Combination Suicide and Cytokine Gene Therapy for Hepatic Metastases of Colon Carcinoma: Sustained Antitumor Immunity Prolongs Animal Survival

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ABSTRACT

The effectiveness of combination therapy using a suicide gene and cytokine genes for the treatment of metastatic colon carcinoma in the mouse liver was investigated. Pre-established hepatic tumors treated with a recombinant adenoviral vector containing the herpes simplex virus thymidine kinase gene (tk) exhibited substantial regression, although all treated animals suffered from subsequent relapses. Although cotreatment with a mouse interleukin 2 (mIL-2)-containing adenoviral vector induced an effective antitumor immune response, the immunity waned with time, and the treated animals eventually succumbed to hepatic tumor relapse or distant metastases. In this study, mouse granulocyte macrophage colony-stimulating factor (mGM-CSF) gene was tested for its ability to further enhance and prolong the antitumoral cellular immunity. A fraction of the animals treated with tk + mIL-2 + mGM-CSF developed long-term antitumor immunity and survived for more than 4 months without recurrence. This long-term antitumor immunity could be enhanced further by subsequent “vaccination” with mIL-2-expressing parental tumor cells. The results indicate that local expression of GM-CSF in the hepatic tumors and prolonged mIL-2 expression are necessary to generate persistent antitumor immunity that is essential for the prevention of tumor recurrence and long-term animal survival.

INTRODUCTION

Metastatic colon carcinoma is the second leading cause of death from malignancy in the United States. Eighty % of the patients who die of colon cancer have metastases in the liver (1). Once hepatic metastases occur, surgery and chemotherapy are the only currently available treatment modalities (2). Thus, the development of new modalities for treatment of metastases is important for patients with metastatic colon cancer.

We have demonstrated previously in a murine model of hepatic metastases of colon cancer that the cytokine gene IL-2 acted synergistically with the suicide gene herpes simplex thymidine kinase (tk) to induce a systemic antitumor immunity that resulted in regression of local tumor and protection against distant site challenge of parental tumor cells. The antitumor immunity was attributed to IL-2-mediated activation and proliferation of CD8+ CTLs (3). However, the antitumor immunity waned with time, resulting in relapse of tumors in the liver and distant sites. To achieve long-term protection against recurrences and metastases, it is imperative for this protective immunity to be maintained. Therefore, identification of other cytokines that can enhance and prolong antitumor immunity in combination with IL-2 is critically important for this therapeutic approach.

Tumor cell killing by tk/GCV results in the uptake of tumor cell-derived peptides by APCs, which then present the tumor antigens to the CD4+ T lymphocytes that in turn activate tumor-specific CD8+ cytolytic T cells. In the presence of elevated concentrations of IL-2 secreted locally by tumor cells, tumor rejection is achieved through enhanced antigen-specific T-cell response (4). Improvement of antigen processing and presentation to tumor-specific T-cell precursors complements IL-2 action and may further enhance the induction of antitumor immune response. GM-CSF is the best-characterized cytokine that promotes the activation and maturation of specialized APCs (5). Dranoff et al. (6) had demonstrated previously that GM-CSF was the most potent cytokine tested in “cancer vaccine” models utilizing irradiated tumor cells engineered to secrete cytokines in the induction of protective antitumor immunity in vivo.

Using appropriate adenoviral vector systems, various doses of cytokine genes can be delivered directly to the tumor cells in vivo for in situ expression and secretion. This permitted us to explore the therapeutic effect of localized expression of GM-CSF, in combination with the tk suicide gene therapy in the treatment of hepatic metastases of colon cancer. The results indicated that GM-CSF stimulated a long-lasting antitumor response and, in conjunction with subsequent vaccination with irradiated tumor cells transduced with cytokines, achieved long-term survival of tumor-bearing animals.

MATERIALS AND METHODS

Construction of Recombinant Adenoviral Vectors. Construction of a replication-defective adenoviral vector containing the tk gene under the transcriptional control of the RSV long-terminal repeat (ADV/tk) has been reported (7). A replication-defective adenoviral vector containing the mIL-2 cDNA under the transcriptional control of the RSV long-terminal repeat promoter (ADV/mIL2) was constructed (3) and plaque purified. A partial-length mGM-CSF cDNA encoding the mature peptide was obtained from R & D Ltd. A 105-bp leader sequence was synthesized with overlapping oligonucleotide fragments that covered the leader sequence, the initiation codon, and Kozak consensus sequence for ribosome binding and was utilized to construct a full-length mGM-CSF cDNA. To ensure correct nucleotide sequence, the entire cDNA was subsequently sequenced. Construction of ADV/mGM-CSF was as described for ADV/mIL-2. The control adenovirus DL-312 with E1 region deletion was obtained from Dr. Tom Shenk of Princeton University. The viral constructs are summarized in Table I. The viral titer (pfus/ml) was determined by plaque assay in 293 cells.

Establishment and Treatment of Hepatic Metastasis Model of Colon Carcinoma. Metastatic colon carcinoma was induced in the liver by intraperitoneal implantation of CC36 cells, a chemically induced colon carcinoma line derived from BALB/c mouse (8). Cells (1 × 10⁶) 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 intratumorally in 70 µl of 10 mM Tris-HCl (pH 7.4)/1 mM MgCl2/10% (v/v) glycerol/Polybrene (20 µg/ml). Twelve h after viral injection, the animals were treated i.p. with GCV at 10 mg/kg twice daily for 5 consecutive days.

Histopathological and Immunocytochemical Analyses of Hepatic Tumors. Fourteen days after various gene therapy treatments, the animals were sacrificed, and computerized morphometric analysis of the largest cross-sectional areas of the relapsed hepatic tumors was performed as described previously (3). For immunocytochemical staining, livers from euthanized animals of various treatment groups were collected at day 7 and cut in the

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3 The abbreviations used are: IL, interleukin; mIL, mouse IL; APC, antigen-presenting cell; GM-CSF, granulocyte-macrophage colony-stimulating factor; mGM-CSF, mouse GM-CSF; RSV, Rous sarcoma virus; plu, plaque-forming unit; tk, thymidine kinase; GCV, ganciclovir; ADV, adenovirus.

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Table 1: Summary of variable adenoviral constructs

<table>
<thead>
<tr>
<th>Construct</th>
<th>Deletion and insertion site</th>
<th>Promoter</th>
<th>cDNA</th>
<th>Polyadenylate</th>
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<td>RSV</td>
<td>HSV-tk</td>
<td>HSV-tk</td>
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<tr>
<td>ADV/mIL-2</td>
<td>E1A</td>
<td>RSV</td>
<td>mIL-2</td>
<td>BGH</td>
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<td>mGM-CSF</td>
<td>BGH</td>
</tr>
<tr>
<td>DL-312</td>
<td>E1A</td>
<td>RSV</td>
<td>HSV-tk</td>
<td>BGH</td>
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* BGH, bovine growth hormone.

middle at the site of the original tumor inoculation. One-half of the tissue was fixed in 10% buffered formalin and stained with hematoxylin and eosin for histopathological analysis. The corresponding half was embedded in Tissue Tek OCT embedding medium (Miles, Inc., Elkhart, IN) and frozen immediately in 2-methylbutane in liquid nitrogen for immunocytochemical analyses. The fluorescein-antifluorescein system was used to identify infiltrating inflammatory cells in the liver. Fluorescein-conjugated primary monoclonal antibodies used in the assay were the following: rat antimouse CD4 (L3T4; Life Technologies, Inc., Grand Island, NY), rat antimouse CD8a (ly-2; Life Technologies, Inc.), and rat antimouse macrophage (F4/80; Harlan Bioproducts for Science, Inc., Indianapolis, IN). After reaction with primary antibodies, the sections were incubated with peroxidase-conjugated rabbit anti-FITC (DAKO, Carpinteria, CA). The slides were then incubated in chromogen solution (3 ml 3,3′-diaminobenzidine, 10 ml PBS, 50 µl 8% NiCl2, 1 µl 30% H2O2) and then counterstained with Nuclear Fast Red for observation.

**Distant Site Challenge in Treated Animals with Parental Tumor Cells.**

At different time points after primary tumor inoculation, animals in different vector treatment groups were challenged with tumorigenic doses (1.5 × 10^5) of parental CC36 tumor cells. The presence of s.c. tumors in animals after various gene therapy treatment was observed for 2–3 weeks. The results were analyzed statistically by logistic regression (9).

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**Fig. 1.** Viable tumor size in animals after various gene therapy treatments. Maximal cross-sectional areas of the tumors were measured by computerized morphometric analysis. The virus dose per mouse in each of the treatment groups of 10 animals each was the following: (a) DL-312 (6 × 10^8 pfus); (b) ADV/tk (3 × 10^7 pfus); (c) ADV/tk (3 × 10^7 pfus) + ADV/mIL-2 (3 × 10^7 pfus); (d) ADV/tk (3 × 10^7 pfus) + ADV/mIL-2 (3 × 10^7 pfus); and (e) ADV/tk (3 × 10^7 pfus) + ADV/mIL-2 (3 × 10^7 pfus) + ADV/mGM-CSF (2 × 10^7 pfus). Columns, mean values for each group; bars, SDs.

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**Fig. 2.** Immunocytochemical analyses of the colon carcinoma 7 days after various treatments. A, H&E; B, CD4 (L3T4); C, CD8 (Ly-2); D, macrophage (F4/80). In all images, the tumor is on the left and the liver is on the right. In the samples from animals treated with tk + mIL-2 and tk + mIL-2 + mGM-CSF, no viable tumor remains, so only necrotic tissue is seen. Magnification, ×200. The liver next to the tumor is very inflamed and there is a fatty change of the hepatocytes and a dilation of the sinusoids in the cases with total tumor necrosis.
were increased dramatically in the livers of animals treated with tk + mGM-CSF or tk + mGM-CSF + mIL-2 (Fig. 2D).

**Immunokinetic Study in Animals Receiving Combination Gene Therapy.** To assess the antitumor immune response in the treated animals, a kinetic study of cytolytic activity was performed. There was no significant CTL activity in the splenocytes of the animals treated with tk or tk + GM-CSF, but significant activities were detected at day 14 in the splenocytes of animals treated with tk + mIL-2 or tk + mIL-2 + mGM-CSF (Fig. 3). In the tk + mIL-2-treated animals, the cytolytic activity did not persist. After 25 days, the activity had decreased to the background level. However, in the tk + mIL-2 + mGM-CSF-treated group, cytolytic activity was maintained at high levels for up to 60 days in the surviving animals. These result indicated that CTL activity was prolonged significantly in animals treated with both cytokine vectors. To evaluate further whether this CTL activity resulted in prolongation of systemic antitumoral immunity, protection against challenges by s.c. inoculation of tumorigenic doses of parental tumor cells was evaluated at various time points (Fig. 4). When challenged at day 14 after primary hepatic tumor cell implantation, all animals in the tk treatment group developed palpable s.c. tumors to an average size of $4 \times 10^3$ mm$^2$ at challenge sites after 7 days. Five of five animals treated with tk + mIL-2 and four of five animals treated with tk + mIL-2 + mGM-CSF showed protection against parental tumor challenge. However, when the challenges were performed at subsequent time points, there was a significant reduction in protection in the tk + mIL-2-treatment group; only 50% were protected when challenged at day 28, and no protection was seen at day 75. Conversely, in the tk + mIL-2 + mGM-CSF-treated group, none of the eight animals formed s.c. tumors at day 28, and protection was also seen at day 75 and even at day 150. A significant difference in protection from tumor rechallenge ($P < 0.04$) was demonstrated between tk + mIL-2 and tk + mIL-2 + mGM-CSF by logistic regression.

**Long-Term Survival of Treated Animals.** To assess treatment outcome, animals receiving tk, tk + GM-CSF, tk + mIL-2, and tk + mIL-2 + mGM-CSF were studied for long-term survival (Fig. 5). In group treated with tk, tk + GM-CSF, or tk + mIL-2, all animals developed relapsed tumor and died at 40–60 days. In the tk + IL-2 + GM-CSF-treated group, animals survived significantly longer as compared to those treated with tk + mIL-2 + mGM-CSF (Fig. 2D).

**RESULTS**

**Regression of Hepatic Colon Tumor in Syngeneic Animals after Combination Gene Therapy in Vivo.** An animal model for colon carcinoma in the liver was established by intrahepatic implantation of $1 \times 10^6$ CC36 cells. After 7 days, tumors of $5 \times 4$ mm$^2$ in size were selected for subsequent gene therapy experiments. The animals with hepatic colon carcinoma were divided into five treatment groups, each receiving (a) a control adenoviral vector (DL-312); (b) ADV/tk; (c) ADV/tk + ADV/mGM-CSF; (d) ADV/tk + ADV/mIL-2; or (e) ADV/tk + ADV/mIL-2 + ADV/mGM-CSF. Twelve h after viral inoculation, all animals received i.p. GCV (10 mg/kg twice daily) for 5 consecutive days. Two weeks after viral inoculation, the viable tumor cells in various treatment groups were quantified with computerized morphometric point-count analysis of the maximal cross-sectional area of the viable tumors (Fig. 1). Animals treated with ADV/tk vector alone had an apparent 50% reduction of tumor size as compared with those treated with the control virus DL-312 ($P < 0.05$). In the animal groups treated with both tk and mIL-2 or mGM-CSF vectors, there was a further significant reduction of the residual tumor size as compared to the animal group treated with the tk vector alone ($P < 0.05$). There was, however, no significant further reduction of tumor size in animals treated with ADV/tk and both cytokine vector. There was massive infiltration of inflammatory cells surrounding the necrotic tumor area in the animals treated with tk + cytokine but not in those treated with tk alone (Fig. 2A). Immunocytochemical analyses revealed that the infiltrates were mainly CD8$^+$ lymphocytes in the tumor boundary area of animals treated with tk + mIL-2 or tk + mIL-2 + mGM-CSF (Fig. 2B). The number of CD4$^+$ lymphocytes was approximately equal in the animals treated with tk + mGM-CSF and those treated with tk + mIL-2 + mGM-CSF (Fig. 2C). Macrophages...
Fig. 5. Long-term survival of animals after various gene therapy treatments. The tumor-bearing animals were divided into four treatment groups: (a) ADV/tk. n = 8; (b) ADV/tk + ADV/mGM-CSF. n = 7; (c) ADV/tk + ADV/mIL-2. n = 7; and (d) ADV/tk + ADV/mIL-2 + ADV/mGM-CSF. n = 8. All the animals received GCV treatment 12 h after virus injection and were observed for survival over time. The survival of animals was analyzed by the Wilcoxon test (9).

Fig. 6. Long-term survival of combination gene therapy-treated animals with subsequent immunity boosting. A, hepatic tumors were treated with adenovirus vectors tk + mIL-2 + mGM-CSF and followed by GCV treatment for 5 days. Two weeks after virus inoculation, the immunity of treated animals was boosted with irradiated $1 \times 10^6$ ADV/mIL-2 (n = 16)- or DL-312 (n = 14)-transduced tumor cells at multiplicity of infection 320, or with irradiated tumor cells alone (n = 13). The animals were boosted subsequently three times at 2-week intervals. B, hepatic tumors were treated with adenovirus vectors tk + mIL-2 and received an identical boosting schedule. Mice boosted with ADV/mIL-2-transduced tumors, n = 19; mice boosted with control virus-transduced tumor, n = 16; and mice boosted with irradiated tumor cells alone, n = 12. The survival of animals was analyzed statistically by the Wilcoxon test (9).

DIscussion

Suicide gene therapy and cancer vaccine are promising approaches for cancer gene therapy (11–13). The combination of both approaches has been shown to be more effective than either approach alone, and the synergistic effect on the induction of antitumoral cellular immune response in the recipient animals has been reported previously (3). Although IL-2 has potent effects on tumor-specific T-cell activation and proliferation, there are multiple steps and regulatory cytokines to maximize an immune response in vivo. Simultaneous delivery of the most potent combination of cytokines to achieve maximal antitumoral cellular immunity is therefore critical in the development of an effective cancer treatment strategy. Studies of an approach using a combination of cytokines by recombinant proteins, or ex vivo "cancer vaccine" approach, have been reported for a number of tumor models and have shown some success (14–16), although the details of the underlying immune mechanisms have not been well studied. Recombinant adenoviral vectors are capable of efficient transduction of target cells in vivo. The current study is the first paper on the use of adenoviral vectors to investigate the effect of combination suicide and multiple cytokine gene therapy by direct intratumoral delivery in a pre-established tumor model in vivo. Using a model of hepatic metastasis of colon carcinoma, we showed that mGM-CSF induced tumor regression more efficiently when combined with tk as compared with either treatment alone. Because there was no significant CTL activity detected in animals receiving the tk + mGM-CSF treatment, the synergistic effect in hepatic tumor regression was probably due to immune effector cells other than the T lymphocytes (17, 18). Significant CTL activity was observed only when tk was combined with mIL-2 with or without mGM-CSF. However, it was only with the combination tk + mIL-2 + mGM-CSF treatment that
antitumoral cellular immune response persisted, particularly with subsequent boosting with mIL-2-expressing irradiated tumor cells.

Regarding the immune mechanisms that are responsible for tumor rejection, we hypothesize the following mechanism. The initial tk + GCV treatment causes tumor death in situ, while local mGM-CSF expression enhances the inflammatory response and activates the APCs, thus attracting CD4+ T cells to the local tumor area, as reflected by the results of the immunohistological staining. Locally expressed mIL-2 by transduced tumor cells then act synergistically to activate and enhance the proliferation of cytolytic T cells. Although effective antitumoral immune response can be induced by tk + mIL-2 treatment alone, the effect is only temporary. The capacity of GM-CSF-activated professional APCs to promote activation and persistence of tumor-specific precursor T cells is crucial for prolongation of the CTL response (19, 20). Because the level of tumor antigen and cytokine will decrease with time after primary treatment, prolongation of tumor antigen presentation and cytokine expression are necessary for improvement of long-term animal survival. The “cancer vaccine” approach with IL-2 has been shown to enhance the proliferation of colon tumor-specific CD8+ cytotoxic T cells (21). These educated T cells can also be clonally expanded in the lymphoid organ of a sensitized animal (22).

Thus, in future gene therapy strategies involving tumor immunomodulation, it is imperative to focus on the enhancement and prolongation of cytolytic immune response. Additional investigation to characterize the antitumoral immune regulatory mechanisms enhanced by GM-CSF will also be necessary to refine the future development as a new treatment modality for metastatic colon carcinoma in vivo.

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