Interleukin 2 Gene Therapy of Colorectal Carcinoma with Autologous Irradiated Tumor Cells and Genetically Engineered Fibroblasts: A Phase I Study

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ABSTRACT

The purpose of this study was to determine the safety, toxicity, and antitumor immune response following S.C. immunizations with a mixture of irradiated, autologous tumor cells and autologous fibroblasts that were genetically modified to express the gene for interleukin 2 (IL-2) in patients with colorectal carcinoma. Ten patients were treated with a fixed dose of tumor cells (10^7) and escalating doses of fibroblasts secreting IL-2 (per 24 h): 100 units (three patients), 200 units (three patients), 400 units (three patients), and 800 units (one patient). Pre- and posttreatment peripheral blood mononuclear cells were evaluated for evidence of antitumor immune responses. Fatigue and/or flu-like symptoms were experienced by seven patients and delayed-type hypersensitivity-like skin reactions were observed at the sites of the second or subsequent vaccinations in five patients. Low frequencies of tumor cytotoxic T-cell precursors (range, 1/190,000–1/1,320,000 peripheral blood mononuclear cells) were detected prior to therapy in four of seven patients. There was a 5-fold increase following treatment in the frequency of tumor T-cell precursors in two of six evaluable patients. Some patients with colorectal cancer have low frequencies of tumor cytotoxic T-cell precursors that may be increased by this well-tolerated form of IL-2 gene therapy, which warrants continued clinical evaluation.

INTRODUCTION

Colorectal carcinoma is one of the most common cancers in the United States, with an annual incidence of >150,000 cases. Most patients are treated with tumor resection and do not have clinically detectable tumor following surgery. However, a majority of patients have microscopic metastases and eventually relapse with clinically overt disease in the liver or abdominal cavity. The large number of patients and the small tumor burden following surgical resection makes colorectal carcinoma an attractive candidate for adjuvant immunotherapy trials. It is generally acknowledged that immunotherapies are likely to be most effective when the tumor burden is low. In this regard, the immunomodulator Levamisole is currently approved for the treatment of patients with Duke’s C tumors. In addition, encouraging results have been obtained with an autologous tumor vaccine as an adjuvant therapy following tumor resection (1). Additional studies have suggested that passive and active immunotherapies directed against the tumor-associated antigen recognized by the monoclonal antibodies 17-1A and GA733 have therapeutic efficacy (2, 3). These findings indicate that immunotherapies may have beneficial effects in colon carcinoma and support the development and evaluation of novel immunotherapy approaches for this disorder.

Vaccinations with tumor cells genetically engineered to express cytokine genes have resulted in significant antitumor immune responses in several animal tumor models without the toxicities that may be associated with systemic cytokine administration (4–7). In the studies using IL-23 gene transfer, the vaccinated animals developed systemic antitumor immunity that is capable of eradicating established, unmodified parental tumors (4, 5). Unfortunately, many types of tumors are difficult to establish in culture, and cytokine gene therapies requiring the genetic modification of autologous tumor cells may not be practical for many cancer patients. In contrast, fibroblasts are readily cultured and modified with retroviral vectors to express cytokine genes. We have successfully treated established tumors in a murine model of colorectal carcinoma by immunizations with a mixture of irradiated tumor cells and fibroblasts genetically engineered to express IL-2 (8). These considerations pro-

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3 The abbreviations used are: IL, interleukin; IMDM, Iscove’s modified Dulbecco’s medium; FBS, fetal bovine serum; PBMC, peripheral blood mononuclear cell; DTH, delayed-type hypersensitivity; pCTL, CTL precursor.
vided the rationale for examining this form of IL-2 gene therapy for colorectal carcinoma in a Phase 1 clinical trial.

**PATIENTS AND METHODS**

**Patients**

All patients had histologically confirmed colorectal carcinoma and had failed standard chemotherapy with a 5-fluorouracil-based regimen. Our patients had varying stages of disease at presentation and measurable metastatic disease at the time of treatment. Signed informed consent was obtained from each patient for the clinical protocol approved by our local institutional review board, the Recombinant DNA Advisory Committee, and the Food and Drug Administration.

**Vaccine Preparation and Characterization**

**Genetically Modified Autologous Fibroblasts.** Primary autologous fibroblast cultures were established from 12-mm² skin biopsies. The skin was collected and transported in IMDM supplemented with 10% FBS (Gemini Bioproducts, Calabasas, CA) and the antibiotic gentamicin sulfate (250 µg/ml; Sigma Chemical Co., St. Louis, MO). Prior to processing, scalpels were used to trim fat from the biopsy. Using two scalpsels, we minced the skin into 1-mm³ pieces. The pieces were incubated overnight with 10 ml of 200 units/ml collagenase (Sigma) and 200 units/ml DNase in IMDM with 10% FBS in an upright T25 flask at 37°C and 10% CO₂. The digested tissues were centrifuged at 1000 rpm and washed with serum-free DMEM. The pellet was resuspended in 10 ml of trypsin-EDTA (Sigma) and incubated for 10 min in an upright T25 flask at 37°C and 5% CO₂. The trypsin digestion was stopped by the addition of 2 ml of FBS. The cells were centrifuged at 1000 rpm, and the pellet was resuspended in 15 ml of IMDM supplemented with 10% FBS and gentamicin sulfate (100 µg/ml), placed in a T75 flask, and incubated at 37°C and 10% CO₂.

The cultured fibroblasts were expanded in DMEM supplemented with 10% FBS prior to transduction with the IL-2 retroviral vector LXSN-tIL2. The construction and characterization of this vector have been described previously (9). Standard retroviral gene transfer methods were used to transduce the fibroblast cultures. Fibroblasts (5 x 10⁵) were added to a T75 flasks and incubated overnight in DMEM supplemented with 10% FBS. The medium was then replaced with supernatant from the LXSN-tIL2 packaging cell line in the presence of 8 µg/ml polybrene as described previously (9). Twenty-four h later, the cells were washed and incubated in DMEM supplemented with 10% FBS. The cells were allowed to grow for 48 h before the neomycin analogue G418 was added at a concentration of 50–200 µg/ml to select for transduced cells. Growth of selected cells became apparent 10–14 days after the addition of G418.

The concentration of IL-2 in the supernatants of the transduced fibroblast cultures was measured using an ELISA (Genzyme), as described previously (1 unit = 38 pg of IL-2; Refs. 8 and 9). Briefly, 96-well plastic microtiter plates that were pre-coated with a human IL-2-specific monoclonal antibody to capture the secreted cytokine were incubated with the test sample, washed, and then incubated with the secondary rabbit polyclonal antihuman IL-2 antisera conjugated to peroxidase or alkaline phosphatase. The enzymatic reaction was developed with the appropriate chromagen substrate, and the optical density was read on an ELISA reader. IL-2 standards of known concentration permitted quantitation of IL-2 levels. After transduction, the G418-resistant cells were expanded until 6 x 10⁶ –10 x 10⁶ cells were available for cryopreservation. The genetically modified cells were centrifuged, washed in DMEM, and then cryopreserved at −70°C until they were required for injection in a solution containing 8% DMSO and 30% FBS in DME. The cells were stored in liquid nitrogen until the time of administration. The transduced fibroblasts were then thawed, washed with DMEM, and irradiated with 4000 cGy to minimize the risk of chronic local inflammatory reactions at the site of injection due to continued secretion of IL-2. This dose of radiation has been shown to render fibroblasts incapable of proliferation while having minimal effects on the level of IL-2 secretion by transduced fibroblasts (9). The transduced cells were certified to be free of contaminating replication competent virus and other adventitious agents by criteria recommended by the Food and Drug Administration (10). The generation of the transduced IL-2-secreting fibroblasts required ~8–12 weeks.

**Autologous Tumor Cells.** Clinically indicated colon tumor resections were obtained under sterile conditions. The resected tumor was placed in iced IMDM plus 10% FBS with 250 µg/ml gentamicin. Necrotic tissue was trimmed away from the tumor mass, and the tumor was cut into 1–2-mm³ pieces. The minced tumor tissue was washed three times and then incubated overnight at 37°C in medium containing 100–400 units/ml collagenase and 100–200 units/ml DNase, depending on the tissue consistency. The cells were collected by centrifugation and resuspended in supplemented medium containing 10 ml of trypsin-EDTA and incubated for 10 min in an upright T25 flask at 37°C and 10% CO₂. The trypsin digestion was stopped by the addition of FBS (2 ml). The cells were then expanded in T225 flasks until ~4 x 10⁸ cells were obtained. The harvested cells were then cryopreserved in liquid nitrogen in 10²-cell aliquots suspended in DME supplemented with 30% FCS and 8% DMSO. The cells were stored in liquid nitrogen until the time of administration. After thawing, the tumor cells used for immunizations were treated with 10,000 cGy, mixed with the appropriate number of IL-2-transduced fibroblasts, and resuspended in a normal saline solution for injection.

**Administration of Therapy and Clinical Monitoring**

Patients received at least three S.C. immunizations at least 2 weeks apart with a mixture of irradiated autologous fibroblasts that were genetically modified to express the gene for IL-2 and 10⁷ irradiated autologous tumor cells. The number of fibroblasts was adjusted to escalate the secreted dose of IL-2 (per 24 h: 100 units [three patients], 200 units [three patients], 400 units [three patients], and 800 units [one patient]).

The following clinical evaluations were performed prior to each treatment, 1 month after the completion of therapy and at 3-month intervals thereafter: history and physical examination, complete blood count with differential, platelet count, PT, PTT, glucose, biliary urea nitrogen, creatinine, electrolytes, aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, alkaline phosphatase, bilirubin, uric acid, calcium, total protein, albumin, amylase and lipase, urinalysis, and carcinoembryonic antigen serum levels.
Diagnostic studies including computerized tomography, magnetic resonance imaging, or X-ray evaluations were performed to document and quantitate the extent of disease activity prior to treatment and following the completion of therapy.

**Cellular Immune Responses**

**Precursor Frequency Analysis.** Precursor frequency analysis of cytotoxic effector cells was performed using a previously described limiting dilution method (11). Autologous PBMCs were used as effector cells, and autologous tumor cells or allogeneic PBMCs were used as stimulator/target cells. Stimulator/target cells were incubated for a minimum of 1 week in the absence of FBS to prevent the presentation of bovine antigens. Irradiated stimulator cells (2 × 10^4) were mixed with effector cells at ratios of 5:1, 1.67:1, and 0.56:1 in 96-well flat-bottomed microtiter plates and cultured in RPMI 1640 supplemented with 4% human AB⁺ serum (Gemini Bioproducts) and 5 ng/ml human IL-4 (R&D Systems). Human IL-2 was added to the cultures on day 3 to achieve a concentration of 50 units/ml. On days 7 and 14, the cultures were refed with fresh medium containing 50 units/ml human IL-2, 5 ng/ml human IL-4, and 2 × 10^4 irradiated stimulator cells. On day 21, 70 µl of each well were harvested and used in a chromium release assay with target autologous tumor cells or allogeneic PBMCs to determine the percentage specific lysis. Precursor frequencies were estimated by Poisson distribution and χ² minimization analyses as described (11).

**Generation and Expansion of CTLs.** A limiting dilution culture system was used to generate CTLs, as described above (11). On day 21, one-third of the cells were removed from each well and tested for cytotoxicity using a standard chromium release assay as described below. On day 22, wells demonstrating >10% specific lysis against target cells were transferred into duplicate 96-well flat-bottomed plates and fed with 2 × 10^7 irradiated stimulator cells in culture medium supplemented with 50 units/ml IL-2 and 5 ng/ml IL-4. Cells from one set of the duplicate plates were tested for cytotoxicity employing a standard chromium release assay on day 28. On day 29, cells of interest were transferred into 12-well tissue culture plates and fed with irradiated stimulator cells at a 25:1 ratio in culture medium supplemented with 50 units/ml IL-2 and 5 ng/ml IL-4. Cells were maintained in this manner with fresh medium and stimulator cells were added every 7–14 days, depending upon CTL proliferation.

**Chromium Release Assay.** A standard chromium release assay was used to evaluate cytotoxicity against autologous tumor and fibroblast target cells. Target cells (2 × 10^6) were labeled with 51 Cr by incubating at 37°C overnight in the presence of 250 µCi of Na 51 Cr. The target cells were washed extensively in RPMI 1640 supplemented with 10% FBS prior to use. Effector cells were added to 96-well V-bottomed plates (Costar Inc.). Labeled target cells were then added to the plates at a concentration of 1 × 10^3 cells per well. Spontaneous release was measured in wells containing only target cells and medium. Maximum release was determined from target cells incubated with 2.5 × H2SO4. The plates were centrifuged for 5 min at 100 × g and incubated at 37°C for 4 h. The plates were then centrifuged at 500 × g for 5 min and the 51 Cr radioactivity measured in 100-µl aliquots of the supernatants. The percentage specific lysis was calculated using the formula [(cpm_exp × cpm_bkgd)/(cpm_max × cpm_bkgd)] × 100.

**Humoral Immunity**

Humoral antitumor immune responses were evaluated by comparing the titer of pretreatment and posttreatment sera for tumor reactivity using a previously described cytofluorometric procedure (12). Briefly, tumor cells were incubated with pretreatment, posttreatment, and normal human sera, washed, and then stained with fluorescein-conjugated goat antihuman immunoglobulin antisera. The stained cells were fixed in a 1% solution of formaldehyde and then analyzed on a flow cytometer, as described previously (12).

**Safety Testing for Retroviral Vectors in Vivo**

A PCR assay was used to detect retroviral vector env sequences in the patients’ PBMCs during the course of treatment. In selected patients (1, 2, 3, 4, and 7), Western blot analyses for evidence of antibody to the Env protein were performed with the patients’ sera. The Western blot assays were performed by Microbiological Associates, as described previously (10). These assays were performed with peripheral blood samples obtained prior to the initiation of therapy and at one or more of the following time points: monthly during therapy (weeks 4 and 8) or at 3-month intervals thereafter.

The PCR assays were performed using standard method, as described previously. Briefly, high molecular weight DNA was isolated from PBMCs. DNA extraction was performed using a QiAamp Blood Kit (Qiagen) following the protocol recommended for the appropriate cell density. Briefly, cell pellet samples were resuspended in 200 µl of PBS with 25 µl of Qiagen proteinase K solution and 200 µl of buffer, which were mixed by vortexing to lyse the sample. Samples were incubated at 70°C for 10 min to maximize the DNA yield and protein denaturation. Subsequently, 210 µl of 100% ethanol were added and mixed to adjust binding conditions. The mixture was then transferred onto a QiAamp spin column and centrifuged at 8000 rpm for 1 min, for absorption of the DNA. The column was then washed two times with 500 µl of the washing buffer to remove impurities. DNA was eluted from the column with 200 µl of Qiagen elution buffer.

The extracted DNA was mixed with appropriate PCR primer sequences. DNA dilutions were prepared to obtain a 4-point standard as follows: 600, 60, 6, and 0.6 ng per 5 µl. The computer program Oligo 5.0 was used to design the oligonucleotides based on the sequence containing the env sequences of the murine amphotropic retrovirus 4070A. The expected PCR product was 410 bp: forward oligonucleotide, 5'-CAG AGA GCC CCC ATC AGG TCT TTA-3'; and reverse oligonucleotide, 5'-GGC AAC TTT AGA GCA TCC CGT GTC-3'. The PCR for env was incubated for 35 cycles with a temperature regimen of 94°C denaturation for 30 s, 60°C annealing for 30 s, and 72°C polymerization for 1 min. Globin detection was used as a positive control to confirm the presence of cellular DNA. The PCR for globin was incubated for 30 cycles with a temperature regimen of 94°C denaturation for 1 min, 65°C annealing for 1 min, and 72°C polymerization for 1 min. Following ethanol precipitation, the PCR products were electrophoresed in...
DTH-like Skin Reactions. DTH-like skin reactions at the sites of the second or subsequent vaccinations were observed in 5 of 10 patients, implying induction of immunological memory responses, because these reactions were not observed with the initial vaccination. Typically, erythema, induration, and pruritus developed at the injection site 48–72 h after the second and subsequent immunizations. The erythema and induration regressed 72–96 h thereafter, with a small amount of residual induration resolving over the next 1–2 weeks. The areas of induration ranged from ~1 × 1 cm to 5 × 5 cm in size. Light microscopy evaluation of vaccination site biopsies obtained after the third immunization in patients 1, 3, 5, and 8 revealed S.C. and dermal perivascular lymphocytic and eosinophilic infiltrates, consistent with the histology of DTH reactions. Skin biopsies obtained from the contralateral arm were without these cellular infiltrates. Interestingly, patient 1 did not exhibit a visible DTH-like skin reaction but exhibited microscopic infiltrates of lymphocytes and eosinophils, consistent with a DTH response.  

Adverse Events. A summary of adverse events associated with treatment is provided in Table 1. Mild to moderate fatigue and/or flu-like symptoms were observed in seven patients. It is unclear whether these symptoms reflected the results of therapy, tumor progression, upper respiratory tract infection (patient 2), or a combination of these possibilities. There were no significant treatment-related changes in complete blood counts, serum chemistries, or urinalyses compared to pretreatment values. Safety testing has revealed no evidence for the generation of replication-competent retrovirus in any of transduced cells used for therapy or in the peripheral blood following treatment.

Clinical Course. Clinically, patient 3 had stabilization of previously enlarging abdominal metastases on computerized tomographic scan of ~3-month duration, associated with a decrease in abdominal pain. All of the patients treated in our study eventually developed progressive disease. There were no treatment-related deaths in this study. The time from receipt of the final immunization to patient death is noted in Table 1 (range, 18 days to 15 months). In two patients (patients 7 and 9), tumor progression during the course of treatment precluded administration of the final planned vaccination. Patient 7 was removed from the study following the second of three planned vaccinations when he was treated with radiation therapy for bone pain at a site of known metastasis. Patient 9 was removed from the study after the second of three planned vaccinations when progressive disease resulted in a small bowel obstruction, necessitating hospitalization.

Immune Responses. pCTL frequency analyses were performed to measure cell-mediated immunity. The patients’ autologous tumor cells (were used as stimulator cells and pre- and posttreatment PBMCs were used as effector cells in these assays. As a control to measure general immune competence, precursor frequencies were concurrently measured against allogeneic PBMCs. The results of these analyses are provided in Table 2. Low frequencies of tumor pCTL (range, 1/190,000–1/320,000 PBMCs) were detected prior to therapy in four of seven patients with sufficient cells for evaluation. There was a general correlation between the frequencies of allogeneic and tumor pCTLs prior to treatment because patients with lower...
frequencies of tumor precursors tended to have lower frequencies of allogeneic precursors. There was a 5-fold increase following treatment in the frequency of tumor pCTL in two of six evaluable patients with detectable pretreatment pCTLs. The responding patients (patients 5 and 8) were treated with fibroblasts secreting 200 and 400 units of IL-2 (per 24 h), respectively. The allogeneic precursor frequency was unaffected by therapy in these patients. There was no increase in tumor pCTL frequency with treatment in patients who did not have detectable pretreatment pCTL (patients 1, 7, and 10) or in either patient treated at the 100-unit dose of IL-2 (patients 1 and 3). In patient 3, the precursor frequencies for both autologous tumor and allogeneic pCTL decreased with time, implying worsening general immunosuppression with tumor progression. Cloned T cells were derived from patients' PBMCs that were cytotoxic for tumor cells but not autologous fibroblasts (Fig. 1).

There was no evidence of a humoral antitumor immune response. Incubation of the tumor cells from patients 1, 5, 7, and 10 with pretreatment, posttreatment, and normal human sera followed by fluorescein-conjugated goat antihuman immunoglobulin antisera revealed no differences in tumor cell staining by cytofluorometric analysis.

Table 2  Summary of pCTL frequencies against autologous tumor cells and allogeneic PBMCs

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<th>Week</th>
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Fig. 1  Tumor lysis by cloned T cells. CTLs were generated from the patient’s PBMC by a limiting dilution culture system using irradiated stimulator tumor cells in culture media supplemented with AB serum, recombinant human IL-2, and IL-4. A standard chromium release assay was used to evaluate cytotoxicity against autologous tumor and fibroblast target cells. Cloned CTLs demonstrated selective cytotoxicity for tumor cells without significant lysis of the patient’s matched autologous fibroblasts.

**DISCUSSION**

Recent advances in the characterization of cytokine and tumor antigen genes, coupled with our increasing ability to manipulate gene expression, have fostered a new era of tumor immunotherapy. The aim of this study was to evaluate the safety and antitumor immune responses of a novel method of cytokine immunotherapy using gene transfer to provide continuous, local cytokine administration at the site of tumor vaccination. Our results indicate that cytokine gene therapy with genetically engineered autologous fibroblasts is well tolerated and provides a practical alternative to related gene therapy approaches with
autologous tumor cells that may be difficult to establish in culture for genetic modification.

Several investigations have isolated cytotoxic T cells recognizing tumor-associated antigens in patients with cancer (13–18). However, few studies of this nature have been performed in patients with colorectal carcinoma. In addition, it is unknown whether immunotherapies would be able to increase the frequency of these tumor cytotoxic T cells. Important results from our study indicate that some patients with colorectal cancer have low frequencies of tumor pCTLs that may be increased by this form of IL-2 gene therapy. In our study, the majority of patients tested had low levels of circulating tumor pCTLs prior to the initiation of IL-2 gene therapy. As a measure of general immune competence, allogeneic pCTLs were also quantified in these patients. There was a general correlation between the frequencies of allogeneic and tumor pCTLs prior to treatment. Patients with lower frequencies of tumor precursors also had lower frequencies of allogeneic precursors. In the patients with an increase in cytotoxic tumor precursors following treatment, the allogeneic precursor frequency was unaffected by therapy. Furthermore, cloned T cells derived from the patients were cytotoxic for tumor cells but not autologous fibroblasts. These findings imply that the immune response following IL-2 gene therapy is tumor specific.

However, an increase in the tumor pCTLs was not uniformly observed in all patients. Tumor-associated immunosuppression, the dose of secreted IL-2 and/or tumor cells, and the immune repertoire of the host are among the variables that may have contributed to the absence of a measurable antitumor T-cell response in all patients. In this regard, it is interesting to note that the patients without an increase in tumor T-cell precursor frequency either had no detectable pretreatment tumor or allogeneic T-cell precursors or had a decrease in allogeneic T-cell precursors during the course of treatment. These findings imply that immunosuppression is a potential factor contributing to the absence of a response to IL-2 gene therapy. Because only a small number of patients were evaluated in this study, it will be necessary to examine additional patients in the future to determine the reasons for the observed variability in antitumor immune responses.

With respect to the side effects of treatment, DTH reactions at the sites of the second or subsequent vaccinations were observed in the majority of patients, implying the induction of immunological memory responses, because these reactions were not observed with the initial vaccination. Mild to moderate fatigue and/or flu-like symptoms were observed in seven patients. There were no significant treatment-related changes in complete blood counts, serum chemistries, or urinalyses, compared to pretreatment values. Safety testing revealed no evidence for the development of replication-competent retrovirus in any of transduced cells used for therapy.

In summary, our findings suggest that this form of IL-2 gene therapy was well tolerated and was associated with the generation of a cellular antitumor immune response in a subset of colorectal carcinoma patients. Our findings confirm and extend the results of other investigations of immunogene therapy. Similar cellular antitumor immune responses were reported following IL-2 gene therapy in patients with glioblastoma, melanoma, and renal cell carcinoma (19–22). Our data indicate that some patients with colorectal cancer have low frequencies of tumor pCTLs that may be increased by IL-2 gene therapy that warrants continued clinical development.

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