Gene Modification of Primary Tumor Cells for Active Immunotherapy of Human Breast and Ovarian Cancer

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INTRODUCTION

The role of soluble factors to augment antitumor immune responses has been well recognized in recent years (1, 2). Since most immunomodulators act as local hormones, it was thought that local delivery of these cytokines might have a more pronounced therapeutic benefit (3, 4). The failure to demonstrate any significant immunological response by repeated injection of cytokines at the tumor site initiated the search for an alternate cytokine delivery system which would enable a potent antitumor immune response. Recent studies using animal models have shown that genetically modified tumor cells expressing cytokines such as IL-2, IL-4, IL-6, IFN-γ, or granulocyte-macrophage colony-stimulating factor were capable of inducing an immune response against preexisting tumors (5–13). It has been shown that localized secretions of IL-2 from genetically modified tumor cells mediated tumor rejection and induced immunological memory that protected animals from subsequent tumor challenge (10). The apparent success of using genetically modified tumor cells to induce protective immunity in animal studies has inspired interest in using active immunotherapy strategies in cancer patients.

The development of cytokine gene-modified tumor vaccine strategies for human breast or ovarian cancer therapy has warranted a number of technical advances. The major obstacle is the development of a simple and efficient gene transfer method for use on noncultured primary human tumor cells. Among the existing gene delivery methods for stable or transient expression, retroviral vectors have thus far been widely used to genetically modify tumor cells for immunization studies (14, 15). Despite extensive progress, the retroviral vectors used to deliver gene-modified tumor vaccines suffer from low-expression efficiency and safety concerns (16). The most serious limitation of this vector system is that retroviruses require actively dividing cells for gene expression, which is often difficult or unattainable in breast or ovarian tumor specimens.

We have previously shown that nonviral gene delivery using cationic liposomes can result in efficient gene transfer and high-level transient expression in dividing and nondividing cell types (17). In our recent studies, we have demonstrated that AAV-based plasmids complexed to cationic liposomes achieved not only efficient gene delivery, but also sustained high-level gene expression in primary cell types (18). When combined with cationic liposomes, plasmids containing the AAV ITRs caused
5–10-fold higher expression than plasmids without AAV ITRs. In the present study, cationic liposomes were used to deliver a modified AAV plasmid into freshly isolated breast and ovarian tumor cells. In addition, we demonstrated that the transfected and irradiated primary tumor cells maintain a high level of gene expression.

MATERIALS AND METHODS

Plasmid Constructs. All noncompatible restriction ends were filled in with T4 DNA polymerase before ligation. The CMV immediate early promoter was isolated as an Apal(5') and BamHI(3') fragment by PCR using primers with overhanging restriction sites and cloned into the BamHI-Apal sites of blue-scrip fast KSII+ (Strategene, La Jolla, CA) to give pBSKSII-CMV. This clone contains sequences 173,560–174,328 of human CMV (EMBL-Genbank Accession no. X17403). pOG44 (Strategene) was digested with EcoRI and PstI to give a fragment containing the adenovirus major late and mouse immunoglobulin-intervening sequence (19), which was cloned into the BamHI site in pBSKSII-CMV to give pBSKSII-CMVI. The SV40 polyadenylation signal sequences were obtained by digesting pREP4 (Invitrogen, San Diego, CA) with Xbal and Asp718 and cloned into the Xbal site in pBSKSII-CMVI to yield pMP1.

The complete AAV genome was cloned into a pBR-based vector by Laughlin et al. (20) and was obtained through ATCC (catalogue no. 37216). In this clone, the AAV sequences are flanked by BglII sites. This clone was digested with Ncol, and the larger fragment was purified. A polynucleotide with overhanging Ncol ends was made by annealing two oligonucleotides (CATGGCTGAGTCTAGAGAGCTC\_CCAAGCTI'GGTCTAGAGAGCTC\_CCATGC\_CATTAGC\_GCTGAGTC\_GTATCTAGAC\_GCTGAGTC\_CCATG\_GCTGAGTC\_GCTGAGTC\_CCAT) and cloned at the Ncol site to yield pAl. This clone has 625 bases of the 5' end consisting of left terminal repeat, P5 promoter, and the first 304 bases of the rep 78/68-coding sequence. This clone also has 190 bases of the 3' end of the AAV consisting of left terminal repeat and 45 bases adjoining. Since the pBR origin of replication produces very low plasmid copy numbers, the AAV portion of pAl was transferred into the high copy number bluescript plasmid, pBSAP1, which had been digested with Nhel and BamHI to produce pMP1L-2 and pMP6L-2, respectively. The chloramphenicol acetyl transferase-coding sequence obtained by digesting pcDNA3-CAT (Invitrogen) with HindIII was cloned into the Nhel site in pMP1 and pMP6.

Liposome Preparation. Small unilamellar liposomes were prepared with the cationic lipid DDAB (Sigma, St. Louis, MO) in combination with the neutral lipid DOPE (Avanti Polar Lipids, Alabaster, AL) and Chol (Calbiochem, La Jolla, CA). Lipids were dissolved in chloroform. DDAB was mixed with DOPE in a 1:1 molar ratio to produce DDAB:DOPE. To produce DDAB:Chol:DOPE, equivalent molar amounts of all three lipids were mixed. The lipid mixtures were dried on a rotary evaporator. The lipid film was then rehydrated by adding sterile double-distilled water to yield a final concentration of 1 mM DDAB. This solution was sonicated with a probe sonifier (Branson, Henrietta, NY) until clear. Liposomes were stored at 4°C under argon.

Tumor Cell Lines and Primary Tumor Isolation. The human breast adenocarcinoma cell line MCF-7 was obtained from ATCC. The cell line was maintained in DMEM supplemented with 10% FBS and incubated at 7% CO₂.

Tumor samples, as effusions from breast and ovarian cancer patients, were obtained with informed consent. Pleural or peritoneal effusions were either T-cell depleted using the MicroCELLector-CD5/8 flasks (AIS, Santa Clara, CA) or separated by differential Ficoll sedimentation. For the differential Ficoll separation, cells were layered over a discontinuous gradient of 100% IsoPrep (Robbins Scientific, Sunnyvale, CA) and 75% IsoPrep and centrifuged for 20 min at 2000 rpm. After centrifugation, the tumor cells were removed from the top of the 75% gradient. Tumor cell preparations were stained with anti-CD3 antibody (Becton Dickinson, San Jose, CA), and flow cytometric analysis was performed using a FACScan (Becton Dickinson) to assess lymphocyte contamination. Cells were transfected on the same day of isolation or after 2–4 days in culture in AIM V.

Transfection. MCF-7 cells were harvested and counted immediately before transfection. Cells were plated at 10⁶ cells/100-mm tissue culture dish in 2 ml serum-free DMEM. Ten µg plasmid DNA were mixed with either 20 (DDAB:DOPE) or 30 nmol (DDAB:Chol:DOPE) total lipid. One ml serum-free DMEM was added to the liposome-DNA complex, and then the complex was added to the cells. After incubating the cells at room temperature for 15 min, 3 ml DMEM containing 20% FBS were added to each plate.

Primary breast and ovarian tumor cells were transfected as described above, but transfections were performed in AIM V media. Where irradiation is indicated, transfected cells were γ-irradiated (10,000 rads) the day after transfection.

IL-2 Assay. At various time points after transfection, supernatants were collected and IL-2 was quantitated using a Quantikine IL-2 ELISA kit from R & D Systems (Minneapolis, MN). IL-2 levels are expressed as pg/10⁶ cells/24 h.

Southern Blot Analysis. Genomic DNA was extracted from cells using standard methods. After digestion with PvuII/HindII, 1 µg DNA was loaded onto a 1% agarose gel, electrophoresed, and transferred to a Hybrid Bond N+ nylon membrane (Amersham, Arlington Heights, IL). The membranes were hybridized with an IL-2 gene fragment labeled with ³²P by random priming (Megaprime DNA labeling kit; Amersham) at 65°C in rapid hybridization buffer (Amersham) and washed according to the manufacturer’s instructions. Autoradiograms of these filters.
were exposed on X-ray film (type XAR; Eastman Kodak Co., Rochester, NY).

**Intracellular IL-2 Staining and Flow Analysis.** Transfected cells were stained for intracellular IL-2 protein levels by a modified flow cytometry procedure (23). For 16 h immediately prior to harvesting transfected cells for intracellular IL-2 determination, cells were incubated in culture medium containing 3 μM monensin. Cells were washed in azide buffer and stained for 20 min at 4°C with a cocktail of monoclonal antibodies containing 260F9, 317G5, 520C9, 741F8 (ATCC, Rockville, MD), BT4Z4.1, and BT8IF1.5 (kindly provided by Dr. Robert Bast, M.D. Anderson, Houston, TX) that were raised against human breast cancer cell lines. After washing with standard azide buffer, cells were stained with an R-PE-conjugated goat antimouse F(ab')2 fragment second-step antibody (Caltag, South San Francisco, CA) for 20 min at 4°C. Cells were washed in standard azide buffer and resuspended in 50% FBS-PBS. Ice-cold 70% ethanol was added to the cells with vortexing. After incubation at 4°C for at least 30 min, the cells were washed with standard azide buffer and aliquoted into staining tubes. Nonspecific antibody binding was blocked by incubation with rabbit IgG (Zymed, South San Francisco, CA) for 30 min at room temperature. The cells were washed, then stained with FITC-conjugated antihuman IL-2 antibody (R & D Systems) for 20 min at 4°C. After washing with standard azide buffer, the cells were resuspended and analyzed by flow cytometry.

**RESULTS**

**IL-2 Gene Transfer into a Breast Tumor Cell Line and Primary Breast and Ovarian Tumor Cells.** Fig. 1 illustrates the plasmid DNA constructs used in these studies. Both pMPIIL2 and pMP6IL2 plasmids contain the CMV immediate early promoter sequence and a hybrid intron followed by the IL-2 cDNA. In addition to this expression cassette, pMP6IL2 contains the identical sequences in addition to AAV ITRs at both ends of the expression cassette. pMPIIL2 contains both the right and left AAV ITRs. pMPIIL2 contains an identical expression cassette but lacks the AAV ITRs. Transfections were performed with both pMPIIL2 and pMP6IL2 on a breast tumor cell line, MCF-7, and IL-2 expression was compared. Transfections were achieved by treating cells with a complex composed of plasmid DNA and cationic liposomes in a ratio of 1 μg DNA to 1 nM DDAB. Two different cationic liposomes were tested, DDAB:DOPE and DDAB:Chol:DOPE. Transfected cells were irradiated 24 h after transfection, and supernatants were collected at various time points and assayed for IL-2 using an ELISA. IL-2 expression was consistently higher using the AAV plasmid pMP6IL2 compared to that achieved with the non-AAV plasmid pMPIIL2 (Fig. 2). Although significant levels of IL-2 were produced by MCF-7 after transfection with pMPIIL2, the levels produced by cells transfected with pMP6IL2 were 3–5-fold higher at every time point, regardless of the liposome composition used. The optimal liposome-mediated gene transfer conditions, as determined in this cell line, were applied to determine the ability to transfect freshly isolated human breast and ovarian tumor cells obtained from pleural and peritoneal effusions. The effusion samples were enriched for tumor cells by depleting T lymphocytes using either AIS MicroCELLector-CD5/8 flask or differential Ficoll gradient centrifugation. On average, 86 ± 14% (n = 5) of the T lymphocytes were depleted from the tumor samples as assessed by CD3 monoclonal antibody staining. This enriched population contained 80–90% tumor cells as assessed by light microscopy, flow cytometry, and immunohistochemical staining for oncogene expression (data not shown). The tumor cells were transfected with both the AAV (pMP6IL2) and non-AAV (pMPIIL2) plasmid using both DDAB:DOPE and DDAB:Chol:DOPE liposomes. The cells were irradiated 24 h after transfection, and culture supernatants were tested for IL-2 using an ELISA. Fig. 2b illustrates the data from one representative ovarian transfection experiment. IL-2 expression was significantly higher when pMP6IL2 was used for transfection, regardless of the liposomes used. Similar results were obtained from three breast and five ovarian tumor samples. In both the ovarian and breast primary tumor cells, the expression levels induced by pMPIIL2 were less than 10 pg/10⁶ cells/24 h as opposed to 1000–3000 pg/10⁶ cells/24 h when pMP6IL2 was used. High-level IL-2 expression was observed for up to 10 days after transfection with the AAV plasmid regardless of the liposome composition. Similar results were obtained using Lipofectin and LipofectAMINE (GIBCO-BRL, Gaithersburg, MD; data not shown).
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Fig. 2: IL-2 plasmid constructs with and without AAV ITRs complexed to either DDAB:DOPE (DD:D) or DDAB:Chol:DOPE (DD:C:D) liposomes were compared for their ability to induce gene expression in breast cancer cell line MCF7 (a) or a primary ovarian tumor sample (b). Cells were transfected with plasmid DNA complexed to cationic liposomes at a ratio of 1 µg DNA:1 nm DDAB. The transfected cells were γ-irradiated (10,000 rads) 24 h after transfection. Supernatants were collected after a 24-h media change at various time points and assessed for IL-2 levels using an ELISA. In a, each column represents the mean of three experiments; bars, SD.

Southern Blot Analysis of AAV- and Non-AAV-transfected Primary Tumor Cells. To verify the delivery of the AAV and non-AAV plasmids to cells, Southern blot analysis of genomic DNA from transfected primary tumor cells was performed. Primary breast tumor cells were transfected with either pMP1IL-2 or pMP6IL-2 complexed to DDAB:DOPE liposomes. Seven days after transfection, genomic DNA was prepared from the transfected cells, and Southern blot analysis was
Transfection Efficiency of AAV Plasmids. To determine the AAV plasmid transfection efficiency, individual cells were measured in a CTLL-2 bioassay. The MCF7 tumor cell line was transfected using pMP6IL-2 complexed to DDAB:DOPE liposomes. Transfected cells were treated with monensin 48 h after transfection for 16 h. The cells were then fixed, permeabilized, and treated with FITC-conjugated anti-IL-2 antibody and analyzed by flow cytometry. To identify the tumor cells that expressed IL-2, the transfected cells were also stained with a cocktail of six antibodies that are specific for breast tumor antigens present on a subpopulation of breast tumor cells. As shown in Fig. 6, approximately 50% of the MCF7 and 40% of the primary tumor cells were positive for intracellular IL-2. Although there was a 10-fold difference in IL-2 levels between MCF7 and primary tumor cells, the percentage of cells expressing IL-2 as assessed by intracellular immunostaining was comparable for both cell types. The breast cancer antibody cocktail stained only 40% of MCF7 and 20% of the primary breast tumor cell preparation. Although 40% of the total primary tumor cell preparation expressed IL-2, only 15% was positive for both IL-2 and breast cancer antigens. In comparison, 35% of the MCF7 cells were positive for breast tumor antigens and expressed IL-2. Although the antibody cocktail did not label all of the tumor cells in the sample, double-stained cells provide evidence that approximately 15% of the tumor cells were expressing IL-2.

DISCUSSION

Previous animal studies indicate a potential protective immune response can be generated in vivo using cytokine gene-modified tumor cells (5–12). Local secretion of cytokines such as IL-2 from genetically modified tumors mediated rejection and immunological memory (10). Active immunotherapy of certain human cancers using cytokine gene modification of primary tumor cells is currently being tested in clinical trials. Therefore, it is critical to genetically modify the primary human tumor cells to perform these studies. Although tumor cells gene-modified with the use of retroviral vectors can express the appropriate cytokine at levels shown to induce protective immunity in animal models (10), the retroviral vectors can only transduce dividing cells, a requirement that has made the genetic
modification of many primary human tumors difficult, if not unfeasible. One alternative to the technical difficulty in genetically modifying tumor cells has been to generate fibroblasts expressing cytokine genes and mixing these with unmodified tumor cells for vaccination (24). Another obvious alternative is to explore other methods of gene delivery to human tumor cells.

An ideal alternative method to cytokine gene delivery to human tumor cells would be safe, simple to use, and lead to levels of cytokine gene expression in lethally irradiated cells sufficient to induce systemic immunity. Among the alternative viral vector systems, recombinant adenovirus and pox virus delivery is currently hindered by significant immune responses to the expressed viral proteins. Another alternative, recombinant AAV, has been considered because it has proven to be efficient in infecting both dividing and nondividing cell types (25, 26). In addition, AAV has the ability to accommodate introns and

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**Fig. 4** Duration of IL-2 gene expression induced by AAV plasmid complexed to cationic liposomes was assessed in both the MCF7 cell line (a) and primary breast tumor samples (b). The cells were transfected using pMP6IL-2 plasmid DNA complexed to DDAB:DOPE liposomes at a ratio of 1 µg DNA:1 nM DDAB. The transfected cells were γ-irradiated at 10,000 rads 24 h after transfection. IL-2 secretion over a period of 24 h was measured at various time points up to 28 days. Columns, means of three experiments; bars, SD.
Fig. 5 To determine the effect of irradiation on long-term IL-2 gene expression, IL-2 secretion from the irradiated and nonirradiated transfected MCF7 cell line (a) and primary breast and ovarian tumor cells (b) was determined. All cells were transfected with pMP6IL-2 plasmid DNA complexed to DDAB:DOPE liposomes. One half of the transfected cells were γ-irradiated at 10,000 rads 24 h after transfection. Irradiated and nonirradiated cells were maintained in culture in parallel. Supernatants were collected at various time points and assessed for IL-2 levels using an ELISA. In a, each column represents the mean of three experiments; bars, SD.

expresses far fewer viral proteins, minimizing the AAV-specific immune response that may be elicited. Although AAV vectors are considered safe, their production requires a complex coinfection with adenovirus that must then be purified, which has resulted in relatively low titers of the AAV vector, with the possibility that contaminating adenovirus is present. Therefore, we considered a simple, efficient, and safe transfection system incorporating an AAV plasmid that delivered with the use of carrier molecules such as liposomes.

Cationic liposomes have been shown to mediate transient expression of plasmid DNA in mammalian cell types (27, 28). The levels of IL-2 expressed by the non-AAV plasmids did not
Efficiency of transfection was assessed by intracellular IL-2 immunostaining and flow cytometric analysis. MCF7 and primary breast tumor cells were transfected with pMP6IL-2 complexed to DDAB:DOPE liposomes. Three days after transfection, the cells were immunostained with antibodies against breast cancer antigens, then permeabilized and stained with a human IL-2 antibody. 

**Fig. 6** Efficiency of transfection was assessed by intracellular IL-2 immunostaining and flow cytometric analysis. MCF7 and primary breast tumor cells were transfected with pMP6IL-2 complexed to DDAB:DOPE liposomes. Three days after transfection, the cells were immunostained with antibodies against breast cancer antigens, then permeabilized and stained with a human IL-2 antibody. 

Columns, means of three experiments; bars, SD.
reach the levels that had been shown to induce protective immunity in animal models (10). The use of the AAV plasmids overcomes the low expression levels that have previously limited the use of cationic liposomes for transfection. As an example, a direct comparison was made between a non-AAV plasmid and an AAV plasmid. Using an AAV-plasmid, the level of expression was 3–10-fold higher, and the expression lasted for more than 25 days, both significantly greater than levels and duration of expression with the non-AAV plasmid. Because human gene-modified tumor immunotherapy studies utilize lethally irradiated tumor cells as in vivo stimulants, the adequacy of cytokine expression must be tested following lethal irradiation. Irradiation of AAV plasmid-transfected cells with 10,000 rads did not alter the level and duration of expression of cytokine expression in either cell lines and primary tumor cells, although the dose of irradiation was sufficient to inhibit cell division (22).

Finally, the most stringent assessment of the adequacy of cytokine gene expression from lethally irradiated cells must be measured by its ability to induce systemic antitumor immunity and protection from disease progression in tumor-bearing animals. Lipofection of AAV-based plasmids demonstrated levels of expression that should induce protective immunity, as expression of IL-2 from AAV plasmid-transfected tumor cells was either comparable or surpassed the levels of IL-2 from retrovirally transduced cells (data not shown) that were known to induce protective immunity in animal models. Furthermore, a direct comparison of active immunotherapy with either retroviral-transduced cells or AAV plasmid-transfected cells in a murine model of metastatic breast cancer demonstrated that the AAV plasmid-transfected cells induced systemic immunity that resulted in inhibition of pulmonary metastasis in tumor-bearing mice. 3

In this report, we have demonstrated the ability of AAV plasmid with cationic liposomes to transfect primary tumor cells isolated from pleural and peritoneal effusions, although this same process can be applied to transfect primary tumor cells isolated from solid tumors, as previously described (18). Pleural and peritoneal effusions include lymphocytes, tumor cells, and other cell types. We have successfully enriched tumor cells by depleting the lymphocyte population using the AIS MicroCEL-Lector CD5/CD8 devices or differential Ficoll. Since liposome-mediated gene transfer is efficient in transfecting a wide variety of cell types, the efficiency of tumor cell transfection was assessed by flow cytometry after double staining with IL-2 and breast antigens. Although the final tumor preparation contains other contaminating cell populations, at least 50% of the cells that were positive for breast cancer antigens were also positive for IL-2 as assessed by the double staining, which indicates that tumor cells are indeed transfected and expressing IL-2. In addition, the IL-2 produced as a result of liposome-mediated AAV plasmid transfection of both cell lines and primary tumor cells was shown to have biological activity in a CTL-L-2 proliferation assay.

Primary tumor cells in general are poor transgene expressors, indicating a defect or restriction in transcriptional and/or translational regulation. Thus, it is important to design a plasmid construct that would induce transgene expression in uncultured tumor cells. Although both the AAV and non-AAV plasmids induced high-level IL-2 gene expression in the MCF7 cell line, only the AAV plasmid caused a significant level of expression in primary tumor cells. We demonstrated by Southern blot analysis of genomic DNA that both the AAV and non-AAV plasmid DNA were delivered in comparable amounts into the primary tumor cells. Liposome compositions did affect the delivery of the DNA into primary tumor types, but did not have any effect on the expression levels, indicating that the plasmid, and not the carrier molecule, has the significant role in expression. Despite comparable amounts of DNA in primary tumor cells, significant levels of IL-2 expression were obtained only with the AAV plasmid, suggesting that the difference between the AAV and non-AAV plasmid lies in the level of expression.

One feature of the AAV plasmid that may explain the high levels of expression is the incorporation of the AAV ITRs into the plasmid (18). In addition, the AAV-based expression plasmid described in this report differs from our previously published AAV construct in its ITR sequences and polyadenylation signal, and it contains a hybrid intron. Although the AAV ITR’s biological effects are unknown, it is speculated that they may act as a promoter-enhancers to induce higher levels of mRNA, 4 and thus higher levels of expression. In addition, ITRs may contribute to the plasmid maintenance in the cell and protect the plasmid from degradation. Of interest, however, was that 10–20% of tumor samples transfected with pMP5IL-2 and containing plasmid DNA did not express any detectable levels of IL-2.

We are exploring a variety of possibilities to identify factors resulting in defective expression in these primary tumor cells. In this report, it is demonstrated that efficient gene expression of biologically active cytokine molecules in primary tumor cells can be obtained utilizing a simple liposome-based delivery of AAV plasmid DNA. Furthermore, the transfection process is independent of cell division and liposome compositions, and can be used for more than one tumor type. Similar to breast and ovarian tumor cells, significant levels of gene expression have been obtained for both primary cultured human prostate (22) and melanoma tumor cells. 5 The achievement of nonviral gene transfer into a wide variety of primary tumor cell types would offer greater chances of developing an efficient gene-based immunological treatment for cancer.

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