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Experimental Gene Therapy for Colon Carcinoma both *in Vitro* and *in Vivo* Using a Deoxycytidine Kinase Suicide Gene Together with Cytosine Arabinoside

Motoyuki NAKAMURA[1,2], Yoshinobu MANOME[2], and Tsuneya OHNO[1,2]

[1]*Department of Oncology, The Institute of DNA Medicine, The Jikei Univercity School of Medicine*
[2]*Department of Microbiology, The Jikei University School of Medicine*

ABSTRACT

Numerous gene therapy studies have already been carried out on carcinoma, but attempts at gene therapy for colon carcinoma, in which conventional approaches such as surgery are expected to be effective, are few. Gene therapy may, however, become a useful alternative for the treatment of inoperable cases such as patients with advanced colon carcinoma. Among the various strategies of gene therapy, we considered that a system using a suicide gene is closer to clinical application than other strategies, because anticancer agents, whose clinical efficacy and safety have been proved, are used in this system. In the present study, we employed deoxycytidine kinase (dCK), which activates cytosine arabinoside (ara-C), dCK/ara-C system, to examine whether the suicide gene system is effective for colon carcinoma. The dCK cDNA was inserted into retroviral and adenoviral vectors, and then transduced to MC38 cells, mouse colorectal cancer cells. The feasibility of the clinical application of this gene therapy using a dCK/ara-C system was also evaluated. An *in vitro* cytotoxic assay revealed that the sensitivity of MC38 cells transduced by the retroviral vector to ara-C was 4-10 times higher than that of the control. The sensitivity of MC38 cells transduced by the adenoviral vector to ara-C was approximately 4 and 10 times higher than that of the control at 100 and 500 multiplicity of infection (MOI), respectively. *In vivo* experiments using subdermal tumor models revealed that in the case of retroviral vector, the treatment with ara-C reduced the growth of tumor cells by approximately 80% compared to the control, whereas in the case of adenoviral vector the growth of tumor cells was reduced by 40-50% after the treatment with ara-C. The present results imply that the dCK/ara-C system might be useful as a treatment for colorectal carcinoma. (Jikeikai Med J 2002 ; 49 : 173-81)

Key words : gene therapy, suicide gene, cytosine arabinoside, deoxycytidine kinase, colon carcinoma

INTRODUCTION

Gene therapy is generally believed to offer a promising new approach to the treatment of cancer. A number of effective strategies have been proposed and extensively studied so far, including the transfer of genes that induce antitumor immune responses, regulate growth or sensitize tumor cells to specific prodrugs[1-4]. Clinically effective gene therapy systems for colorectal carcinoma, however, have not been fully studied. We considered that a suicide gene/prodrug system should be tried first for possible

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中村　素行，馬目　佳信，大野　典也
Mailing address : Motoyuki NAKAMURA, Department of Oncology, The Institute of DNA Medicine, The Jikei University School of Medicine, 3-25-8, Nishi-Shimbashi, Minato-ku, Tokyo, 105-8461, Japan.
E-mail address : soko@jikei.ac.jp

clinical application of gene therapy to colorectal carcinoma. This strategy involves the transfer and expression of genes encoding enzymes that convert nontoxic prodrugs into cellular toxins[5–7]. Previously, we reported a marked tumor-killing effect of a suicide gene/prodrug system using deoxycytidine kinase (dCK) gene and cytosine arabinoside(ara-C) against glioma, a solid tumor[8]. It was thought that colorectal carcinoma could also be successfully treated by this gene therapy system. An antimetabolitic drug, ara-C, is one of the most effective agents in the treatment of acute myeloid leukemia[9–11]. Its advantages are as follows : 1) ara-C is pharmacokinetically a classic drug, for which the clinical safety has been well established[12]. 2) High-dose regimens of ara-C have been established and are currently used in clinical settings[13,14]. Although the most frequent and fatal side effect of ara-C is bone marrow suppression, administration of high doses of ara-C has been attempted, while taking bone marrow transplantation into consideration[15]. Ara-C has been reported to show only a limited efficacy against most gastrointestinal tract tumors such as colon carcinomas, probably because of insufficient levels of dCK activity in colon carcinoma cells[16]. Our preliminary works demonstrated that transduction of the dCK gene into brain tumors conferred sensitivity to ara-C and that this dCK/ara-C system seems to have immunological as well as pharmacokinetic advantages[8]. In the present study, we investigated experimentally the feasibility of this system in colon carcinoma. It was demonstrated that transduction of the dCK gene by viral vectors expressing the dCK gene increased the sensitivity of the transfected cells to the cytotoxic effects of ara-C both *in vitro* and *in vivo*.

Materials and Methods

Tumor cells and animals

MC38 murine adenocarcinoma cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum[17].

Retroviral transfection

The amphotrophic PAMP51[18] retrovirus packaging cells were transfected as described previously[8]. A 0.8-kb fragment of the human dCK cDNA was cloned into the *Eco*R I site of the pMV7 retroviral vector. This plasmid was designated pMV7-dCK. In pMV7-dCK, the dCK cDNA is located just 3′, and is transcribed from the murine Moloney leukemia virus long terminal repeat (LTR). The neo gene is transcribed from a thymidine kinase promoter. The supernatant from PAMP51/pMV7 or PAMP51/pMV7-dCK retroviral producer cells was used to

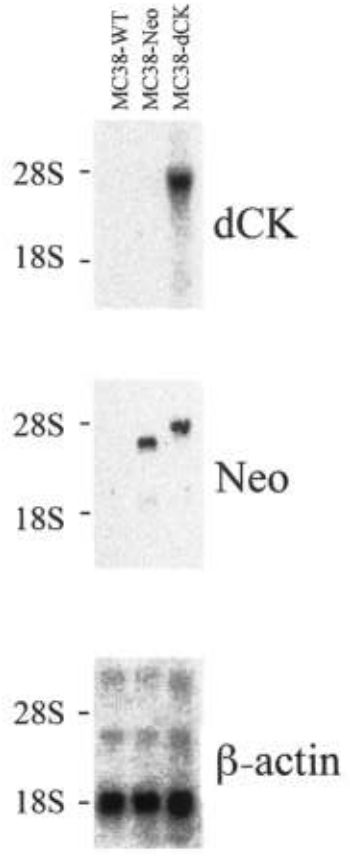

Fig. 1. dCK-Specfic mRNA expression in pMV7-dCK retrofectants. Total RNA (10 μg) was analyzed for expression of the transgenes by Northern blot analysis. The full length dCK cDNA was used to probe for expression of dCK mRNA. The neomycine 3′phosphotransferase (neo) cDNA was used as a control to show expression of neo mRNA in both MC38-Neo and MC38-dCK cells. Hybridyzation to the β-actin probe demonstrates equal loading of the lanes.

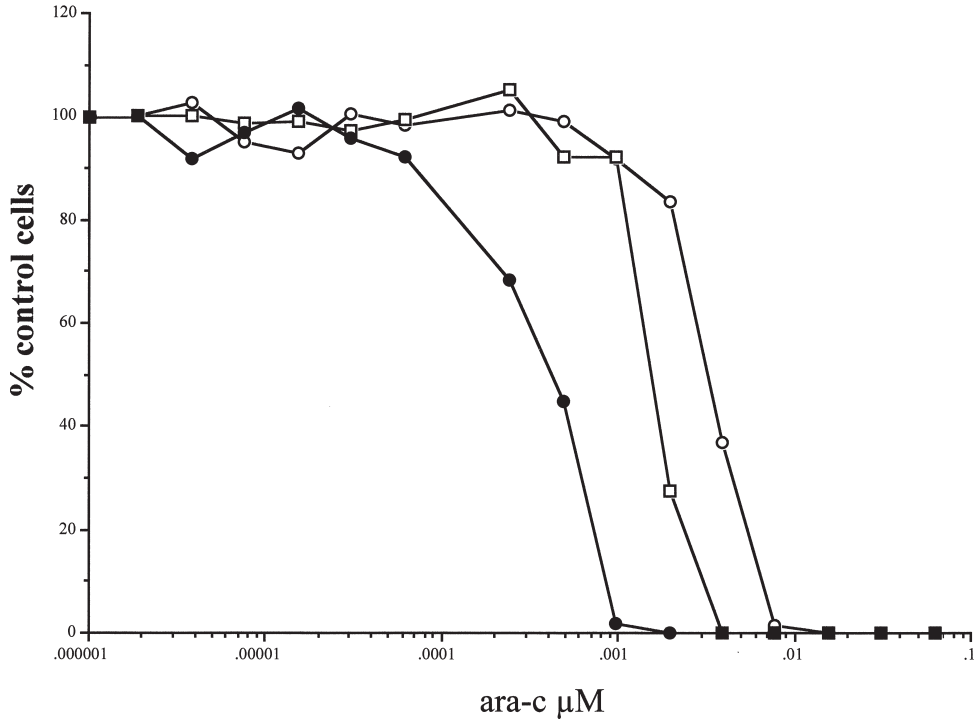

Fig. 2. Sensitivity of retrofectants to the cytotoxic effects of ara-C. Cells were exposed to the indicated concentrations of ara-C for 72 h. Cytotoxity was determined by fixation, staining with methylene blue, and monitoring absorbance at 600 nm. Symbols: MC38-WT(○); MC38-Neo(□); MC38-dCK(●). The MC38-dCK cells were significantly more sensitive to ara-C than the MC38-WT or MC38-Neo cells.

transfect MC38 target cells. After infection, MC38 cells were selected with 800 μg/ml geneticine sulfate (GIBCO BRL, Gaithersburg, MD, USA). MC38-Neo (retrofected by pMV7) and MC38-dCK (retrofected by pMV7-dCK) were characterized further.

Recombinant adenovirus

The constructions of the recombinant adenoviruses Ad.CMV-β-gal (kindly provided by Ronald Crystal) and Ad.CMV-dCK were previously described[8]. The virus stocks were purified by cesium chloride ultracentrifugation, dialyzed against 10% glycerol, 10 mM Tris (pH 8.0) and 1 mM MgCl2 (viral vehicle), and stored at −80°C. Virus titers were determined as PFU (plaque forming units) assayed in semisolid cultures of 293 cells[8]. The virus titer was 2×10^8 PFU.

Northern blot analysis

Total cellular RNA was purified from MC38-Neo and MC38-dCK using RNA ZOL B (TEL-TEST, Friendswood, TX, USA). MC38 cells were infected with Ad.CMV-dCK in 1 ml medium at a MOI of 100. Forty-eight hours later, total RNA was extracted by RNA ZOL B. The RNA (10 μg) was separated by electrophoresis on 1% agarose-formaldehyde gels. It was then transferred to nitrocellulose filters and hybridized to the following ^{32}P-labeled DNA probes: 1) a 0.8-kb *Nco* I and *Bam*H I fragment of the dCK gene from the pET3d-dCK plasmid[8]; 2) a 1.3-kb *Hind*III fragment from the p1Aneo plasmid (kindly provided by Earl Ruley, Massachusetts Institute of Technology) containing the neomycin 3′-phosphotranspherase cDNA sequence; 3) a 3.5-kb *Not* I fragment from the pcDNA3-LacZ vector (kindly provided by Dr. Mitsuyoshi Yamazoe, Kumamoto University) containing the β-galactosidase gene; and 4) a 1.5-kb

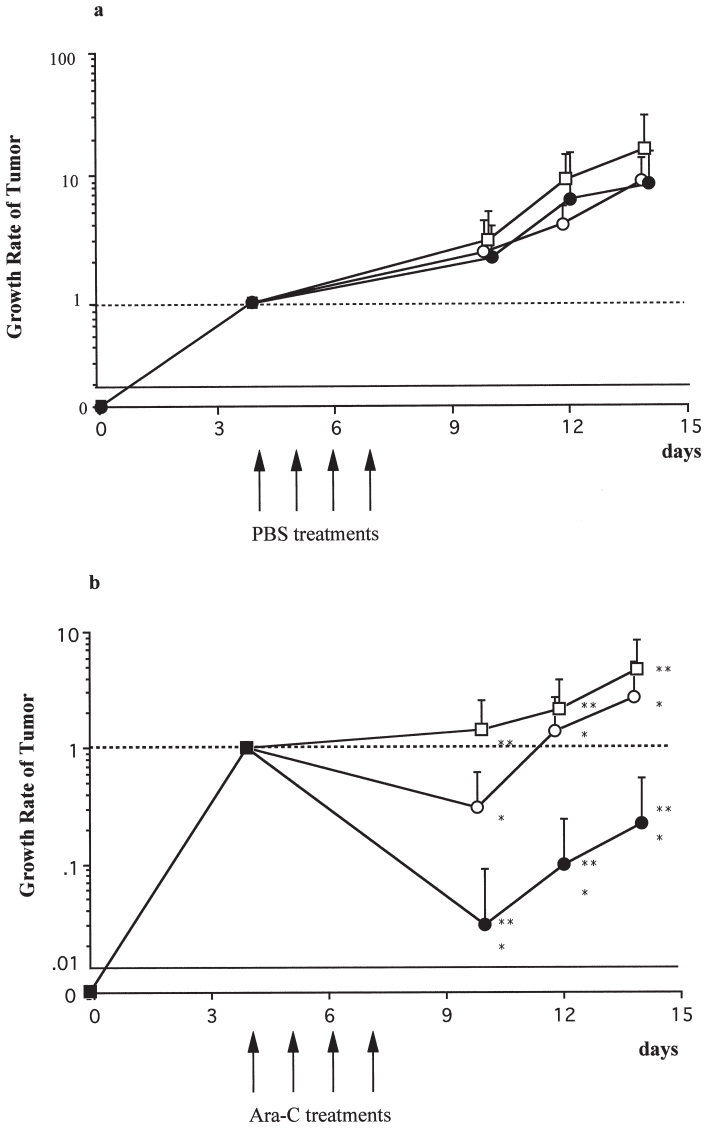

Fig. 3. *In vivo* growth of subdermal retrofected MC38 cells treated with ara-C. MC38-WT, MC38-Neo, or MC38-dCK cells (2×10^6) were inoculated subdermally on day 0. On day 4, mice were treated with PBS (a), or ara-C (b). Symbols: MC38-WT (○), MC38-Neo (□), MC38-dCK (●). The results, expressed as the means±S.D. of tumors treated with ara-C compared with the other groups, were statistically significant (*, **, $p<0.05$; t-test).

*Eco*R I insert of the human β-actin gene from the pBlue script SK(−) vector (American Type Culture Collection, Rockville, MD). Hybridization was performed at 42°C for 12 hrs, and signals were detected using a Bas 2000 phosphorimager (Fuji Film, Tokyo, Japan).

In vitro cytotoxic assay

The cytotoxic assay was performed as described previously[8]. Briefly, cells (5×10^3) were seeded into individual wells of a 96-well microtiter plate (Corning, NY, USA) and treated with various doses of ara-C for 72 hrs. The cells were fixed for 15 min after ara-C exposure by adding 10 μl of 25% glutaraldehyde to each well. After several washes with running water,

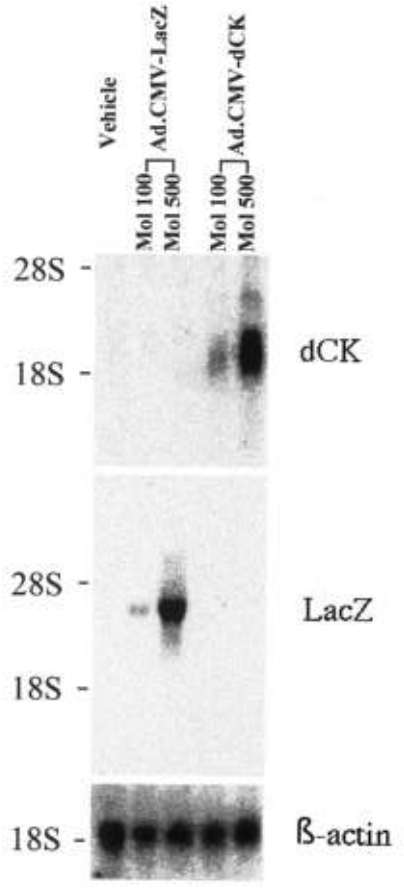

Fig. 4. Northern analysis of dCK expression in MC38 cells transduced with the Ad.CMVdCK. Forty-eight hours after transduction, cells were harvested and total RNA (10 μg) was analyzed for expression of transgenes. MC38 cells were transduced at the indicated MOIs in order to assess the effects of viral titer on the level of gene expression. Hybridization to the β-actin probe demonstrates equal loading of lanes.

the cells were stained with 200 μl of 0.05% methylene blue. The dye was eluted by agitation with 0.33N HCl for 15min, and the absorbance was measured with a microplate reader (MTP-100; CORONA ELECTRIC, Ibaragi, Japan) at 600 nm wavelength.

Inhibition of tumor growth in vivo

MC38, MC38-Neo, or MC38-dCK cells (2×10^6) were injected subdermally into the left flanks of C57 BL/6 mice. Having confirmed the tumor formation after 4 days, the mice received intraperitoneal injections of high-dose Ara-C (250 mg/kg) daily for 4 consecutive days[19]. Tumor sizes were determined as [shorter axis (mm)]$\times$[longer axis (mm)]^2/2[20].

In gene transfer experiments with adenoviral vectors, MC38 cells (2×10^6) were injected subdermally into the left flanks of C57 BL/6 mice. Six days later, a viral vehicle or 2×10^8 PFU of either the Ad.CMV-β-gal or Ad.CMV-dCK vector was directly injected into the tumors. This injection method was determined in preliminary studies using Ad.CMVβ-gal to obtain sufficient expression of the transduced gene. We had determined that the β-gal gene was expressed most successfully in the tumor tissue when the virus was injected at 3 different depths (data not shown). Forty-eight hours later, the mice received intraperitoneal injections of either PBS or ara-C (250 mg/kg) for 4 consecutive days. Tumor sizes were also evaluated.

Results

Stable expression of dCK in retrofected MC38 cells

Northern blot analysis showed that parental MC38 cells (MC38-WT) and MC38-Neo cells expressed undetectable levels of dCK (Fig. 1). In contrast, MC38 cells trancduced by pMV7-dCK (MC38-dCK) stably expressed high levels of the dCK transcript. The neomycin 3′-phosphotransferase gene was expressed in both the MC38-Neo and MC38-dCK cells. The neo gene is transcribed as a full-length message (5′-LTR to 3′-LTR) of the entire genomic region of the retrovirus in the pMV7 vectors, which explains why the transcript in the MC38-dCK cells is larger than the transcript from the MC38-neo cells (Fig. 1).

Ara-C sensitivity of retrofected cells

Growth of the MC38-WT, MC38-Neo, and MC38-dCK cells was identical *in vitro* in the absence of ara-C (doubling time, 20 hours). Since these cells showed the same growth rate, we next assessed the sensitivity of the cells to ara-C *in vitro*. Cytotoxic assays showed that while MC38-WT and MC38-Neo cells were relatively resistant to the drug ($IC_{50}=0.004\ \mu$M and 0.002 μM, respectively), stable transductions of

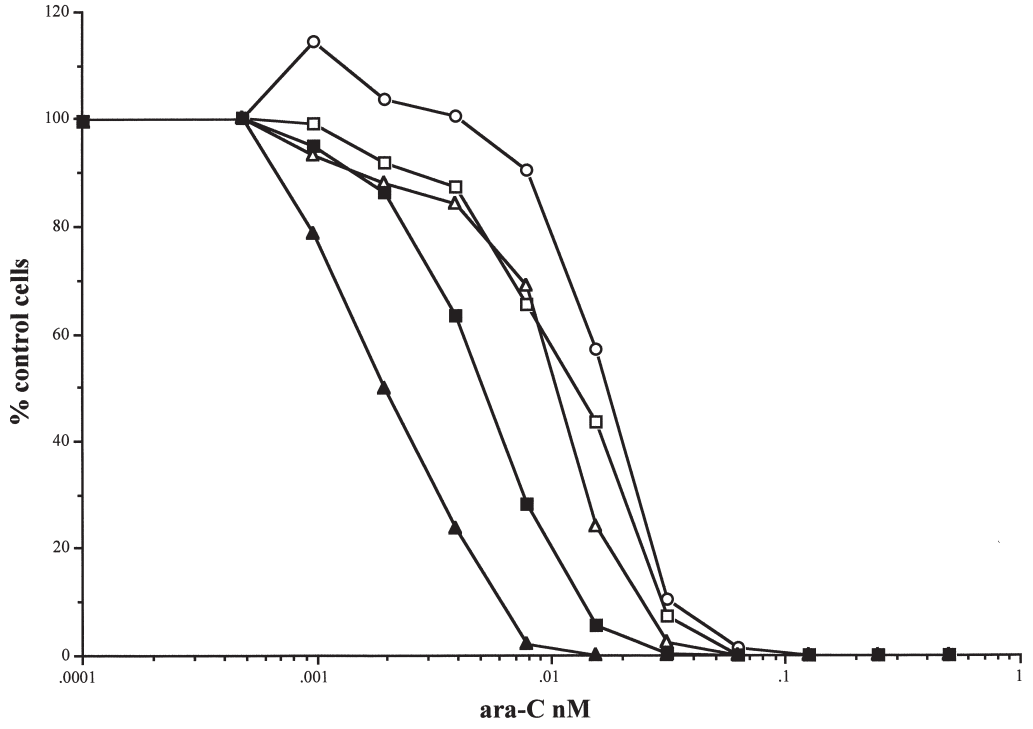

Fig. 5. *In vitro* sensitivity to ara-C following transduction with dCK-expressing or control, virus. MC38 cells were infected as follows: Ad.CMV-βgal at MOIs 0 (○), 100 (□), and 500(△); and Ad.CMVdCK at MOIs 100 (■), and 500 (▲). After 48hrs, cells were exposed to the indicated concentrations of ara-C for 24hrs. Cytotoxicity was determined by staining with methylene blue. The difference in cells killed was statistically significant for cells treated with Ad.CMVdCK at an MOI of 500.

dCK gene (MC38-dCK) and MC38-dCK cells were 4 times and 10 times more sensitive to ara-C (IC_{50} = 0.0005 μM), respectively (Fig. 2).

Treatment of subdermal tumors of retrofected MC38 cells

Based on the cytotoxic assay data, we proceeded to evaluate the *in vivo* sensitivity of these cells to ara-C using subdermal tumors. When treated with PBS, we did not observe any differences among the growth of MC38-WT, MC38-Neo, and MC38-dCK cells (Fig. 3a, b). In contrast, ara-C treatment suppressed the growth of MC38-dCK tumors by about 80% compared to controls on day 10 (Fig. 3b) ($p < 0.05$; t-test).

Gene transfer experiments using an adenoviral vector system

Wild-type MC38 cells were infected with Ad. CMV-dCK (MOI=100, 500) for 48hrs, and expression of the dCK gene was evaluated by Northern blotting. The cells infected by Ad.CMV-dCK expressed the expected size of dCK mRNA (Fig. 4). Expression of dCK mRNA was increased when infected with a higher MOI. MC38 cells treated with a viral vehicle or Ad.CMV-β-gal did not express detectable levels of dCK mRNA.

In the cytotoxic assay, we confirmed an MOI-dependent increase in sensitivity to ara-C for cells infected with Ad.CMV-dCK (at MOI=100, and 500, IC_{50}=0.0055 nM, and 0.0020 nM, respectively), while increased sensitivity to ara-C was not observed for the mock or β-gal control (Fig. 5).

In *in vivo* experiments, injection with Ad.CMV-dCK followed by ara-C administration resulted in significant suppression of tumor growth by 40-50% compared to controls on day 15 (Fig. 6a, b) ($p < 0.05$; t-test).

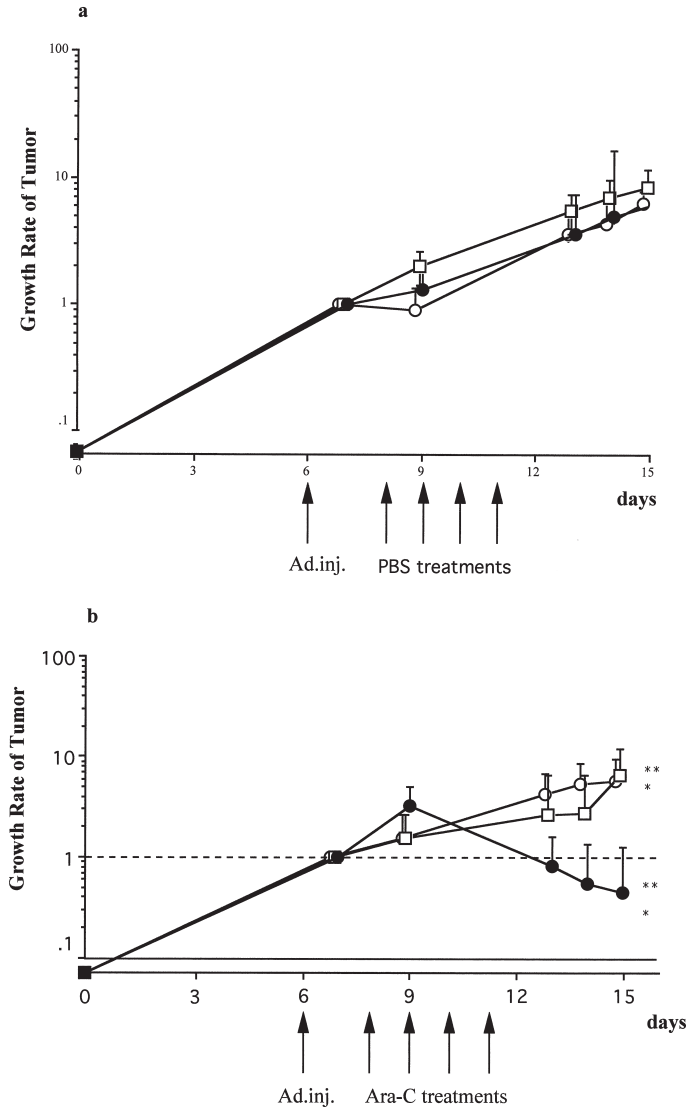

Fig. 6. *In vivo* growth of subdermal adeno-infected MC38 cells treated with ara-C. MC38 cells (2×10^6) were injected subdermally into the left flank of C57 BL/6 mice. Six days later, a viral vehicle, or 2×10^8 PFU (plaque forming units) of either the Ad.CMV-βgal or Ad.CMV-dCK vector, was directly injected into the tumors at three different depth. Forty-eight hours later, mice received i.p. injections of either PBS (a) or ara-C (250 mg/kg) (b) for four successive days. Symbols: Vehicle injection (○), Ad.CMV-βgal infection (□), Ad.CMVdCK infection (●) ($n=8$). The results expressed as the means±s.d. of tumors treated with AdCMVdCK/ara-C compared with the other groups, were statistically significant (*, **, $p<0.05$; t-test).

Discussion

We demonstrated that transduction of the dCK gene enhances its cytotoxicity to colon carcinoma with ara-C *in vitro*. We also found significant growth suppression of the dCK gene transfected tumors induced by ara-C treatment *in vivo*.

In everyday clinical practice, the first choice for the medical treatment of colon cancer is an operation and its outcome is respectably high. When radical cure-operation of colon cancer is performed, its prognosis is good[21]. However, we find it impossible to perform radical treatment in patients who have complications such as invasion to other organs, lymphnode metastasis, carcinomatous peritonitis and metastasis in the liver. Although these patients receive treat-

ment such as chemotherapy and immunotherapy, it is hard to say whether a dramatic medical effect is achieved by the treatment[22–24]. Therefore, we considered that an alternative treatment to colon cancer needed to be developed for the future. Thus, we started to study gene therapy as a new method to treat advanced stages of colorectal carcinoma. As a clinically well-characterized drug is used in the method using a suicide gene, this method was considered to be the most appropriate as a first step among the strategies of gene therapy. In the present study, we examined the effects of the dCK/ara-C system on colon carcinoma. Ara-C is one of the most effective agents in the treatment of acute myeloid leukemia[9–11]. On the other hand, even a high dose of ara-C was not effective against colon carcinoma in a clinical setting[25], probably because of its lower level of dCK activity[16] and the existence of cytidine deaminase, a catabolizing enzyme. Transduction of the dCK gene should, in theory, sensitize colon carcinoma cells to ara-C. A murine colorectal carcinoma cell line that stably expresses the dCK gene was constructed by transfection with a retroviral vector (Fig. 1). This cell line showed remarkably increased sensitivity to ara-C both *in vitro* and *in vivo* (Fig. 2, 3). These findings support that the dCK/ara-C system can be applied to colorectal carcinoma. Therefore, we explored the possibility of using this system in a more clinically relevant setting, that is, gene transfer experiments using an adenoviral vector. In the *in vitro* cytotoxic assay, MOI-dependent increases were observed in the expression level of the dCK gene (Fig. 4) and in the sensitivity to ara-C (Fig. 5). Reduction of tumor growth by 40–50% was observed *in vivo* (Fig. 6), indicating good tumor supression effects.

Our present system has various advantages as follows: First, ara-C is pharmacokinetically a classic drug, for which the clinical safety has been well established. Second, adenoviral vector can be directly injected into tumors, because colorectal tumors can be approached by colonoscopy. This would minimize the possibility for the adenoviral vector to induce immune responses. Immune responses are one of the major drawbacks of adenoviral vectors which limit their clinical application[26,27]. Third, the dCK gene of this system is of human origin. Immune responses to the protein derived from this gene would, therefore, be negligible compared to suicide gene products such as herpes thymidine kinase and Escherichia coli-derived cytosine deaminase providing a superior expression of the introduced gene. Fourth, the prodrug doses used in this study have clinical relevance. The clinical dosage of ara-C is 20 mg/kg/day and the plasma level ranges from 1.5×10^{-8} M to 10^{-7} M[28]. The IC_{50} value of ara-C in this study (2×10^{-12} M in Fig. 5 (MOI = 500)) was far smaller than these values. Furthermore, in the *in vivo* study, tumorcidal effects were observed at a dose of 250 mg/kg/day, corresponding to the high dose schedule of the clinical regimen.

Accordingly, the present study suggests that our gene therapy strategy using the dCK/ara-C system might have a significant potential to improve of the treatment of colon carcinoma.

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