INTRODUCTION

Prostate cancer is the most common malignancy in men, and in 1998, it accounted for approximately 40,000 deaths in the United States (1). Virtually all prostate cancer fatalities are due to the development of HRPC.3 Whereas the treatment of metastatic HRPC often involves cytotoxic therapy, it is clear that novel agents and approaches are required (2). One such approach that is being evaluated is the enhancement, either specific or nonspecific, of anticancer immune responses.

GM-CSF is a potent cytokine capable of inducing tumor necrosis factor and interleukin-1 expression as well as activation of macrophage and dendritic cell antigen activity (3, 4). GM-CSF appears to enhance antitumor immunity through direct activation of dendritic cells as well as indirect T-cell activation via interleukin-1 release. GM-CSF also enhances antitumor effector mechanisms by activating macrophages and inducing tumor necrosis factor release. The immune response induced by immunization with tumor cells genetically engineered to locally secrete cytokines has been evaluated (5). In systematic testing of various cytokines administered in a paracrine fashion, GM-CSF stands out as the most effective cytokine in inducing systemic immune responses against a nonimmunogenic melanoma cell line, B-16 (6). In this model, there is histological evidence of an inflammatory infiltrate consisting primarily of activated macrophages.

The efficacy of autocrine GM-CSF secretion in the setting of prostate cancer has been studied in the Dunning rat prostate carcinoma model. The treatment of rats with irradiated prostate cancer cells engineered to secrete human GM-CSF resulted in a longer disease-free survival compared to untreated animals or rats vaccinated with untransfected cells and then injected with soluble human GM-CSF (7). These studies have been the basis of vaccine trials in patients with prostate cancer using autologous or allogeneic prostate cancer cells stably transfected with the GM-CSF gene.

The microenvironment and/or tissue concentration of GM-CSF that is required for an antitumor effect in patients with prostate cancer is unknown. In animal models, coinjection of tumor cells with GM-CSF did not result in apparent antitumor...
activity. However, it is possible that a single short-term systemic exposure to GM-CSF is not necessarily an adequate test of its antitumor activity. Whereas systemic administration of GM-CSF in patients with advanced prostate cancer has not been reported, a similar approach in patients with metastatic renal cell carcinoma (8, 9) and metastatic melanoma (10) has suggested that GM-CSF has some, albeit minor, antitumor activity.

To test the hypothesis that GM-CSF has anti-prostate cancer activity, we evaluated the efficacy of exogenously administered GM-CSF in two cohorts of patients with advanced HRPC enrolled in sequential prospective Phase II trials. In addition, we performed a series of in vitro studies to examine the effect of GM-CSF on tumor cell proliferation and PSA production in prostate cancer cells.

PATIENTS AND METHODS

Patients. Eligibility criteria included histological diagnosis of adenocarcinoma of the prostate and the presence of disease that was progressive despite androgen deprivation. Progressive disease was defined by objective evidence of disease progression on any imaging study or at least two consecutive PSA levels at least 2 weeks apart, each of which demonstrated a >50% increase above the nadir level achieved with the most recent therapeutic manipulation. For patients with bone-only disease, a minimum PSA value of 10 ng/ml was required. Tumor progression after conventional hormonal therapy followed by antiandrogen withdrawal was required. Prior second- and third-line hormonal therapy, chemotherapy, immunotherapy, radiation therapy, or therapy with other experimental agents was permitted, provided that treatment was completed at least 4 weeks before enrollment and that disease progression had occurred despite that therapy. A life expectancy of at least 4 months and a KPS of 60% or higher were required. Required values for initial laboratory data included: (a) a WBC count between 2,000 and 20,000 cells/μl; (b) an ANC between 1,000 and 15,000 cells/μl; (c) a platelet count of >100,000 cells/μl; (d) hemoglobin ≥ 9 g/dl; (e) serum creatinine ≤ 2 mg/dl; (f) testosterone level ≤ 50 ng/ml; and (g) a total bilirubin, aspartate aminotransferase, and alanine aminotransferase of ≤ 2 mg/dl; and (h) a total bilirubin, aspartate aminotransferase, and alanine aminotransferase of ≤ 2 mg/dl.

Response to therapy was assessed with serum PSA measurements and imaging studies. For patients with bidimensional measurable disease, standard response criteria were used. All patients, including those with bone-only disease, were evaluated for changes in serum PSA levels.

Patients continued on therapy until disease progression was documented or until the patient withdrew or was withdrawn from the study. Progression was defined as either any increase of ≥25% in the product of the perpendicular diameters of any measured lesion or the appearance of a new lesion on any imaging study, including bone scan. Progression by PSA criteria required a climb in PSA on two consecutive occasions, at least 2 weeks apart, each 50% above the PSA nadir or baseline, whichever was lowest. All patients were followed for survival.

Cell Culture and Treatment. The human androgen-dependent prostate carcinoma cell line LNCaP was obtained from the American Type Culture Collection (Manassas, VA) and grown as a monolayer in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 2 mm l-glutamine, 10% (v/v) heat-inactivated certified fetal bovine serum, and 10 μg/ml gentamicin at 37°C in 5% CO₂. Cells at passage 29 were plated at 3 × 10⁴ cells/well into 12-well plates and allowed to attach for 48 h. GM-CSF was obtained from Immunex. The agent was prepared as a stock solution in sterile dH₂O and then serially diluted in culture medium to produce final concentrations of 0.45, 1.5, and 5.0 ng/ml. Cells were treated with 1 ml of freshly diluted drug or control (culture medium alone) daily.

PSA Quantification. Every 24 h, the supernatants were collected from one plate and frozen at −20°C. Adherent cells were trypsinized and counted on a Coulter Z1 counter (Coulter...
Electronics, Hialeah, FL). The amount of PSA secreted into the supernatant was measured using the Tandem-E PSA assay (Hybritech, San Diego, CA). The amount of PSA secreted per cell was calculated for every 24-h period.

**Immunoblot Analysis.** After treatment with GM-CSF or control for 120 h, adherent cells were collected by trypsinization, cytosolic protein extracts were prepared in the presence of protease inhibitors according to standard methods, and protein concentrations were quantitated using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Equal amounts of protein were separated on 10% polyacrylamide-SDS gels and then transferred to nitrocellulose. The blot was developed according to the Western Light chemiluminescent detection system (Tropix, Bedford, MA) using a 1:10 dilution of PSA antibody (Cappel, Aurora, OH) followed by incubation in a 1:500 dilution of alkaline phosphatase-conjugated antiumouse IgG (H+L) (Promega, Madison, WI).

**Quantitative Reverse Transcription-PCR.** LNCaP cells were plated into 12-well plates at a density of 3.0 × 10^4 cells/well. After a 48-h incubation, the cells were treated with 0.45, 1.5, and 5.0 ng/ml GM-CSF diluted in culture medium for 0.5, 1, 3, 6, or 9 h. At each time point, the supernatant was aspirated, and the adherent cells were lysed in 250 μl of Trizol (Life Technologies, Inc.). Total RNA was extracted according to the manufacturer’s protocol. The RNA pellet was resuspended in diethyl pyrocarbonate (Sigma, St. Louis, MO)-treated water, and contaminating genomic DNA was digested with 4 units of DNase I (Ambion, Austin, TX) at 26°C for 15 min. First-strand cDNA was prepared using 0.5 μg of oligodeoxythymidylic acid primer and 200 units of Superscript II reverse transcriptase (Life Technologies, Inc.). Each cDNA:RNA hybrid was digested with 2 units of RNase H at 37°C for 20 min followed by heat inactivation at 70°C for 10 min. The final cDNA product was diluted 3-fold in 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

PSA and AR primers were synthesized by BioServe Biotechnologies (Laurel, MD) and are as follows: (a) PSA 614, 5'-ATGGGCTCTTCAAGAATCGATTCCCTC-3' (sense); (b) PSA 1154, 5'-AGTTTGGGGTCTTCAATTTCCAAGGT-3' (antisense); (c) AR 2381, 5'-TGGGCTCATGTTGTGTTG-3' (sense); and (d) AR 2881, 5'-CAGAAAAGGACTTTTG-3' (antisense). Primers are numbered to correspond to the base in the published sequence in which the primer anneals. HMR GAPDH amplimers were purchased from Clontech (Palo Alto, CA) and will amplify GAPDH sequences from human, mouse, and rat species. For each pair of primers, the sequence to be amplified was subcloned into pCR-Script AMP SK(+) (Stratagene, San Diego, CA) to serve as a positive control for PCR. PCR was performed on 2 ng of positive control template or 1 μl of diluted cDNA in the presence of 0.5 mM Tris-HCl (pH 8.3; 25°C), 250 μg/ml BSA, 2 mM MgCl2 (for AR) or 3 mM MgCl2 (for PSA) and HMR GAPDH, 0.5% (w/v) Ficoll 400, 1 mM tartrazine, 500 μM of each deoxyribonucleotide triphosphate, 1 μM of each primer, and 0.5 unit of AmpliTaq DNA polymerase (Perkin-Elmer, Foster City, CA) in a total volume of 10 μl. Thermal cycling parameters for each template and primer set were optimized to be within a linear amplification range. The thermal cycling program consisted of an initial denaturation at 94°C for 15 s followed by several cycles of denaturation at 94°C for 0 s, annealing at the optimized temperature for 0 s, and extension at 72°C for 30 s. PCR products (7 μl) were separated on 1.3% agarose gels in Tris-borate-EDTA buffer. The gels were stained with SYBR Green I (Molecular Probes, Eugene, OR) and scanned on a Fluorimagier SI (Molecular Dynamics, Sunnyvale, CA). Bands were quantitated using NIH Image 1.6. Differences in RNA isolation and cDNA synthesis efficiencies between time points and duplicates were accounted for by the normalization of each band to the level of GAPDH detected at the same time point. Induction was determined relative to the initial time point (t = 0).

**Statistical Design.** The original trial (cohort I) was a Phase II study with a one-stage sample design. This design required a total sample size of 20 patients to test the null hypothesis that the response rate was 15% or less. If one patient or no patients had a response, the trial ended, and the drug was rejected. The sample design provided for a power of 95% with a one-sided level of significance of 5%. A patient withdrawal from the study before a scheduled response evaluation was considered a nonresponse. After fluctuating PSA values were observed in a number of patients in cohort I, an additional 13 patients were enrolled on a second study (cohort II) evaluating the effects of maintenance GM-CSF.

Progression-free survival and overall survival were determined from the start of protocol therapy and analyzed by the product-limit method of Kaplan and Meier.

Differences in progression-free survival and overall survival between PSA responders and nonresponders were compared using the log-rank test. The survival and response proportion distributions of pretherapy and posttherapy variables were compared using the log-rank test. Analysis of characteristics associated with survival was conducted using a Cox proportional hazards model.

**RESULTS**

**Patient Characteristics.** A total of 36 patients were enrolled, 23 patients in cohort I and 13 patients in cohort II. Twenty-two of 23 cohort I patients were fully evaluable for efficacy and toxicity. One patient had a cerebral vascular accident after agreeing to participate in the trial but before receiving any therapy; as a consequence, this patient was excluded from analysis. Thirteen patients were enrolled in cohort II, and 11 of these patients were fully evaluable for efficacy and toxicity. One patient developed worsening fatigue 3 weeks after beginning GM-CSF therapy and discontinued participation. A second patient developed a new supraventricular tachycardia 1 day after beginning GM-CSF and was taken off study without further treatment. These two patients are included in the analysis and were scored as nonresponders. Patient characteristics are summarized in Table 1.

In cohort I, the median age of patients was 71.5 years. Twenty-one of 22 patients had positive bone scans. The single patient with a negative bone scan had bidimensionally measurable disease in pelvic lymph nodes. A total of 11 patients had bidimensionally measurable disease, mostly in regional lymph nodes (Table 1). The median pretreatment PSA level was 276 ng/ml (range, 19.2–3676 ng/ml), the median pretreatment hemoglobin was 12.0 g/dl, the median creatinine was 1.1 mg/dl, and the median KPS was 80%. Thirteen patients (59%) had
received prior chemotherapy or suramin. Twenty-one of the 22 patients had received three or more prior hormonal therapies; antiandrogen withdrawal was not considered a hormonal manipulation. All patients had undergone prior antiandrogen withdrawal.

Cohort II patient characteristics were generally similar to those in cohort I (Table 1), although there were fewer patients with measurable disease or exposure to prior chemotherapy or suramin. Ten of 13 patients had positive bone scans. Two patients had progressive disease as determined by a rising PSA level alone, without radiographic evidence of metastases. In addition, the median PSA of 81 ng/ml (range, 8.9–240 ng/ml) was lower in the patients in cohort II, possibly reflecting a lower tumor burden in this group.

Table 1  Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>Cohort I (n = 22)</th>
<th>Cohort II (n = 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median Age (yr; range)</td>
<td>71.5 (58–83)</td>
<td>71.0 (63–89)</td>
</tr>
<tr>
<td>Median KPS (range)</td>
<td>80% (60–100%)</td>
<td>90% (60–100%)</td>
</tr>
<tr>
<td>Median pretreatment PSA (ng/ml; range)</td>
<td>276 (192–3676)</td>
<td>81 (8.9–240)</td>
</tr>
<tr>
<td>Median pretreatment hemoglobin (g/dl)</td>
<td>12.0</td>
<td>12.7</td>
</tr>
<tr>
<td>Patients with positive bone scan</td>
<td>21/22 (96%)</td>
<td>10/13 (77%)</td>
</tr>
<tr>
<td>Patients with measurable disease</td>
<td>11/22 (50%)</td>
<td>2/13 (15%)</td>
</tr>
<tr>
<td>Patients with measurable disease only</td>
<td>1/22 (05%)</td>
<td>1/13 (08%)</td>
</tr>
<tr>
<td>Prior systemic therapy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemotherapy or suramin</td>
<td>13/22 (59%)</td>
<td>4/13 (31%)</td>
</tr>
<tr>
<td>Patients with ≥ three prior hormone treatments</td>
<td>21/22 (95%)</td>
<td>11/13 (85%)</td>
</tr>
</tbody>
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Fig. 1  PSA levels from four representative patients from cohort I who developed oscillating PSA values while receiving GM-CSF therapy.

Efficacy. In cohort I, 10 of 22 patients had a PSA versus time plot with a sawtooth pattern, with PSA predictably declining after 2 weeks on GM-CSF and then climbing during the off-therapy period (Fig. 1). Because of this sawtooth pattern, it was less common to see patients with two consecutive declines in PSA. Nevertheless, five patients experienced two consecutive declines in PSA. Declines in PSA were seen while patients were receiving drug. Ultimately, however, all patients had an upward trend in their PSA level. The median maximal PSA decline was 37% (range, −5.8% to −64%). Overall, 5 of 22 patients had a >50% decline on at least one occasion; however, as noted above, these declines were generally not sustained. Six patients were removed from therapy for progressive disease by PSA criteria alone, whereas the remaining patients had objective
evidence of disease progression. No patient developed objective evidence of progression in the face of a falling or stable PSA. The median response duration was 3.5 months (range, 2–9 months).

In cohort II, oscillating PSA values were noted in two patients, but the fluctuations were less prominent and of briefer duration than those observed in cohort I patients. A third patient had a dramatic decline in PSA from 77 to 0.1 ng/ml accompanied by an improvement in bone scan, which persists at 14+ months (Fig. 2). All but one patient in cohort II experienced a decline in PSA (median decline, 32.4%), but a PSA decline of >50% that was sustained for more than 6 weeks was seen in only one patient (Fig. 2).

The median survival for all patients was 15.8 months (Fig. 3).

**Toxicity.** Toxicity from the administration of GM-CSF was minimal. The most common toxicity was grade 1 malaise and fever that was self-limited, lasting less than 6 h. Two patients in cohort I and four patients in cohort II developed transient grade 2 injection site reactions with erythema and swelling, but these reactions generally resolved within 24 h. No patient had a grade 3 or grade 4 toxicity attributed to the administration of GM-CSF, and no patient discontinued GM-CSF therapy due to toxicity. No patient had GM-CSF held due to excessive leukocytosis.

**In Vitro PSA Analysis.** The PSA pattern observed was reminiscent of a pattern previously thought to be consistent with suppression of PSA production with no cytotoxic effect (11, 12). To explore this possibility, we tested GM-CSF in vitro using the androgen-dependent cell line LNCaP (13). GM-CSF treatment resulted in a 10.3–15.2% decrease in cell number compared to controls after 120 h of treatment (Fig. 4A). GM-CSF treatment also resulted in an 11.2–73.2% increase in PSA secretion per cell (Fig. 4B). Immunoblots of cell extracts prepared from LNCaP cells treated with GM-CSF for 120 h showed a modest decrease in intracellular PSA (Fig. 5). The largest decline (25%) was observed at the highest tested concentration of GM-CSF (5.0 ng/ml). The effect of GM-CSF on the transcription of both PSA and the AR was quantitated using reverse transcription-PCR. GM-CSF had a modest effect (<20% decrease) on the
transcription of these genes (Fig. 6). The possibility that GM-CSF interfered directly with the PSA assay was tested by mixing GM-CSF with control serum before PSA testing. The possibility that GM-CSF interfered indirectly with PSA measurement by inducing the expression of another factor that confounded the PSA assay was tested by determining PSA levels in serum from a prostate cancer control patient before and after the addition of varying amounts of serum obtained from the responding patient in cohort II. There was no effect of either GM-CSF or the responding patient’s serum on PSA concentration (data not shown).

DISCUSSION

Systemically administered GM-CSF resulted in a fairly typical pattern of PSA suppression in patients with metastatic HRPC, with nearly 50% of patients in cohort I having an initial sawtooth pattern in their PSA versus time plot, with the decline occurring while patients were receiving the drug. Furthermore, approximately 25% of patients had a >50% decline in PSA. Median response duration was brief at 3.5 months, although some patients had response durations exceeding 9 months. Other than PSA data, however, in this group of patients there was no compelling evidence of antitumor activity. However, in cohort II, one patient had an impressive and durable PSA response associated with bone scan improvement. Overall, GM-CSF was well tolerated and easy to administer.

Several potential mechanisms can be postulated that would explain some of the PSA patterns observed in this trial. First, the intrinsic variability in the PSA assay may result in some fluctuation in PSA values. This does not appear to be the case in this series, however, because fluctuations observed in this trial were generally of a magnitude much larger than those reported with assay variability, and all patients had persistently rising PSA levels before GM-CSF therapy. This observation, coupled with the fact that PSA levels began to climb in a consistent fashion without downward perturbations once the GM-CSF was discontinued, suggests that the PSA perturbations observed were not due to simple assay variability.

A second potential explanation for decline in PSA while on GM-CSF is direct interference by GM-CSF with the PSA assay. This explanation seems unlikely, given the ultimate rise in PSA values in some patients even while on GM-CSF. Nevertheless, we tested this possibility in vitro by measuring PSA values in serum samples (obtained from patients with prostate cancer who were not part of this trial) before and after adding GM-CSF. Adding GM-CSF had no effect on PSA values. In addition, serum from the responding patient in cohort II did not interfere with the PSA assay, suggesting that GM-CSF does not simply induce the expression of other factor(s) that inhibit PSA measurement.

It is possible that activation of macrophages and macrophage-like cells resulted in increased clearance of PSA. Whereas there is no direct way of testing this possibility, it has previously been suggested that GM-CSF can stimulate increased clearance of other molecules such as cholesterol (14). We prospectively serially measured fasting cholesterol, high-density lipoprotein, low-density lipoprotein, and triglyceride levels in all patients enrolled on this study every 2 weeks and found no change in these levels during treatment (data not shown). However, this does not rule out increased PSA clearance as an explanation for the PSA patterns observed.

Finally, it is possible that there is down-regulation in the expression of PSA by GM-CSF with no cytotoxic effect. For example, suramin is a drug that in tissue culture has been reported to result in decreased PSA production without inhibition of prostate cancer cells (11). The effect of GM-CSF was tested in an in vitro system using the androgen-dependent cell line LNCaP. The results observed in our experiments suggest that clinically relevant concentrations of GM-CSF did not decrease PSA expression to a degree that could account for the declines we observed in these patients.

There are several implications of these findings. An im-

Fig. 6 Amplifications of cDNA isolated from LNCaP cells treated with 0.45, 1.5 and 5.0 ng/mL of GM-CSF. Each cDNA was amplified within a linear amplification range for a) PSA, 540 bp and b) AR, 500 bp. Equivalent volumes of each amplification reaction were loaded into each well of the gel.
GM-CSF in Advanced Prostate Cancer

important observation from this trial is that patients treated with GM-CSF may experience transient declines in PSA levels. Despite its limitations, the use of PSA as a marker for activity of new therapeutic approaches or as a surrogate end point for survival has gained wide acceptance (15, 16). One of the major difficulties with using PSA as an end point is the confounding effect of a variety of interventions that perturb the PSA level. This includes the effect of antiandrogen withdrawal (17) and the use of concurrent medications such as corticosteroids that could result in a decline in PSA values. GM-CSF must now be added to this list of confounding variables. If GM-CSF is used as a growth factor to prevent therapy-induced leukopenia or as an immune adjuvant, its potential effects on PSA must be accounted for in evaluating the activity of the treatment regimen.

Whether GM-CSF exerts its effects on prostate cancer through immune mechanisms remains speculative. However, the prolonged serological and bone scan response we observed in one patient cannot be attributed to any intervention other than GM-CSF therapy. The mechanism by which GM-CSF produced a response in this patient or resulted in PSA declines in other patients requires further investigation and is almost certainly multifactorial. The activation of both macrophage and dendritic cell antitumor activity has been attributed to GM-CSF in vitro and may play a role in our observations. As a vaccine adjuvant, GM-CSF can enhance the growth and antigen-presenting function of macrophages and dendritic cells and can lead to direct T-cell activation (3). The extent to which GM-CSF can achieve these effects in the absence of presentation of a specific antigen or immune effector cell by a vaccine or dendritic cell infusion is unknown.

In summary, the results of this study suggest that systemically administered GM-CSF may have biological activity in patients with prostate cancer that extends beyond simple suppression of PSA values. These results suggest that a trial of GM-CSF in less heavily pretreated patients with a smaller tumor burden may be warranted. Further investigation of the mechanism by which GM-CSF produces this effect and its relevance to the treatment of prostate cancer is required. At the very least, the concurrent use of GM-CSF should be viewed as a confounding variable when PSA responses are used as an end point in clinical trials for the treatment of advanced prostate cancer.

REFERENCES
