Allogeneic Granulocyte Macrophage Colony-Stimulating Factor–Secreting Tumor Immunotherapy Alone or in Sequence with Cyclophosphamide for Metastatic Pancreatic Cancer: A Pilot Study of Safety, Feasibility, and Immune Activation

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Abstract

Purpose: The combination of chemotherapy and immunotherapy has not been examined in patients with advanced pancreatic cancer. We conducted a study of two granulocyte macrophage colony-stimulating factor–secreting pancreatic cancer cell lines (CG8020/CG2505) as immunotherapy administered alone or in sequence with cyclophosphamide in patients with advanced pancreatic cancer.

Experimental Design: This was an open-label study with two cohorts: cohort A, 30 patients administered a maximum of six doses of CG8020/CG2505 at 21-day intervals; and cohort B, 20 patients administered 250 mg/m^2 of cyclophosphamide i.v. 1 day before the same immunotherapy given as in cohort A. The primary objective was to evaluate safety and duration of immunity. Secondary objectives included time to disease progression and median overall survival.

Results: The administration of CG8020/CG2505 alone or in sequence with cyclophosphamide showed minimal treatment-related toxicity. Median survival values in cohort A and cohort B were 2.3 and 4.3 months, respectively. CD8$^+$ T-cell responses to HLA class I–restricted mesothelin epitopes were identified predominantly in patients treated with cyclophosphamide + CG8020/CG2505 immunotherapy.

Conclusion: Granulocyte macrophage colony-stimulating factor–secreting pancreatic cancer cell lines CG8020/CG2505 alone or in sequence with cyclophosphamide showed minimal treatment-related toxicity in patients with advanced pancreatic cancer. Also, mesothelin-specific T-cell responses were detected/enhanced in some patients treated with CG8020/CG2505 immunotherapy. In addition, cyclophosphamide-modulated immunotherapy resulted in median survival in a gemcitabine-resistant population similar to chemotherapy alone. These findings support additional investigation of cyclophosphamide with CG8020/CG2505 immunotherapy in patients with advanced pancreatic cancer.

Pancreatic cancer remains the fourth leading cause of cancer-related deaths in the United States (1). Despite efforts to develop new therapies, locally unresectable and metastatic disease have a median survival of 10 to 12 months and 3 to 6 months untreated, respectively (2). Gemcitabine is considered the standard first-line therapy for patients with advanced pancreatic cancer (3). More recent strategies have focused on improving efficacy by combining gemcitabine with other chemotherapy agents or with small-molecule drugs (4–12). However, the benefits have been modest. For patients who will inevitably develop disease progression on first-line chemotherapy, 5-fluorouracil is the only other approved chemotherapy. However, the additional benefits have been questioned with a median survival of <3 to 4 months (13–15). There have been a number of other chemotherapy agents tested for gemcitabine-refractory disease. The results have also been of limited benefit to date (16–20).

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Received 2/12/07; revised 8/12/07; accepted 1/11/08.

Grant support: National Cancer Institute grants K23CA093566-01A1 (D.A. Laheru), P50CA62924 (E.M. Jaffee), and RO1CA88058 (E.M. Jaffee); and Cell Genesys, Inc., South San Francisco, CA.

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Conflict of interest: Under a licensing agreement between the study sponsor Cell Genesys and the Johns Hopkins University, the university is entitled to milestone payments and royalty on sales of the vaccine product described in this article. The terms of this arrangement are being managed by the Johns Hopkins University in accordance with its conflict of interest policies.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi:10.1158/1078-0432.CCR-07-0371

Immunotherapy, in theory, promises unlimited capacity to recognize specific motifs expressed by tumor cells relative to their normal cellular counterparts. A number of proteins are overexpressed by most pancreatic cancers (21–26). Cellular immunotherapies and antibodies designed to target these antigens have been tested in early-phase clinical trials (10, 27–32). Some studies have shown posttreatment immune responses to the relevant peptides or whole proteins. However, significant clinical responses have not yet been observed.

Because few tumor antigens expressed by pancreatic tumors have been identified, the whole tumor cell has been the best source of immunogen. We have previously reported the results of a phase I study of an allogeneic, granulocyte macrophage colony-stimulating factor (GM-CSF)–secreting whole-cell tumor immunotherapy approach tested in sequence with adjuvant chemoradiation in patients with resected pancreatic adenocarcinoma (33). This approach is based on the concept that certain cytokines are required at the site of the tumor to effectively prime cancer-specific immunity. This study showed a direct correlation between postimmunotherapy *in vivo* delayed-type hypersensitivity responses to autologous tumor and postimmunotherapy T-cell responses to a candidate pancreatic tumor antigen mesothelin (34).

More recent data from our group and others suggest that immunomodulating doses of cyclophosphamide given 1 day before immunotherapy could enhance treatment-induced antitumor immune responses by inhibiting the $CD4^+/CD25^+$ regulatory T cells (34–37). Here, we report the first evaluation of GM-CSF–secreting pancreatic cancer lines as immunotherapy given either alone or in combination with immunomodulating doses of cyclophosphamide in patients with advanced pancreatic cancer.

Patients and Methods

Patient selection

Fifty patients with advanced pancreatic cancer were enrolled at two sites (Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins and Mary Crowley Medical Research Center) between June 6, 2002, and October 16, 2003. Main eligibility criteria included histologic diagnosis of pancreatic adenocarcinoma; at least one measurable lesion; a Karnofsky performance status of ≥70; $CD4^+$ lymphocytes >200 cells/mm^3; and normal hematologic, renal, and liver functions. Key exclusion criteria included non–protocol-specified treatment within 4 weeks of the first treatment, prior cancer vaccine therapy, HIV seropositivity, systemic corticosteroid use within 4 weeks of the first vaccination, and evidence of brain metastases.

Study design

The first 30 patients enrolled in the study were included in cohort A (six treatments alone repeated every 3 weeks). All subsequently enrolled patients were included in cohort B (six treatments, preceded by 250 mg/m^2 of cyclophosphamide 1 day before immunotherapy). Following completion of final treatment and follow-up, patients were followed monthly for a maximum of 9 monthly visits. The interventions and data collection schedule are diagrammed in Supplementary Fig. S1.

Treatment

CG8020 and CG2505 were formulated as directly injectable products. CG8020 and CG2505 were administered on an outpatient basis. On the day of immunotherapy, vials of CG8020 and CG2505 were removed from the freezer and thawed in a 37°C water bath, drawn into labeled syringes, and kept on ice until administration. All injections were given within 60 minutes of thawing. Each treatment consisted of four to eight intradermal injections (0.5 mL/injection) of CG8020 to deliver a total of $\sim 2.5 \times 10^8$ cells, and four to eight intradermal injections (0.5 mL/injection) of CG2505 to deliver $\sim 2.5 \times 10^8$ cells, for a combined total of $\sim 5 \times 10^8$ cells per dose.

Assessments of toxicities

All patients underwent toxicity monitoring every 3 weeks to include history and physical exam, complete blood count, a complete chemistry profile, and serum amylase. Serum was collected for GM-CSF levels before (day 0) and at days 1, 2, 3, and 4 after treatments 1 and 3 for a subset of patients. All adverse events were collected from the day of the first dose of cyclophosphamide in cohort B, until 4 weeks after the last treatment. Intensity was evaluated using the National Cancer Institute Common Toxicity Criteria (version 2.0).

Immune monitoring studies

Peripheral blood lymphocytes. Peripheral blood (100 mL) was obtained before the first, third, and sixth treatment and at follow-up visits 1, 3, 6, and 9 for immune analysis. Prevaccine and postvaccine peripheral blood lymphocytes (PBL) were isolated by density gradient centrifugation using Ficoll-Hypaque (Pharmacia). Cells were washed twice with serum-free RPMI 1640. PBL were stored frozen at -180°C in 90% AIM-V medium containing 10% DMSO until the day of analysis.

Enrichment of PBL for $CD8^+$ T cells. $CD8^+$ T cells were isolated from thawed PBL using CD8-negative isolation kits according to the manufacturer's directions (Dynal Biotech). Cells were fluorescently stained with CD8-phycoerythrin antibody (Becton Dickinson) to confirm that the population contained $CD8^+$ T cells.

ELISPOT assay. Multiscreen 96-well filtration plates (Millipore) were coated overnight at 4°C with 60 μL/well of 10 μg/mL anti-hIFNλ mouse monoclonal antibody 1-D1K (Mabtech). Wells were then washed thrice each with 1× PBS and blocked for 2 hours with T-cell medium. T-2 cells (1×10^5) pulsed with peptide (2 μg/mL) in 100 μL of T-cell medium were incubated overnight with 1×10^5 thawed PBL that was enriched for $CD8^+$ T cells in 100 μL of medium on the ELISPOT plates in replicates of three. Following overnight incubation at 37°C in 5% CO_2, the ELISPOT assays were completed as previously reported (34). All time points were assayed in three replicates and reported as the mean number of mesothelin-specific $CD8^+$ T cells per 10^6 total $CD8^+$ T cells.

Pharmacokinetic analysis of serum GM-CSF levels. Serum was separated from whole blood by centrifugation for 10 minutes and frozen in 1-mL aliquots at -80°C until the day of testing. Serum GM-CSF levels for all collection time points were determined by ELISA (Quantikine Systems) as previously reported (33).

Statistical considerations. Analyses were done using data from patients with advanced pancreatic cancer treated either with immunotherapy alone or in sequence with cyclophosphamide. Summary statistics include exact 95% confidence intervals for categorical data and medians, confidence intervals, and ranges for continuous outcomes. The longevity of serum concentrations of GM-CSF were evaluated by plotting the mean concentration with 95% confidence intervals over time for a subset of the population. Progression-free survival and overall survival were calculated from the date of the start of treatment. Patients withdrawing from the study for reasons unrelated to progression-free survival or overall survival were considered censored for that survival outcome at the time of their last visit. The survival outcomes (progression-free survival and overall survival) were analyzed using the Kaplan-Meier technique that allows for adjustment of the estimators due to censoring. For each treatment regimen, the median of each survival outcome was reported to be within 95% confidence intervals and ranges. The two treatments were compared for each outcome using a log-rank test with a significance level of 0.05.

Peptides. The mesothelin peptides used in these studies were identified by methods previously reported (34). All peptides were purified to >95% and synthesized by the Johns Hopkins University Oncology Department Peptide Synthesis Facility according to published sequences. The HLA-A1–binding peptides used were mesothelin $A1_{(310\text{-}318)}$ peptide

EIDESLIFY and mesothelin $A1_{(429-437)}$ peptide TLDTLTAFY. The HLA-A2 and HLA-A3–binding mesothelin and HIV control peptides have been previously reported (34). Stock solutions (10 mg/mL) of peptides were prepared in 100% DMSO (JT Baker) and stored at -80°C until further dilution into culture medium before each assay.

Tetramer studies. The HLA-A2 tetramers used in these studies were manufactured by Beckman Coulter, Inc. Phycoerythrin-conjugated tetramers were constructed for the HLA-A2–binding mesothelin peptides $MesoA2_{(20-28)}$ (SLLFLLFSL) and $MesoA2_{(531-539)}$ (VLPLTVAEV) and the HLA-A2–binding tyrosinase peptide YMDGTMSQV. Tetramer staining was done only when pretreatment and posttreatment PBL were available. For each time point analyzed, 1×10^6 freshly thawed PBL were resuspended in 50 μL of each HLA-A2 tetramer diluted from 1:10 to 1:60 in PBS/2% fetal bovine serum (Atlas) and incubated at 4°C for 40 minutes in the dark. After 40 minutes, 10 μL of FITC-conjugated anti-CD8 monoclonal antibody (BD PharMingen) were added and the samples were incubated for another 20 minutes at 4°C in the dark. Stained PBL were washed twice in 3 mL of PBS before being fixed in PBS/0.5% formaldehyde (JT Baker) and analyzed on a FACSCanto flow cytometer (Becton Dickinson) at the Johns Hopkins University Human Immunology Core Facility. Flow data were further analyzed using Flow Jo (Tree Star).

Results

Patient characteristics. A total of 50 patients were enrolled in the study and received study treatment (Table 1): 30 patients in cohort A (CG8020/CG2505 only) and 20 patients in cohort B (cyclophosphamide 1 day before CG8020/CG2505). The median age was 55 years (56 years in cohort A and 61 years in cohort B), with a range of 38 to 81 years. At screening, 94% of the patients had stage IV disease. Fifteen of 30 (50%) patients in cohort A and 13 of 20 (65%) patients in cohort B had a Karnofsky performance status of ≥90. Prior pancreatic cancer therapies included pancreaticoduodenectomy surgery (39 of 50 patients, 78%), radiation (13 of 50, 26%), and at least one gemcitabine-containing chemotherapy (41 of 50, 82%).

GM-CSF–secreting cell lines as immunotherapy is feasible and safe to administer to patients with advanced metastatic pancreatic cancer. A summary of all treatment-related adverse events are described in Table 2. All 50 patients received at least one treatment of CG8020 and CG2505, and, overall, patients received a median of two treatments (two for cohort A and three for cohort B). Twenty-six of 26 evaluable patients in cohort A and 20 of 20 patients in cohort B developed erythema/induration or pain/soreness at the treatment sites following immunotherapy, similar to what was observed in phase I testing (33). These reactions were expected and self-limiting, lasting up to 1 week.

Pharmacokinetics of serum GM-CSF. We previously reported the detection of low serum GM-CSF levels that peaked at 48 hours after treatment with the GM-CSF–secreting tumor lines in patients receiving the highest dose of cells as adjuvant therapy in the phase I study (33). Correlative data from the phase I study provide strong evidence that low serum GM-CSF levels peaking at 24 to 48 hours following treatment provide an important measure of the bioactivity of this immune-based therapy (38). Serum GM-CSF levels were therefore assessed in 16 of the patients (5 from cohort A and 11 from cohort B) after treatment 1, and in 5 patients (from cohort B only) after treatment 3 (Supplementary Fig. S2). The results were similar for each cohort. Mean serum GM-CSF reached peak levels of 26 pg/mL. Measurable levels of GM-CSF were sustained for 4 days after treatment in the majority of patients both after the first and third treatments.

Clinical responses. This was a two-cohort, nonrandomized study. Of the 50 patients, 13 (26.0%) had stable disease for a duration of 18 weeks (5 of 30, 16.7% from cohort A; 8 of 20,

Table 1. Patient demographics

	Cohort A (n = 30)	Cohort B (n = 20)	Total (N = 50)
Median age (range)	56 (37-88)	61 (38-81)	56 (37-88)
Gender			
Male	19 (63%)	11 (55%)	30 (60%)
Female	11 (37%)	9 (45%	20 (40%)
Ethnic origin			
1	27 (90%)	20 (100%)	47 (94%)
2	1 (3.3%)	0	1 (2%)
3	1 (3.3%)	0	1 (2%)
4	1 (3.3%)	0	1 (2%)
KPS			
70	3 (10%)	2 (10%)	5 (10%)
80	12 (40%)	5 (25%)	17 (34%)
≥90	15 (50%)	13 (65%)	28 (56%)
Stage			
III	3 (10%)	0	3 (6%)
IV	27 (90%)	20 (100%)	47 (94%)
Prior surgery	24 (80%)	15 (75%)	39 (78%)
Prior XRT	7 (23%)	6 (30%)	13 (26%)
Prior chemotherapy regimens			
0	6 (20%)	3 (15%)	9 (18%)
1	8 (27%)	12 (60%)	20 (40%)
2	11 (37%)	2 (10%)	13 (26%)
3	5 (17%)	3 (15%)	8 (16%)

NOTE: Ethnic origin: 1, White Caucasian; 2, African American; 3, Hispanic; 4, Asian.
Abbreviation: KPS, Karnofsky performance status.

Table 2. Summary of treatment-related adverse events

(A) Injection site reactions (per vaccine)

Vaccine 1	Cohort A (*n* = 30)			Cohort B (*n* = 20)		
Site reaction	**Grade 1**	**Grade 2**	**Grade 3/4**	**Grade 1**	**Grade 2**	**Grade 3/4**
Erythema/induration	18	8	0	18	2	0
Pruritus	20	0	0	16	0	0
Pain/soreness	11	0	0	7	0	0
Vaccine 2	**Cohort A (*n* = 15)**			**Cohort B (*n* = 15)**		
Site reaction	**Grade 1**	**Grade 2**	**Grade 3/4**	**Grade 1**	**Grade 2**	**Grade 3/4**
Erythema/induration	11	4	0	15	0	0
Pruritus	8	0	0	9	0	0
Pain/soreness	8	0	0	3	0	0
Vaccine 3	**Cohort A (*n* = 9)**			**Cohort B (*n* = 10)**		
Site reaction	**Grade 1**	**Grade 2**	**Grade 3/4**	**Grade 1**	**Grade 2**	**Grade 3/4**
Erythema/induration	8	1	0	9	1	0
Pruritus	5	0	0	5	0	0
Pain/soreness	2	0	0	2	0	0
Vaccine 4	**Cohort A (*n* = 7)**			**Cohort B (*n* = 7)**		
Site reaction	**Grade 1**	**Grade 2**	**Grade 3/4**	**Grade 1**	**Grade 2**	**Grade 3/4**
Erythema/induration	5	0	0	6	0	0
Pruritus	5	0	0	6	0	0
Pain/soreness	0	0	0	2	0	0
Vaccine 5	**Cohort A (*n* = 5)**			**Cohort B (*n* = 4)**		
Site reaction	**Grade 1**	**Grade 2**	**Grade 3/4**	**Grade 1**	**Grade 2**	**Grade 3/4**
Erythema/induration	4	1	0	4	2	0
Pruritus	5	0	0	2	0	0
Pain/soreness	1	0	0	1	0	0
Vaccine 6	**Cohort A (*n* = 3)**			**Cohort B (*n* = 4)**		
Site reaction	**Grade 1**	**Grade 2**	**Grade 3/4**	**Grade 1**	**Grade 2**	**Grade 3/4**
Erythema/induration	2	1	0	4	0	0
Pruritus	2	0	0	3	0	0
Pain/soreness	1	0	0	0	0	0
Vaccine 6	**Cohort A (*n* = 3)**			**Cohort B (*n* = 4)**		
Site reaction	**Grade 1**	**Grade 2**	**Grade 3/4**	**Grade 1**	**Grade 2**	**Grade 3/4**
Erythema/induration	2	1	0	4	0	0
Pruritus	2	0	0	3	0	0
Pain/soreness	1	0	0	0	0	0

(B) All other treatment-related adverse events

Organ class	All grades			Grade 3/4		
	Cohort A	**Cohort B**	**Total**	**Cohort A**	**Cohort B**	**Total**
	***n* = 30 (%)**	***n* = 20 (%)**	***N* = 50 (%)**	***n* = 30 (%)**	***n* = 20 (%)**	***N* = 50 (%)**
Metabolism						
Dehydration	1 (3.3)	0 (0)	1 (2)	1 (3.3)	0 (0)	1 (2)
Nervous system						
Dizziness	0 (0)	1 (5)	1 (2)	0 (0)	0 (0)	0 (0)
Headache	0 (0)	1 (5)	1 (2)	0 (0)	0 (0)	0 (0)
Vascular						
Hot flashes	0 (0)	1 (5)	1 (2)	0 (0)	0 (0)	0 (0)
Gastrointestinal						
Nausea	1 (3.3)	1 (5)	2 (4)	0 (0)	0 (0)	0 (0)
Vomiting	0 (0)	1 (5)	1 (2)	0 (0)	0 (0)	0 (0)

(*Continued on the following page*)

Table 2. Summary of treatment-related adverse events (Cont'd)

(B) All other treatment-related adverse events

Organ class	All grades			Grade 3/4		
	Cohort A	Cohort B	Total	Cohort A	Cohort B	Total
	n = 30 (%)	*n* = 20 (%)	*N* = 50 (%)	*n* = 30 (%)	*n* = 20 (%)	*N* = 50 (%)
Skin						
Dry skin	1 (3.3)	0 (0)	1 (2)	0 (0)	0 (0)	0 (0)
Night sweats	1 (3.3)	1 (5)	2 (4)	0 (0)	0 (0)	0 (0)
Pruritus	1 (3.3)	1 (5)	2 (4)	0 (0)	0 (0)	0 (0)
Rash	1 (3.3)	2 (10)	3 (6)	0 (0)	0 (0)	0 (0)
Generalized itching	1 (3.3)	0 (0)	1 (2)	0 (0)	0 (0)	0 (0)
Musculoskeletal						
Arthralgia	1 (3.3)	2 (10)	3 (6)	0 (0)	0 (0)	0 (0)
Myalgia	0 (0)	2 (10)	2 (4)	0 (0)	0 (0)	0 (0)
Extremity pain	0 (0)	1 (5)	1 (2)	0 (0)	0 (0)	0 (0)
General disorders						
Asthenia	6 (20)	7 (35)	13 (26)	1 (10)	1 (5)	2 (4)
Chills	1 (3.3)	5 (25)	6 (12)	0 (0)	0 (0)	0 (0)
Fatigue	4 (13.3)	2 (10)	6 (12)	1 (3.3)	1 (5)	2 (4)
Inflammation	0 (0)	1 (5)	1 (2)	0 (0)	0 (0)	0 (0)
Injection site discoloration	0 (0)	1 (5)	1 (2)	0 (0)	0 (0)	0 (0)
Injection site vesicles	0 (0)	1 (5)	1 (2)	0 (0)	0 (0)	0 (0)
Pyrexia	5 (16.7)	3 (15)	8 (16)	0 (0)	0 (0)	0 (0)
Overall	24	34	58	3	2	5

40.0% from cohort B). The median time to death, as measured from administration of the first treatment dose, was 69 days for cohort A and 130 days for cohort B (Supplementary Table S1). Kaplan-Meier estimates of survival (time from first treatment), by treatment cohort, are shown in Supplementary Fig. S3. Of interest, there was one patient in cohort B with a history of resected pancreatic cancer followed by disease progression to involve the left lobe of her lung; the patient was enrolled with progressive disease on gemcitabine chemotherapy. This patient's progression stabilized on study. She completed all six vaccinations and had continued stable disease radiographically. Given the fact that she had disease involving only the left lower lobe of her lung with no interval disease at other sites following treatment, she underwent a lower left lobectomy with pathology consistent with pancreatic cancer. Subsequent follow-up scans continued to show no evidence of recurrent disease. She eventually died of non-pancreatic cancer–related illness 25 months after completing immunotherapy.

Mesothelin-specific immune responses were detected in patients with metastatic pancreatic cancer. We previously reported that detection of postvaccination mesothelin-specific T-cell responses correlated with other measures of immune activation and prolonged disease-free survival for patients with resected pancreas cancer treated with immunotherapy (34). We therefore assessed whether this immunotherapy could induce mesothelin-specific T-cell responses in patients with metastatic pancreatic cancer. These patients with pancreatic cancer have advanced disease that is thought to impede the induction and maintenance of effective immune responses. Thawed and enriched $CD8^+$ T cells were assayed for the production of γ-IFN in response to T2 cells pulsed with HLA class I–restricted mesothelin epitopes using ELISPOT. Lymphocytes for postimmunotherapy analysis were available only for patients who did not have disease progression before treatment 3 (11 of 30 patients in cohort A and 12 of 20 patients in cohort B). Reagents were available for HLA-$A1^+$, HLA-$A2^+$, and HLA-$A3^+$ patients (8 of 11 patients in cohort A and 10 of 12 patients in cohort B). A summary of the ELISPOT data for all 18 patients analyzed is shown in Table 3. Baseline mesothelin-specific $CD8^+$ T cells were detected at low levels in the peripheral blood in most patients before the first cycle of immunotherapy. However, $CD8^+$ T-cell responses to HLA-restricted mesothelin epitopes were augmented after cycles 3 and 6 of the therapy, predominantly in patients treated with cyclophosphamide + immunotherapy (9 of 10) compared with patients treated with immunotherapy alone (4 of 8). $CD8^+$ T-cell responses were detected against the positive control CMV/EBV/influenza A (CEF peptide) pool in all patients treated in both cohorts (Fig. 1). Although the analysis to date is limited to HLA-$A1^+$, HLA-$A2^+$, and HLA-$A3^+$ patients, median survival from this subset of patients with induction or enhancement of mesothelin-specific T-cell responses treated with immunotherapy alone is 7.6 versus 10.4 months for patients treated with cyclophosphamide + immunotherapy.

To further evaluate the quality of mesothelin-specific T-cell responses in these subjects, mesothelin-specific T cells from a subset of HLA-$A2^+$ subjects were assessed for avidity by dilutional HLA-A2/$Meso_{(20-28)}$ and HLA-A2/$Meso_{(531-539)}$ tetramer analysis. Tetramer staining was done at a range of concentrations (1:10 to 1:60) on freshly thawed PBL when banked pretreatment and posttreatment PBL were available (3 of 5 HLA-$A2^+$ subjects in cohort A and 6 of 7 HLA-$A2^+$ subjects in cohort B). In concordance with the ELISPOT results, mesothelin-specific T cells were detected in both pretreatment and posttreatment PBL. Similarly, changes in mesothelin-specific T-cell frequencies were also observed. The flow cytometry results for three representative subjects are shown in Supplementary Fig. S4. Tetramer analysis of both cohort A subject 7 and cohort B subject 7 showed an increase in the frequencies and avidity of mesothelin-specific T cells in

Table 3. Summary of mesothelin-specific T-cell responses in the HLA-A1$^+$/HLA-A2$^+$/HLA-A3$^+$ patients treated with the vaccine alone or in sequence with cyclophosphamide

Patient/HLA	Mesothelin-specific T cells/10^6 CD8 T cells						Survival (mo)
	Pre	Vaccine 3	Vaccine 6	Follow-up	Follow-up 2	Follow-up 3	
Cohort A (patients given immunotherapy alone)							
1/(2)	40	110	NA	NA	NA	NA	3.36
2/(1)	10	0	0	NA	NA	NA	7.1
3/(3)	20	10	NA	NA	NA	NA	3.36
4/(3)	20	50	100	30	50	NA	7.9
5/(1)	90	160	110	NA	NA	NA	17.6
6/(2)	40	60	NA	NA	NA	NA	6.1
7/(2)	60	0	NA	NA	NA	NA	1.7
8/(2)	0	0	NA	NA	NA	NA	2.86
Cohort B (patients given immunotherapy + Cytoxan)							
1/(2)	50	230	NA	NA	NA	NA	3.23
2/(1)	10	60	NA	NA	NA	NA	22.5
3/(2)	110	270	NA	NA	NA	NA	6
4/(2)	240	400	NA	NA	NA	NA	7.73
5/(3)	0	0	210	70	NA	NA	25+
6/(3)	70	130	190	NA	NA	NA	8.13
7/(2)	0	0	10	10	NA	NA	13.07
8/(2)	130	350	50	NA	NA	NA	3.7
9/(2)	30	60	50	30	40	110	12.3
10/(2)	50	100	NA	NA	NA	NA	2.6

NOTE: The ELISPOT assay was used to determine the number of mesothelin-specific CD8$^+$ T cells specific for the HLA-A1$^+$ epitope, MesoA1$_{(20-28)}$, HLA-A2$^+$ mesothelin epitope MesoA2$_{(530-539)}$, or the HLA-A3 mesothelin$_{(225-234)}$ epitope. All time points for each patient were assayed simultaneously in six replicates and reported as the mean number of mesothelin-specific CD8$^+$ T cells per 10^6 total CD8$^+$ T cells. Background spots were determined using a negative peptide control known to bind to HLA-A1 HIV-Nef$_{(73-87)}$ (QVPLRPMTY), HLA-A2 HIV-gag$_{(77-85)}$ (SLYNTVATL), and HLA-A3 HIV-1NEF$_{(94-102)}$ (QVPLRPMTYK6). Background spots ranged from 0 to 10 spots per well. A CEF pool was used as a positive control. The CEF pool contains epitopes from cytomegalovirus, EBV, and influenza A virus proteins that bind to most HLA class I molecules. The CEF pool was obtained from NIH AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH (CEF control peptide pool, 9808). Positive control spots ranged from 40 to 1,300 per well.
Abbreviations: Pre, pretreatment 1; treatment cycle 1, posttreatment 1; vaccine 3, posttreatment 3; vaccine 6, posttreatment 6; NA, not available due to patient progression.

posttreatment PBL compared with PBL isolated before treatment. In contrast, a decrease in posttreatment mesothelin-specific T-cell frequencies was measured in cohort B subject 8. Although changes in frequencies of mesothelin-specific T cells were detected, changes in frequencies of T cells specific for tyrosinase, an irrelevant melanoma antigen, were not detected in any of the nine subjects evaluated. This suggests that the changes measured were not due to time point–related differences in nonspecific tetramer staining. Furthermore, mesothelin and tyrosinase-specific T cells were barely detectable in a healthy HLA-A2$^+$ donor resembling the levels of tyrosinase-specific T cells observed in the PBL of subjects on this study (Supplementary Fig. S4). The highest dilution of tetramer at which staining was no longer detectable is also shown for all subjects tested (Table 4). Although the analysis was only done on a small number of subjects, it is interesting that the posttreatment MesoA2$_{(531-539)}$ tetramer titration correlated with overall survival. Importantly, these tetramer changes seem to be antigen specific because time point differences in tyrosinase tetramer titrations were not observed and were lower than those observed for mesothelin peptide tetramers.

Discussion

This study of allogeneic GM-CSF–secreting cell lines as immunotherapy supports the following three conclusions. First, immunotherapy given alone or in combination with immunomodulating doses of cyclophosphamide showed minimal treatment-related toxicity and was feasible to administer to patients with advanced pancreatic cancer following progression on first-line chemotherapy. Second, there is a suggestion of enhanced activity when cyclophosphamide is given before immunotherapy based on the higher rate of induction of mesothelin-specific T-cell responses as well as longer progression-free and overall survival in the cyclophosphamide plus immunotherapy cohort. However, this study was not designed to formally test for cohort differences. Third, mesothelin-specific CD8$^+$ T cells could be detected in patients with advanced pancreatic cancer, and enhanced number and avidity may be associated with longer survival.

This is the first clinical trial testing GM-CSF–secreting tumor cell lines as immunotherapy in patients with metastatic pancreatic cancer. Despite the short survival expected in this patient population, it was possible to evaluate the safety and induction of immune responses to this experimental therapy. The most common adverse event was injection site reactions observed in all assessed patients. Other than injection site reactions, the most frequently reported adverse events involved the gastrointestinal system (e.g., nausea, abdominal pain, and vomiting) and general disorders (e.g., fatigue and pyrexia).

Serum GM-CSF levels were also assessed pretreatment and posttreatment for a subset of patients who received either immunotherapy alone or immunotherapy + cyclophosphamide

as an indirect measure of the longevity of the treatment. Serum GM-CSF levels were similar in both cohorts, reaching peak levels 2 days posttreatment. These findings are similar to what was observed in resected subjects receiving this immunotherapy approach and in preclinical models evaluating the mechanism of action of this immune-based therapy (33, 38–40). These peak levels have also correlated with a systemic eosinophilia that occasionally is associated with biopsy-proven systemic rashes due to eosinophil and lymphocyte infiltration (33). Importantly, peak serum GM-CSF levels were consistent following repeated administrations, suggesting that the pancreatic cancer cell lines are not rapidly cleared by a repetitive treatment administered every 3 weeks. Additional clinical studies evaluating a larger cohort of patients is necessary to confirm the stability of the pancreatic cancer cell lines at the treatment site following repeated immunizations. It is also possible that patients with advanced pancreatic cancer have a more global immunosuppression and are unable to mount an allogeneic immune response capable of rapidly rejecting repetitively injected allogeneic pancreatic cancer cells. This is an unlikely circumstance because mesothelin-specific T-cell responses are detectable in some of these patients. Furthermore,

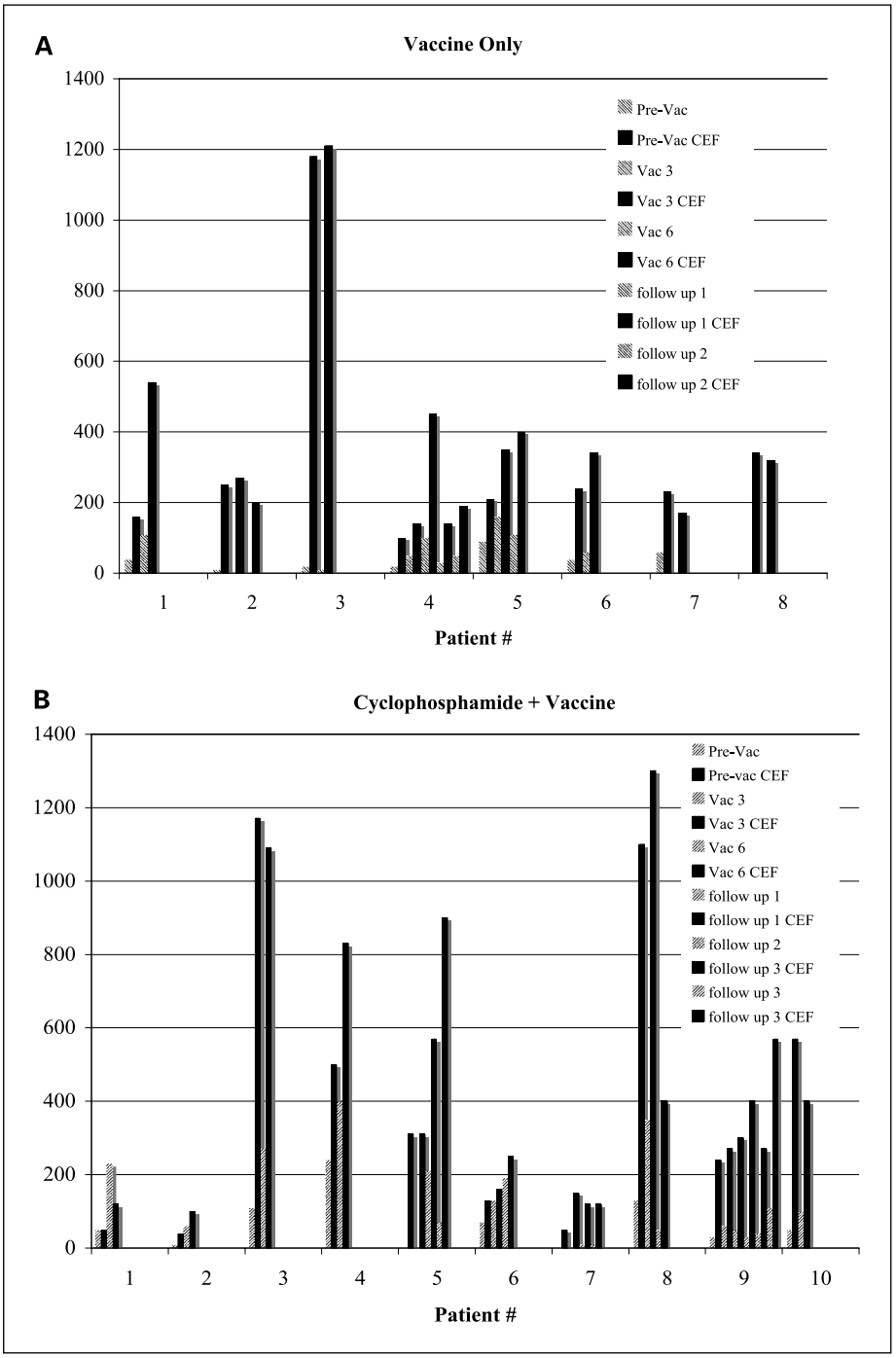

Fig. 1. Comparison of the magnitude of $CD8^+$ T cells specific for mesothelin versus the CEF pool of peptides. An ELISPOT analysis was done to determine the number of IFN-γ–secreting T cells specific for mesothelin as described in Table 3. All patient lymphocytes were also assessed for recognition of a HIV-negative control peptide and a positive antigen control pool of peptides (CEF pool). Background spots ranged from 0 to 10 spots per well as described in Table 3. Mesothelin-specific T-cell response data presented in Table 3 and the corresponding CEF pool–specific $CD8^+$ T-cell responses are graphed. *A,* immunotherapy-only cohort. *B,* cyclophosphamide + immunotherapy cohort. Striped columns, mesothelin data. Black columns, CEF pool data.

Table 4. Summary of HLA-A2 tetramer titrations in a subset of HLA-A2$^+$ patients treated with the vaccine alone or in sequence with cyclophosphamide

Patient	MesoA2$_{(531-539)}$		Tyrosinase		Survival (mo)
	Pre	Vaccine 3	Pre	Vaccine 3	
Tetramer titration (patients without cytoxan)					
4	1:60	1:60	1:10	1:10	7.9
6	1:40	>1:60	1:20	1:20	6.1
7	1:20	1:40	1:10	1:20	1.7
Tetramer titration (patients given cytoxan)					
1	1:10	1:40	1:10	1:10	3.23
4	1:20	>1:40	1:20	1:20	7.73
7	1:40	1:60	1:20	1:20	13.07
8	>1:60	1:20	1:10	1:10	3.7
9	>1:60	>1:60	1:20	1:20	12.3
10	1:60	<1:60	1:10	1:10	2.6

NOTE: Patient PBL were labeled with HLA-A2 tetramers as described in Patients and Methods. The tetramer dilutions at which detectable tetramer staining was lost are shown.
Abbreviations: Pre, prevaccine 1; vaccine 3, prevaccine 3.

local treatment site reactions, a result of infiltrating lymphocytes and antigen-presenting cells, increased with each immunotherapy (33).

This study was not powered to detect significant differences in progression-free and overall survival between the two cohorts. The cohorts are not matched and, as such, there are imbalances with respect to performance status and prior treatment. With the caveat that the sample size for each group is small, 65% of patients in cohort B versus 50% of patients in cohort A had a Karnofsky performance status of $\geq$90. This would be expected to slightly favor cohort B. The majority of patients in both cohort A (24 of 30) and cohort B (17 of 20) had at least one prior chemotherapy with 16 of 30 patients in cohort A and 5 of 20 patients in cohort B receiving second- or third-line chemotherapy. In addition, the majority of patients for both cohorts had stage IV disease. Although there was no statistical difference in overall survival and 12-month survival between cohorts, the 1 year survival of 20% and median overall survival of 4.3 months for subjects in cohort B is at least consistent with published data for second-line therapy for advanced pancreatic cancer (13–20).

In this study, we also show the feasibility of detecting CD8$^+$ T-cell responses to HLA-restricted mesothelin epitopes. Posttreatment responses were measured predominantly in patients who were treated with combined cyclophosphamide + immunotherapy and who have also shown prolonged survival. Interestingly, baseline T-cell responses to mesothelin were detected in a number of patients but did not predict survival benefit. Baseline T-cell responses to pancreatic cancer antigens such as mesothelin are likely secondary to the large tumor antigen load from the pretreatment tumor burden. It is also interesting to note that the baseline response to mesothelin was higher in cyclophosphamide-treated patients and associated with longer survival and may suggest that these cells can be activated. These data are in contrast with our previous findings in patients with resected pancreas cancer who are at risk for recurrent cancer (33). In those patients, baseline mesothelin-specific T cells were not detected. In addition, it is possible that the vaccine, if given with immunomodulating agents such as cyclophosphamide, could alter the avidity of the T-cell responses in favor of higher activity even when the total number of T cells is low. The metastatic patient population is one of the more difficult subjects in which to study immune responses due to their advanced stage of disease and rapid progression. However, despite this challenge, this study shows the ability to detect and sometimes enhance the avidity of pancreatic cancer antigens. This study provides the feasibility on which to begin combining other more specific and potent immune-enhancing agents with this vaccine. Additional clinical studies are also warranted to determine whether the induction or change in mesothelin-specific T-cell responses could predict which patients will benefit from this immune-based therapy. Additional studies are also required to assess whether there are functional differences between the baseline and posttherapy mesothelin-specific T-cell populations.

In conclusion, this immune-based therapy is safe and feasible to administer to patients with advanced pancreatic cancer who have progressed on first-line chemotherapy. These data suggest that CG8020/CG2505 given in sequence with cyclophosphamide results in antitumor activity that is similar to reported second-line chemotherapy. In addition, mesothelin-specific CD8$^+$ T-cell responses can be detected in patients with stage IV disease and may correlate with overall survival. This study provides the foundation for integrating immunotherapy with other targeted therapies for the treatment of patients with advanced pancreatic cancer.

Acknowledgments

We thank Dr. Ferdynand Kos and the Sidney Kimmel Comprehensive Cancer Center at John's Hopkins Human Immunology Core for assistance with the tetramer analyses, and Dr. Sanje Khare and the Sidney Kimmel Comprehensive Cancer Center at John's Hopkins Peptide Synthesis Facility for assistance with synthesizing the peptides used in the immunologic studies.

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Allogeneic Granulocyte Macrophage Colony-Stimulating Factor–Secreting Tumor Immunotherapy Alone or in Sequence with Cyclophosphamide for Metastatic Pancreatic Cancer: A Pilot Study of Safety, Feasibility, and Immune Activation

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Clin Cancer Res 2008;14:1455-1463.