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Dose of Adenoviral Vectors Expressing Interleukin-2 Plays an Important Role in Combined Gene Therapy with Cytosine Deaminase/5-Fluorocytosine: Preclinical Consideration

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Using a syngeneic murine model, we investigated the therapeutic efficacy of combined gene therapy using adenoviral vectors expressing murine interleukin-2 (AdmIL-2) and *Escherichia coli* cytosine deaminase (AdCD). In a subcutaneous tumor model, tumor-bearing mice were treated with an intratumoral injection of adenoviral vectors and received an intraperitoneal administration of 5-fluorocytosine (5-FC). Only the mice treated with AdCD (2×10^8 pfu) and an intermediate dose of AdmIL-2 (1×10^6 pfu) survived significantly longer than mice treated with AdCD alone ($P<0.01$). Moreover, 40% of these treated mice obtained complete remission from tumor-bearing status. The cytotoxicity of splenocytes obtained from the treated mice was related to the survival period. Tumor-specific cytotoxic T lymphocyte assay showed that the cell-mediated cytotoxic response was specific for parental tumor cells. In a hepatic metastasis model, mice treated with an intravenous administration of both AdCD (2×10^8 pfu) and an intermediate dose of AdmIL-2 (1×10^6 pfu) demonstrated the most significant reduction of metastatic foci and the longest survival following a 5-FC administration. These results suggest that gene therapy combined with AdmIL-2 and AdCD may be a promising strategy for clinical application and, in addition, that translation of combined gene therapy from murine models into the clinical setting will require careful attention to the variables of cytokine expression levels in the design of clinical trials and in the evaluation of treatment efficacy.

Key words: Preclinical study — Combined gene therapy — Suicide gene — Cytokine gene — Adenoviral vector

Immunogene therapy for malignancies has been well investigated, and many groups have demonstrated that cytokine-gene-transduced cells (e.g., tumor cells, fibroblasts, and tumor-infiltrating lymphocytes (TIL)) enhanced the antitumor response, resulting in tumor reduction, especially in the case of interleukin-2 (*IL-2*) gene.[1–5] Indeed, the antitumor effect of IL-2 has been shown to be primarily based on T cell-dependent and natural killer (NK) cell-mediated killing activity.

For *in vivo* gene transfer strategies, adenoviral vectors may be more suitable than other vectors (e.g., retroviral vectors, adeno-associated viral vectors, and liposomes), since they can produce viruses at high titers and are highly efficient for transduction in a variety of cell types with high levels of transgene expression.[6–8] However, the effects of *in vivo* treatment with adenoviral vectors expressing IL-2 were reported to be associated with the degree of IL-2 toxicity.[9] These studies indicated the limitations of immunogene therapy using *IL-2* and probably also other cytokine genes.[10] Moreover, it has been reported that the IL-2 level produced by genetically modified tumor cells or fibroblasts was critically important to determine the protective efficacy.[2,11] In the development of therapeutic strategies to circumvent these problems, alternative gene transfer methods able to overcome the limitation of a single use of immunogene therapy must be identified.

Interesting candidates for genes to be used in combination with *IL-2* are those encoding suicide genes, and two of the most widely studied methods involve the use of the viral enzyme thymidine kinase (TK) with the prodrug ganciclovir (GCV) or the bacterial enzyme cytosine deaminase (CD) with the prodrug 5-fluorocytosine (5-FC).[12–14] Moreover, it was recently reported that a combination of *IL-2* and *TK* genes provided a more potent therapeutic

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benefit by the induction of antitumor immunity for the *in vivo* treatment of primary or metastatic carcinoma.[15–19]

However, a recent comparison of suicide gene therapy suggested that the CD/5-FC system is superior to the TK/GCV system in terms of its strong bystander and neighbor cell-killing effects.[20–22] Furthermore, it has also been reported that the CD/5-FC system generated immunological memory for parental tumor cells,[23–25] and a synergistic effect of its combination with interferon-γ or granulocyte macrophage-colony stimulating factor (*GM-CSF*) gene therapy was described.[26, 27]

In the present study, we examined combined murine IL-2 (mIL-2) with *CD/5-FC* gene therapy using an adenoviral vector for a subcutaneous tumor mouse model of colon carcinoma, and we found that treatment with the combination of adenoviral (Ad) CD and AdmIL-2 resulted in an intermediate dose of IL-2 expression, which should eliminate the IL-2-related toxicity while retaining the ability to induce complete tumor remission in this subcutaneous tumor model and also a hepatic metastasis model examined here.

MATERIALS AND METHODS

Mice and cell lines BALB/c (H-2^d) female mice (obtained from Crl SLC, Inc., Atsugi) were bred under specific pathogen-free conditions and used for experiments at the age of 7 to 10 weeks. CT26 (H-2^d), a subline of the N-nitroso-N-methylurethane-induced BALB/c undifferentiated colon adenocarcinoma (C26), was obtained from Hoffman-La Roche Co. (Kamakura). The MC38 tumor cell line is a nonmetastatic dimethylhydrazine-induced colon adenocarcinoma derived from C57BL/6 mice. Meth-A (H-2^d), a methylcholanthrene-induced sarcoma cell line, was purchased from Riken Gene Bank (Ibaraki). The 293 cell line, an Ad5 E1-transformed human embryonic kidney cell line, was purchased from the American Type Culture Collection (Rockville, MD). Cells were grown in Dulbecco's modified Eagle's medium (Nissui Pharmaceutical Co., Tokyo) supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 m*M* L-glutamate at 37°C in a humidified 5% CO_2 atmosphere. Supplements were purchased from GIBCO-BRL (Grand Island, NY).

Preparation of recombinant adenoviral vectors Recombinant replication-defective adenoviral vectors were prepared according to the methods described previously.[28] Briefly, an expression cosmid cassette was constructed by inserting the expression unit composed of a cytomegalovirus immediate early enhancer and a modified chicken β-actin (CA) promoter, a cDNA coding sequence, and rabbit β-globin poly(A) signal sequence[29] into the *Swa*I site of pAxcw, which is a 42-kb cosmid containing the 31-kb adenovirus type 5 genome lacking *E1A*, *E1B*, and *E3* genes. The expression cosmid cassette and adenoviral DNA-terminal protein complex were cotransfected into 293 cells by the calcium phosphate precipitation method. The incorporation of the expression cassette into the isolated recombinant virus was confirmed by digestion with appropriate restriction enzymes. Thus, three vectors were constructed: AdmIL-2, an adenoviral vector with murine *IL-2* gene; AdCD, an adenoviral vector with *Escherichia coli CD* gene; and Admock, a control adenoviral vector lacking *E1A*, *E1B*, and *E3* genes. All recombinant viruses were propagated with 293 cells, purified by two rounds of CsCl density centrifugation, dialyzed, and stored at −80°C as previously described.[30] The titers of viral stocks (pfu/ml) were determined by plaque assay on 293 cells. All viral stocks were checked for the presence of replication-competent adenoviral vector by polymerase chain reaction (PCR).[31] None of the stocks of adenoviral vectors used in this study was detectably contaminated with replication-competent viruses.

***In vitro* murine IL-2 production assay** For the *in vitro* transduction of adenoviral vectors, medium was discarded from the cells seeded in 12-well culture plates, and 150 μl of viral stock was added to each well. After incubation for 1 h at 37°C, growth medium was added and the cells were cultured for 2 days. Briefly, CT26 cells (5×10^5 per well) were transduced *in vitro* with the adenoviral vectors, AdmIL-2 alone or both AdmIL-2 and AdCD, at the multiplicity of infection (MOI) range from 1 to 50. The supernatants were collected at 48 h after plating 5×10^5 cells in a 10-cm dish containing 10 ml of medium, and we quantitated the amount of IL-2 by enzyme-linked immunosorbent assay (ELISA) (Endogen, Cambridge, MA).

Direct intratumoral injection studies of an adenoviral vector expressing mIL-2 and CD against subcutaneous tumor The mice receiving a subcutaneous inoculation of 5×10^5 CT26 cells in 100 μl of Hank's balanced salt solution (HBSS) were randomly divided into the following five groups ($n=10$ per each group): group 1, AdCD (2×10^8 pfu); group 2, AdCD (2×10^8 pfu) plus AdmIL-2 (5×10^5 pfu); group 3, AdCD (2×10^8 pfu) plus AdmIL-2 (1×10^6 pfu); group 4, AdCD (2×10^8 pfu) plus AdmIL-2 (2.5×10^7 pfu); group 5, AdCD (2×10^8 pfu) plus AdmIL-2 (2×10^8 pfu). At 10 days after the tumor inoculation, all of the mice were intratumorally administered an appropriate concentration of adenoviral vectors in a volume of 50 μl, and the mice then received intraperitoneal administration of 5-FC (500 mg/kg) daily for 7 days.

***In vitro* cell-mediated cytotoxicity assays** Fourteen days after the adenoviral vector administration, all of the mice were sacrificed and their spleens were harvested. Splenocytes were cocultured with mitomycin-C (100 μg/ml)-treated parental CT26 tumor cells on 24-well plates at a concentration of 6×10^6 cells/ml (responder/stimulator ratio of 10) in complete medium supplemented

with recombinant murine IL-2 (20 U/ml). After 5 days of cocultivation, the sensitized splenocytes were assayed for specific cell lysis against parent CT26 tumor cells in a ^{51}Cr-release assay. Briefly, 1×10^6 parental CT26 cells were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ for 1 h at 37°C. The cells were then washed five times, and 1×10^4 of labeled cells were added to each well of a 96-well V-bottomed plate. Effector cells were added to triplicate wells at various effector/target (E/T) cell ratios in a final volume of 200 μl/well. The plates were incubated at 37°C for 4 h. Supernatant (100 μl) was collected from each well and counted in a γ-counter. The percentage of specific cytotoxicity was calculated as follows:

$$\%\ \text{cytotoxicity} = (\text{experimental counts} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release}) \times 100.$$

NK cell assays were performed using the NK cell-sensitive cell line YAC-1 as the target cell line as described above.

Tumor-specific cytotoxic T lymphocyte (CTL) assay The CT26, MC38, AdCD-transduced CT26 (AdCDCT26), and Admock-transduced CT26 (AdmockCT26) cells were used as nonradiolabeled blocking cells (cold targets). Constant numbers of effector cells (50 μl at 1×10^7/ml) and different numbers of cold targets were dispensed into each well of a 96-well V-bottomed plate in a total volume of 100 μl/well. The plates were incubated at 37°C for 30 min, and then $Na_2{}^{51}CrO_4$-labeled target cells (100 μl at 1×10^5/ml) were added to each well at the E/T cell ratio of 50. The cold/hot target cell ratio varied from 5/1 to 20/1. The plate was then processed for a standard 4-h ^{51}Cr-release assay.

Moreover, since CTL epitope of CT26 colon tumors was reported by Huang *et al.*[8] to be gp70 of an endogenous retrovirus, we evaluated whether CTL generated in this study recognized the same epitope or not. Briefly, CT26, Meth-A and Meth-A pulsed with AH-1 (100 μg/1×10^6) were used as target cells. Peptide AH-1 (SPSYVYHQF), an immunodominant antigen identified in CT26 cells, was synthesized by TaKaRa Biochemicals (Ohtsu) to >99% purity. CTL assays were performed as described above.

Treatment of a hepatic metastasis model with intravenous administration of adenoviral vectors To evaluate the efficacy of combined gene therapy using AdCD and AdmIL-2 for hepatic metastasis of colon carcinoma, we anesthetized syngeneic BALB/c mice with an intraperitoneal administration (0.3 ml per mouse) of 2.5% solution of a mixture of 2,2,2-tribromoethanol and *tert*-amyl alcohol (Wako Pure Chemical Industries, Osaka). After laparotomy, CT26 (1×10^4 cells) were inoculated into the portal vein. At 5 days after this tumor cell inoculation, the mice were divided into five groups (n=5 per group), and the adenoviral vectors were administered intravenously into the tail vein as follows: group A, HBSS as the control; group B, AdCD (2×10^8 pfu) followed by HBSS; group C, AdCD (2×10^8 pfu) followed by 5-FC; group D, AdCD (2×10^8 pfu) plus AdmIL-2 (1×10^6 pfu) followed by 5-FC; group E, AdCD (2×10^8 pfu) plus AdmIL-2 (2×10^8 pfu) followed by 5-FC. The mice received the intraperitoneal administration of 5-FC (500 mg/kg) or HBSS daily for 7 days. All of the mice were sacrificed on the 21st therapeutic day, and their livers were removed and fixed in 10% formalin neutral-buffered solution (pH 7.4). The numbers of metastatic foci on the liver surface and the weight of the liver were measured. To evaluate whether the therapeutic effects of the reduction of metastatic foci were reflected by a prolongation of the animals' survival, we inoculated and treated an additional set of mice according to the protocol described above (n=10 in each group) and evaluated their survival.

Statistical analysis Quantitative results are expressed as mean±standard deviation of the mean. The statistical analysis was performed by ANOVA and Fisher's test, with the exception of the survival data, that were analyzed by the Kaplan-Meier plot and the log-rank (Mantel-Cox) test using Statview 5.0 software (Abacus Concepts, Berkeley, CA). P values less than 0.01 were considered significant.

RESULTS

Kinetics of IL-2 production of transduced tumor cells With the reporter adenoviral vector AdlacZ, CT26 cells could be transduced at nearly 100% efficiency at the MOI of 100 (data not shown). To determine whether CD over-

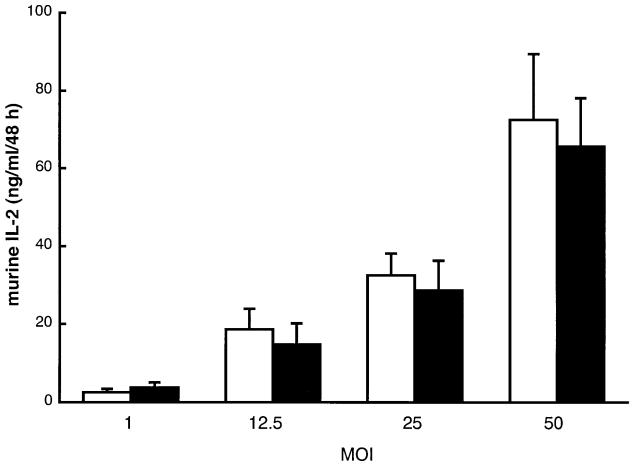

Fig. 1. Murine IL-2 production of AdmIL-2- and AdCD-transduced CT26 tumor cells. Cells were transduced with AdmIL-2 alone (□) or with AdmIL-2/AdCD (■) at the same MOI range from 1 to 50. After 2 days, the supernatants were collected and the IL-2 production was quantitated by ELISA.

expression can interfere with the production of mIL-2, we quantified mIL-2 production of AdmIL-2- and AdCD-transduced cells by ELISA. As shown in Fig. 1, the IL-2 production was not reduced in the supernatants of cells transduced with both AdCD and AdmIL-2, suggesting that the cellular events triggered by CD did not affect the IL-2 production.

Dose effects of combined adenoviral *mIL-2* gene therapy with the CD/5-FC system An animal model of colon cancer was established by administering a subcutaneous inoculation of CT26 cells (5×10^5). After 10 days, subcutaneous tumors were palpable at 2×2 mm^3 in size, and we administered the adenoviral vectors intratumorally. Starting on the day after the adenoviral vector injection, all of the mice were treated with an intraperitoneal administration of 5-FC (500 mg/kg) for 7 consecutive days. For the assessment of the therapeutic effects, the survival of the mice was observed (Fig. 2). In the two groups that received a combination with a high dose of AdmIL-2 (group 4: 2.5×10^7 pfu and group 5: 2×10^8 pfu), all of the mice relapsed and died, and in the mice treated with a low dose of AdmIL-2 (group 2: 5×10^5 pfu), the therapeutic effect was transient (P=0.0477 vs. group 1 as control), and all of these mice died. However, the group with an intermediate dose of AdmIL-2 (group 3: 1×10^6 pfu) survived significantly longer than the mice treated with AdCD/5-FC alone (P=0.0015). Moreover, 40% of these mice obtained complete remission from tumor-bearing status (Table I). For the determination of whether systemic antitumor protection was obtained in these groups, lethal doses of parental CT26 tumor cells were challenged in an opposite flank site 14 days after an adenoviral administration, and all of the tumor-free mice in group 3 were able to reject this parental tumor re-challenge (Table I).

In addition, to show the range of the therapeutic window of AdmIL-2 in combination with AdCD/5-FC, we have compared six different doses between 5×10^5 pfu and 2.5×10^7 pfu. As summarized in Table II, the administration of 1×10^6 pfu of AdmIL-2 in combination with AdCD/5-FC induced complete remission in 40% of

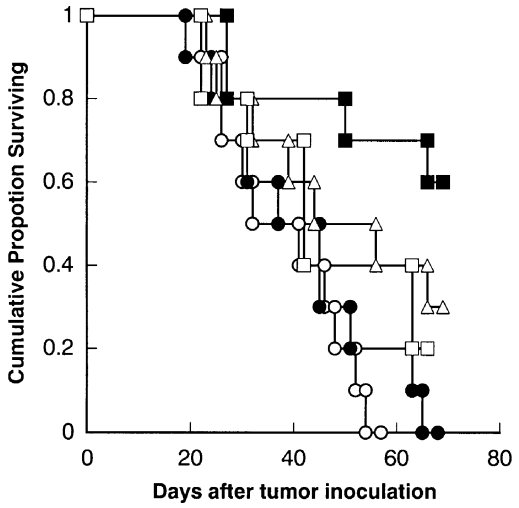

Fig. 2. Survival of mice treated with adenoviral vectors (Kaplan-Meier plots). The subcutaneous tumor-bearing mice were divided into five groups (n=10 per group): group 1 (○), AdCD (2×10^8 pfu); group 2 (△), AdCD (2×10^8 pfu)+AdmIL-2 (5×10^5 pfu); group 3 (■), AdCD (2×10^8 pfu)+AdmIL-2 (1×10^6 pfu); group 4 (□), AdCD (2×10^8 pfu)+AdmIL-2 (2.5×10^7 pfu); group 5 (●), AdCD (2×10^8 pfu)+AdmIL-2 (2×10^8 pfu). All of the mice received an intraperitoneal administration of 5-FC and were observed for survival.

Table I. Therapeutic Response to Intratumoral Injection of Combined AdCD/5-FC and AdmIL-2

	AdCD	AdmIL-2	Tumor-free	Rejection
group 1	2×10^8	(–)	0/10	no
group 2	2×10^8	5×10^5	0/10	no
group 3	2×10^8	1×10^6	4/10	yes
group 4	2×10^8	2.5×10^7	0/10	no
group 5	2×10^8	2×10^8	0/10	no

The subcutaneous tumor-bearing mice were intratumorally administered with the appropriate dose of adenoviral vectors and then received intraperitoneal administration of 5-FC (500 mg/kg) daily for 7 days. Fourteen days later, parental CT26 cells were challenged in an opposite flank site. group 1, AdCD (2×10^8 pfu); group 2, AdCD (2×10^8 pfu)+AdmIL-2 (5×10^5 pfu); group 3, AdCD (2×10^8 pfu)+AdmIL-2 (1×10^6 pfu); group 4, AdCD (2×10^8 pfu)+AdmIL-2 (2.5×10^7 pfu); group 5, AdCD (2×10^8 pfu)+AdmIL-2 (2×10^8 pfu).

Table II. The Therapeutic Window of AdmIL-2 Combined Therapy with AdCD/5-FC

AdmIL-2	Partial response[a)] (%)	Complete remission[b)] (%)
(–)	0/15	0/15
5×10^5	3/15 (20)	0/15
1×10^6	4/15 (27)	6/15 (40)
2×10^6	2/15 (13)	0/15
5×10^6	0/15	0/15
1×10^7	0/15	0/15
2.5×10^7	0/15	0/15

The subcutaneous tumor-bearing mice were intratumorally administered with the appropriate dose of adenoviral vectors and then received intraperitoneal administration of 5-FC (500 mg/kg) daily for 7 days.

a) Tumors completely regressed for at least 2 weeks but reoccurred.

b) Tumors completely regressed and did not reoccur.

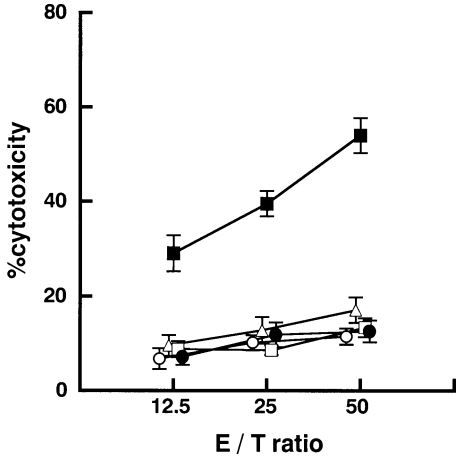

Fig. 3. Induction of cell-mediated cytotoxicity by combined *CD/5-FC* and *IL-2* gene therapy. The splenocytes from mice treated with different protocols were incubated for 5 days with mitomycin C-treated parental CT26 cells. After incubation, the adherent cells were collected and the cytotoxicity against CT26 cells was calculated in a ^{51}Cr-release assay. Each group consists of 5 animals. Data was expressed as % mean±standard deviation. group 1 (○), AdCD (2×10^8 pfu); group 2 (△), AdCD (2×10^8 pfu)+AdmIL-2 (5×10^5 pfu); group 3 (■), AdCD (2×10^8 pfu)+AdmIL-2 (1×10^6 pfu); group 4 (□), AdCD (2×10^8 pfu)+AdmIL-2 (2.5×10^7 pfu); group 5 (●), AdCD (2×10^8 pfu)+AdmIL-2 (2×10^8 pfu).

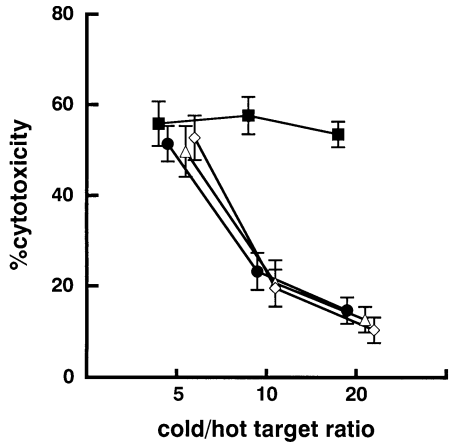

Fig. 4. Cold target competition assay. The CT26 (◇), MC38 (■), AdCD-transduced CT26 (AdCDCT26, ●), and Admock-transduced CT26 (AdmockCT26, △) cells were used for nonradiolabeled blocking cells (cold targets). The cold/hot target cell ratios varied from 5/1 to 20/1. The plate was then processed in a standard 4-h ^{51}Cr-release assay.

tumors (6/15), and an additional 27% of treated mice (4/15) showed a partial response resulting in disappearance of tumor mass for at least 2 weeks before tumor regrowth. The combined administration of 5×10^5 pfu (half the dose) or 2×10^6 pfu (double the dose) of AdmIL-2 induced only transient tumor regression. In contrast, in the mice treated with AdCD/5-FC alone, or those treated with AdCD/5-FC plus a higher dose of AdmIL-2 (5×10^6 pfu, 1×10^7 pfu, and 2.5×10^7 pfu), the tumors grew progressively following treatment and none underwent complete remission.

Induction of cell-mediated immune response by combined gene therapy To evaluate whether survival prolongation was associated with antitumor immunity, we tested the CTL activity against parental tumor cells. After a mixed lymphocyte reaction (MLR), there was no remarkable cytotoxic activity against parental CT26 tumor cells in the splenocytes from mice treated with AdCD/5-FC alone, or those treated with AdCD/5-FC plus high or low AdmIL-2 therapy (groups 1, 2, 4, and 5). However, the splenocytes induced from mice treated with combined AdCD/5-FC and an intermediate dose (1×10^6 pfu) of AdmIL-2 showed the most potent cytotoxicity against parental tumor cells ($P<0.01$, Fig. 3). Cytotoxicity against YAC-1 (NK-susceptible) cells in the splenocytes from all of the mice was not shown (data not shown).

Induction of tumor-specific immunity in the treated mice We next performed the cold target competition assay to determine whether the specific CTL response was induced against parental CT26 tumor cells. Against ^{51}Cr-labeled parental CT26 target cells, nonradiolabeled CT26, AdCDCT26, and AdmockCT26 cold target cells inhibited the cytotoxicity of CTL, whereas MC38 cold target cells had no influence on the cytotoxicity (Fig. 4). Moreover, no remarkable differences were observed among the cytotoxicity curves of nonradiolabeled CT26, AdCDCT26, AdmockCT26 used as cold targets at all cold/hot target ratios.

In addition, to test whether the immunodominant antigen epitope of CT26 cells is recognized or not, we evaluated CT26-specific activity using AH-1 peptide. Splenocytes were obtained from the group that had been treated with AdCD and an intermediate dose of AdmIL-2. These effector cells exhibited specific cytotoxicity of CT26 cells or Meth-A sarcoma cells pulsed with AH-1, but not parental Meth-A sarcoma cells (Fig. 5).

Therapeutic efficacy of combined gene therapy by intravenous administration of adenoviral vector for hepatic metastasis We tested whether combined gene therapy by an intravenous administration of adenoviral vectors was effective for hepatic metastasis of colon carcinoma. Twenty-five days after a 1×10^4 tumor cell inoculation into the portal vein of mice, hepatic metastasis of colon carcinoma could be observed macroscopically on the surface of the liver (data not shown). After an intravenous viral delivery via the tail vein, mice were treated

with HBSS (as the control) or 5-FC (500 mg/kg) intraperitoneally for 7 consecutive days. All mice were sacrificed on the 21st day after the adenoviral delivery and the number of metastatic foci on the liver surface and the weight of the liver were measured. The mice treated with an intermediate dose (1×10^6 pfu) of AdmIL-2 and AdCD (group D) demonstrated the most significant reduction of metastatic foci (Table III), and there was no significant difference in body weight compared to other groups. To evaluate further whether the therapeutic effects of the reduction of metastatic foci were also reflected by a prolongation of the animals' survival, we treated an additional set of mice by the same protocols for the purpose of survival evaluation. As shown in Fig. 6, the mice treated with combined gene therapy showed long survival; those treated with 1×10^6 pfu of AdmIL-2 and AdCD (group D) demonstrated significant survival prolongation compared to the other groups ($P=0.0061$ vs. group A).

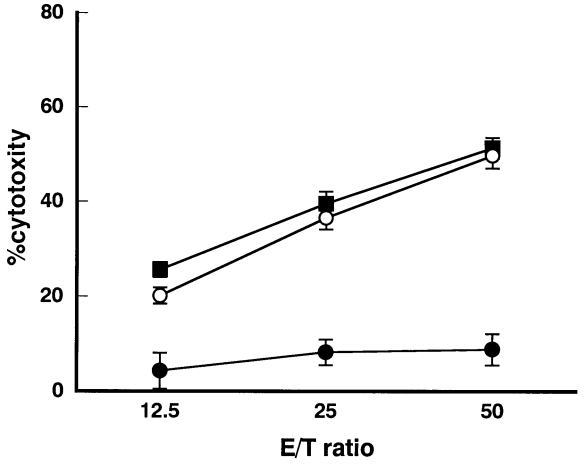

Fig. 5. Induction of tumor-specific CTL response after intratumoral administration of AdCD and an intermediate dose of AdmIL-2. The splenocytes from the treated mice were incubated for 5 days with mitomycin C-treated parental CT26 cells. After incubation, the adherent cells were collected and the cytotoxicity against target cells was calculated in a ^{51}Cr-release assay. CT26 (■), Meth-A (●) and Meth-A cells pulsed with AH-1 (100 μg/1×10^6) (○) were used as target cells. Peptide AH-1 (SPSYVYHQF) is an immunodominant antigen identified in CT26 cells. Each group consists of 5 animals. Data are expressed as % mean±standard deviation.

able III. Effect of Combined AdCD/5-FC with AdmIL-2 Gene herapy for Hepatic Metastases

Treatment			Number of hepatic metastases	Liver weight (g)
AdCD	5-FC	AdmIL-2		
(–)	(–)	(–)	7.0±2.0	1.86±0.28
2×10^8	(–)	(–)	7.6±1.8	1.75±0.51
2×10^8	(+)	(–)	6.6±1.5	1.73±0.89
2×10^8	(+)	1×10^6	1.0±0.6*	1.44±0.10
2×10^8	(+)	2×10^8	6.4±2.3	1.72±0.67

CT26 cells (1×10^4) were inoculated into the portal vein of syngeneic BALB/c mice on day 0. Five days later, various doses of adenoviral vectors were administered intravenously into the tail vein. On the 21st therapeutic day, all the mice were sacrificed and their livers were removed. Each group consists of 5 animals. * Significant difference was observed compared to other groups ($P<0.01$).

DISCUSSION

Although current gene therapy techniques have been shown to be potentially powerful therapeutic tools, their practical clinical application remains to be demonstrated.[32] Most of the protocols are cytokine gene therapies; however, it is possible that excessive cytokine produced from gene-modified cells may induce toxicity rather than an antitumor immune response. Moreover, when cytokine genes were transferred for the therapy of established cancer, the antitumor immunity of the host was augmented, but was usually too weak to eradicate established tumors.

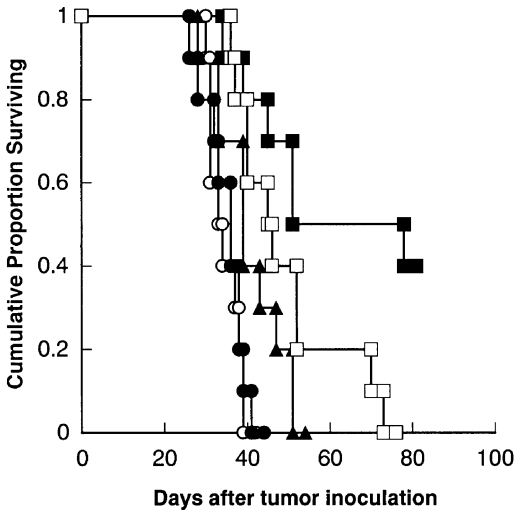

Fig. 6. Survival of mice with hepatic metastasis of colon carcinoma (Kaplan-Meier plots). The mice bearing hepatic metastasis of colon carcinoma were treated with an intravenous administration of adenoviral vector as follows: group A (○), HBSS as the control; group B (●), AdCD (2×10^8 pfu) followed by HBSS; group C (▲), AdCD (2×10^8 pfu) followed by 5-FC; group D (■), AdCD (2×10^8 pfu)+AdmIL-2 (1×10^6 pfu) followed by 5-FC; group E (□), AdCD (2×10^8 pfu)+AdmIL-2 (2×10^8 pfu) followed by 5-FC. The mice then received an intraperitoneal administration of 5-FC (500 mg/kg) or HBSS daily for 7 days.

Another approach for gene therapy is the use of suicide genes. It has been demonstrated that killing tumor cells *in vivo* with suicide gene therapy such as the TK/GCV system or CD/5-FC system can lead to the generation of T cell-dependent and NK cell-mediated antitumor immunity.[33–35] When suicide gene was transduced *in vivo* followed by the administration of the prodrug, the pre-established tumors were easily eradicated; however, the system lacked the ability to induce an efficient antitumor immune response in the host. Hence, effective cancer therapies involving gene transfer may prove to be most effective through the use of several modalities that complement and interactively enhance the activity of each form of treatment.

In this report, we further examined the efficacy of combined gene therapy using mIL-2 and the CD/5-FC system for obtaining antitumor response. First, adenoviral vectors expressing CD and mIL-2 were prepared and transduced alone or in combination directly into subcutaneous tumors of murine colon carcinoma. Our *in vivo* results demonstrated that the most potent efficacy of combined gene therapy was achieved at a dose of adenoviral vectors producing intermediate IL-2 levels, and that lower IL-2 secretion reduced the number of successfully treated mice, whereas the high level expression of IL-2 rather abolished the protection against tumor growth. In addition to the therapeutic effect, the cell-mediated cytotoxicity assays also provided evidence that the induction of an immune response was most potent when CT26 tumor tissues were treated with AdCD and an intermediate dose of AdmIL-2. These results indicated that tumor regression was achieved when combined gene therapy was associated with the development of a specific immune response. Moreover, the cold target competition assay revealed that the immune response was due to a CTL induction to the parental CT26 tumor cells.

In this study, we used murine CT26 colon cancer model. In normal mice, CT26 is poorly immunogenic, and does not induce detectable tumor-specific CTLs.[1] It has been reported that CT26 cells express gp70, an envelope protein of an endogenous ectropic murine leukemia virus (MuLV). This viral antigen, gp70, that is not expressed in the normal tissue of BALB/c mice acts as the immunodominant antigen.[36] Therefore, CT26 tumor model is suitable for investigation of combined therapy with immunogen and a suicide gene. In this study, we have demonstrated that CTLs obtained from the group treated best recognized the same epitope.

Regarding the immune mechanism for tumor rejection, we hypothesize that tumor cell killing by the CD/5-FC system results in the processing of tumor antigen peptides by antigen-presenting cells, and this activates the T lymphocyte response. In the tumor microenvironment, an adequate concentration of IL-2 was essential to enhance the proliferation and cytotoxic activity of the tumor-specific CTLs by tumor antigen peptides. An intermediate dose of adenoviral vectors expressing IL-2 was most effective in the induction of CTLs, consistent with the view that a low concentration of IL-2 may preferentially stimulate antigen-specific CTLs through binding to high-affinity IL-2 receptors.[37, 38]

Furthermore, an *in vivo* virus-mediated oncolysate may also act as a potent vaccine. The *in vivo* destruction of cells leads to efficient uptake and presentation of tumor antigens by immune effector cells. Evidence supports an improved antitumor effect in an immune-competent mouse compared to a T cell-deficient mouse, using the CD/5-FC system, even though gene expression should be higher and more prolonged in the immune-deficient mouse.[25, 39] CTLs appear to be responsible for bystander killing and immune memory.[40] It is possible that a distant uninfected tumor may be responsive to immune destruction if tumor-specific CTLs are generated. These CTLs may also protect against the growth and development of metastatic disease.

Next, for the possible clinical application of these strategies, we investigated whether an intravenous administration of adenoviral vectors enhances the therapeutic efficacy of combined gene therapy in a hepatic metastasis model of colon carcinoma. The intravenous administration of adenoviral vectors results in over 90% of the vector accumulating in the liver, mainly in the hepatocytes.[41, 42] Thus, unlike the subcutaneous tumor model, the selective transduction of exogenous genes to tumor cells by an intravenous administration of adenoviral vectors cannot be expected in a hepatic metastasis model. However, tumor cells are usually more susceptible to chemotherapeutic drugs than are normal cells,[43] and the therapeutic strategy for hepatic metastasis is focused on the hepatocytes expressing the *CD* gene or *IL-2* gene. We speculate that the mechanism of tumor rejection in a hepatic metastasis model may be conversion of the 5-FC to 5-fluorouracil (5-FU) and the secretion of sufficient amounts of 5-FU to promote toxicity, and sufficient IL-2 to induce antitumor immunity against nearby tumor cells without concomitant hepatocyte toxicity.

In summary, our findings indicate that the dose of an adenoviral vector expressing mIL-2 is an important variable in the optimization of combined gene therapy with CD/5-FC and mIL-2. Different doses or promoters result in different levels of IL-2 secretion, which in turn activate different effector components of antitumor immune response. Further studies will be needed to dissect the relationships between promoter activity, cytokine secretion, and the recruitment of different immune system effector mechanisms in order to optimize the treatment of cancer with combined gene therapy with cytokine and suicide gene. The applicability of this observation to humans is not clear. In an attempt to enhance the *in vivo* bystander

effect, investigators have combined suicide gene systems with cytokine genes to enhance the immune response.[44)]

In conclusion, we have shown that the use of a combination of *CD* and *IL-2* gene therapy can overcome the drawbacks of suicide gene therapy or cytokine gene therapy, which were proved to be unsatisfactory when used alone. This combination therapy may thus be a good candidate for the treatment of subcutaneous tumors and hepatic metastases of colon carcinomas.

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