

Cancer Therapy: Clinical

Inhaled Granulocyte-Macrophage Colony Stimulating Factor for First Pulmonary Recurrence of Osteosarcoma: Effects on Disease-Free Survival and Immunomodulation. A Report From the Children's Oncology Group

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Abstract

Purpose: Osteosarcoma most commonly recurs in the lung. Based on preliminary data on the anti-tumor effects of granulocyte-macrophage colony stimulating factor (GM-CSF) in animal models, and promising phase I trials, we embarked on a feasibility study of inhaled GM-CSF in patients with first isolated pulmonary recurrence of osteosarcoma.

Experimental Design: Forty-three eligible patients received inhaled GM-CSF at doses from 250 to 1,750 µg twice daily on alternate weeks. Following two cycles, patients underwent thoracotomy to resect tumor and analyze pulmonary nodules for expression of Fas/Fas ligand (Fas/FasL), and the presence of dendritic cells by immunostaining for CD1a, clusterin, and S100. Following surgery, patients received 12 additional cycles of therapy on alternating weeks or until progression. Event-free survival and survival, and feasibility of therapy delivery were evaluated.

Results: Dose escalation to 1,750 µg twice daily was feasible with no dose-limiting toxicity. Mean scores for Fas/FasL in nodules from patients with bilateral recurrence who underwent unilateral thoracotomy pretreatment (using a scoring system of 0-3) were 1.3 and 0.88, respectively, compared with 0.78 and 0.62 in nodules resected following two cycles of therapy. Only 11 of 30 nodules postinhalation were positive for CD1a, 4 of 30 for S100, and 6 of 30 for clusterin. Event-free and overall survival at 3 years were 7.8% and 35.4%, respectively.

Conclusions: Inhalation of GM-CSF at doses from 250 to 1,750 µg twice daily on alternate weeks was feasible with low toxicity. However, no detectable immunostimulatory effect in pulmonary metastases or improved outcome postrelapse was seen. *Clin Cancer Res*; 16(15): 4024-30. ©2010 AACR.

The most common site for the recurrence of osteosarcoma is the lung. Patients who develop pulmonary recurrence have a poor outcome, with a 5-year survival ranging from 23% to 44%, resectability and time from completion of initial therapy to relapse being the most important prognostic factors (1-9). The role of chemotherapy in recurrent osteosarcoma is controversial, with the

largest retrospective study suggesting that chemotherapy may "contribute to limited improvements of outcome" while acknowledging physician bias in which patients received chemotherapy (5). Moreover, most patients have received all agents that have shown activity during their initial treatment of osteosarcoma. This limits therapeutic options after recurrence because of cumulative organ toxicity concerns and presumed resistance to previously used chemotherapeutic agents.

An immunostimulatory approach that directly targets the lung is an attractive alternative to explore for patients with first pulmonary recurrence of osteosarcoma. Based on preliminary animal data on the antitumor effects of granulocyte-macrophage colony stimulating factor (GM-CSF; ref. 10, 11) and initial phase I data on inhalational GM-CSF in humans (12-14), we embarked on a trial of inhaled GM-CSF for first pulmonary recurrence of osteosarcoma (AOST0221). The ability of osteosarcoma cells to metastasize to and grow in the lung has been shown to inversely correlate with their cell surface expression of Fas and is

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Translational Relevance

Inhalational therapy is an effective means to deliver therapeutic agents to the lung, which is the most common "target organ" for metastases in osteosarcoma. Based on preliminary data on the antitumor effects of granulocyte-macrophage colony stimulating factor (GM-CSF), we embarked on a study of inhaled GM-CSF and measured the immunostimulatory effects of this therapy by doing immunostains for dendritic cells on resected tumor nodules, and evaluated them for Fas/Fas ligand expression. Although there was no evidence of invasion of dendritic cells into the lung nodules, and no increased expression of Fas/Fas ligand posttherapy, this study provided proof of principle that it is feasible to deliver aerosol therapy with a biological agent to patients with osteosarcoma. Given preliminary data from other investigators that early lymphocyte recovery is related to prognosis in osteosarcoma and Ewing sarcoma, future directions could focus on stimulation of the immune system both locally and systemically to improve lymphocyte numbers.

linked to the constitutive expression of Fas ligand in the lung (15). Moreover the induction of Fas on established lung metastases using aerosol therapy results in tumor regression (16). We had previously shown that osteosarcoma lung metastases from patients with osteosarcoma were Fas negative (17). We therefore investigated whether aerosol GM-CSF affected Fas expression in the patients' nodules.

The aims of this study were to provide a descriptive analysis of histologic findings in pulmonary metastases resected following two cycles of therapy with inhaled GM-CSF; to analyze resected nodules for expression of Fas/Fas ligand (Fas/FasL), presence of dendritic cells, and infiltration by macrophages; to estimate the event-free survival (EFS) of patients receiving inhaled GM-CSF as given in this study; and to determine if the maximum dose utilized in the adult trial of inhaled GM-CSF for melanoma is tolerable in pediatric patients.

Materials and Methods

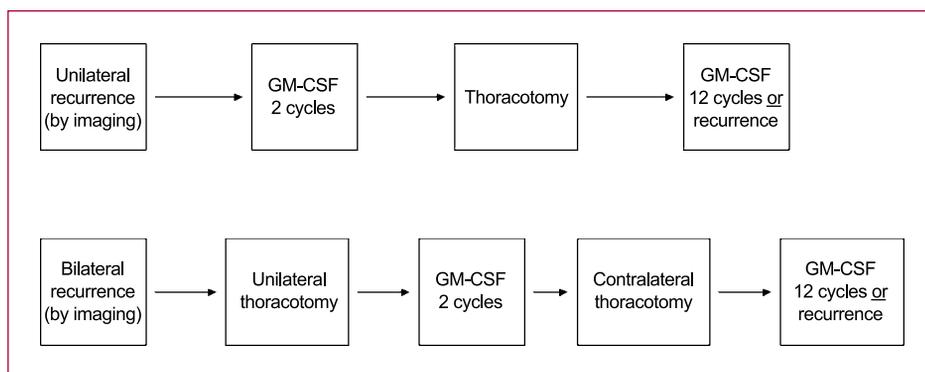
Patients

Patients less than 40 years of age with suspected first isolated resectable (defined as able to be removed without pneumonectomy) pulmonary recurrence of osteosarcoma after initial complete remission of osteosarcoma were eligible. Previous thoracotomies for initial diagnosis of osteosarcoma did not render a patient ineligible as long as they fulfilled all other eligibility criteria. Other eligibility requirements included life expectancy ≥ 8 weeks, ability to withstand surgery and to cooperate with and perform inhalational therapy, Eastern Cooperative Oncology Group performance status of 0, 1, or 2 or Karnofsky ≥ 50 , no pleural effusion, at least one parenchymal nodule (not only pleural-based nodules), only one prior treatment regimen for osteosarcoma, no evidence of dyspnea at rest, no exercise intolerance, pulse oximetry of $>94\%$ in room air, baseline forced expiratory volume in one second (FEV1) $\geq 80\%$ of predicted, no prior history of asthma, reactive airway disease or bronchospasm, negative pregnancy test in females with childbearing potential, not breast feeding, willingness to use effective contraception for sexually active patients of childbearing potential, institutional review board approval, and written signed informed consent.

Treatment program

Patients were assigned to one of two treatment schedules, depending on whether they had unilateral or bilateral lung metastases as determined by the institutional radiologist at enrollment. Figure 1 shows the experimental design schema. Patients with imaging consistent with unilateral recurrence underwent inhalation of GM-CSF (kindly provided by Genzyme) twice daily, on days 1 to 7 and 15 to 21, followed by thoracotomy on day 22 or as soon as possible following day 21 treatment (within 24-72 hours). Patients resumed treatment on day 29, or as soon as they were able to within 7 to 14 days postsurgery. The treatment schedule continued on an alternate week schedule for a maximum of 12 additional cycles (24 weeks) after thoracotomy, or until evidence of disease progression at any site. Patients who progressed in the lung during the initial two cycles of treatment prior to thoracotomy were eligible

Fig. 1. Experimental design schema.



to remain on study as long as the nodules did not become unresectable. One cycle of therapy was considered one week of therapy plus one week rest (except during the cycle immediately prethoracotomy in which patients went to surgery as soon as possible after their last dose of GM-CSF).

Patients with imaging consistent with bilateral recurrence underwent a unilateral thoracotomy first, prior to any inhalation treatment, and then followed the same schedule above (two cycles of inhalation therapy followed by thoracotomy on the other side). Median sternotomy was allowed if this was the institutional practice, but thoracoscopic procedures were not allowed.

The initial trial design called for 40 eligible patients to be treated with 250 μg of GM-CSF twice daily based on the phase I study. Simultaneous ongoing studies of the treatment of melanoma showed that doses of 2,000 μg twice daily could be safely delivered in adults. The study was amended to add two dose levels of 1,000 μg twice daily and 1,750 μg twice daily. Six patients were to be enrolled at 1,000 μg and the safety profile of the agent was to be assessed. If the dose could be feasibly given, the dose was to be escalated to 1,750 μg . If this dose could be feasibly administered in six patients, a total of 40 evaluable patients were to be enrolled.

To administer the drug, the 500- μg vial of lyophilized GM-CSF was reconstituted with 1 mL of sterile water. The appropriate dose of GM-CSF was withdrawn and then saline was added to the nebulizer cup to administer a total volume of 2.5 mL for the 250 μg dose, 3 mL for the 1,000 μg dose, and 3.5 mL for the 1,750 μg dose. Patients were instructed in administration technique in the office prior to going home, and were instructed to do the inhalation in the sitting position using a nebulizer with appropriate particle size. Patients were instructed to do daily peak flows by peak flow meter and to contact their physician if the peak flow fell below 80% of their personal best for three or more days that week, or immediately if peak flow fell below 50% of their personal best. For airway irritation secondary to inhalation, the use of albuterol inhalation prior to GM-CSF inhalation was recommended. Patients were monitored with chest computed tomography (CT) scans at entry into study, prethoracotomy, weeks 8 and 16, end of therapy (week 24), every 2 months thereafter for 1 year after completion of therapy, every 4 months for the second year, and every 6 months during the third to fifth years after completion of therapy.

Statistical analysis

The goal was to enroll 40 eligible patients who were evaluable for the biological end points of expression of Fas/FasL in resected nodules, presence of dendritic cells, and infiltration of macrophages. Forty patients also allowed estimation of 2-year EFS and overall survival with a maximum SE of 8%. EFS was taken to be the time from enrollment until disease progression, diagnosis of a second malignant neoplasm (SMN), death, or last patient contact, whichever occurred first. Patients who experi-

enced disease progression, SMN, or death were considered to have experienced an event; otherwise the patient was considered as censored at last contact. Survival was the time from enrollment to death or last patient contact, whichever occurred first. Patients who died were considered to have experienced an event; otherwise the patient was considered as censored at last contact. The risk for the outcomes as a function of time was estimated by the method of Kaplan and Meier (18). Confidence intervals were derived using the asymptotic distribution of the complementary log-log transformation of the Kaplan-Meier estimate of the survivor function (18).

Patients who became unresectable at the end of two cycles were considered feasibility failures, and patients who experienced grade III or IV toxicity during the same period were considered feasibility failures. A dose was considered feasible if 5 of the first 6 patients tolerated the dose and were able to undergo resection.

Assessment of biological end points

Methods for assessment of Fas utilizing monoclonal mouse anti-human Fas antibody, as well as evaluation for dendritic cells using an immunohistochemical panel including S-100 protein, CD1a, and clusterin, have been previously described (17). Tissues were stained for FasL similarly as described for Fas, except that the antibody used was from Santa Cruz BioTechnology, Inc., rabbit polyclonal anti-human FasL (N-20) with 1:50 dilution. Secondary antibody was horseradish peroxidase-labeled goat anti-rabbit IgG from The Jackson Laboratory with 1:500 dilution. Immunohistochemical results for S100, CD1a, and clusterin were reported as positive or negative, whereas Fas/FasL results were scored as 0, +1, +2 or +3. There was not enough tissue to do duplicate stains. Fas/FasL was reviewed by one investigator (NK) and immunohistochemical stains were reviewed by another investigator (CI). If there was not enough tissue available for both Fas/FasL and dendritic cell analysis, priority was given to analysis for Fas/FasL.

Results

Eligibility/evaluability

AOST0221 was open for enrollment in July 2004 and closed in December 2008. Data for analysis were taken current to April 2009. Forty-nine patients were enrolled in this study but six were ineligible: three did not have their pulse oximetry done prior to enrollment, two had baseline FEV1 values that were <80% of the predicted value, and one patient did not have a chest CT done within two weeks of starting therapy as required. These six patients were excluded from further analysis.

Of the 43 eligible patients, all were evaluable for the feasibility end point. During the dose escalation portion of the study, none of the first six patients enrolled at the 250- μg dose level, one of the first six enrolled at the 1,000- μg dose level, and none of the first six enrolled at the 1,750- μg dose level were feasibility failures. The one

Table 1. Patient characteristics

	<i>n</i> (%) [*]
No. of eligible patients	43
Age at initial diagnosis (y)	
Median (range)	15 (7-25)
Tumor stage at initial diagnosis	
Metastatic	34 (79%)
Nonmetastatic	9 (21%)
Age on study (y)	
Median (range)	16 (8-29)
Sex	
Male	26 (60.4)
Female	17 (39.6)
Race	
White	34 (79.0)
Black	6 (14.0)
Other	2 (4.7)
Unknown	1 (2.3)
Type of recurrence	
Unilateral	27 (62.8)
Bilateral	16 (37.2)
Number of metastatic lesions (by imaging)	
Unilateral	
Median (range)	1 (1-5)
Bilateral	
Median (range)	4 (2-15)
Mean	5.2
Overall	
Median (range)	2 (1-15)
Mean	3.0
Interval from initial diagnosis of osteosarcoma to study enrollment	
Median (range)	17.7 months (8.6-65)

^{*}Percentages based on eligible patients.

patient who was a feasibility failure at the 1,000- μ g dose level became unresectable after the first two cycles. After the first six patients at the 1,750- μ g dose level were evaluated for feasibility, two later patients were feasibility failures: one due to toxicity and one due to becoming nonresectable.

Six patients were not evaluable for the biological end point. Of these six, two did not have osteosarcoma at the time of thoracotomy. These two patients were excluded from the estimation of EFS and overall survival. Two went off protocol therapy for toxicity prior to surgery, and one became unresectable during the first two cycles. The remaining patient had initial thoracotomy documenting recurrence on one side, was enrolled on study for bilateral disease, but on subsequent bilateral thoracotomies had nodules removed that were not consistent with osteosarcoma.

In summary, 43 patients were eligible and considered in the evaluation of toxicity and feasibility. Forty-one were considered for the evaluation of disease outcome. However, only 37 were evaluable for the biological end points.

Patients and treatment

The characteristics of eligible patients, including age, gender, race, metastatic status at initial diagnosis, and type of recurrence (unilateral versus bilateral) are shown in Table 1.

Nineteen patients (44%) completed 10 to 14 cycles of therapy, 13 (30%) completed 6 to 8 cycles, 5 (12%) completed 4 cycles, and 6 (14%) completed only 2 cycles. Fifteen patients were treated at the starting dose of 250 μ g twice daily on alternate weeks, 7 patients at 1,000 μ g, and 21 patients at 1,750 μ g. The median interval from the last dose of GM-CSF during the first two cycles to thoracotomy was 1 day (range, 0-10). Only three patients had intervals of longer than three days from last dose of GM-CSF to thoracotomy.

Nine patients had grade 3 respiratory toxicity presumed secondary to GM-CSF, resulting in the removal of two from protocol therapy. At the dose level of 250 μ g, one patient developed grade 3 bronchospasm, wheezing, and dyspnea. The treating physician removed the patient from protocol therapy because it was considered in the patient's best interests, although the protocol-specified criteria for dose-limiting toxicity had not been met. One patient developed grade 3 decreased vital capacity and FEV1 which began to resolve by the time the next dose was due, but the patient relapsed so drug was not restarted. There were no grade 3 toxicities at the 1,000- μ g dose level. At the 1,750- μ g dose level, five patients developed grade 3 pulmonary toxicities. One patient developed decreased vital capacity and FEV1 shortly after thoracotomy, which resolved and GM-CSF was resumed; one patient developed pulmonary embolism unrelated to study drug; two patients developed FEV1 decrease or grade 3 cough and dyspnea which were relieved by pretreatment with bronchodilator. The fifth patient developed grade 3 dyspnea, decreased FEV1 associated with diffuse bilateral infiltrates and pleural effusions, probably related to therapy, and was removed from protocol therapy. A sixth patient who received a dose of 1,750 μ g developed hypotension, respiratory failure, and cardiac failure several days after thoracotomy. This toxicity was believed by the treating institutional investigator to be related to anthracycline cardiotoxicity from a prior treatment regimen and not due to GM-CSF. This same patient was also unable to have complete resection of metastases.

Among the 27 patients with unilateral disease, 24 had a thoracotomy after their second GM-CSF cycle. The 24 procedures included (a) unilateral thoracotomy ($n = 20$), (b) bilateral thoracotomy ($n = 2$), (c) median sternotomy ($n = 1$), and (d) a video-assisted thoracoscopic resection ($n = 1$). Three discontinued protocol therapy prior to thoracotomy (one became unresectable, one due to toxicity, and one by physician choice in the patient's best interest). Among the 16 patients with bilateral disease, all had their

Table 2. Fas/FasL results pretreatment/posttreatment by number of nodules

	Score	Nodules pretreatment, n (%)	Nodules posttreatment, n (%)
Fas	0	5 (26)	28 (60)
	1	7 (37)	9 (19)
	2	4 (21)	2 (4)
	3	3 (16)	8 (17)
FasL	0	10 (52)	31 (66)
	1	5 (26)	7 (15)
	2	1 (5)	5 (11)
	3	3 (16)	4 (9)

initial thoracotomy before treatment and their subsequent thoracotomy after two cycles of treatment. Fifteen had a unilateral thoracotomy in one lung then a contralateral thoracotomy after two GM-CSF cycles. The 16th had a unilateral thoracoscopy before treatment and median sternotomy after two cycles.

Fas/FasL staining

Sixteen patients with bilateral disease had 19 samples from pulmonary nodules examined after the first thoracotomy (and before treatment with GM-CSF). For patients with more than one sample tested it was not always possible to determine whether samples were from the same nodule or from separate nodules in the same patient. Details of the Fas/FasL results pretreatment are presented in Table 2. Following therapy, 47 resected nodules proven to be osteosarcoma from 37 patients were examined for Fas/FasL. Details of the Fas/FasL results posttreatment are also presented in Table 2. Only 8 of 47 nodules had a Fas score of 3 posttherapy, and 4 of 47 had FasL scores of 3 posttherapy.

Staining for dendritic cells

There were 37 nodules from 32 patients examined for CD1a, S100, and clusterin. Only nine were positive for two or more markers (only one was positive for all three). The results are shown in Table 3. Only 11 of 30 nodules postinhalation were positive for CD1a, 4 of 30 for S100, and 6 of 30 for clusterin. Five patients had nodules evaluated both preinhalation and postinhalation. Of these, one patient had two markers converted from focal positivity to negative, one converted from all three markers being negative to all being focally positive,

two had no change with all markers remaining negative both pretherapy and posttherapy, and one had reversal of two markers from positive to negative and negative to positive.

Patient outcome

Thirty-seven patients had a second recurrence of osteosarcoma. The median follow-up of those patients without an EFS event was 41.1 months (3.4 years). The estimated median time to an event was 4.3 months. The majority of recurrences were within one year of study enrollment. No second malignancies were reported. The estimated 2- and 3-year EFS from time of enrollment were 12.9% [95% confidence interval (95% CI), 4.8-25.3%] and 7.8% (95% CI, 2.0-18.7%), respectively (Fig. 2).

Twenty patients in this study died during follow-up. The median follow-up of those patients alive at last contact was 21.8 months (1.8 years). The estimated median overall survival time was 26.4 months (2.2 years). The estimated overall survival rates from time of enrollment were 63.1% (95% CI, 44.8-76.9%) at 2 years and 35.4% (95% CI, 17.3-54.2%) at 3 years.

Discussion

GM-CSF stimulates proliferation and differentiation of hematopoietic cells and augments functional activities of neutrophils, monocytes, macrophages, and dendritic cells (13). Dranoff et al. showed that vaccination with irradiated tumor cells engineered to secrete murine GM-CSF stimulated potent specific and long-lasting immunity against three different tumor models. They evaluated 10 different immunomodulatory proteins and found that GM-CSF

Table 3. Results of dendritic cell staining pretreatment and posttreatment

	CD1a positive (%)	S100 positive (%)	Clusterin positive (%)
Pretreatment	2/7 (29)	3/7 (43)	1/7 (14)
Posttreatment	11/30 (37)	4/30 (13)	6/30 (20)

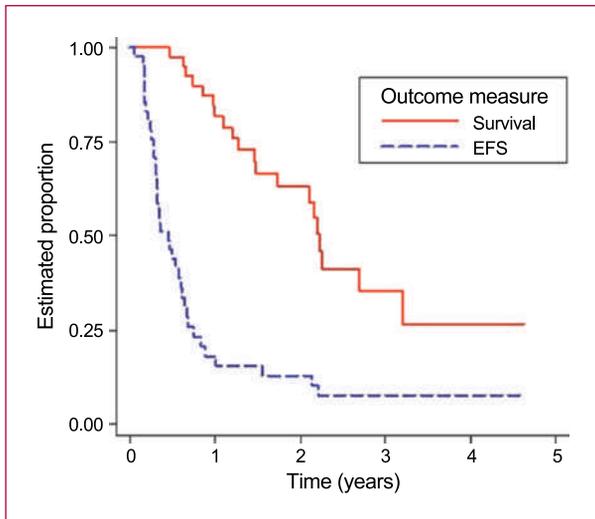


Fig. 2. Survival and event-free survival.

most significantly protected mice from subsequent tumor challenges (10). In a study of GM-CSF-transformed melanoma cells, secretion of GM-CSF facilitated killing of non-transformed bystander tumor cells (11). GM-CSF also has a number of actions on immune function that may contribute toward immune recognition and/or tumor destruction in hosts who were previously not able to contain pulmonary metastases. Included in these immunomodulatory and immunostimulatory effects are promotion of increased numbers of and cytotoxicity of activated macrophages (19), CD4 T cells (20), improved accessory cell function (21, 22), increased natural killer cell activity (23), and facilitation of immune responsiveness via dendritic cells (24). We had previously shown that most pulmonary nodules resected from patients who had not had chemotherapy prior to surgery showed no or only very weak Fas expression and showed only small numbers of antigen-presenting dendritic cells. However, elevated levels of Fas were observed in those patients who received chemotherapy prior to the lung resection (17). In preclinical animal studies with aerosol gemcitabine, increased expression of Fas receptor was also found after treatment (25). The inhibition of the Fas signaling in osteosarcoma tumor cells impaired the response of metastases to aerosol gemcitabine treatment, which indicates the significant contributory role of Fas in response to the treatment (16). Based on these findings, levels of Fas/FasL and dendritic cells were used in this study as markers of the response and mechanism of the GM-CSF activity.

In the first phase I study of inhaled GM-CSF, dose escalation up to 250 μg twice daily on alternate days resulted in no toxicity with only minor changes in pulmonary function tests. One patient with Ewing sarcoma was felt to have had a radiographic complete response, one patient with melanoma had a radiographic partial response, and three patients

(leiomyosarcoma, osteosarcoma, and melanoma) had radiographically stable disease for two to six months (12). Subsequently, an additional 45 patients were treated (not on protocol) with 250 μg twice daily on alternate weeks, of whom 24 had disease stabilization ($n = 21$) or partial regression ($n = 3$). One patient with melanoma and stable disease had a 10-fold increase in certain melanoma-specific T lymphocytes after therapy (26). In a subsequent much larger phase I trial of 40 patients with metastatic melanoma, dose escalation up to 2,000 μg twice daily on alternate weeks was feasible without dose-limiting toxicity. However, no GM-CSF dose was capable of inducing antitumor immunity in the majority of patients. Nonetheless, five patients developed an immune response to one or more melanoma-specific antigens (27).

We had hoped to capitalize on the preliminary data on the immunomodulatory effect of GM-CSF to achieve upregulation of Fas/FasL in tumor cells and migration of dendritic cells to the pulmonary metastases. Despite promising preliminary data on the immunomodulatory effect of GM-CSF there was no evidence of biological activity or upregulation of Fas/FasL on osteosarcoma in lung nodules. There was also no evidence of presence of dendritic cell recruitment as measured by immunostains for CD1a, clusterin, and S100. Overall survival was similar to that described in multiple previous studies (1–6, 8, 9). Our study again provided proof of principle that inhalation therapy for malignancy is a feasible and nontoxic route of administration of GM-CSF, which has shown some activity in other human tumor models (27). Doses of GM-CSF of 1,750 μg twice daily, however, have not been previously administered to pediatric and adolescent patients. One of the limitations of this study is that we did not prove that GM-CSF actually reached its target of lung metastases. This was not feasible in the current study as it would have required radiolabeling of drug. Moreover, it is not possible to determine whether the lack of recruitment of macrophages or upregulation of Fas/FasL was secondary to lack of drug reaching its target, inadequate drug dosing, or, if the dosing was adequate and drug reached its target, if GM-CSF did not have the hypothesized activity. Chemotherapy may possibly be a better means to upregulate Fas expression. Fas upregulation on osteosarcoma lung nodules has been described after aerosol gemcitabine (25, 28). Although GM-CSF, an agent to increase macrophage activation and numbers, did not affect outcome in our study, other means of stimulating immune function could possibly provide benefit. Because a more rapid absolute lymphocyte count recovery after chemotherapy for Ewing sarcoma and osteosarcoma is associated with significantly better survival, future aerosol and/or immune therapy efforts against sarcoma lung metastases possibly may be more effective using agents to improve lymphocyte numbers (29, 30). In addition, better methods to determine at initial diagnosis which patients are at highest risk for recurrence and therefore the best candidates for new approaches to therapy are needed, in addition to new approaches for patients with recurrent disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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