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Combination gene therapy for liver metastasis of colon carcinoma *in vivo*

(cancer gene therapy/metastatic colon carcinoma/suicide gene/cancer vaccine/recombinant adenoviral vectors)

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ABSTRACT **The efficacy of combination therapy with a "suicide gene" and a cytokine gene to treat metastatic colon carcinoma in the liver was investigated. Tumor in the liver was generated by intrahepatic injection of a colon carcinoma cell line (MCA-26) in syngeneic BALB/c mice. Recombinant adenoviral vectors containing various control and therapeutic genes were injected directly into the solid tumors, followed by treatment with ganciclovir. While the tumors continued to grow in all animals treated with a control vector or a mouse interleukin 2 vector, those treated with a herpes simplex virus thymidine kinase vector, with or without the coadministration of the mouse interleukin 2 vector, exhibited dramatic necrosis and regression. However, only animals treated with both vectors developed an effective systemic antitumoral immunity against challenges of tumorigenic doses of parental tumor cells inoculated at distant sites. The antitumoral immunity was associated with the presence of MCA-26 tumor-specific cytolytic CD8$^+$ T lymphocytes. The results suggest that combination suicide and cytokine gene therapy *in vivo* can be a powerful approach for treatment of metastatic colon carcinoma in the liver.**

Metastatic colon carcinoma is second only to lung cancer as a cause of death from malignancy in the United States, accounting for 60,000 fatalities a year. Eighty percent of the patients who die of colon cancer have metastases in the liver, and half of them have only liver metastases at the time of death (1). Once metastases to the liver have occurred, current treatments including surgery and chemotherapy have been disappointing (2). Recent advances in science and technology for direct gene transfer into living animals have provided opportunities to develop new treatment modalities of malignancies by somatic gene therapy. Among the various paradigms for cancer gene therapy, "suicide gene" and "cancer vaccine" represent two of the most promising approaches.

The best-studied suicide gene to date is the herpes simplex virus thymidine kinase (HSV-tk) gene. As first proposed by Moolten in 1986 (3), HSV-tk converts the pro-drug ganciclovir (GCV) to a form that is cytotoxic for dividing cells (4). This treatment modality was first demonstrated to be effective in causing brain tumor regression *in vivo* (5). Critical to the successful results was the so-called "bystander" effect that confers cytotoxicity to the neighboring nontransduced cells as well. This observation is extremely important as it alleviates the necessity to deliver the suicide gene to every tumor cell *in vivo*. This approach has recently been demonstrated to be efficacious in causing regression of other solid tumors, including metastatic colon carcinoma in the rat liver (6).

The current strategy for the cancer vaccine approach involves the transduction of established tumor cell lines with various cytokines or histocompatibility genes *in vitro*, followed by transplantation of the transduced cells into syngeneic animals (7). Recipient animals were protected against subsequent challenges by tumorigenic doses of parental tumor cells inoculated at distant sites. This approach has been reported to prevent tumor formation in a number of models, including colon carcinoma (8–10). Thus, there is increasing evidence to support the concept of gene therapy for primary and metastatic tumors.

In previous reports (5–11), recombinant retroviral vectors were used to deliver the HSV-tk or cytokine genes into the tumors. While efficacious, the retroviral vector system is limited by low titers and low efficiency for gene transfer *in vivo*. The recombinant adenoviral system, on the other hand, is characterized by extremely high titer and efficiency for direct gene delivery into a variety of cell types *in vivo* (12, 13). This efficient vector system permits *in vivo* testing of both the suicide gene and cancer vaccine approaches for tumor rejection. The present study assesses the efficacy of combination therapy with HSV-tk and mouse (m) interleukin 2 (IL-2) genes in a liver metastasis model of colon carcinoma.

MATERIALS AND METHODS

Construction of Recombinant Adenoviral Vectors. Construction of a replication-defective adenoviral vector containing the HSV-tk gene under transcriptional control of the Rous sarcoma virus (RSV) long terminal repeat (ADV/RSV-tk) has been reported (14, 15). A replication-defective adenoviral vector containing the mIL-2 cDNA under the transcriptional control of the RSV long terminal repeat promoter (ADV/RSV-mIL-2) was similarly constructed and plaque purified. The viral titer [plaque-forming units (pfu)/ml] was determined by plaque assay.

Establishment and Treatment of Hepatic Metastasis Model of Colon Carcinoma. Metastatic colon carcinoma was induced in the liver by intrahepatic implantation of cells of the MCA-26 line, which is a chemically induced, poorly differentiated colon carcinoma cell line derived from BALB/c mice (16). MCA-26 cells (3×10^5) were injected at the tip of the left lateral liver lobe of syngeneic mice. At day 7, various titers of recombinant adenoviral vectors were injected directly into the hepatic tumors in 70 μl of 10 mM Tris·HCl, pH 7.4/1 mM $MgCl_2$/10% (vol/vol) glycerol/Polybrene (20 μg/ml). Twelve hours after viral injection, the animals were treated intraperitoneally with GCV at 35 mg/kg twice daily for 6 consecutive days.

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Abbreviations: HSV-tk, herpes simplex virus thymidine kinase; GCV, ganciclovir; IL-2, interleukin 2; mIL-2, mouse IL-2; RSV, Rous sarcoma virus; moi, multiplicity of infection; pfu, plaque-forming unit(s); β-gal, β-galactosidase; CTL, cytotoxic T lymphocytes.

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Histopathological and Morphometric Analysis of Residual Tumors. After various gene therapy treatments, computerized morphometric analysis of the largest cross-sectional areas of the residual tumors was performed. The point-counting method using a computer-assisted digitizing system with BIOQUANT software (BQ MEG IV; R&M Biometrics, Nashville) was chosen for morphometric analyses as viable tumor cells were not always contiguous. Briefly, >1600 predetermined points in the region of the tumor were counted. The proportion of viable tumor cells in the nodule equaled the sum of the points of viable tumor cells divided by the total number of points. The functional area of viable tumor cells among the groups was compared statistically by Student's *t* test (17).

Distant Site Challenge in Treated Animals with Parental Tumor Cells. One day after completion of GCV treatment, animals in all treatment groups were challenged with tumorigenic doses of the parental tumor cells (MCA-26) as well as a heterologous but syngeneic breast tumor cell line (MOD). MCA-26 cells (1×10^5) were injected subcutaneously at a single site on the right flank of the animals, and 2×10^6 MOD cells were injected subcutaneously on the contralateral site. The presence of subcutaneous tumors in animals after various gene therapy treatments was observed for 4 weeks. The results were analyzed statistically by the Fisher exact test (17).

Cytotoxic T-Lymphocyte (CTL) Assay. The CTL assay was performed according to published procedures (18). Viable splenocytes were isolated from various animal treatment groups at 3 days after completion of GCV treatment. *In vitro* stimulation was performed for 5 days in 24-well plates; each well contained 6×10^6 splenocytes and 5×10^5 MCA-26 cells that had received 15,000 rads (1 rad = 0.01 Gy) of radiation plus recombinant mIL-2 (20 units/ml). The stimulated effector cells were then used in CTL assays against target cells. Data represent the mean of triplicate cultures; the SEM was <7% in all assays.

RESULTS

Functional Characterization of Recombinant Adenoviral Vectors. To determine whether introduction of the HSV-tk gene would render the MCA-26 colon tumor cells susceptible to killing by GCV, the replication-defective recombinant adenoviral vector ADV/RSV-tk was used to transduce the colon carcinoma cell line *in vitro* (Fig. 1*A*). After transduction with the recombinant vector and subsequent treatment with PBS, the cells were completely viable even at a multiplicity of infection (moi) of 2400. After subsequent GCV treatment, however, only ≈10% of the cells were viable at a moi of 50, and there were few surviving cells at a moi of 200. To illustrate the bystander effect in MCA-26 cells, ADV/RSV-tk-transduced MCA-26 cells were mixed in various proportions with nontransduced cells prior to GCV treatment. The results demonstrated that only 3.3% of the tk-transduced cells is sufficient to confer GCV toxicity to >90% of the parental tumor cells *in vitro* (Fig. 1*B*).

The functionality of the replication-defective mIL-2 adenoviral vector (ADV/RSV-mIL-2) was illustrated by transduction of mouse B16 cells *in vitro*, followed by demonstration of mIL-2 activity in the conditioned medium using a T-cell proliferation assay (18). mIL-2 activity of 750 units/ml was present in the conditioned medium of cells after transduction with the mIL-2 adenoviral vector at a moi of 200, but no mIL-2 activity was detected in cells transduced with a control adenoviral vector (data not shown).

Regression of Hepatic MCA-26 Tumors in Syngeneic Animals After Combination Gene Therapy *in Vivo*. An animal model for metastatic colon carcinoma in the liver was established by intrahepatic implantation of 3×10^5 MCA-26 cells. After 6 days, 5×7 mm^2 tumors were present in the liver of 60–70% of the animals. This was the tumor size selected for all

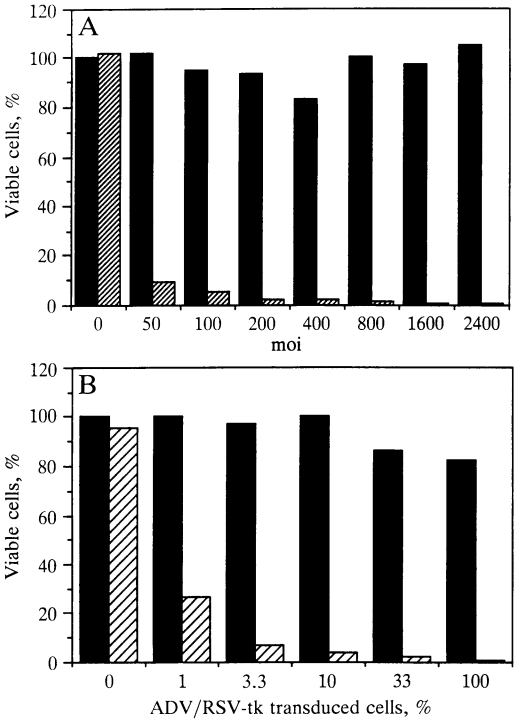

FIG. 1. Functional characterization of the recombinant adenoviral vectors. (*A*) GCV susceptibility of ADV/RSV-tk-transduced MCA-26 colon tumor cell lines. MCA-26 cells were transduced with ADV/RSV-tk at various moi followed by treatment with either PBS (solid bars) or GCV at 10 μg/ml (hatched bars) 12 hr later. After 3 days, the viable cells were determined by trypan blue staining. (*B*) Various proportions of ADV/RSV-tk-transduced cells (at a moi of 3000) were mixed with nontransduced MCA-26 cells prior to treatment with PBS (solid bars) or GCV at 10 μg/ml (hatched bars). After 3 days, the viable cells were again determined by trypan blue staining.

subsequent gene therapy experiments. BALB/c mice with hepatic colon carcinoma were divided into five treatment groups: 1, ADV/RSV-β-gal; 2, ADV/RSV-mIL-2; 3, ADV/RSV-β-gal plus ADV/RSV-mIL-2; 4, ADV/RSV-tk; and 5, ADV/RSV-tk plus ADV/RSV-mIL-2. The residual solid tumors in various animal treatment groups were measured and examined histopathologically (Fig. 2). The animals that were treated with the β-galactosidase (β-gal) vector had large nodules of actively growing undifferentiated carcinoma (Fig. 2 *A* and *B*). The animals that were treated with the mIL-2 vector had large nodules composed of mostly viable tumor cells with mitotic activity equal to the β-gal vector-treated mice (Fig. 2 *C* and *D*). Animals treated with a combination of β-gal and mIL-2 vectors appeared to have more tumor necrosis with infiltration of inflammatory cells, but plenty of viable tumor cells remained (Fig. 2 *E* and *F*). The tk vector treatment group had abundant tumor necrosis, but actively replicating neoplastic cells still remained (Fig. 2 *G* and *H*). All animals that were treated with the tk plus mIL-2 vectors exhibited massive tumor necrosis surrounded by inflammatory cells. In some livers, no viable malignancy remained (Fig. 2 *I* and *J*). The large zone of necrosis included a mixture of completely destroyed tumor (white regions), hemorrhage (dark pink), and ischemic liver damage (large light pink zone). The higher magnification pictures showed the presence of numerous inflammatory cells throughout. Seven of the 10 animals in the tk plus mIL-2 combination treatment group had few residual tumor cells present, and the remaining 3 animals appeared to be tumor free.

To quantify the effectiveness of tk plus mIL-2 combination gene therapy in causing tumor regression, residual tumor sizes were measured by computerized morphometric point count

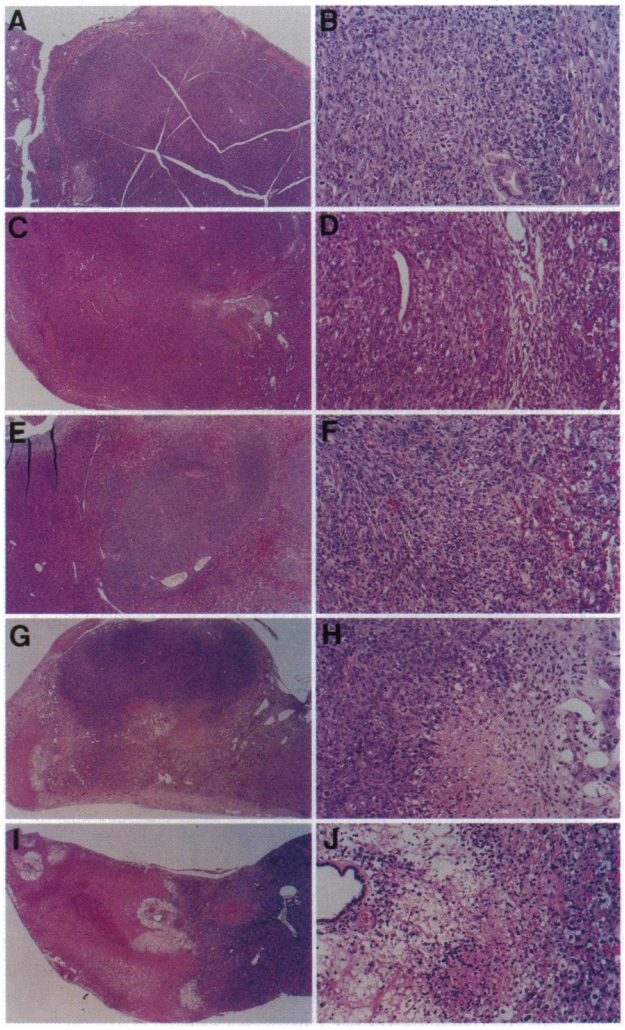

FIG. 2. Histopathological analysis of hepatic tumors in the recipient BALB/c mice at 1 day after completion of GCV treatment. Formalin-fixed and paraffin-embedded sections were stained with hematoxylin/eosin. Magnifications of ×15 (*Left*) and ×120 (*Right*) of representative sections in each of the animal treatment groups are shown. (*A* and *B*) ADV/RSV-β-gal (3×10^9 pfu). (*C* and *D*) ADV/RSV-mIL-2 (6×10^8 pfu). (*E* and *F*) ADV/RSV-β-gal (3×10^9) pfu plus ADV/RSV-mIL-2 (6×10^8 pfu). (*G* and *H*) ADV/RSV-tk (3×10^9 pfu). (*I* and *J*) ADV/RSV-tk (3×10^9 pfu) plus ADV/RSV-mIL-2 (6×10^8 pfu).

analyses of the maximal cross-sectional area of the solid tumors (Fig. 3). All animals treated with the β-gal or the mIL-2 vector developed large tumors. Animals treated with the β-gal and mIL-2 vectors together had an apparent 60% reduction of tumor size ($P < 0.05$). Those animals treated with the tk vector alone exhibited extensive tumor necrosis, and the residual tumor size was ≈5-fold smaller than those treated with the β-gal vector ($P < 0.01$). In the animal group treated with both tk and mIL-2 vectors, there was an apparent further reduction of the residual tumor size as compared to the animal group treated with the tk vector alone ($P < 0.03$).

Systemic Antitumoral Immunity in Animals Treated with tk Plus mIL-2 Vectors. To test whether there was any antitumoral immunity in the animals that underwent various gene therapy treatments, protection against secondary challenges by subcutaneous inoculation of tumorigenic doses of MCA-26 cells was performed at the conclusion of GCV administration. All animals in the β-gal vector treatment group developed palpable subcutaneous tumors at the challenge sites of 4×5 mm^2 in size after 7 days and 6×7 mm^2 in size after 14 days (Fig. 4). Five of six mIL-2 vector-treated animals and four of six

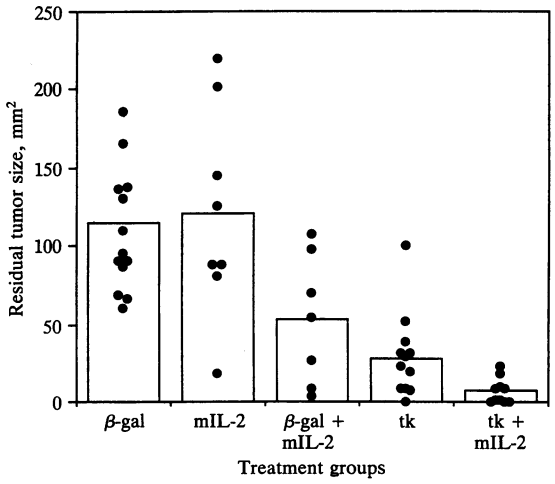

FIG. 3. Residual tumor sizes in animals after various gene therapy treatments. Maximal cross-sectional areas of the tumors were measured by computerized morphometric analysis. The cumulative number of animals in the five treatment groups from two separate experiments were 13 (β-gal), 8 (mIL-2), 7 (β-gal plus mIL-2), 12 (tk), and 10 (tk plus mIL-2). Data points for individual animals in each treatment group are plotted, and the bars represent mean values for each group.

β-gal plus mIL-2 vector-treated animals also developed subcutaneous tumors of similar sizes at the challenge site. The difference, however, was not statistically significant ($P > 0.4$). Interestingly, all six of the tk vector-treated animals developed palpable substaneous tumors in 1 week, and none of the six tk plus mIL-2 vector-treated animals developed subcutaneous tumors even after 4 weeks (Fig. 4). The results indicate that protection against distant site challenge is mIL-2 dependent ($P < 0.005$ between the tk and tk plus mIL-2 treatment groups). The systemic antitumoral immunity in these animals also appeared to be MCA-26 cell specific, as no protection against distant site challenge by tumorigenic doses of a heterologous but syngeneic breast tumor cell line (MOD) was observed in the same animals (Fig. 4).

To critically evaluate whether tumor rejection was associated with sensitization of host effector cells, an *in vitro* cytotoxic T-lymphocyte assay was performed. There was no significant CTL activity against MCA-26 target cells in the splenocytes of the untreated normal mice, as well as those animals in treatment groups 1–4. There was, however, a

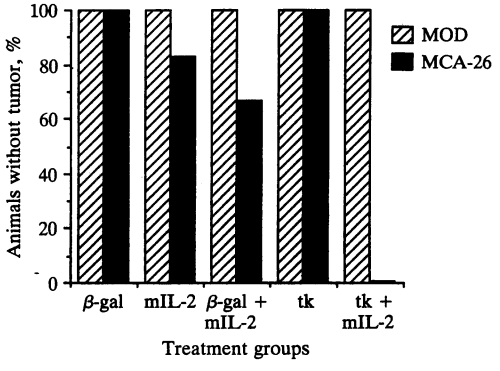

FIG. 4. Systemic antitumoral immunity against parental tumor cell challenges at distal sites. Tumorigenic doses of MCA-26 and MOD cells were inoculated subcutaneously in two flanks of the animals. Presence of 4×5 mm^2 subcutaneous palpable tumors in normal animals was noted after 1 week, which grew to 6×7 mm^2 in size after 2 weeks. The number of animals challenged with MCA-26 was six in each group, and those challenged with MOD ranged between two and four per group.

dramatic increase in MCA-26-specific CTL activity in the splenocytes of animals treated with both the tk and mIL-2 vectors (Fig. 5*A*). In contrast, splenocytes of tk plus mIL-2-treated mice failed to lyse BALB/c-derived MOD breast tumor cells or YAC1 target cells (data not shown).

To determine whether the CTL response reflected activity of $CD4^+$ and/or $CD8^+$ T cells, monoclonal antibodies against either CD4 or CD8 were incorporated as blocking reagents in the ^{51}Cr release assay. The monoclonal antibody against CD8 was effective in complete abolition of the CTL response against MCA-26 cells, while the monoclonal antibody against CD4 was ineffective (Fig. 5*B*). These experiments provided strong evidence that $CD8^+$ T cells from tk plus mIL-2 vector-treated animals were responsible for MCA-26-specific cytotoxicity.

DISCUSSION

Recent developments of gene transfer technologies have provided new possibilities for the treatment of malignancies. Suicide gene therapy and cancer vaccines are two of the most promising approaches. These methods have been attempted in the treatment of a number of tumors, including colon carcinoma, with varying degrees of success in laboratory animals. In the original report by Culver *et al.* (5) as well as many others (19–23), recombinant retroviral vectors were used to deliver the HSV-tk gene into the tumors. Because the recombinant retroviral vector can only be produced at relatively low titers, the virus-producing cells must be injected into the solid tumors rather than the viral isolate itself. This particular limitation can be readily resolved with the use of a recombinant adenoviral vector containing the same suicide gene. While the recombinant retroviral vectors have titers of 10^6–10^7 pfu/ml, recombinant adenoviral vectors are available at titers of 10^{11}–10^{12} pfu/ml. A second rationale for the use of recombinant retroviral vectors in suicide gene therapy is the fact that this viral vector will transduce only dividing cells, which provides a margin of safety to the surrounding normal tissues that are nondividing. However, GCV, upon phosphorylation by HSV-tk, becomes a substrate for DNA synthesis, which causes death in dividing cells (4). Thus, the use of this particular suicide gene system in an adenoviral vector provides the same margin of safety to surrounding normal tissues that are quiescent.

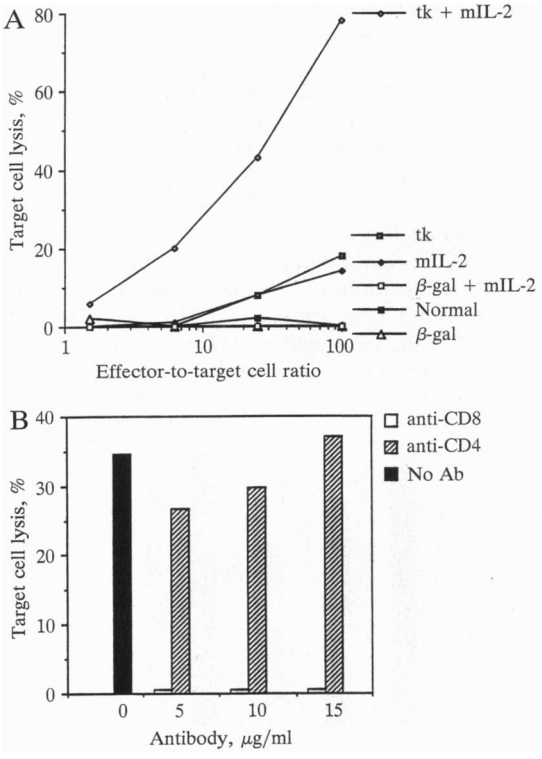

FIG. 5. (*A*) Cellular immune response in animals after various gene therapy treatments using a CTL assay as described in *Materials and Methods*. (*B*) *In vitro* blocking of the CTL response using monoclonal antibodies against either CD4 or CD8. Splenocytes from animals treated with ADV/RSV-tk plus ADV/RSV-mIL-2 were stimulated *in vitro* and then incubated with various concentrations of purified, sodium azide-free antibodies (RM4-5, monoclonal antibody to CD4, or 63-6.7, monoclonal antibody to CD8a; PharMingen) at 37°C for 30 min. The ^{51}Cr release assay was performed at an effector-to-target cell ratio of 50:1. Ab, antibody.

Cancer vaccines represent another general strategy in the treatment of metastatic tumors, which involves the transduction of established tumor cell lines with various cytokine genes using recombinant retroviral vectors *in vitro*, followed by transplantation of the transduced cells into immunocompetent syngeneic animals. Either live or killed tumor cells are used for vaccine purposes. The recipient animals are then challenged with tumorigenic doses of parental tumors cells at distant sites. This strategy has been reported for a number of tumor cells, including IL-2-transformed colon carcinoma (8–11, 24, 25). One of the reasons for the *ex vivo* strategy in cancer vaccine development lies in the fact that direct intratumoral delivery of cytokine genes with recombinant retroviral vectors resulted in few transduced cells *in vivo*. On the other hand, tumor cells transduced *in vitro* with retroviral vectors containing the neomycin-resistance gene can be selected with G418, and all transplanted tumor cells will express the transduced cytokine genes. This limitation can again be resolved by using the adenoviral vector system for efficient *in vivo* gene delivery.

The efficient adenoviral vector systems also provide an opportunity to combine the suicide gene and cancer vaccine approaches, which may be more effective than either approach alone in cancer gene therapy. In the current study, we find that the direct delivery of the HSV-tk and mIL-2 genes in recombinant adenoviral vectors to metastatic colon carcinoma in the liver resulted in their regression *in vivo*. Neither the β-gal vector nor the mIL-2 vector alone was capable of arresting tumor growth in this model. The tk vector alone did cause significant hepatic tumor necrosis, but the treated animals did not develop an effective antitumoral immune response. Combination therapy with both the tk and mIL-2 vectors was not only efficacious in causing hepatic tumor regression with few viable tumor cells remaining but also resulted in the establishment of a systemic antitumoral immunity that effectively protected against tumorigenic doses of the parental tumor cells inoculated at distant sites. The specificity of this antitumoral immunity against the parental tumor cells was further illustrated by the fact that there was no antitumoral immunity against tumorigenic doses of a heterologous breast carcinoma cell line inoculated in the same animals. Finally, it was demonstrated that the antitumoral immunity in these animals was associated with the presence of MCA-26 tumor-specific $CD8^+$ CTLs.

The generation of an effective systemic antitumoral immune response in the tk plus mIL-2-treated animals is a critical observation that may have important implications for gene therapy of metastatic colon carcinoma in the future. The fact that this response is dependent on mIL-2 suggests that local production of mIL-2 may be important. It may be hypothesized that tumor cell killing by tk/GCV resulted in the uptake of tumor cell-derived peptides by antigen-presenting cells, which then presented the tumor antigens to the T lymphocytes. The tumor antigen-specific T cells were better able to proliferate and to express genes important for cytolytic activity in the presence of elevated local concentrations of mIL-2. The enhancement of tumor antigen-specific T-cell populations would then mediate the systemic antitumoral effect against distant site challenges of parental tumor cells. The hypothesis

will need to be tested by detailed analyses of the inflammatory cell types at the challenge site, as well as *in vivo* depletion experiments using antibody preparations against various immune cells.

While the results are encouraging, much more scientific progress and technologic development must be achieved before the goal of gene therapy for metastatic colon carcinoma can be realized. A major limiting issue lies in the lack of long-term survival of the treated animals. All animals that were treated with either the β-gal or mIL-2 vector as well as four out of five animals treated with the mIL-2 plus β-gal vectors died of primary hepatic lesions between day 19 and 23 after MCA-26 cell implantation. This observation was expected as the histopathological analyses demonstrated that their tumors exhibited aggressive proliferation. Even the animal groups that were treated with the tk vector alone succumbed to liver tumors a few days later, as MCA-26 is a very aggressive tumor line and the residual viable tumor cells rapidly developed relapses. Of the five animals that were treated with tk plus mIL-2 vectors, however, only two died of liver tumor relapses, at 20 and at 30 days after MCA-26 cell implantation. The remaining three animals were sacrificed due to the presence of metastatic lesions in alternative organs: one at day 37 due to a large subcutaneous tumor, one at day 46 due to a tumor in the heart, and one at day 54 due to metastatic lung tumor. They were, however, free of hepatic tumors at the time of their sacrifice. Thus, while combination gene therapy was effective in causing complete tumor regression in the liver in three of the five animals that developed $CD8^+$ T lymphocytes against the parental tumor cells, the resulting antitumoral immunity in these animals was apparently insufficient to abolish metastatic tumors in other organs. While IL-2 is a powerful cytokine for T-cell proliferation involved in the development of an effective CTL response, granulocyte/macrophage colony-stimulating factor is also an effective cytokine for development of systemic antitumor immunity in syngeneic animals (9, 10). These reports strongly implicate the involvement of granulocytes and macrophages in tumor rejection. Thus, future inclusion of other cytokine genes in a combination adenoviral-mediated gene delivery protocol with IL-2 and tk may result in a more effective and long-lasting antitumoral effect against colon carcinoma *in vivo*. Continuous progress in these investigations in the future will be necessary for the successful development of a new treatment modality for metastatic colon carcinoma by *in vivo* gene therapy.

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