Aerosol Gene Therapy with PEI:IL-12 Eradicates Osteosarcoma Lung Metastases

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ABSTRACT

Purpose: We determined whether polyethylenimine (PEI), a polycationic DNA carrier, can be used to deliver the interleukin (IL) 12 gene by aerosol to treat established osteosarcoma (OS) lung metastases in a nude mouse model.

Experimental Design: Tumor response was assessed using our OS lung metastases model. Treatment with aerosolized PEI containing the murine IL-12 gene (PEI:IL-12; 600 μl PEI and 2 mg IL-12) was given twice weekly for 5–6 weeks.

Results: Aerosol therapy for 2 weeks resulted in high expression of both the p35 and p40 subunits of IL-12 in the lungs but not in the livers of mice. Peak IL-12 mRNA expression was seen 24 h after a single aerosol PEI:IL-12 treatment. This expression gradually decreased with continued detection for up to 7 days. IL-12 protein was not detectable in plasma even after 6 weeks of aerosol therapy. The number of lung metastases in mice treated with aerosol PEI:IL-12 was decreased significantly (median, 0; range, 0–33) compared with mice that received PEI alone (median, 37.5; range, 11–125; P = 0.002). Nodule size was also significantly smaller in the aerosol PEI:IL-12 group with 87% of the nodules measuring ≤0.5 mm in diameter compared with 65% in the aerosol PEI group. Mice that received aerosol PEI alone had numerous large lung nodules (3–5 mm). In the aerosol PEI:IL-12 group, no nodules were >1 mm. Weekly aerosol PEI:IL-12 therapy was as effective as twice weekly therapy.

Conclusions: Aerosol therapy resulted in selective gene expression and protein production in the tumor area. Aerosol PEI:IL-12 may avoid the systemic toxicities described previously in patients treated with i.v. IL-12. Because OS metastasizes almost exclusively to the lung, aerosol PEI:IL-12 therapy may provide a therapeutic option, which may be especially valuable.

INTRODUCTION

The lung is the most common site for metastases in patients with OS, with >30% of patients developing lung metastases despite aggressive combination chemotherapy and surgery (1–4). Patients who present with pulmonary metastases have a high rate of treatment failure, with <20% achieving long-term, metastasis-free survival. Salvage chemotherapy for OS lung metastases has been disappointing, showing questionable benefit in terms of improvement in disease-free survival. By contrast, surgical excision appears to have the highest impact on patient prognosis (5). For these reasons, our laboratory has focused on identifying new therapeutic approaches for patients with OS lung metastases. Particularly intriguing is the concept of concentrated, specific drug delivery to the lung.

Because the lung is the most common and often the only site for metastatic spread, aerosol therapy may provide a unique and effective way to treat patients with OS lung metastases. Aerosol therapy delivers the agent directly to the lung, increasing the concentration of the agent in the lung area and potentially decreasing systemic toxic effects. We recently developed a reproducible OS lung metastasis model using human SAOS-LM6 cells in nude mice (6). Using this model, we demonstrated that intranasal mIL-12 gene therapy resulted in the regression of established pulmonary metastases (7). Whereas these results were encouraging and validated the concept of regional therapy for OS, such an approach has technical limitations for use in human patients. Distribution after intranasal instillation does not reach the more peripheral regions of the lung. Additionally, the use of viral vectors, such as the adenovirus, adds another spectrum of problems. Immune responses and possible pathological consequences remain a concern for clinical applications using adenoviral vectors, particularly when repeated treatment is necessary (8).

Nonviral vectors are advantageous in that they avoid the strong immune response that limits gene expression, making them an attractive candidate for aerosol therapy. PEI is an organic macromolecule with a high cationic charge density potential and has been shown to be an efficient vector for gene transfer in vivo (9–12). This high cationic charge allows reten-
tion of its cationic state at the physiological pH level. The substantial buffering capacity of PEI may also permit endosome buffering, which protects the construct from DNA degradation by the lysosomes (13). PEI-DNA complexes are stable during the process of nebulization and deposit uniformly throughout the lung (14, 15). We have demonstrated that aerosol delivery of PEI-DNA complexes results in significant levels of gene expression in the lungs of mice, with minimal inflammatory response (15). In a study by Densmore et al. (16), aerosol PEI-p53 therapy resulted in highly significant reductions in the number and size of OS lung metastases.

For these reasons, we examined the effect of aerosol PEI-IL-12 as gene therapy for OS lung metastases. Our data indicate that PEI is an effective delivery vehicle for aerosol IL-12 gene therapy. Mice treated with aerosol PEI-IL-12 had significant IL-12 expression in lung tissue, but not in other organs, with fewer and smaller pulmonary nodules.

MATERIALS AND METHODS

Reagents. 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 (Walkersville, MD). Fetal bovine serum was purchased from Atlanta Biologicals (Norcross, GA). All of the reagents were free of endotoxin as determined using the Limulus amebocyte lysate assay (sensitivity limit, 0.025 ng/ml) purchased from Sigma Chemical Co. (St. Louis, MO). PEI (M₉ 25,000 branched form) was purchased from Aldrich Chemical (Milwaukee, WI). A stock solution of PEI (pH 7.0–7.5) was prepared at a concentration of 4.3 mg/ml (0.1 M in nitrogen) in PBS.

Vector. The pCAGG plasmid containing mIL-12 (pCA-IL-12) was a gift from Dr. Jun-ichi Miyazaki (Osaka University Medical School, Yamadaka, Japan). The plasmid was purified commercially by Bayou Biolabs (Harahan, LA), was endotoxin free, and was quantitated using UV absorbance. Agarose gel analysis showed primarily supercoiled plasmids with a small amount of nicked plasmid.

PEI:IL-12 complexes were prepared as described previously (17) at a ratio of 10:1 for PEI nitrogen:DNA phosphate and 1.29:1 for PEI:DNA weight ratio. Two mg of IL-12 plasmid in 5 ml of water was added slowly into 600 ml of stock PEI solution in 5 ml of water and was vortexed. Ten ml of mixed solution for one nebulizer dose per chamber was incubated at room temperature for 15–20 min before use.

Cell Lines. SAOS-LM6, a cell line derived from SAOS-2 human OS cells, was developed by repetitive cycling through the lungs of nude mice (6). SAOS-LM7 cells were derived from the lung metastasis of a mouse injected with SAOS-LM6 cells. These cells were maintained in Eagle’s MEM supplemented with 1 mm nonessential amino acids, 1 mm sodium pyruvate, 2 mm L-glutamine, two-fold MEM vitamins, and 10% heat-inactivated (56°C for 30 min) fetal bovine serum. The monolayer culture of cells was maintained in 75-cm² tissue culture flasks (Costar, Cambridge, MA) at 37°C in a humidified 5% CO₂ incubator and was tested periodically for Mycoplasma contamination by RT-PCR (primer purchased from Sigma Genosys, The Woodlands, TX). i.v. injection of 10⁶ SAOS-LM6 cells results in microscopic lung metastases by 5–7 weeks and macroscopic disease at 8 weeks. Injection of 10⁶ SAOS-LM7 cells results in microscopic lung metastases by 3–5 weeks with macroscopic disease at 6 weeks.

Mice. 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 for Accreditation 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 NIH. Mice were housed 5 to a cage and were kept in a laminar flow cabinet under specific pathogen-free conditions, with a 12-h dark cycle at least 1 week before initiation of therapy studies.

In Vivo Transfection with PEI:IL-12 by Aerosol. To determine whether PEI:IL-12 aerosol gene therapy resulted in transduction of lung tissue, 2 6-week-old mice per group were placed into plastic cages (13 × 17 × 30 cm) that were sealed with tape. PEI:IL-12 was aerosolized using the Aerotech II nebulizer (AT II; CIS-US, Inc., Bedford, MA) at a flow rate of 10 liters/min. Aerosol was delivered using air containing 5% CO₂ and was passed through the sealed plastic cage that housed the mice (15, 16). Air exited this chamber through a HEPA filter, and the entire apparatus was situated under a laminar flow hood vented through additional HEPA filtration. The exposure required ~30 min, during which time the mice were allowed to move freely in the cages. The mice were treated twice weekly for 2 weeks. Control groups included 2 untreated mice and 2 mice treated with PEI alone. Dosage was 2 mg of plasmid (in 10 ml of aerosolized solution) per nebulization chamber at an PEI nitrogen:DNA phosphate ratio of 10:1. Mice were sacrificed 24 h after the last aerosol therapy. The lungs and livers were removed and homogenized. Total RNA was extracted using TRIzol reagent, and IL-12 expression was determined by RT-PCR.

To determine how long IL-12 expression could persist in
the lung, 10 mice were given only one PEI:IL-12 aerosol treatment, and then 2 mice per group were sacrificed at 24, 48, 72, or 96 h, or 1 week later. The 2 lungs per group were removed and homogenized. Total RNA was extracted using TRIzol reagent, and IL-12 expression was determined by RT-PCR.

RT-PCR. RT-PCR was performed by using PTC-200 DNA Engine Peltier Thermal cycler (MJ Research, Inc., Waltham, MA). One μg of total RNA extracted from lungs and livers was reverse transcribed with 15 units of avian myeloblastosis virus reverse transcriptase in 20 μl of reverse transcription reaction system (Promega, Madison, WI) at 42°C for 15 min and then 95°C for 5 min. Two μl of cDNA samples were amplified by PCR for 30 cycles; each cycle included a denaturing step at 94°C for 1 min, an annealing step at 60°C for 1 min, and an extending step at 72°C for 1 min with 2.5 units of TaqDNA polymerase (Roche Diagnostics Co., Indianapolis, IN), 1 μl of 10 mM deoxynucleotide triphosphate mix, and 5 μl of a 10 mM concentration of each murine IL-12 primer: sense (forward primer) 5’-ATGTGTCCTCAGAAGCTAAC, and antisense 5’-TCCTAGGATCGGACCCTG for murine IL-12 p40 (18). As an internal control, classic II 18 s primer/competimer (18s internal standards, 324 bp; Ambion, Austin, TX) was amplified. Total volume was 50 μl. The primer was designed to produce a 648-bp fragment for mL-12 p35 and 1010-bp for mL-12 p40. The PCR products were isolated by electrophoresis on 2% agarose gel stained with ethidium bromide and were visualized under UV light. The results were quantified using a Quantity one machine (Bio-Rad, Hercules, CA).
Table 2 Effect of PEI:IL-12 aerosol therapy on osteosarcoma lung metastasis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Median # pulmonary nodules (range)</th>
<th>Median lung weight (mg) (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9/10a</td>
<td>28.5 (0–134)</td>
</tr>
<tr>
<td>PEI</td>
<td>8/8</td>
<td>37.5 (11–125)</td>
</tr>
<tr>
<td>PEI:IL-12</td>
<td>2/9</td>
<td>(0–33)b</td>
</tr>
</tbody>
</table>

a The number of mice with visible lung metastases/total number of mice injected with tumor cells.

b PEI:IL-12 versus PEI, P = 0.002.

c PEI:IL-12 versus PEI, P = 0.021.

Table 3 Effect of weekly versus twice weekly aerosol PEI:IL-12 on osteosarcoma lung metastases

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor incidence</th>
<th>Median # pulmonary nodules (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9/9a</td>
<td>51 (1–69)</td>
</tr>
<tr>
<td>PEI</td>
<td>99</td>
<td>27 (1–42)</td>
</tr>
<tr>
<td>PEI:IL-12 once weekly</td>
<td>2/9</td>
<td>0 (0–3)b</td>
</tr>
<tr>
<td>PEI:IL-12 twice weekly</td>
<td>3/9</td>
<td>0 (0–5)b</td>
</tr>
</tbody>
</table>

a The number of mice with lung metastases/total number of mice injected with tumor cells.

b PEI:IL-12 versus PEI, P = 0.003.

Fig. 4 Expression of IL-12 mRNA in lungs of mice after weekly versus twice weekly aerosol PEI:IL-12 therapy. Mice were given aerosol therapy with PEI:IL-12 weekly or twice weekly for 5 weeks. Mice were sacrificed 1 day after therapy completion. Lungs were removed, and total RNA was extracted for RT-PCR analysis. LM6-#6 cells were used as a positive control (Fig. 1). One of 2 lungs were analyzed.

**In Vivo Production of IL-12.** For analysis of in vivo IL-12 protein production, 2 mice/group were treated with aerosol PEI or PEI:IL-12 twice weekly for 2 weeks. After completion of therapy, blood was collected from the renal artery under anesthesia at the indicated times. The isolated serum was aliquoted and stored at −80°C. The lungs were removed and washed twice with PBS (4°C), and protein was extracted by homogenizing with lysis buffer (20 mM Tris (pH 8), 137 mM NaCl, 10% (w/v) glycerol, 1% Triton, 2 mM ethylenediaminetetraacetic acid (pH 8), 0.1 mM phenylmethylsulfonyl fluoride, 1 µg of aprotinin, 1 µg/ml of leupeptin, and 1 µg/ml of pepstatin). These homogenates were vortexed, incubated on ice for 20 min, and then spun. The supernatants were collected and stored at −80°C. The IL-12 protein level in cells, serum, and homogenate supernatants from the lungs were quantified by ELISA (Endogen, Inc., Woburn, MA) according to the manufacturer’s recommendations.

**Immunohistochemistry.** Two mice were treated with aerosol PEI:IL-12 twice weekly for 2 weeks. Mice were sacrificed 1 day after therapy completion; their lungs were removed, washed with PBS, and snap frozen in liquid nitrogen. Frozen sections were stained with a rat antimouse monoclonal IL-12 antibody (1:100 dilution; Biosource International, Inc., Camarillo, CA). Secondary antibody used was a goat antirat IgG antibody (1:200 dilution; Jackson ImmunoResearch, West Grove, PA). After three rinses with PBS, sections were incubated with diaminobenzidine substrate (Research Genetics, Huntsville, AL) for 10–20 min. The sections were rinsed with distilled water and counterstained with Gill’s hematoxylin.

**Aerosol PEI:IL-12 Therapy.** SAOS-LM6 cells in exponential growth were harvested by trypsinization. Single-cell suspensions of 1 × 10⁶ cells in 0.2 ml HBSS (4°C) were injected into nude mice via the lateral tail vein. Seven weeks later, mice were placed into plastic cages and treated with aerosol PEI alone or aerosol PEI:IL-12. Ten mice were treated together during nebulization. One nebulizer dose per chamber consisted of 2 mg of plasmid IL-12 and 600 µl of stock PEI solution prepared as described above. Therapy was given twice weekly for 6 weeks. Mice were sacrificed 1 day after completion of therapy. Blood was collected from the renal artery under anesthesia; the serum was isolated, aliquoted, and stored at −80°C. Lungs were removed, weighed, and fixed in Bouin’s solution. The number of metastatic nodules was then quantified. To examine IL-12 expression in the lungs and livers, organs were homogenized, and RT-PCR was performed as detailed above.

At the completion of the above aerosol studies using SAOS-LM6 cells we successfully isolated SAOS-LM7 cells from the lung metastases of a mouse injected with SAOS-LM6 cells. As we have demonstrated previously with additional lung cycling (6), this new cell line induced lung metastases more quickly after i.v. injection. SAOS-LM7 cells yielded metastases 2 weeks earlier than the SAOS-LM6 cells. We elected to use SAOS-LM7 cells for the comparison of weekly versus twice-weekly therapy. Four weeks after i.v. injection of 10⁶ SAOS-LM7 cells (a time when microscopic disease is present), mice received aerosol PEI:IL-12 therapy once or twice weekly for 5 weeks. Mice were sacrificed as described above.

**Toxicity Studies.** Three mice/group were treated with aerosol PEI:IL-12 or PEI alone twice weekly for 6 weeks. Mice were sacrificed 1 day after completion of therapy. The lungs,
livers, heart, brains, spleens, and kidneys were removed and examined histologically.

**Statistical Analysis.** A two-tailed Student’s *t* test was used to compare a control mean with several treatment means (*P* < 0.05 was considered significant).

**RESULTS**

**IL-12 Expression in Lung Tissue after Aerosol PEI:IL-12.** Mice were treated with aerosol PEI:IL-12 twice weekly for 2 weeks and then sacrificed 24 h after final treatment; lungs and livers were removed. As shown in Fig. 1, lung tissue of mice that received aerosol PEI:IL-12 (Fig. 1, Lanes 3 and 4) demonstrated expression of both the p40 and p35 subunits of IL-12. The induction of IL-12 expression in the lungs after aerosol treatment of PEI:IL-12 was not secondary to the aerosol procedure or to a nonspecific effect of PEI lung irritation, because PEI alone (Fig. 1, Lane 2) failed to induce IL-12 expression in the lung. These data indicated that aerosol PEI:IL-12 effectively delivered the IL-12 gene into the lungs. By contrast, no IL-12 expression was detected in liver tissues (Fig. 1, Lanes 5 and 6). Furthermore, IL-12 protein was undetectable in the serum at the end of PEI:IL-12 aerosol therapy (data not shown). Together, these data indicate specific tissue delivery to the lung, with minimal systemic spillover. Immunohistochemical analysis was performed on lungs from mice that received aerosol PEI:IL-12 or PEI alone twice weekly for 2 weeks. As shown in Fig. 2, IL-12 protein was demonstrated in lung tissue of mice receiving aerosol PEI:IL-12 (Fig. 2C), and no IL-12 was detected in lungs from control animals (Fig. 2A) or in mice treated with PEI alone (Fig. 2B). Additionally, IL-12 protein quantified by ELISA (Table 1) showed that both p40 and p70 IL-12 in lung tissue were higher in the PEI:IL-12-treated mice than in the PEI-treated or control mice.

We next determined how long IL-12 expression persisted in the lung after aerosol PEI:IL-12. Two mice/group were sacrificed at 24, 48, 72, or 96 h, or 1 week after a single dose of PEI or PEI:IL-12. Lungs were removed, and IL-12 expression was quantified. As shown in Fig. 3, both p35 and p40 IL-12 expression were demonstrated at 24, 48, and 72 h after aerosol delivery of a single dosage of PEI:IL-12 (Fig. 3, Lanes 3–5). IL-12 expression persisted for up to 7 days (Fig. 3, Lane 7). There was no IL-12 expression in the lungs of control mice or in mice treated with PEI alone (Fig. 3, Lanes 1 and 2).

**Aerosol PEI:IL-12 Inhibits OS Lung Metastases.** Having shown that mIL-12 can be expressed specifically in the lung after aerosol PEI:IL-12 treatment, the effect of aerosol PEI:IL-12 therapy on the formation of pulmonary OS metastases was assessed. Seven weeks after SAOS-LM6 cell injection (a time when there is microscopic metastatic disease in the lung), mice were given aerosol therapy with PEI or PEI:IL-12 twice weekly for 6 weeks. Mice that received PEI:IL-12 had significantly fewer metastatic nodules (Table 2) than did those that received PEI (median, 0 and range, 0–33 versus median, 37.5 and range, 11–125; *P* = 0.002). In addition, lung weights were lighter in the PEI:IL-12-treated mice (median, 282 mg; range, 169–325) compared with mice receiving PEI alone (median, 332 mg; range, 256–431 mg; *P* = 0.021). The size of the metastatic nodules was also smaller in the PEI:IL-12-treated mice (86.8% measuring <0.5 mm in diameter compared with 65.5% in the PEI group). Mice that received PEI alone developed numerous and large pulmonary tumors throughout the lung, ranging in size from 3 to 5 mm. In the PEI:IL-12 group, only two nodules measured ≥1 mm. At the end of the 6-week therapy, we were able once again to detect IL-12 mRNA in the lungs but not in the livers (data not shown).

As shown in Fig. 3, aerosol treatment with a single dose of
PEI:IL-12 resulted in IL-12 expression for up to 7 days. For this reason, we investigated the effect of weekly PEI:IL-12 aerosol therapy. As shown in Fig. 4, IL-12 expression in lung tissue from mice treated twice a week for 5 weeks was greater than in mice treated once a week for 5 weeks. However, weekly aerosol PEI:IL-12 for 5 weeks was as effective as twice weekly therapy against pulmonary metastases (Table 3). The incidence of lung metastases was significantly lower in both PEI:IL-12 treatment groups compared with the control or PEI groups. The number of lung metastases was lower and the size of the nodules smaller in mice treated with aerosol PEI:IL-12 (Fig. 5). There was no evidence of organ toxicity or inflammation in mice who were treated with aerosol PEI:IL-12 twice weekly for 6 weeks (data not shown).

DISCUSSION

The present study demonstrated that PEI could be used to deliver the IL-12 gene to the lung by aerosol administration, resulting in selective IL-12 expression with no spillover to the blood stream. Aerosol PEI:IL-12 resulted in IL-12 expression in the lung within 24 h, which persisted for up to 7 days. No IL-12 expression was seen in the liver. Similarly, IL-12 protein was undetectable in the plasma, even after 6 weeks of twice weekly PEI:IL-12 administration. Our data additionally demonstrated that aerosol PEI:IL-12, given either once weekly or twice weekly for 5–6 weeks, resulted in the regression of pulmonary OS metastases (Tables 2 and 3).

The mechanism of the antitumor activity of IL-12 is not fully understood and may involve several different activities, including T-cell activation, natural killer cell activation, and antiangiogenic activity (18–26). Preclinical studies in several different tumor models have demonstrated that IL-12 may be a powerful therapeutic option for cancer treatment (23–26). Unfortunately, severe toxic effects have been associated with the systemic administration of IL-12 (27–29). What is intriguing about our studies is that aerosol PEI:IL-12 resulted in selective delivery of the gene to the lung area with selective protein production. We hypothesize that this local targeting can, therefore, result in a higher concentration of IL-12 protein at the metastatic site, thus avoiding the toxic side effects associated with systemic delivery of the protein. Aerosol gene therapy is an appealing approach for OS, because the lung is the most common and often the only site of metastasis. The concentration of a drug or cytokine and the level of gene transfer at a metastatic site plays a critical role in inducing tumor regression.

Finding a suitable vector for aerosol gene therapy is also crucial to the success and implementation of gene therapy in clinical practice. The majority of gene therapy trials have used adenoviral or retroviral vectors for this task. However, viral vectors have limitations because of their potential for promoting genomic rearrangement and stimulating an immune response (30). In addition, there may also be a pathological effect associated when repeated treatment with viral vectors is necessary. Immune response induced by an adenovirus can lead to the rapid elimination of the vector, resulting in reduced effectiveness on subsequent administration (9). Nonviral vectors offer several advantages, one of which is avoiding the strong immune response. PEI, a cationic polymer, has been shown to be an effective vector for gene delivery in vivo (10, 11, 13–15). PEI-DNA complexes are stable during jet nebulization and deposit uniformly throughout the lung (14, 15). Our data demonstrated that multiple treatments of aerosol PEI:IL-12 resulted in the eradication of OS metastases with no histological evidence of organ toxicity in brain, lung, liver, kidneys, heart, or spleen. These data suggest that this approach has merit in the treatment of OS patients with lung metastases.

Because relapsed OS is difficult to treat and is often resistant to salvage chemotherapy regimens, alternative approaches are warranted. Our data demonstrate that aerosol PEI is a feasible way to deliver the IL-12 gene to the lung and to get induction of IL-12 protein in the tumor-organ environment with subsequent tumor regression. Therefore, we conclude that aerosol therapy may offer a novel therapeutic approach for OS and other diseases that metastasize to the lung, and that PEI can serve as the vector system for aerosol gene delivery.

REFERENCES


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