Efficient Therapeutic Gene Delivery after Systemic Administration of a Novel Polyethylenimine/DNA Vector in an Orthotopic Bladder Cancer Model


ABSTRACT

Successful systemic gene therapy has been hindered by vector-related limitations, including toxicity and inefficient gene delivery to tumor cells after i.v. administration. To circumvent these problems, we developed a novel formulation between the polycation polyethyleneimine and DNA that mediates high-level tumor cell transduction in vitro and efficient i.v. gene delivery in that greater reporter gene expression occurred in tumor than in lung. Strikingly, administration of just 6 μg of the polyethyleneimine/DNA-p53 vector every 3 days for 3 weeks indicated restoration of normal cell cycle regulation and apoptotic mechanisms as demonstrated by efficient p53 expression, increased apoptosis, and a 70% reduction in tumor size in an orthotopic bladder cancer model. This novel vector formulation represents a new method to increase i.v. delivery of genes to tumors.

INTRODUCTION

Mutations in the p53 gene are the most common genetic defect in human bladder cancer (1). Of particular significance, alterations in p53 are linked to cancer progression and response to radiation and chemotherapy (2). In many human tumor cells, including bladder cancer, reintroduction of wild-type p53 into tumor cells is incompatible with a tumorigenic phenotype, and as a result, tumorigenicity in nude mice is reduced. As a consequence, replacement of p53 has become a new therapeutic approach. Although the delivery of p53 appears to be feasible, the efficacy of gene therapy remains to be realized. However, as delivery systems improve, we may be able to capitalize on the proapoptotic and antiangiogenic effects of p53 and incorporate the use of p53 gene therapy into novel therapeutic strategies for advanced bladder and other cancers. To address the issue of gene delivery, we developed a novel formulation between PEI© and DNA that mediates high-level tumor cell transduction and efficient gene delivery via an i.v. route.

MATERIALS AND METHODS

Cell Lines and Vector Preparation. The human bladder cancer cell lines were obtained from Drs. William F. Benedict, Colin P. N. Dinney, and Grossman (The University of Texas, M. D. Anderson Cancer Center, Houston, TX) and were grown as a monolayer in complete media. Undifferentiated normal urothelial cells were kindly donated by Dr. Barton Grossman (The University of Texas, M. D. Anderson Cancer Center). Differentiated normal urothelial cells were produced by switching the cell culture media of undifferentiated cells (third passage) to DMEM containing 10% FCS 24 h before transduction. All cell lines were plated 48 h before transduction so that a 75% confluency on a 12-well plate was obtained on the day of transduction. PEI/DNA vector for in vitro and in vivo use was prepared by adding 10 μg of PEI directly to 6 μg of DNA in 60 μl of HEPES Buffer (10 mM), followed by a 10-min incubation at room temperature. The vector (2.5 μg of DNA) was then either analyzed for particle charge (Delsa particle charge analyzer; Coulter, Inc.) or added to cells in vitro. For in vivo studies, the same formulation was used, but 5 min after PEI and DNA were combined, polyethylene glycol and dextrose were added to final concentrations of 5 and 4%, respectively. The vector was then incubated for an additional 5 min and then injected i.v. via the tail vein using a 28-gauge needle.

In Vitro and in Vivo Transduction Analysis. In vitro transduction by the PEI/DNA vector containing the plasmid pCMV/β-gal (3) was measured in bladder cancer cell lines and normal urothelial cells. To determine the optimal transduction efficiency, PEI was mixed with pCMV/β-gal at a p:peptide ratio of 2.7:1 and 9:1 and incubated with cells for 3 h in medium without FCS. After this, vector-containing medium was replaced with complete medium, and cells were stained for β-gal expression 24 h later. All in vitro transduction experiments were performed twice using duplicate samples. The UM-UC-3 human bladder carcinoma cell line, which carries a large deletion in the p53 locus (4) and showed high transduction efficiency in vitro, was used for all in vivo studies. To initiate tumor growth, 50,000 viable cells in 50 μl of HBSS were injected into the bladder wall of male athymic BALB/c mice (day 0). Four days later, vector (6 μg of DNA) containing the plasmid pCMV/β-gal was administered on 3 consecutive days via the tail vein to animals bearing palpable bladder tumors. Twenty-four h later, tumors and lungs were removed (n = 5 animals), completely homogenized, and β-gal expression was determined using the Galacto-Light assay (Tropix, Bedford, MA). The analysis of tumor tissue (n = 5 animals) by PCR, Western blotting, and TUNEL used more established tumors (10 days after tumor inoculation) that were treated on 3 consecutive days with vector (6 μg DNA/injection) containing either the plasmid pCMV/βgal or the plasmid cytomegalovirus enhancer/promoter-driving human p53 gene expression (3) and then harvested 24 h later.

In Vivo Systemic Gene Therapy of Orthotopic Bladder Tumors. Athymic nude mice injected with UM-UC-3 tumor cells as outlined above were randomized into three groups (n = 10/group): (a) control; (b) PEI/DNA/β-gal treated; or (c) PEI/DNA-p53 treated. A single injection of PEI/DNA vector (6 μg) was started on day 4 after tumor cell injection and then an injection was performed every 3 days for a total of seven treatments. At this point, the control animals had palpable tumors. Twenty-four h after the last treatment, all animals were euthanized, and the bladders and tumors were removed and weighed. The other major organs, including the lungs, were also harvested and frozen at −80°C. The intra-abdominal lymph nodes were also removed, fixed in 10% buffered formalin, stained with H&E, and examined for the presence of metastases.

PCR Detection of PEI/DNA-β-gal Delivery in Vivo. Total DNA was isolated using Trizol according to the manufacturer’s protocol (Life Technologies, Inc., Gaithersburg, MD). Primers flanking the junction of the CMV enhancer/promoter and β-gal gene were used to detect plasmid, whereas β-actin-specific primers were used as controls.

Western Blotting. Tumors from control and PEI/DNA-p53-treated animals were harvested and homogenized in lysis buffer (20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin, and 0.15 unit/ml aprotinin) and centrifuged to recover insoluble protein. Protein (30 μg) was then resolved by SDS-PAGE and transferred to nitrocellulose membranes, which were then probed with mouse monoclonal anti-human p53 antibody (Oncogene Research Products,
RESULTS AND DISCUSSION

Use of the novel PEI/DNA-β-gal vector at an a:p ratio of 2.7:1 produced low toxicity transduction as high as 78% across many bladder cancer cell lines (Fig. 1A). This was as much as 30 times higher than levels obtained with the commonly used, more toxic 9:1 ratio. In contrast, the transduction rate was only <1% in normal urothelial cells (both differentiated and undifferentiated). This suggests that the cancer cells may promote internalization of this vector over normal cells (Fig. 1A). This has been demonstrated by other groups in the transduction of tumor cells by PEI-based vectors (5).

Gene delivery and expression in bladder tumors and lungs from mice injected i.v. with the PEI/DNA-β-gal vector were analyzed by PCR analysis and luminescent detection of β-gal expression. PCR analysis of DNA prepared from the bladder tumors of PEI/DNA-β-gal-treated mice confirmed local plasmid delivery (Fig. 1B). In addition, expression of β-gal was 14-fold higher in bladder tumors than in lungs from animals treated with the PEI/DNA-β-gal vector (Fig. 1C). To determine whether this level of transduction would be sufficient to obtain a therapeutic effect, the vector was used to deliver the tumor suppressor p53 gene in an orthotopic bladder cancer xenograft model produced with the cell line UM-UC-3, which has a large deletion in the p53 gene (4). Analysis of treated animals identified that bladder tumors from control and PEI/DNA-β-gal-treated mice had median weights of 616 and 537 mg, respectively (Table 1). In contrast, the median weight of bladder tumors from animals treated with the PEI/DNA-p53 vector was 126 mg ($P = 0.004$ compared with control groups, Mann Whitney U test). Of additional importance, lymph node metastasis occurred in all control mice, 5 of 6 PEI/DNA-β-gal-treated mice, but in only 2 of 10 PEI/DNA-p53-treated mice ($P = 0.001$, $\chi^2$ test). Although this reduction in lymph node metastasis may reflect the smaller tumor burden in these animals, the possibility of a systemic therapeutic effect on metastatic cells cannot be ruled out. To measure the expression of wild-type p53 as well as normal p21 expression in the bladder tumors from animals treated with the PEI/DNA-p53 vector, Western blot analysis was performed. Because the UM-UC-3 cell line carries a large deletion in the p53 gene, wild-type p53 expression was not detected (Fig. 2A). However, increased p53 expression that contributed to an up-regulation of normal p21 expression was identified in bladder tumors from PEI/DNA-p53-treated animals (Fig. 2A). In addition, in keeping with the proapoptotic function of wild-type p53, the number of apoptotic cells was increased in tumors from animals treated with the PEI/DNA-p53 vector (Fig. 2B). This low p53 protein level, disproportionate increase in the p21 protein level, and enhanced apoptosis were also observed in human clinical trials with adenoviral p53 therapy and may be explained by the short half-life and instability of wild-type p53 (6, 7). Thus, it can be concluded that the significant reduction in the growth and metastasis of orthotopic human bladder tumors was attributed to the p53 gene delivery obtained with our novel formulation. Additional analysis of these tumor samples will also allow us to determine whether the resulting inhibition of tumor growth was due primarily to the proapoptotic nature of p53, its ability to mediate an antiangiogenic effect, or a combination of the two.

The use of PEI in this formulation is critical based on its polycationic, branched structure that allows for DNA binding/condensation as well as an intrinsic endosmolytic activity (8). Every third atom in the PEI molecule is an amino nitrogen atom that can be protonated, allowing PEI to act as a proton sponge at virtually any pH (8). In this
study, we used an a:p ratio of 2.7:1 for in vitro and in vivo transduction. At this ratio, the PEI/DNA vector has a marginally positive ζ potential (data not shown) and thus should minimally interact with complement and other protein species, making it ideal for in vivo delivery (9, 10). This may also contribute to the preferential gene expression in tumor tissue we observed after systemic administration of the PEI/DNA vector, which agrees with recent observations that a PEI/DNA vector with a positive ζ potential is likely to predominantly mediate lung transduction, whereas formulations with near-neutral ζ potentials such as we used selectively transduce tumors (10). Furthermore, although PEI/DNA vectors usually become trapped in the pulmonary vascular beds (11) and transduce lung cells, the differential gene expression and PCR analysis suggest that our vector formulation mediates greater transduction of bladder tumor cells. Although this observation will require additional analysis, the differential transduction between normal cells and tumor cells that we observed in culture may contribute to this result by allowing more vector to pass through the lung. In addition, the marginally positive ζ potential may promote binding of the PEI/DNA vector to the negatively charged glycoproteins on the tumor cell surface and may also favor better interaction with integrins that can be increased in proliferating tumor cells, leading to endocytosis and increased gene delivery (8, 12, 13). These cellular components have been implicated in the uptake mechanisms of other PEI/DNA formulations (5).

Interestingly, our novel formulation is capable of efficient i.v. delivery to a tumor when just 6 μg of DNA is used. This low vector dose and low a:p ratio of 2.7:1 (three times lower than the commonly used 9:1 ratio) contribute to the lack of toxicity seen in our experiments. In contrast, a recent study using linear PEI (a version thought to be less toxic than the branched form) demonstrated efficient gene delivery only when doses over 50 μg were used, but this was always accompanied by high-level toxicity and animal death (14). Toxicity has also been observed when high molecular weight PEI (M, 800 Kda) has been used in PEI/DNA vector preparations (9, 10). In our study, M, 25 Kda PEI was used, and no toxicity was observed. Other groups using this size of PEI have observed cytotoxicity, which results from using high concentrations and doses well above those we have used for optimal transduction (8). We therefore postulate that the lack of toxicity in our study is related to the low overall doses of PEI and DNA used. It is also interesting to note that such a potent antitumor effect was obtained using the p53 gene. This is clearly attributable to the replacement of p53 expression in the tumor as determined by Western blotting and the increase in p21 expression that results from increased p53 expression (15). In addition, the restoration of wild-type p53 function restored normal cell cycle regulation and reestablished DNA repair and apoptotic mechanisms (15) as seen by increased apoptosis in the tumor. However, an antiangiogenic effect cannot be ruled out. The use of p53 tumor suppressor gene therapy is a valid approach in treating bladder cancer as mutations in the gene for p53 are one of the most common genetic abnormalities in bladder cancer and are a critical molecular determinant for the acquisition of an invasive phenotype (1, 2, 16). Although the success of this approach will certainly require transduction of a large number of tumor cells to completely inhibit tumor growth, recent observations of p53’s bystander effect by inhibiting angiogenesis may reduce this requirement (17). We are now in the process of determining the degree to which angiogenesis was affected by p53 expression in tumor samples from the PEI/DNA-p53-treated animals.

This is the first article documenting the ability of PEI to deliver a tumor suppressor gene such as p53 by an i.v. route to a distant tumor. Other groups have attempted to deliver the p53 gene to tumors by i.v. administration of liposome/DNA vectors but have had mixed results in that the vector either becomes trapped in the lung (18) or delivery to tumor cells is sufficient to produce a high therapeutic effect when combined with treatments such as radiation (19). The polycationic vector system described here promises to overcome some of the limitations of traditional viral and nonviral vectors (20) in that it is inexpensive, simple to prepare/administer, and can accommodate large plasmids containing multiple genes based on its self-assembling properties. In contrast to viruses, PEI should not elicit an immune response, allowing repeated administration to extend transgene expression. The low a:p ratio and dose of our formulation should also

Fig. 2. A, representative Western blot analysis for p53 and p21 expression in cell lysates prepared from UM-UC-3 orthotopic bladder cancer xenografts in animals treated with either PEI/DNA-β-gal (Lane 1) or PEI/DNA-p53 (Lanes 2–5). B, representative TUNEL analysis for apoptotic cells in UM-UC-3 orthotopic bladder cancer xenografts from mice treated with either (a) PEI/DNA-βgal or (b) PEI/DNA-p53. Magnification, ×100.
reduce toxicity that has been observed with repeated dosing of other vector formulations. In addition, unlike the heterogeneous pattern of gene delivery in a tumor after direct intratumoral injection of adenoviral vectors, systemic administration of our formulation should achieve more homogeneous gene distribution (7). Just as important, viral vectors for bladder and other cancer therapies.

REFERENCES


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