Intranasal Therapy with an Adenoviral Vector Containing the Murine Interleukin-12 Gene Eradicates Osteosarcoma Lung Metastases

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INTRODUCTION

Most patients with OS have pulmonary micrometastases at diagnosis. Despite aggressive chemotherapy and surgical resection of the primary tumor, 30–40% of patients will relapse with pulmonary metastases (1–3). Surgery and salvage chemotherapy have not significantly changed the metastases-free survival rate (4, 5).

Because of the poor response rate of patients with relapsed OS, we previously developed an experimental mouse model to assess the efficacy of new therapeutic agents against pulmonary metastasis (6). In this study, we used this model to study the effects of IL-12 on pulmonary metastases.

IL-12 is a heterodimer composed of two subunits with molecular masses of 35 and 40 kDa that are linked by a disulfide bond (7, 8). IL-12 exerts a variety of biological effects on NK and T cells. It activates the cytotoxic activity of NK cells (8) and facilitates the induction of CTLs (9). IL-12 stimulates the production of IFN-γ by T cells and NK cells, regulates induction of the Th1 response from naïve T cells (10, 11), and has antitumor activity (12). Many of these activities are implicated in the antitumorigenic activities of IL-12.

Tumor regression and antitumorigenic activities are found when IL-12 is administered intratumorally and systemically to mice with Renca cell carcinoma, Lewis lung carcinoma, reticulum cell sarcoma, and melanoma (13, 14).

Initial studies of the effect of IL-12 given s.c. to patients with renal cell carcinoma (15) or melanoma (16) found that the drug was well tolerated, with some responses observed. IL-12 has also been given i.v. to patients with advanced melanoma, renal cell cancer, or colon carcinoma in a Phase I trial (17). Severe toxic effects were associated with its systemic delivery (18).

Given the toxic effects associated with systemic therapy, we hypothesized that localized therapy to the lungs might deliver a high drug concentration to the site of metastatic disease in OS while avoiding the toxic effects associated with systemic delivery. Because the amount of IL-12 at the tumor site is critical for tumor regression (19), we sought the optimal method to concentrate IL-12 in the lung. Previous studies in animals showed that the optimal route of administration to the lung for transfection efficiency of an adenoviral vector is the intranasal route (20). Consequently, we investigated the effect of intranasal delivery of IL-12 in an adenoviral vector on experimental pulmonary OS metastasis in nude mice.

MATERIALS AND METHODS

Reagents and Drugs. Eagle’s MEM, HBSS without Ca²⁺ or Mg²⁺, nonessential amino acids, sodium pyruvate, MEM, vitamins, l-glutamine, and 2.5% trypsin were purchased from Whittaker Bioproducts (Walkerville, 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).

Cell Lines. Human embryonic kidney cells transformed with adenovirus type 5 (293 cells) were obtained from American Type Culture Collection (Manassas, VA). SAOS-LM6, a human
OS cell line, was developed by repetitive cycling through the lungs of nude mice (6). SAOS-LM6 cells are maintained Eagle’s MEM supplemented with nonessential amino acids, sodium pyruvate, L-glutamine, and 10% fetal bovine serum. These cells were cultured periodically for mycoplasma contamination and verified to be free of pathogenic murine viruses (M. A. Bioproducts, Walkersville, MD).

**Mouse Model.** Male, 4–6-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 the United States Department of Agriculture, the Department of Health and Human Services, and the NIH. Mice were housed five to a cage and kept in a laminar flow cabinet under specific pathogen-free conditions for 2 weeks before use.

Injection of $10^6$ SAOS-LM6 cells i.v. resulted in microscopic metastases by 5 weeks, with visible lung nodules by 8 weeks (6).

**Vectors and Cell Transfection.** SAOS-LM6 cells were transfected with a pcAGG plasmid containing mIL-12 (a gift from Jun-ichi Miyazaku; Osaka University Medical School, Osaka, Japan) or a neomycin control plasmid using from Jun-ichi Miyazaku; Osaka University Medical School, transfected with a pcAGG plasmid containing mIL-12 (a gift

**Experimental OS Pulmonary Metastases.** The SAOS-LM6 (21) were injected via the lateral tail vein. Six weeks later, twice-weekly intranasal therapy with 25 µl of undiluted virus [1 × 10^10] pfu/ml Ad.mIL-12 or 5 × 10^9] pfu/ml AdΔβgal (the maximal concentration of each virus available) was introduced in a dropwise manner to both nostrils.

**RESULTS**

**Effect of mIL-12 Transfection on the Development of Experimental OS Pulmonary Metastases.** The SAOS-LM6 cells were transfected with a plasmid containing the gene for mIL-12 or a neomycin control plasmid. SAOS-LM6 that were not transfected and those transfected with the neomycin control plasmid did not produce the p35 or p40 mIL-12 subunits (data

**Western Blot Analysis.** To evaluate mIL-12 production, SAOS-LM6 cells (2 × 10^5) were seeded on 100-mm plates 1 day before treatment and then incubated with Ad.mIL-12 or AdΔβgal for 48 h. Cells were washed with cold PBS and lysed with buffer containing the protease inhibitors aprotonin (2 µg/ml), leupeptin (2 µg/ml), pepstatin A (1 µg/ml), and phenylmethylsulfonyl fluoride (100 µg/ml). Lysates were passed 10 times through a 25-gauge needle. Fifty µg of protein were solubilized in SDS sample buffer (24), boiled for 5 min, loaded onto a 7.5% SDS-PAGE gel, and transferred to a nitrocellulose membrane. Specific protein detection was performed with a monoclonal sheep anti-mIL-12 antibody (Genetics Institute, Cambridge, MA) using the enhanced chemiluminescence Western blotting analysis system (Amersham) according to the manufacturer’s instructions. Densitometric analysis was performed, and values were normalized to β-actin densities.
not shown). Several mIL-12-transfected clones were isolated. These clones expressed various amounts of both IL-12 subunits and produced mIL-12 protein (>1000 pg/ml) as assayed by ELISA.

Neomycin-transfected SAOS-LM6 cells and IL-12-transfected SAOS-LM6 cells were injected into nude mice to assess the effect of local IL-12 production on the development of metastases. The mice were examined for evidence of metastases 12 weeks later, a time when macroscopic disease is expected to be present. Mice injected with cells that secreted IL-12 did not produce pulmonary metastases. By contrast, mice injected with SAOS-LM6 neomycin-transfected cells had visible lung metastases in four of five mice.

In Vivo Expression of mIL-12 after Intranasal Administration of Ad.mIL-12. Having documented that SAOS-LM6 cells could be infected in vitro with Ad.mIL-12, we assessed the ability of intranasal Ad.mIL-12 to induce the production of mIL-12 in the lung. Mice were treated intranasally with Ad.mIL-12 on days 1 and 4 and then sacrificed on day 7. The lungs were removed and homogenized, and RNA was extracted. As shown in Fig. 1, there was substantial mIL-12 expression in the lung after the intranasal instillation of Ad.mIL-12. Mice sacrificed 24 h after only one treatment with Ad.mIL-12 showed no mIL-12 expression in the lung (data not shown). Livers from animals treated intranasally on days 1 and 4 and sacrificed on day 7 showed no evidence of mIL-12 expression (data not shown).

Effect of Intranasal Ad.mIL-12 on Lung Metastases. Having shown that mIL-12 can be expressed in the lung after intranasal Ad.mIL-12, the effect of Ad.mIL-12 intranasal therapy on the formation of pulmonary OS metastases was assessed. Mice that had received intranasal therapy with Ad.mIL-12 had a significantly lower median number of lung metastases (Fig. 2) than did control mice that received no therapy (P = 0.016) or mice treated with Adβgal (P = 0.04). In addition, the metastases that did develop were smaller. Control animals had tumors that ranged in size from <0.5 to 5 mm. Animals that received intranasal therapy with Adβgal developed tumors that ranged in size from >0.5 to 3 mm. Animals that received intranasal therapy with Ad.mIL-12 developed pulmonary tumors that ranged in size from <0.5 to 1.0 mm. The majority of these nodules were ≤0.5 mm, with only three animals having a single nodule measuring 1.0 mm.

Serum levels of mIL-12 and IFN-γ were examined. Mice received no therapy or were treated with intranasally with Ad.mIL-12 or Adβgal on days 1 and 4 and then sacrificed on day 7. Mice that received no therapy or received intranasal Adβgal had low median serum levels of IFN-γ at 164 pg/ml (range, 150–178 pg/ml) and 126 pg/ml (range, 120–537 pg/ml), respectively, compared with mice treated intranasally with Ad.mIL-12 (median serum level, 2680 pg/ml; range, 400 to >6000 pg/ml). Serum levels of mIL-12 were undetectable in mice treated intranasally with Adβgal. However, mice treated with Ad.mIL-12 had a median serum IL-12 level of 26 pg/ml (range, 3–35 pg/ml).

Serum levels were also measured at the end of therapy. The majority of the mice (15 of 20 mice) that received intranasal Ad.mIL-12 twice weekly for 5 weeks had detectable serum levels of mIL-12 (median level, 9.25 pg/ml; range, 0–26.5 pg/ml), whereas mice that received Adβgal had no detectable serum levels of IL-12. Lung homogenates obtained at the end of therapy from the mice that received Ad.mIL-12 showed mIL-12 expression, whereas liver homogenates from these animals showed no mIL-12 expression as measured by Western blotting (data not shown).

Distribution of mIL-12 Expression in the Lung. Fig. 1 shows that intranasal administration of Ad.mIL-12 results in mIL-12 expression in the lung but does not address the distri-
bution of IL-12 expression. Although intranasal administration of Ad.mIL-12 substantially reduced the number of metastases, some animals still had metastases. Metastases could be found in the periphery of the lung and near the major airways and vessels. We were concerned that intranasal Ad.mIL-12 therapy did not result in adequate distribution of the gene to all parts of the lung. Therefore, the distribution of mIL-12 expression was examined using immunohistochemistry. Mice were treated as described above. At the end of therapy, the lungs were sectioned for histological examination. The top panels in Fig. 3 are H&E-stained sections of lungs from animals injected with SAOS-LM6 cells and then treated twice-weekly beginning at week 6 with Adβgal (Fig. 3A) or Ad.mIL-12 (Fig. 3B) for 5 weeks. A characteristic pulmonary metastasis is seen in Fig. 3A. Staining of sections of the lung with an antibody for IL-12 showed no expression of IL-12 in animals treated with Adβgal (Fig. 3C), whereas lungs from mice treated with intranasal Ad.mIL-12 showed mIL-12 expression throughout the lung (Fig. 3D). Sections of the lung more distal to major airways stained less intensely for IL-12.

Lung sections were also stained for NK cells using a Pan NK antibody. Lungs from mice treated with inhalation therapy with Adβgal (Fig. 3E) do not show infiltration with NK cells. Lungs from mice treated with Ad.mIL-12 inhalation therapy (Fig. 3F) show the presence of NK cells.

DISCUSSION

The present study demonstrated the antitumor effect of IL-12 in a murine OS lung metastasis model. Transduction of human SAOS-LM6 OS cells with the mIL-12 gene decreased the ability of these cells to form lung metastases after i.v. injection. Although we used human cells in these studies, we selected the mIL-12 gene instead of the human IL-12 gene because we were looking for an in vivo effect on the host microenvironment. Murine cells do not respond to the human IL-12 protein (26).

The amount of IL-12 available at the tumor site is critical for tumor regression (19). Systemic administration of IL-12 and other cytokines often results in unacceptable toxicity before an effective drug concentration can be achieved in the tumor area. Direct delivery of the cytokine to the tumor or to the surround-
ing microenvironment may offer an alternative approach. Systemic toxic effects may be decreased or even avoided if cytokine exposure can be limited to only a few organ areas. Because the lung is the most common and often the only site of relapse for OS, we reasoned that direct gene delivery to the lung might be able to achieve the high concentration of IL-12 necessary for an antitumor effect.

Intranasal instillation has been shown to be an effective route for the delivery of other genes to the lung (20). For lung targeting, the intranasal route was far superior to the i.v. route. In this study, we administered an adenoviral vector containing the mL-12 gene via the intranasal route to mice with established microscopic SAOS-LM6 OS metastases. Intranasal delivery of Ad.mIL-12 resulted in transduction of lung cells after two administrations, with no evidence of viral spread or IL-12 production in the liver. Furthermore, after twice-weekly intranasal Ad.mIL-12 therapy for 5 weeks, mice that had been injected with the tumor cells had fewer pulmonary metastases. One week after the treatment ended, lung homogenates from animals receiving Ad.mIL-12 but not Ad-βgal expressed mIL-12. Once again, there was no mIL-12 expression in the liver. IL-12 was detected in the serum of Ad.mIL-12-treated mice 7 days after the administration of two doses and at the end of the 5-week treatment period, but the concentrations (3–35 pg/ml at the beginning of treatment and 0–26.5 pg/ml 1 week after the completion of therapy) were considerably lower than the serum concentrations (1000 pg/ml) seen in humans after the i.v. administration of IL-12 (17), arguing that local IL-12 gene delivery may indeed decrease the systemic toxic effects.

The mechanism by which IL-12 exerts its antitumor activity is not fully understood. T cells are thought to play a critical role in mediating the antitumor activity of IL-12 in several different animal tumor models (14). Because the studies described here use nude mice, it is unlikely that activated T cells played a role in the antitumor activity described. NK cells are functional in nude mice and are also activated by IL-12. As seen in Fig. 3, mice who received Ad.mIL-12 intranasally had infiltration of NK cells in the lung. NK cells were not demonstrated in the animals that received intranasal Adβgal. NK cells may therefore play a role in this antitumor activity. IL-12 stimulates the release of IFN-γ from NK cells (10) and stimulates inducible protein 10, which may be mediating an antitumor response. We have demonstrated that sera obtained from mice that received intranasal Ad.mIL-12 have an increase in IFN-γ.

When intranasal Ad.mIL-12 was given to mice with microscopic lung metastases, tumor growth was inhibited. The few tumor nodules that developed were smaller than those in control animals were. This observation may indicate an effect on tumor angiogenesis. Inhibiting the growth of new vasculature around the tumor can impact the growth rate and size of the individual nodules. Indeed, the antiangiogenic activity of IL-12 has been documented in various tumor models (27, 28). IL-12 has been shown to completely inhibit corneal neovascularization induced by basic fibroblast growth factor in immunocompetent SCID mice, nude T-cell-deficient mice, and NK cell-deficient beige mice (12). We are currently investigating the role of IL-12 as an antiangiogenic factor and the role of NK cells in the mouse model that we used here. Although this is not an immunocompetent model, and T cells play a significant role in antitumor activity, the goal of these studies is to investigate the role that IL-12 plays in eradicating tumors in nude mice.

In summary, we have demonstrated that transduction of human OS cells with the IL-12 gene reduced the ability of these cells to form lung metastases in nude mice after i.v. injection of the tumor cells. Nasal delivery of an adenoviral vector containing the IL-12 gene caused local production of the IL-12 protein and inhibited the development of pulmonary metastases. Nasal delivery is an ideal therapeutic approach for patients with OS because the lung is the primary and often the only metastatic site. The data presented here indicate that the intranasal route may be a viable way to deliver IL-12 gene therapy for OS lung metastases. Direct gene delivery to the lung could offer a new therapeutic approach for relapsed patients with metastatic pulmonary nodules.

REFERENCES
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