Efficient Gene Transfer with Adeno-associated Virus-based Plasmids Complexed to Cationic Liposomes for Gene Therapy of Human Prostate Cancer

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ABSTRACT

We have shown previously that treatment of rats bearing the Dunning R3327 MatLyLu prostatic tumor with human interleukin 2 (IL-2) gene-modified tumor cell preparations induces potent antitumor immunity in the animal. To test the clinical feasibility of using genetically modified tumor vaccines for the treatment of prostate cancer, we have explored the use of a simplified gene delivery system based on liposomes to introduce and express the IL-2 gene in the Dunning rat R3327 MatLyLu prostatic tumor cell line (MatLyLu) and in short-term cultures of primary human prostatic tumor cells. Liposome-DNA complexes containing the adeno-associated virus inverted terminal repeats exhibited 3-10-fold higher levels of gene transfer and IL-2 expression than did liposome complexes with non-adeno-associated virus containing plasmids. Single transfections resulted in IL-2 expression for an extended period of time that exceeded severalfold the amount of IL-2 secreted from retrovirally transduced MatLyLu cells. X-irradiation of cells (4000 rads) prior to transfection did not affect cytokine secretion, indicating that liposome-mediated gene transfer does not depend on cell proliferation. High levels of gene transfer and IL-2 expression were also achieved in short-term cultures of primary human prostatic tumor cells established from tumor specimens obtained following radical prostatectomy of cancer patients. Depending on the type of liposome used, IL-2 levels secreted from the human prostatic tumor cells were comparable to or exceeded the levels of IL-2 secreted from retrovirally transduced MatLyLu cells, which induced antitumor immunity in the rat model. The ability to culture and expand ex vivo human prostatic tumor cells, and the use of a simple and highly efficient gene transfer method to generate genetically modified tumor vaccines, set the stage for clinical exploration of gene-based immunotherapy of prostate cancer.

INTRODUCTION

Cancer of the prostate is the most frequently diagnosed cancer and the second leading cause of cancer-related deaths in Western civilization. The disease has an improved prognosis when discovered early, but most often the cancer has already spread, and there is no effective treatment at that stage (1). Progression of prostate cancer in humans is not accompanied by a vigorous antitumor immune response, although weak immune responses have been documented occasionally in some patients (2). Several attempts to actively immunize patients with adenocarcinoma of the prostate have shown little or no therapeutic benefit (3, 4). Thus, like most other human neoplasms, prostate cancer is largely a nonimmunogenic cancer.

Recent studies using rodent tumor models with little or no intrinsic immunogenicity have shown that genetically modified tumor cell preparations consisting of irradiated tumor cells transduced with and expressing cytokines such as IL-2, IL-6, IFN-γ, or GM-CSF were capable of inducing the regression of preexisting tumors and in some instances of curing animals of their disease (5-11). We have explored previously the use of cytokine-secreting tumor vaccines for the treatment of advanced prostate cancer in the nonimmunogenic Dunning rat R3327-MatLyLu (MatLyLu) prostatic tumor model (10). The MatLyLu line is an anaplastic androgen-independent tumor that metastasizes to the lymph nodes and the lung (12). This, and the fact that it does not exhibit any signs of intrinsic immunogenicity in the tumor-bearing rat (10), suggest that the MatLyLu tumor is an excellent model to explore immunotherapeutic protocols for the treatment of metastatic human prostatic cancer. We have shown that IL-2-secreting, irradiated, tumor cell preparations were able to cure all animals with s.c. established tumor and induce immunological memory that protected animals from subsequent tumor challenge. GM-CSF was less effective than IL-2 since only a fraction of animals was cured, and no immunological memory was observed. Immunotherapy was, however, less effective when tumors were induced orthotopically; nevertheless, it led to improved outcome and significantly delaying and occasionally preventing recurrence of tumors after resection of the cancerous prostate (10). The induction of a potent immune response in tumor-bearing animals against the nonimmunogenic MatLyLu tumor supports the view that active immunotherapy warrants further investigation as a potential therapeutic approach to prostate cancer.

One rate-limiting step in the development and use of genetically modified tumor vaccines for the treatment of patients with cancer of the prostate is the procurement of sufficient tumor tissue for vaccine preparation. Peelh (13) developed a method to culture and expand ex vivo prostatic tumor cells obtained from patients undergoing radical prostatectomies. Another approach to culture human prostatic tumor cells was developed by Roberson and Robertson and was used in our studies.

A second major hurdle facing the development of immunotherapeutic strategies utilizing genetically modified tumor vaccines is gene transfer into primary human tumor cells. The important attributes of a clinically useful gene transfer method are: (a) high efficiency of gene transfer into human tumor cells; (b) simplicity of the manufacturing process and use; (c) cost effectiveness; and (d) safety. Retroviral vectors have been used, thus far, the method of choice to stably and efficiently introduce genes into mammalian cells, including tumor cells. For example, Jaffee et al. (14) have used a murine retroviral vector to transduce over 50% of cells in short-term cultures established from a variety of human tumors and were able to express physiologically relevant levels of human GM-CSF. Likewise, Sando

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2 To whom requests for reprints should be addressed, at Duke University Medical Center, Box 3619, Durham, NC 27710.
et al. (15) have used the same retroviral vector system to introduce and express GM-CSF in primary cultures established from human prostatic tumors. The use of retroviral vectors to derive genetically modified tumor vaccines suffers, however, from several drawbacks: (a) the generation and manufacturing of retroviral vectors is complex and not inexpensive; (b) general safety concerns are associated with the use of any viral-based vector system, including replication defective murine retroviral vectors, a fact which adds to the complexity and cost of manufacturing; and (c) retroviral vectors currently used are able to transduce only dividing cells (16). This is perhaps the most serious drawback of using retroviral vectors, since the establishment of even short-term cultures of proliferating human tumor cells is often difficult or unattainable. Clearly, simplified methods to introduce and express cytokine genes in primary human tumor cells that are compatible with clinical procedures have to be developed. In this report, we summarize our ongoing efforts to develop efficient and simple methods to introduce genes into rat and human prostatic tumor cells using liposomes as vehicles for gene delivery.

Liposomes are noninfectious and nonimmunogenic and are therefore unlikely to have serious adverse effects on the patient, in particular in a vaccination setting where the tumor cell vaccines are treated with liposomes ex vivo. In addition, liposomes are simple to manufacture and use and are therefore cost effective. Liposomes, especially pH-sensitive liposomes, have been shown to efficiently encapsulate and deliver nucleic acids into cells (17). Recently, positively charged cationic liposomes were also shown to efficiently introduce genes into cells by forming complexes with the plasmid DNA in solution, facilitating fusion of the liposome DNA complex with the cell membrane (18, 19). In contrast to retroviral or AAV-based gene deliveries, liposome-based gene delivery (like other nonviral systems) is expected to result in transient expression of the heterologous gene due to the episomal nature of the transfected plasmid. In a recent report, Philip et al. (20) have shown that using plasmids containing the ITRs from AAV, the efficiency of cationic lipid-mediated gene transfer was augmented significantly, and long-term expression of the heterologous gene was observed in dividing and nondividing cells.

In this study we show that cationic liposome-mediated gene transfer of AAV-based plasmids results in efficient gene transfer and expression of human IL-2 in the rat MatLyLu prostatic tumor cell line and in short-term cultures established from human prostatic tumors.

**MATERIALS AND METHODS**

### Plasmids and Cell Lines

Table 1 shows the DNA constructs used in these studies.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Promoter</th>
<th>Gene</th>
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<tr>
<td>AAV/TK-LacZ</td>
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<td>LacZ*</td>
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<tr>
<td>pGEM/CVM-IL-2</td>
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</tbody>
</table>

* LacZ, E. coli β-galactosidase; IL-2, human interleukin 2; LTR, long terminal repeat, from Moloney murine leukemia virus; CMV, human cytomegalovirus, immediate/early promoter; SV40, simian virus 40, early promoter; hADA, human adenosine deaminase promoter; Neo, bacterial Tn5-derived neomycin phosphotransferase.

AAV/ADA-LacZ were derived by cloning the bacterial LacZ gene fused to the TK promoter or the human ADA promoter, into the AAV-derived psub201 vector (21).

#### Isolation and Culture of Human Primary Prostate Cancer Cells

Human prostatic epithelial cells were isolated and cultured from 1-cm³ surgical specimens obtained from patients undergoing radical prostatectomy. Prior to culture, an adjacent 5-mm section was stained with hematoxylin and eosin for determination of morphology by a certified pathologist. Briefly, prostate samples were minced into small fragments and digested with the use of a collagenase-hyaluronidase procedure that was optimized for dissociation of prostatic acini from stromal cells. Isolated cells were grown in a serum-free medium developed specifically for prostatic epithelial cells. Immunohistochemical characterization of prostatic cells was performed with a panel of mAbs to cytokeratins, prostate-specific antigen, and prostate acid phosphatase. More detailed information on ex vivo culture conditions of these cells will be published elsewhere.4

#### Liposome Preparations

The following liposome preparations were used in these studies: (a) lipofectamine reagent (GIBCO BRL, Gaithersburg, MD) is a polycationic lipid composed of a positively charged lipid, DOSPA, and the neutral lipid, DOPE, in a 3:1 molar ratio; (b) the cationic lipid, DDAB, used in combination with DOPE at 2:1 or 0.6:1.0 ratios (20); and (c) DMIHE in combination with DOPE in a 1:1 molar ratio (26), obtained from VICAL Corp. (San Diego, CA). Liposome reagents were stored at 4°C prior to transfection.

#### Cellular Transfection Procedure

R3327 MatLyLu and human primary prostatic adenocarcinoma cells were plated on 60-mm dishes at a density of 5 x 10⁵ cells/dish. Various amounts of cationic liposomes (5–20 μl) and plasmid DNA (5–20 μg) were diluted in serum-free medium to a total volume of 100 μl and then gently admixed in polystyrene tubes. The solution was then allowed to form complexes at room temperature for 15 min. Cells were then washed twice with serum-free medium. The DNA-liposome complex was diluted in serum-free media and gently added to the adherent monolayers. After an incubation period of 60 min, another 2 ml of medium were added to the cells. For MatLyLu cells, FCS was added to yield a final concentration of 10%. Twenty-four h following transfection, or as indicated, supernatant was collected and IL-2 levels were measured with the use of ELISA (R&D Systems, Inc., Minneapolis, MN).

Cellular transfection efficiency of lacZ-containing vectors was measured by FDG staining with the use of flow cytometry (FACStar; Becton Dickinson, San Jose, CA). Briefly, cells from exponentially growing cultures were harvested by trypsinization. Single cell suspensions were washed in PBS and brought to a

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5 R. Philip et al., manuscript in preparation.
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A. Bacterial plasmid

Fig. 1. AAV and non-AAV expression plasmids. A, a non-AAV expression plasmid consists of a eukaryotic promoter fused to a heterologous "gene" closed into a plasmid capable of replication in bacteria. Not shown, the gene (in fact, a cDNA) also contains a polyadenylation signal and (not always) an intron which can be situated 5' or 3' to the coding sequence. B, an AAV-based expression plasmid is derived from AAV by replacing the viral genes rep (Rep) and capsid with a heterologous gene. The AAV-based expression plasmid, therefore, differs from the non-AAV expression plasmid by the presence of the two viral inverted terminal repeats and some adjacent viral sequences (thick bars), which presumably play no functional role but may have some adverse effects on the expression of the heterologous genes.

RESULTS

Liposome-mediated Gene Transfer in Rat MatLyLu Prostatic Tumor Cells. Table 1 describes the DNA constructs used in these studies. We tested human IL-2, Escherichia coli lacZ, and Tn5 transposon neo-containing plasmids, the expression of which was driven by various promoters as indicated. One group of expression cassettes was contained within AAV vectors as described in "Materials and Methods." A schematic diagram of the structure of AAV and non-AAV-based vectors is shown in Fig. 1.

The efficiency of short- (transient) and long-term (stable) gene transfer was tested with the use of lacZ- and neo-containing plasmids, respectively. Various DNA constructs were mixed with a liposome preparation consisting of DOSPA/DOPE (3:1) and applied to monolayer cultures of MatLyLu cells as described in "Materials and Methods." The efficiency of transient gene transfer was determined by counting the number of cells expressing lacZ 48 h posttransfection (Fig. 2A). We have observed consistently that transfection with AAV-based plasmids was significantly more efficient than gene transfer with non-AAV-based plasmids. As shown in Fig. 2A, up to 78% of cells became lacZ-positive following transfection with the two AAV-based plasmids, AAV/TK-lacZ and AAV/ADA-lacZ, compared to 5% or less lacZ-positive cells obtained following transfection with the 2 non-AAV based plasmids, pJ3/lacZ and E2A/lacZ. For comparison, 2 days following infection with a high titer lacZ-containing retroviral vector, 17% of cells expressed lacZ, and over 85% of a retrovirally transduced FDG-selected clone were lacZ positive in this assay. Similarly, stable gene transfer, as measured by the appearance of G418-resistant colonies 2-3 weeks following transfection of neo-containing plasmids, was markedly increased in cells transfected with the AAV-based plasmid pAlTNeo compared to the non-AAV-based plasmids, N2A and pSVNeo (Fig. 2B).

Next, we compared the transfection efficiency of several liposome preparations using an AAV-based IL-2 expression plasmid, pSSV9/CMV-IL-2 (20). MatLyLu cells were irradiated 24 h after transfection, and IL-2 secretion into the medium over a period of 24 h was determined with the use of an ELISA assay 48 h after irradiation. As shown in Fig. 3A, DOSPA-based liposomes [DOSPA/DOPE (3:1 or 1:1)] were superior to other liposomal preparations [DMRIE/DOPE (1:1) or DDB/DOPE (1:2 and 1:1)]. Fig. 3B shows the levels of IL-2 secreted from clonal cell lines infected with various IL-2-containing retroviral vectors that have been used in previous studies and have shown therapeutic benefit in animal tumor models (8, 10, 11). Comparison of Fig. 3, A and B, shows that more IL-2 was secreted from

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* Unpublished data.
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A. Transient - Liposomes

![Graph showing comparison between transient transfection with various liposomes and retroviral transduction]

B. Stable - Retroviral Vectors

![Graph showing comparison between retroviral transduction with various vectors and transient transfection]

Fig. 3. Comparison between MatLyLu cells transduced transiently with various liposome preparations and retrovirally transduced clonal isolates. A, cells were transfected with various liposomes complexed with the AAV-based pSSV9/CMV-IL-2 plasmid at optimal liposome/DNA ratio. Cells were irradiated 24 h following transfection, and IL-2 secretion into the medium was determined during a period of 24 h. Transfections were carried out in duplicate cultures. SD, <5%. I, DDAB/DOPE (1:2); II, DDAB/DOPE (1:1); III, DOSPA/DOPE (3:1); IV, DOSPA/DOPE (1:1); V, DMRIE/DOPE (1:1). B, retrovirally transduced MatLyLu clones with various IL-2 gene-containing retroviral vectors described previously (8, 10). VI, DC/AD/R/IL-2; VII, DC/TK/IL-2; VIII, N2/IL-2. Cells were irradiated, and IL-2 secretion in the medium was determined 24 h later.

To determine the optimal conditions for liposomal transfection of the AAV-based IL-2 expression plasmid pSSV9/CMV-IL-2, DNA and liposome concentrations were varied. Optimal conditions for IL-2 gene transfer and expression were found to be 2-5 µg of plasmid DNA and 10 µl of DOSPA/DOPE (3:1)/ml reaction for 5 x 10⁵ cells (Fig. 4, A and B). To test whether liposomes can mediate gene transfer into nondoning cells, transfection efficiency was measured before or following irradiation of MatLyLu cells. No difference in transduction efficiency of pSSV9/CMV-IL-2 plasmid was noted whether the cells had or had not been irradiated (data not shown).

The AAV-based IL-2 expression plasmid pSSV9/CMV-IL-2 contains the 5' portion of the viral rep gene (Fig. 5A), which other studies have suggested could be inhibitory to the expression of heterologous genes encoded in AAV-based vectors (27, 28). We therefore tested another AAV-based IL-2 expression plasmid, pMP6-IL-2. As shown in Fig. 5A, all rep-coding sequences have been deleted in pMP6 plasmid. In addition, the CMV-driven IL-2 expression cassette in pMP6-IL-2 contains a different polyadenylic acid sequence, and the intron (which was also different) precedes the IL-2-coding sequences.

The transfection efficiency of the two AAV-based IL-2 expression plasmids was tested in the presence of DOSPA/DOPE (3:1). For comparison, a non-AAV-based plasmid was also included. As shown in Fig. 5B, 3-fold higher levels of IL-2 were secreted from cells transfected with the rep-negative pMP6-IL2 plasmid at optimal DNA concentrations. These IL-2 levels exceeded the levels of IL-2 secreted from a clonal population of retrovirally transduced cells.

To determine the length of time IL-2 is secreted from irradiated MatLyLu cells transfected with the pMP6-IL-2 plasmid in the presence of DOSPA/DOPE (3:1), secretion of IL-2 was assayed at various times after irradiation of the transfected cells, as shown in Fig. 6. IL-2 secretion is lost rapidly in nonirradiated cultures (Fig. 6A) at a rate that is inversely proportional to the rate of cell proliferation (Fig. 6B), most certainly reflecting the transient nature of the gene transfer.

Fig. 4. Effect of liposome and DNA concentration on IL-2 expression in MayLyLu cells. A, MatLyLu cells were transfected with DOSPA/DOPE (3:1; □) or DMRIE/DOPE (1:1; ○) complexed to various concentrations of pSSV9/CMV-IL-2 plasmid. B, cells were transfected with various concentrations of DOSPA/DOPE (3:1) complexed to 1 (▲), 4 (●), or 10 (■) µg pSSV9/CMV-IL-2 DNA.
Gene Transfer and IL-2 Expression in Primary Human Prostatic Tumor Cells. Having determined the optimal conditions for liposome-mediated gene transfer and expression of IL-2 in an established rat-derived prostatic tumor cell line, we sought to determine whether this simple gene transfer method can be used to introduce and express the IL-2 gene in human prostatic tumor cells. Short-term cultures of prostatic tumor cells were established from patients undergoing radical prostatectomy.\textsuperscript{4} Fig. 7 shows the amount of IL-2 secreted from human prostatic tumor cells obtained from a patient with Gleason grade 3 disease following transfection with the pMP6-IL-2 plasmid and DOSPA/DOPE (3:1). At optimum DNA concentration, the amount of IL-2 secreted from the primary human tumor cells was comparable to the amount of IL-2 secreted from retrovirally transduced MatLyLu cells. Primary human prostatic tumor cultures frequently grew in patches and were not dispersed readily into single cell suspensions. Consequently, we were unable to accurately determine IL-2 expression per cell. We have therefore carried out the transfections when cell confluency was between 30–60\%, which corresponded to approximately 2–3 × 10\(^5\) cells/60-mm plate. Table 2 shows the results of 6 consecutive transfection experiments performed with specimen obtained during a 3-week period. The ages of the patients from which the samples were derived ranged from 63 to 71 years, and cells were cultured between 23 and 91 days before transfection. Efficient delivery and expression of IL-2 was achieved in 5 of the 6 short-term cultures obtained from patients with Gleason 3 or 4 disease.

Next, we compared several liposome formulations in primary human prostatic tumor cultures. As shown in Fig. 8, while DDAB/DOPE (0.6:1.0) liposomes were inferior to DOSPA/DOPE (3:1) liposomes in promoting IL-2 gene transfer and expression, DMRIE/DOPE (1:1) formulations were superior to DOSPA/DOPE (3:1) at all liposome/DNA ratios tested. This observation is in stark contrast with the finding that in the rat-derived MatLyLu cells, DOSPA/DOPE formulations were consistently superior to DMRIE/DOPE (Fig. 3). Thus, in primary human prostatic tumor cells we were also able to achieve levels of IL-2 secretion that exceeded the levels of IL-2 secreted from a retrovirally transduced MatLyLu clone.

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DISCUSSION

Gene delivery into nonimmortalized human tumor cells is a rate-limiting step in the development of genetically modified tumor vaccines. In this study we have shown that liposome-mediated gene transfer is a simple and highly efficient method to introduce and express genes in culture-established rat prostatic tumor cells, as well as in primary cultures of human prostatic tumor cells derived from tumor specimens obtained during radical prostatectomies of patients with prostate cancer.

We found that incorporation of AAV ITRs into expression plasmids significantly enhances the transfection efficiency in MatLyLu cells (Fig. 2). This observation confirms and extends a previous observation reported by Philip et al. (20). While the mechanism underlying the AAV ITR-mediated stimulation of gene transfer is not known, it is tempting to speculate that the presence of the ITRs facilitates the nuclear accumulation of the transfected plasmid. Liposomal gene transfer of the AAV-based IL-2 expression plasmid pSSV9/CMV-IL-2 into MatLyLu cells resulted in high levels of IL-2 expression that were comparable to the amount of IL-2 expressed from retrovirally transduced cells (Fig. 3). This is potentially significant because the retrovirally transduced IL-2-secreting cells were shown previously to have significant therapeutic effect in the treatment of tumorbearing animals (10). With the use of a new and improved AAV vector design, pMP6, even higher levels of IL-2 expression were achieved that exceeded 3–10-fold the amount of IL-2 expressed from retrovirally transduced cells (Fig. 5). The fact that growth-arrested cells can be transduced efficiently with liposomes, as indicated by the fact that irradiated cells are susceptible to liposomal gene transfer, contrasts with murine retroviral vectors, which are able to introduce genes only into dividing cells (16). Consequently, liposomal gene transfer will not necessitate the establishment of ex vivo tumor cultures and, therefore, will significantly extend the use of genetically modified tumor vaccines to a wide range of cancers.

An important feature of retroviral gene transfer is that it leads to stable integration and continuous expression of the foreign gene in the cell. In contrast, the liposome-transfected genes exist in the cell in a nonintegrated form as episomes and are lost when cells proliferate. However, when cells growth was arrested by irradiation shortly after transduction with liposomes, the transected IL-2 gene continued to be expressed at high levels for at least 15 days (Fig. 6). This will be more than adequate for vaccination purposes because the genetically modified, irradiated tumor vaccines administered to the patient are expected to persist for a similar or lesser period of time before being eliminated by an inflammatory reaction and/or an immune response.

The ability to efficiently transfet primary human prostatic tumor cells with heterologous genes is an important step toward implementing a gene-based immunological treatment of prostatic cancer. Liposomal gene transfer was found to be very efficient when applied to primary human prostatic cells (Figs. 7 and 8; Table 2). Unlike rat MatLyLu prostatic cells, in which DOSPA/DOPE-based liposomes were most effective in gene transfer and IL-2 expression, in primary human prostatic tumor culture DMRIE/DOPE (3:1) formulations led to the highest levels of IL-2 expression, which exceeded the amount of IL-2 expressed from retrovirally transduced MatLyLu cells (Fig. 8).

There is no reason to believe that prostatic tumor cells of rat or human origin are unique in their susceptibility to liposomal gene transfer. Indeed liposomes were found to be equally effective in

Table 2 IL-2 secretion from human prostate cancer cells transfected with DOSPA/DOPE (3:1)-pMP6-IL-2 plasmid complexes

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>IL-2 [ng/24 h]</th>
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* A confluent 60-mm plate of MatLyLu cells contains 1–2 x 10⁶ cells.
introducing and expressing the IL-2 gene in the murine MBT-2 bladder tumor cell line. Moreover, in preliminary studies liposomes were consistently effective in transducing primary human tumor cells obtained from patients with ovarian, breast, and lung cancer and melanoma. The fact that ovarian, breast, and lung cancer cells were obtained from pleural effusions and did not proliferate ex vivo underscores the ability of liposomal gene delivery system to access nondividing cells that are refractory to retrovirus-mediated gene transfer.

In summary, high efficiency of gene transfer, independence from cell division, persistence of expression in growth-arrested cells, together with simplicity, cost effectiveness, and minimal safety concerns associated with this procedure, suggest that liposome-mediated gene transfer may be a superior method to derive genetically modified tumor vaccines.

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