not detect any tumor-specific CTLs. However, histological examination of the tumor-bearing mice treated with Ad.mGM-CSF indicated that immune cells such as neutrophils, histiocytes, lymphocytes, and

eosinophils were increased at the periphery of tumor and between tumor cells (Figure 5A). Consequently these cells were associated with apoptotic cell death of tumor (Figure 5B and C). Tissue sections from GM-CSF producing melanoma cells excised from inoculation sites of the mice showed a dense inflammatory infiltrate composed of neutrophils, tissue macrophages, numerous CD4- and CD8-positive lymphocytes, large number of dendritic cells and cells expressing the B7-2 costimulatory molecule (2). This and our results suggest that antitumor effect of GM-CSF may result from a recruitment of various immune cells including dendritic cells at tumor sites.

Although we observed an antitumor effect of mGM-CSF in Meth A tumor model, antitumor effect of Ad.mGM-CSF could not be observed in CT26 colon adenocarcinoma model (data not shown). A reason may be a poor transduction efficiency of Ad.mGM-CSF into CT26 cells. Another reason may result from different characteristics of Meth A and CT-26 tumor cells. CT-26 cells are aggressive and nonimmunogenic (9). Meth A tumor cell line is derived from fibrosarcoma that was induced in BALB/C mice by treatment with methycholanthrene (7). Meth A cells grow aggressively in vivo like CT-26 cells, but immunogenic (22). A fibrosarcoma derived from C3Hf mouse has also moderate immunogenicity and grows progressively (20). Small size inocular ($10^1 \sim 10^3$ cells) of Meth A cells from a murine fibrosarcoma induced immunologic tolerance and moderate size inocula ($10^4 \sim 10^6$ cells) were immunogenic, but more than 10^6 cells were again tolergenic when injected subcutaneously into C3Hf mice (19). We used 10^6 Meth A cells derived from BALB/C mouse to generate subcutaneous tumors in mice. Thus, relatively high transduction efficiency of Meth A cells with the recombinant adenovirus and moderate immunogenicity of Meth A cells depending on inocular size may have resulted in a significant inhibition of tumor development in mice by a single intratumoral injection of Ad.

mGM-CSF. This character of Meth A cells may also account for spontaneous regression of tumor in mice of the control groups at low level (Table 1). In conclusion, our results confirm the effectiveness of GM-CSF and adenovirus vector for in vivo cancer gene therapy, and expand the antitumor effect of Ad.mGM-CSF to a fibrosarcoma model.

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