Increased Fas Expression Reduces the Metastatic Potential of Human Osteosarcoma Cells

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ABSTRACT

Purpose: **The process of metastasis requires the single tumor cell that seeds the metastatic clone to complete a complex series of steps. Identifying factors responsible for these steps is essential in developing and improving targeted therapy for metastasis. Resistance to receptor-mediated cell death, such as the Fas/Fas ligand pathway, is one mechanism commonly exploited by metastatic cell populations.**

Experimental Design and Results: **LM7, a subline of the SAOS human osteosarcoma cell line with low Fas expression, was selected for its high metastatic potential in an experimental nude mouse model. When transfected with the full-length *Fas* gene (LM7-Fas), these cells expressed higher levels of Fas than the parental LM7 cells or LM7-neo control-transfected cells. These cells were also more sensitive to Fas-induced cell death than controls. When injected intravenously into nude mice, the LM7-Fas cell line produced a significantly lower incidence of tumor nodules than control cell lines. Lung weight and tumor nodule size were also decreased in those mice injected with LM7-Fas. Levels of Fas were quantified in osteosarcoma lung nodules from 17 patients. Eight samples were Fas negative, whereas the remaining 9 were only weakly positive compared with normal human liver (positive control).**

Conclusions: **Our results demonstrate that altering Fas expression can impact the metastatic potential of osteosarcoma cells. We conclude that the increase of Fas on the surface of the LM7 osteosarcoma cells increased their sensitivity to Fas-induced cell death in the microenvironment of the lung, where Fas ligand is constitutively expressed. Thus, loss of Fas expression is one mechanism by which osteosarcoma cells may evade host resistance mechanisms in the lung, increasing metastatic potential. Fas may therefore be a new therapeutic target for osteosarcoma.**

INTRODUCTION

The lung is the most common site for metastatic spread in patients with osteosarcoma. Indeed, the majority of osteosarcoma patients have pulmonary micrometastases at presentation. Thirty to forty percent of newly diagnosed patients will relapse, despite aggressive combination chemotherapy and surgical resection of the primary tumor (1–3). Salvage chemotherapy has been disappointing, showing questionable benefit in terms of long-term disease-free survival, so new treatments must be developed and tested. As a first step, an experimental osteosarcoma lung metastasis mouse model was developed to assess the efficacy of new therapeutic agents (4). This model also allows the evaluation of specific factors that may contribute to the metastatic potential of osteosarcoma cells. This, in turn, may identify possible targets for therapy.

One such target is the Fas/Fas ligand (FasL) pathway. Loss of Fas or the function of Fas has been implicated in tumor progression of several cancers (5, 6). Furthermore, loss of Fas expression in primary tumors has been shown to correlate with disease progression and metastasis (6). Osteosarcoma metastasizes most often to the lung, an organ that constitutively expresses FasL, which can induce cell death in Fas-expressing cells. Fas expression on osteosarcoma cells may therefore play a pivotal role in the elimination of these tumor cells when they metastasize to the lung. Specifically, decreased Fas may prevent tumor cell elimination through the Fas/FasL-mediated pathway. To understand the mechanism by which osteosarcoma cells avoid Fas-induced cell death and thereby survive, thrive, and proliferate in the lung, it is critical to characterize the Fas expression of osteosarcoma cells in relation to metastatic potential.

To study the role of the Fas/FasL system and its relevance in osteosarcoma lung metastases, we used our experimental human osteosarcoma lung metastases model that was developed by repeated cycling of SAOS-2 human osteosarcoma cells through the lungs of nude mice (7, 8). The resultant SAOS-LM7 cell line (referred to henceforth as LM7) produces macroscopic pulmonary metastases 6 weeks after its intravenous injection into nude mice. This model has been used to evaluate the *in vivo* activity of chemotherapy and biological response modifiers (8, 9).

With this model, we have demonstrated previously that Fas expression inversely correlates with metastatic potential (10). In this earlier study, the LM7 subline had a significantly lower level of Fas expression than the sublines with lower metastatic potential. In the present report, we further demonstrate the importance of Fas expression to the metastatic phenotype of

Received 2/24/04; revised 8/20/04; accepted 9/2/04.

Grant support: National Institutes of Health grants CA 42992 (E. Kleinerman) and CA 16672 Cancer Center Support Core Grant (to The University of Texas M. D. Anderson Cancer Center) and the American Legion Auxiliary Cancer Research Fellowship (E. Lafleur).

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osteosarcoma cells by characterizing the effect of increased Fas expression on the metastatic potential of LM7 cells *in vivo*. Transfection of LM7 cells with the full-length *fas* gene yielded a cell line (LM7-Fas) whose injection showed a significantly reduced tumor incidence. For those mice that did develop tumors, tumor size and lung weight were also decreased compared with those of the controls. These data support the hypothesis that LM7 cells transfected with Fas are eliminated in the lung by a Fas/FasL-mediated pathway.

MATERIALS AND METHODS

Cell Lines. The human osteosarcoma cell line SAOS-2 was obtained from American Type Culture Collection (Manassas, VA). The LM7 cell line was derived from SAOS-2 cells by initial selection in 0.9% agarose followed by repeated intravenous recycling through the lungs of nude mice (8). Pulmonary metastases are evident 6 weeks after intravenous injection of LM7 cells. Parental SAOS-2 cells do not form metastases (8). Cells were maintained in Eagle's minimum essential medium supplemented with nonessential amino acids, sodium pyruvate, L-glutamine, and 10% fetal bovine serum. Cells were periodically screened for *Mycoplasma* contamination and verified to be free of pathogenic murine viruses (M. A. Bioproducts, Walkersville, MD).

For *in vivo* injection, cells in mid-log growth were treated with 0.25% trypsin/0.02% EDTA (w/v) and then resuspended in supplemented medium. The cells were then washed and resuspended in Hanks' balanced salt solution with Ca^{2+} or Mg^{2+} (HBSS) at a concentration of 2×10^6 cells/mL, and then 1×10^6 cells in 0.2 mL were injected into the left lateral tail vein of nude mice. At the designated time, mice were anesthetized and sacrificed. The lungs were weighed and fixed in Bouin's solution, and metastases were counted and measured with the aid of a dissecting microscope.

Reagents and Drugs. Eagle's minimum essential medium, HBSS, nonessential amino acids, sodium pyruvate, minimal essential medium vitamins, L-glutamine, and 2.5% trypsin were purchased from Whitaker Bioproducts (Walkersville, MD). Fetal bovine serum was purchased from Intergen Co. (Purchase, NJ). All reagents were free of endotoxin as determined by the *Limulus amebocyte* lysate assay (sensitivity limit, 0.025 ng/mL) purchased from Sigma Chemical Co. (St. Louis, MO).

Fluorescence-Activated Cell Sorting, Staining, and Flow Cytometry. Indirect staining and flow cytometric analyses were carried out as described previously (11). Cells (5×10^6) were plated in 6-well plates, incubated for the appropriate periods, collected, and incubated with either biotinylated Fas antibody (clone DX2; PharMingen, San Diego, CA) or isotype-matched, control mouse antihuman IgG1 antibodies (Sigma) and streptavidin-conjugated phosphotidylethenolamine. Samples were analyzed with a FACScan (Becton Dickinson, Mountain View, CA).

Cytostasis Assay. Cytostasis was quantified by measuring [^{3}H]thymidine incorporation, as described previously (12). Cell death was initiated by incubating the cells with an antihuman, anti-Fas antibody (clone CH11; Medical and Biological Laboratories, Nagoya, Japan) that induces signaling by trimerizing Fas molecules on the cell surface. Briefly, 5×10^3 LM7 cells were plated in each well of a 96-well plate and fed with either medium alone, medium containing 1 ng/mL Fas antibody (clone CH11), or medium containing 1 ng/mL mouse IgM (PharMingen). The plates were incubated for 60 hours; the cultures were labeled with 0.2 μCi/well [^{3}H]thymidine during the last 24 hours. The cells were then washed twice with HBSS and lysed with 0.1 mL of 0.1 N KOH. Radioactive incorporation was quantified, and cytostasis was calculated.

Vectors and Cell Transfection. LM7 cells were transfected with a cytomegalovirus promoter-based pcDNA3 plasmid containing human *fas* (a gift from L. Owen-Schaub; University of California Riverside, Riverside, CA) or a neomycin control plasmid using the liposomal transfer reagent FuGENE (Roche Molecular Biochemicals, Indianapolis, IN; refs. 13 and 14), which is known to be effective in SAOS cells. To obtain stably transfected clones, we cultured the transfected cells in medium containing G418 (0.25 mg/mL) for 3 months, and the resistant (*i.e.,* surviving) clones were propagated separately. Expression of Fas in LM7-Fas clones was confirmed by fluorescence-activated cell-sorting analysis.

Animal Studies. Male 4- to 6-week–old specific pathogen-free athymic nude mice were purchased from Charles River Breeding Laboratories (Kingston, MA). The mice were maintained in an animal facility approved by the American Association of Laboratory Animal Care in accordance with current regulations and standards of the United States Department of Agriculture, the Department of Health and Human Services, and the National Institutes of Health. Mice were housed five to a cage and kept in a laminar flow cabinet under specific pathogen-free conditions for 2 weeks before use. Intravenous injection of 10^6 LM7 cells results in the development of macroscopic lung metastases in nude mice within 6 weeks.

Immunohistochemistry of Patient Samples for Fas. Tissue blocks derived from 18 patients with osteosarcoma pulmonary metastases were obtained from the M. D. Anderson Cancer Center Tissue Bank. Five-micrometer tissue sections were deparaffinized in xylene, rehydrated, and then examined by immunohistochemistry for Fas expression. Sections were incubated with 3% H_2O_2 for 12 minutes to block exogenous peroxidase and then incubated with PBS containing 10% normal horse serum. Monoclonal mouse antihuman Fas (BD PharMingen, San Diego, CA) diluted to 2.5 μg/mL was applied and left overnight at 4°C. The secondary antibody labeled with horseradish peroxidase was then applied for 2 hours at room temperature. The slides were then developed with 3,3′-diaminobenzidine as a substrate and lightly counterstained with hematoxylin. Negative controls were prepared by omission of the primary antibodies. Because Fas is constitutively expressed in the liver (15), normal human liver tissue was used as a positive control in these studies.

Statistics. For the *in vivo* studies, lungs from the nude mice were resected and weighed, and tumor nodules were counted. To demonstrate any significant differences in the number of metastatic nodules, the Mann-Whitney rank-sum test was used, which allows for the calculation of statistical significance regardless of any outlying data points.

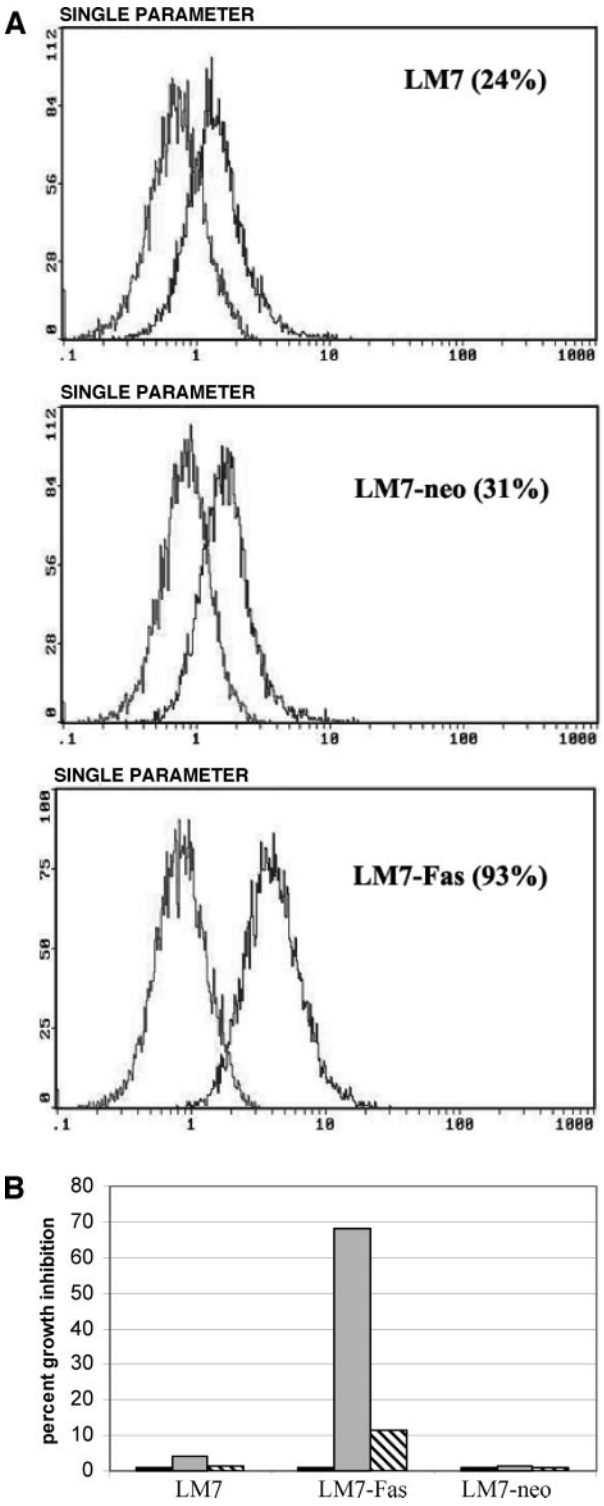

Fig. 1 Tranfection of *fas* into the SAOS-LM7 cell line. *A*. LM7 cells and LM7 cells stably transfected with *fas* (LM7-Fas; 2.5×10^5) or a neomycin control plasmid (LM7-neo) were stained with biotinylated antibodies to Fas, treated with streptavidin-phycoerythrin, and analyzed by flow cytometry. *B*. The above-mentioned cell lines were plated on a 96-well plate in 250 μL of medium and treated as indicated with medium alone (■), 1 ng/mL Fas antibody (CH11; ▨), or 1 ng/mL IgM isotype control (▧). After 60 hours, growth inhibition was assayed by [^{3}H]thymidine incorporation.

RESULTS

Generation and Characterization of Stable Transfectants. Successfully transfected LM7-Fas cells possessing the *neo* resistance gene were selected in a G418-containing medium. Stable transfectants were tested for Fas expression by fluorescence-activated cell-sorting analysis (Fig. 1*A*). Additionally, the LM7-Fas cells had a greater sensitivity to Fas-induced cell growth inhibition than the parental LM7 cells, as demonstrated by [^{3}H]thymidine incorporation (Fig. 1*B*). This demonstrates that the Fas expressed on the surface of transfectants was functional. The doubling times *in vitro* for parental LM7, LM7-neo, and LM7-Fas transfected cells were similar (data not shown).

Metastatic Potential in Nude Mice. Athymic nude mice received injection with either LM7, LM7-neo, or LM7-Fas cells. Each mouse received 10^6 cells via tail vein. After 10 weeks, mice were sacrificed, the lungs were resected and weighed, and tumor nodules were measured and counted. Mice receiving LM7-Fas had fewer pulmonary metastases than those receiving LM7 or LM7-neo cells (Fig. 2). Tumors from mice receiving LM7 or LM7-neo cells were larger than those in mice receiving injections of the LM7-Fas cells (Table 1). The Mann-Whitney rank-sum test showed that tumor incidence was significantly lower in mice receiving LM7-Fas cells compared with mice receiving parental LM7 cells ($P = 0.0002$) or LM7-neo control cells ($P < 0.0001$).

Expression of Fas in Human Osteosarcoma Lung Tumors. Sections from osteosarcoma lung metastases from 18 patients were stained using immunohistochemical methods for Fas expression. Of 17 specimens with viable tumor, 8 were Fas

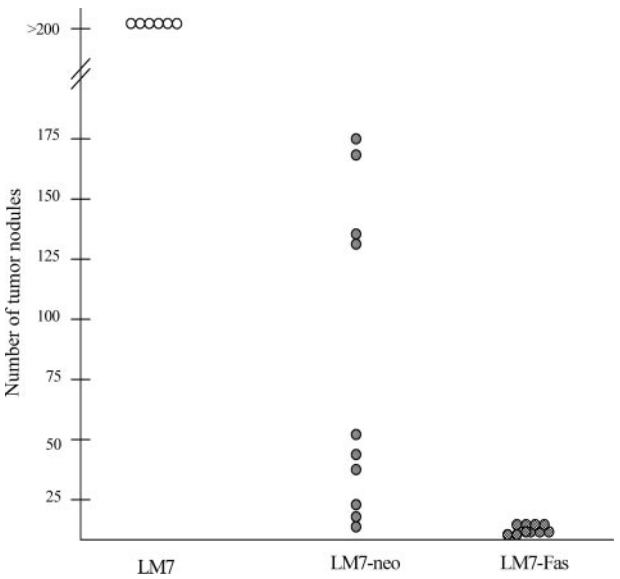

Fig. 2 Effect of *fas* transfection on the metastatic potential of LM7 cells. Mice received intravenous injection with 1×10^6 LM7 cells, LM7 *neo* transfectants (LM7-neo), or LM7 *fas* transfectants (LM7-Fas). The mice were sacrificed 10 weeks later, the lungs were fixed in Bouin's solution, and the metastases were counted. LM7-Fas cells resulted in fewer nodules compared with LM7 cells ($P = 0.0002$) and LM7-neo cells ($P < 0.0001$).

Table 1 Effect of *Fas* transfection on the metastatic potential of LM7 cells

LM7	7.0/>200	5.5/>200	6.0/>200	5.5/>200	6.5/>200	9.0/>200				
LM7-neo	>0.5/170	>0.5/128	>0.5/49	4.0/121	2.5/167	>0.5/15	>0.5/37	>0.5/8	>0.5/20	>0.5/28
LM7-Fas	0/0	<0.5/1	<0.5/2	<0.5/3	0/0	<0.5/2	0/0	<0.5/3	0/0	<0.5/4

NOTE. Mice received intravenous injection with 1×10^6 LM7 cells, LM7 *neo* transfectants (LM7-neo), or LM7 *fas* transfectants (LM7-Fas). Ten weeks later, the mice were sacrificed, the lungs were fixed in Bouin's solution, and the metastases were counted and measured. Data represent size (in mm)/number of nodules.

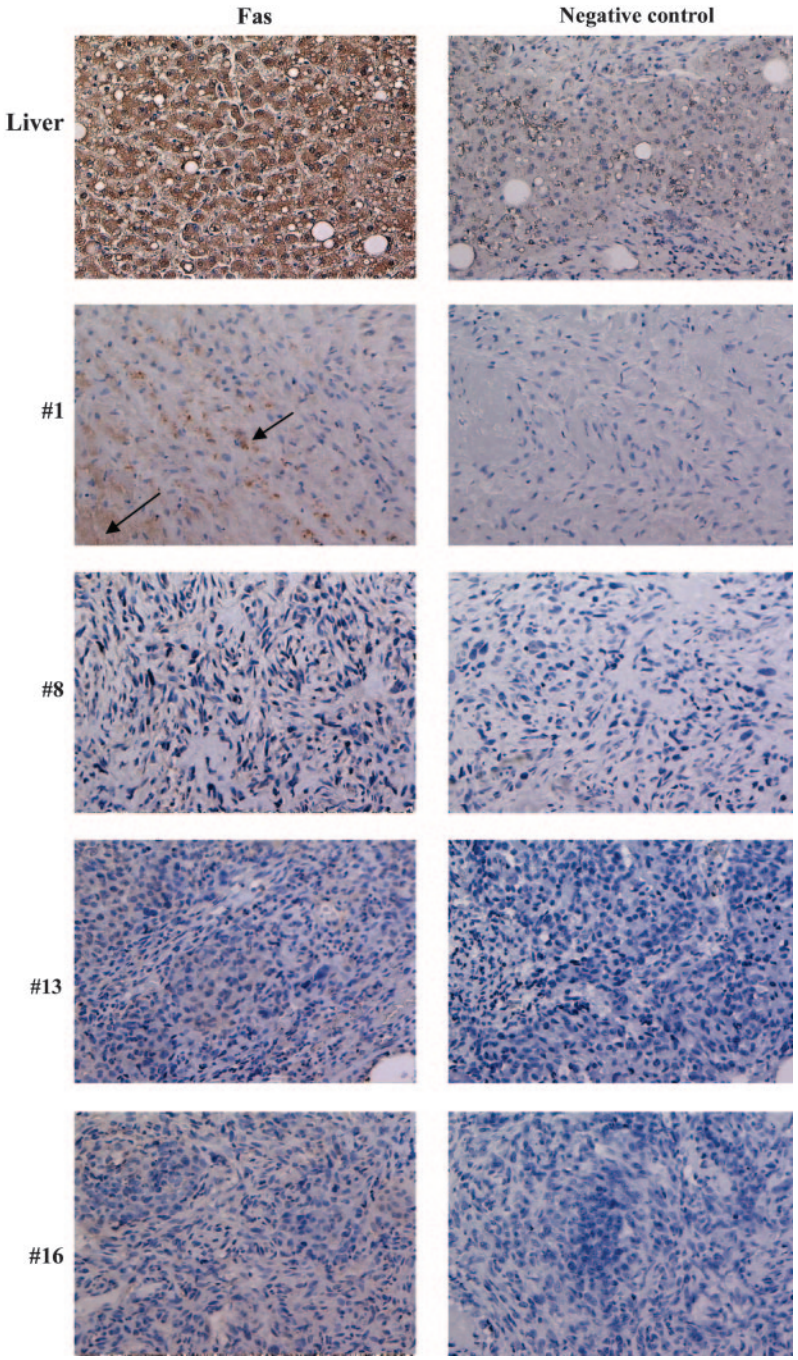

Fig. 3 Fas expression in lung metastases from patients with osteosarcoma. Paraffin-embedded tissue sections were prepared and stained as described in Materials and Methods. Representative slides from patients 1, 8, 13, and 16 are shown. Anti-Fas immunoreactivity is shown as the *brown-stained areas*, whereas areas that were not reactive to the anti-Fas antibody are indicated by the *blue counterstain* (hematoxylin). *Arrows* indicate positive staining for Fas in the patient sample. Human liver was used as a positive control. Original magnification, ×100.

negative (Fig. 3, patients 8, 13, and 16). The remaining nine specimens had low to moderate staining (Fig. 3, patient 1), compared with normal human liver tissue. Elevated Fas expression was seen in the interstitial areas and often correlated with the necrotic tumor areas. None of the specimens showed uniform expression of Fas in the tumor.

DISCUSSION

The present study demonstrates that altering Fas expression impacts the ability of osteosarcoma cells to metastasize to the lung. Previously, the LM7 subline has been shown to have significantly lower levels of *Fas* expression than the parental SAOS-2 cell line and sublines with low metastatic potential (10). Transfection of the *Fas* gene into LM7 cells augmented cell surface Fas expression, increasing susceptibility to Fas-induced cell death *in vitro* without affecting the cell growth rate *in vitro* (Fig. 1). The LM7-Fas cells showed a decrease in metastatic potential after intravenous injection into athymic nude mice. In mice receiving LM7-Fas cells, the number and size of lung tumors were reduced compared with those in animals receiving the untransfected or control-transfected LM7 cells (Table 1).

This decrease in metastases substantiates our hypothesis that Fas expression plays a critical role in the metastatic potential of osteosarcoma cells. Cells with high Fas expression will have low metastatic potential, whereas those with low Fas expression will form metastases. Because Fas is a cell surface protein capable of inducing ligand-mediated apoptosis, the ability of tumor cells to modulate the Fas system would be a distinct advantage for cell types that commonly metastasize to the lung, such as osteosarcoma. Resident lung cells express FasL on their surfaces. Therefore, tumor cells that possess intact Fas pathways and have significant Fas cell surface expression may have difficulty thriving in the lung because they can be eliminated by cytotoxic natural killer cells or resident FasL-positive cells in this animal model. We hypothesized that cells expressing high levels of Fas would be eliminated upon arrival in the lung. Indeed, LM7-Fas cells produced fewer and smaller pulmonary metastases after injection than either LM7 or LM7-neo cells. We also found that Fas expression was absent or weak in the viable tumor areas of the lung nodules excised from 17 osteosarcoma patients (Fig. 3). Our interpretation is that Fas-positive cells have difficulty thriving in the lung, an organ that constitutively expresses FasL. Cells with high Fas expression will be eliminated, whereas those with low or no Fas will form metastases.

Identifying agents that can up-regulate Fas expression may therefore aid in the treatment of osteosarcoma pulmonary metastases. We have demonstrated previously that interleukin (IL)-12 up-regulated Fas expression in osteosarcoma cells and altered metastatic potential (9, 16). SAOS-LM cells transfected with the *IL-12* gene had a decreased metastatic potential compared with SAOS-LM-*neo*–transfected cells. Furthermore, intranasal IL-12 gene therapy induced tumor regression in mice with SAOS-LM lung metastases (9). We hypothesize that the increased Fas expression induced by IL-12 increased the susceptibility of the osteosarcoma cells to ligand-induced apoptosis by the resident lung cells expressing FasL. However, IL-12 has many different effects *in vivo* including inhibition of angiogenesis, stimulation of T cells and natural killer cells, regulation of adhesion molecules, and induction of interferon-γ production in addition to its ability to up-regulate Fas expression (17, 18). Some or all of these effects may have contributed to the ability of IL-12 to alter metastatic potential. The data presented here demonstrate that specific up-regulation of Fas expression *alone* significantly influenced the metastatic potential of osteosarcoma cells *in vivo* and therefore indicate that the Fas/FasL pathway plays a critical role in the ability of osteosarcoma cells to metastasize to the lung.

By identifying the factors in tumor cells that increase metastatic potential, we can more efficiently develop and direct targeted therapies for a specific disease. Here, we have shown that Fas expression may be critical to the ability of osteosarcoma to survive in the lung environment. Manipulating Fas expression with agents such as IL-12 may induce the Fas-mediated cell death of tumors cells in the lung and produce tumor regression.

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Clin Cancer Res 2004;10:8114-8119.