Comparative evaluation of viral, nonviral and physical methods of gene delivery to normal and transformed lung epithelial cells

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Few studies have directly compared the efficiencies of gene delivery methods that target normal lung cells versus lung tumor cells. We report the first study directly comparing the efficiency and toxicity of viral [adeno-associated virus (AAV2, 5, 6) and lentivirus], nonviral (Effectene, SuperFect and Lipofectamine 2000) and physical [particle-mediated gene transfer (PMGT)] methods of gene delivery in normal mouse lung cells and in mouse adenocarcinoma cells. Lentivirus pseudotyped with the vesicular stomatitis virus glycoprotein was the most efficient gene transfer method for normal mouse airway epithelial cells [25.95 (±3.57) %] whereas AAV6 was most efficient for MLE-12 adenocarcinoma cells [68.2 (±3.2) %]. PMGT was more efficient in normal mouse airway epithelial cells than AAV5, Lipofectamine 2000 and SuperFect. AAV5 displayed the lowest transfection efficiency at less than 10% in both cell types. PMGT was the only method that resulted in significant toxicity. In summary, for all of the gene delivery methods examined here, lung tumor cells were transfected more easily than normal lung cells. Lipofectamine 2000 is potentially highly selective for lung tumor cells whereas AAV6 and lentivirus vesicular stomatitis virus glycoprotein may be useful for gene delivery strategies that require targeting of both normal and tumor cells. *Anti-Cancer Drugs* 19:783–788 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Lung cancer is the leading cause of cancer-related deaths with 1.2 million cases diagnosed worldwide each year [1]. The increasing ageing population means that the incidence of lung cancer is likely to increase further. Despite its prevalence, progress in improving treatment strategies for lung cancer has been relatively poor. Advances in conventional chemotherapy, radiation therapy and surgical strategies have had a minimal effect on survival rates, which remain at less than 15% [1]. Increased understanding of the molecular basis of lung cancer has stimulated research efforts to develop gene therapy strategies to treat this disease. However, efforts to date to deliver genes to lungs have largely focused on cystic fibrosis as a disease target rather than lung cancer. As a result, relatively few studies have compared gene delivery methods to normal versus lung tumor cells.

As with other types of cancer, gene therapy strategies to treat lung cancer include introduction of tumor suppressor genes and induction of apoptosis, immunogenicity and drug sensitivity [1]. However, in common with gene therapy approaches to other diseases, efficient gene delivery to target tumor cells has proven difficult. Although several viral vectors such as adenovirus and adeno-associated virus (AAV) have a natural tropism for lung epithelial cells, receptors for viral entry are often downregulated in cancer cells [1]. Immunoreactivity can also produce serious side effects. Similarly, while liposome toxicity may be less of an issue for lung cancer treatment than cystic fibrosis treatment for example, the efficiency rates of liposomal vectors are still relatively low. Specific targeting of lung tumor cells also remains a major challenge for both viral and nonviral approaches.

The respective merits and difficulties associated with viral and lipid-based/polymer-based gene delivery vectors have been well documented [2,3]. Gene delivery to the lungs poses further distinct challenges for gene therapeutics as these organs are well equipped to combat infection and expel foreign material [3]. Limited success with viral and lipid-based approaches has led to the exploration of alternative methods to improve gene delivery and a variety of physical methods aimed at improving delivery to lung cells have recently received new or renewed attention. Particle-mediated gene transfer (PMGT) uses physical force to deliver material into cells. PMGT has a number of advantages over chemical, biological and other physical transfer methods. This direct transfer method does not rely on expression of

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cell surface receptors, cell division or membrane porosity. PMGT has primarily been used with cells that are difficult to transfect and it has also been used to transfect tissues such as liver *in vivo* [4].

Most gene therapy strategies to treat lung cancer will need to maximize delivery to tumour cells while minimizing targeting of their normal counterparts. Some strategies, however, may require delivery to normal cells also. Progress in identifying lung stem cells and lung cancer stem cells has been made in recent years. It has been proposed that both normal airway and alveolar epithelial cells and adenocarcinomas arise from the same population of cells in distal mouse airways, termed as bronchioalveolar stem cells [5]. To assess the ability of different delivery methods to specifically transfect lung tumour cells, we carried out an extensive evaluation of the relative efficiencies and toxicities of viral, nonviral and physical modes of gene delivery in normal mouse airway cell cultures and in MLE-12 cells, which are derived from mouse lung adenocarcinomas.

Few studies have been carried out to date in any cell type to directly compare the transfection efficiencies of viral, liposome and physical methods of gene delivery. Furthermore, compared with adenovirus, the ability of AAV vectors to transfect lung cells has not been widely examined [1]. A study of AAV1, 2, 4, 5, 6, lentivirus pseudotyped with the vesicular stomatitis virus glycoprotein (HIV-VSV-G) and Lipofectin (Invitrogen, Paisley, UK) vectors in rat mesenchymal stem cells found that HIV-VSV-G was most efficient in these cells [6]. Another study using rat cardiomyocytes compared AAV2, electroporation and a range of polysomal and liposomal methods of gene delivery [7]. Lipofectamine 2000 and AAV2 were the most efficient nonviral and viral methods of delivery, respectively, whereas electroporation was more efficient than liposomes [8]. To our knowledge, no such study has been carried out comparing normal and transformed cells using a wide range of delivery methods or that uses any type of lung cells.

Materials and methods

Cell culture

Normal primary mouse lung cells were freshly isolated from female C3H-Hen mice as described previously [9]. These mouse airway epithelial cell (MAEC) cultures comprise approximately 80% Clara cells and 20% ciliated cells. MAECs are isolated in clumps and therefore cell counting using a hemocytometer is not feasible. To ensure equal seeding densities, an aliquot of cell isolate was taken before seeding and an absorbance value (A_{450}) was obtained using the Cell Titer 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega Corp, Madison, Wisconsin, USA). Cell suspensions were diluted appropriately based on A450 values to obtain equal seeding densities. MLE-12 cells were obtained from ATCC (Manassas, Virginia, USA). Cells were seeded in culture medium [1:1 Hams F12:M199 (Gibco, Glasgow, UK), 10% fetal bovine serum, 2 mmol/l L-glutamine (Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco)].

Adeno-associated virus transfections

AAV vector production was carried out as described previously [6]. Cells were plated at a concentration of 3.5×10^3 cells/well in 24-well tissue culture plates (Sarstedt, Wexford, Ireland). Following 48 h attachment, supernatants were removed and cells were rinsed once in OptiMEM (Gibco). OptiMEM (400 μl) containing 5×10^7, 5×10^8 or 5×10^9 plaque-forming units of AAV2-green fluorescent protein (GFP), AAV5-GFP or AAV6-GFP was added and cells were incubated for 5 h at 37°C, 5% CO_2 and 95% air. Supernatants were removed and fresh culture medium was added. After 24 or 48 h, cells were harvested by trypsinization and resuspended in phosphate-buffered saline containing 1% formaldehyde. GFP expression was quantified using a FACScan Flow Cytometer (Becton Dickinson, Oxford, UK).

Lentiviral transfections

Third generation rHIV-1-based lentivirus pseudotyped with VSV-G envelope and expressing GFP under the control of phosphoglycerate kinase promoter was prepared as described previously [6]. Cells were seeded at 3.5×10^3 cells/well in a 24-well plate and transduced 48 h later. Cells were transduced with 7.5×10^4, 1.5×10^5 or 2.25×10^5 plaque-forming units in 400 μl OptiMEM. GFP analysis was performed as described above for AAV-GFP.

Lipid transfections

Cells were seeded at 1.92×10^4 cells/well and transfected 48 h later. Lipofectamine 2000 (Invitrogen) was complexed to pMGFP (Promega) in a ratio of 0.4 μg: 0.75 μg (lipid: DNA). pMGFP was incubated in 100 μl OptiMEM at room temperature, lipid was added and the lipoplex was allowed to form by incubating for 30 min at room temperature. The lipoplex solution was added to each well and OptiMEM was added to a final volume of 200 μl. For Effectene transfections, for each well, a solution containing 0.66 μg pMGFP, 100 μl Buffer EC (Qiagen, Sussex, UK) and 1.28 μl of Enhancer (Qiagen) was incubated for 5 min at room temperature. Effectene (1.6 μg) (Qiagen) was added and incubated for a further 30 min. The volume was increased to 200 μl with OptiMEM and added to cells. For SuperFect transfections, for each well, 0.578 μg pMGFP in 100 μl OptiMEM was incubated at room temperature for 5 min. SuperFect (4 μl) was added and incubated for a further 30 min. OptiMEM was added to a final volume of 200 μl and added to cells. For all lipid transfections, cells were incubated for 6 h at 37°C, 5% CO_2 and 95% air after

which 200 µl culture medium was added. At 24 and 48 h, cells were harvested and GFP analysis was performed as described above for AAV-GFP.

Gene gun bombardment

MAECs were seeded into 35-mm^2 tissue culture dishes (NalgeNunc, New York, USA). Cells required for subsequent analysis by fluorescence microscopy were grown on glass cover slips placed in the culture dishes. Cells were seeded at a density of 0.1 absorbance unit per dish as determined by the Cell Titer 96 AQ_{ueous} One Solution Cell Proliferation Assay described above. Bombardment was carried out 24 h later. pMGFP was precipitated onto gold microcarriers as follows: briefly, 50 µl pMGFP (1 µg/µl), 50 µl 2.5 mol/l $CaCl_2$, 20 µl 0.1 mol/l spermidine and 3 mg 1.6 µm gold particles (BioRad, California, USA.) were mixed then rinsed once with 70% EtOH, once with 100% EtOH and resuspended in 60 µl 100% EtOH. Aliquots (6 µl) were spread onto macrocarriers (BioRad). Cells were bombarded with a Biolistic PDS-1000/He gun (BioRad) using 900 psi rupture discs and a vacuum of 15 in Hg. The macrocarrier and stopping screen assembly was placed on the top shelf and the tissue culture dish was also placed on the top shelf such that distances from the tissue culture dish to the stopping screen and from the stopping screen to the rupture disc were approximately 3.0 and 2.5 cm, respectively.

Cell viability

For viability assessment of cells following viral and nonviral methods of transfection, cells were harvested by trypsinization at each time point and viability counts were carried out using ethidium bromide/acridine orange staining with an ultraviolet microscope. For viability assessment following gene gun bombardment, cells were analyzed by flow cytometry. Viable cells were gated as determined by cell size and granularity. Cells outside of this population were deemed nonviable.

Results and discussion

We compared the transfection efficiencies and toxicities of a range of the most commonly used viral vectors (AAV2, AAV5, AAV6 and HIV-VSV-G), commercial nonviral vectors [Effectene (Qiagen), SuperFect (Qiagen) and Lipofectamine 2000 (Invitrogen)] and the physical method of gene gun bombardment using GFP reporter transgenes. Preliminary experiments were carried out using wide ranges of concentrations of each viral and nonviral vector to determine narrower ranges of optimal transfection efficiencies, and to minimize toxicity in the case of the nonviral vectors (data not shown). Optimal parameters for gene gun bombardment were also determined before this study (data not shown). For viral and nonviral methods, GFP expression was determined at 24 and 48 h time points post-gene delivery so that toxicity, differences in vector expression time, and variations in cell cycle rates between the cell types could be taken into account. Because cell damage was high using the PMGT method, GFP expression was measured at 24 h only for this method.

Viral and nonviral delivery at 24 h. An increase in GFP expression was observed with increasing titers of all viral vectors in both cell types at both 24 and 48 h time points except for HIV-VSV-G in MAECs at 24 h. The highest level of transgene expression in primary MAECs at 24 h post transfection was achieved with AAV6, which transfected 23 (± 7.29) % of cells (Fig. 1a). HIV-VSV-G ranked second best in MAECs with an efficiency of 10.8 (± 1.59) % followed by Effectene, which was the most efficient nonviral vector at 7 (± 1.9) %.

AAV6 was also the most efficient vector in MLE-12 cells at 68.2 (± 3.2) %. Lipofectamine 2000 was next best and was the most efficient nonviral vector at 55.89 (± 4.07) %. AAV5 performed poorly in both cell types with GFP detected in less than 10% of cells.

Cell viability was determined in each cell line after transfection with each vector. No significant toxic effect was observed with any of these delivery methods at 24 h (data not shown).

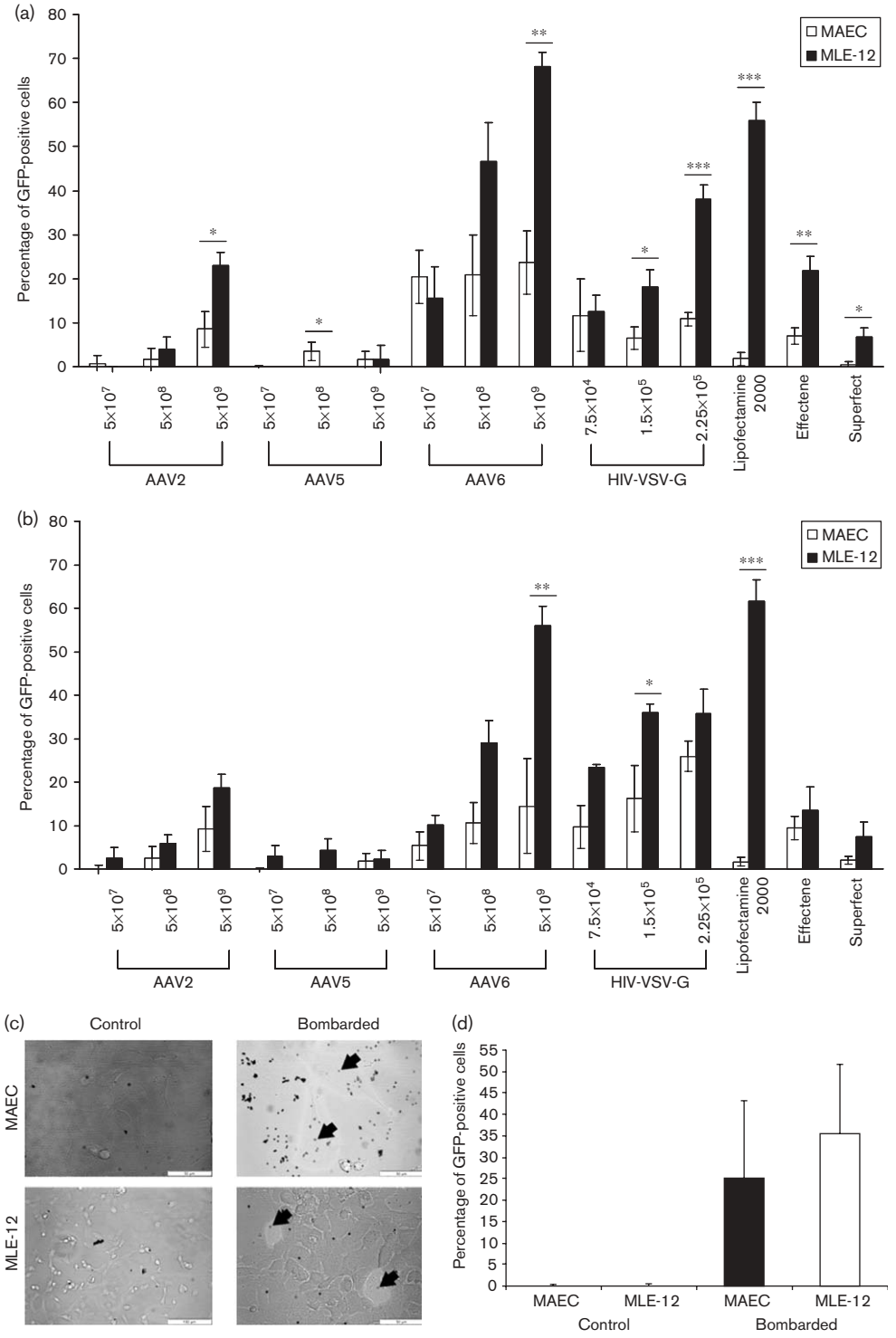
Viral and nonviral delivery at 48 h. At 48 h, the percentage of GFP-positive MAECs transfected using AAV6 had decreased to 14.44 (± 10.93) % (Fig. 1b). In contrast, the percentage of GFP-positive MAECs transfected using HIV-VSV-G had increased from 10.79 (± 1.58) % at day 1 to 25.95 (± 3.57) % at day 2. This was the highest transfection rate achieved in MAECs at either time point with any method.

In MLE-12 cells, the level of AAV6-GFP expression was also reduced from 24 h to 56.02 (± 4.39) %. A slight increase in GFP expression from 24 h with Lipofectamine 2000 transfection was observed in MLE-12 cells with a transfection efficiency of 61.49 (± 5.05) %.

No significant toxic effect was observed with any of these delivery methods at this time point (data not shown).

Particle-mediated gene transfer. Bombardment was carried out in 35-mm^2 tissue culture dishes. Cells were approximately 90% confluent. The target area of the gene gun was calculated to be approximately 20% of the surface area of the dish. Therefore, 20% of the total cell population was targeted. Whereas MLE-12 cells were bombarded once per experiment, MAECs were bombarded twice because primary cells are more difficult to transfect by this method and no transgene expression was detected after a single bombardment (data not

Fig. 1



Transfection efficiencies of viral, nonviral and physical gene delivery methods in mouse airway epithelial cells (MAECs) and MLE-12 cells. (a) 24 h post-transfection. AAV6 was the most efficient vector in both cell types at this time point although at the highest titer of AAV, the green fluorescent protein (GFP) reporter was expressed in three times as many MLE-12 cells as MAECs. The greatest difference in transfection efficiency between cell types occurred with Lipofectamine, which transfected 10 times more MLE-12 cells than MAECs. (b) 48 h post-transfection. Compared with the 24 h time point, a reduction in GFP expression was evident with all vectors except HIV-VSV-G and Lipofectamine 2000 at 48 h with the latter producing both the highest overall rate of transfection in MLE-12 cells and the greatest difference in transfection efficiency between the cell types. Student's *t*-test was used to determine significance, $*P<0.05$; $**P<0.01$; $***P<0.001$. (c) Gene gun bombardment of MAECs and MLE-12 cells. GFP fluorescence was observed in both cell types 24 h post-transfection, arrows indicate gold particles. (d) GFP expression was detected in approximately 25 and 35% of targeted MAECs and MLE-12 cells, respectively, with the gene gun bombardment method. AAV, adeno-associated virus.

shown). 24 h after bombardment, cells were analyzed by fluorescence microscopy and flow cytometry for GFP expression.

The targeted area was identifiable using a microscope due to the presence of gold particles (Fig. 1c). Within this area, GFP expression was observed in both cell types. Total cell populations were harvested from culture dishes by trypsinization and analyzed by flow cytometry. GFP expression in the total MAEC and MLE-12 populations was 5.04 (± 3.6) % and 7.11 (± 3.2) %, respectively. However, because only 20% of the total population had been targeted, these figures can be adjusted 25.2 (± 18) % and 35.55 (± 16) %, respectively (Fig. 1d). This means that PMGT was almost as efficient as HIV-VSV-G, although more toxic than the viral method. Because MAECs were bombarded twice, the procedure was relatively harsh on these cells with only 58.98 (± 9.15) % of cells remaining viable 24 h post bombardment. The treatment was less damaging to MLE-12 cells, which were only bombarded once, with 86.13 (± 4.17) % of cells remaining viable at 24 h (data not shown).

Primary MAECs, MLE-12 cells, AAV6 vectors and HIV-VSV-G all have been used in only a small number of lung gene delivery studies to date. As expected, primary MAECs were generally more difficult to transfect than transformed MLE-12 cells. Negligible levels of transfection were achieved in MAECs using AAV5, Lipofectamine 2000 and SuperFect, with the nonviral vectors performing particularly poorly. With Effectene, GFP expression was detected in approximately 10% of cells at 48 h. The highest levels of transfection in MAECs were achieved with AAV6 at 24 h and HIV-VSV-G at 48 h with efficiency levels approaching 30% and no significant toxicity. Compared with primary MAECs, MLE-12 cells were markedly easier to transfect with AAV6, HIV-VSV-G and, most strikingly, Lipofectamine 2000. AAV6 performed the best in these cells. These data indicate that AAV6 or HIV-VSV-G may serve as suitable vectors to target both normal and tumour cells, whereas Lipofectamine 2000 may be useful to achieve selective targeting of tumour cells over normal cells.

Previous studies report that AAV5 and AAV6 transfect lung cells more efficiently than AAV2 [10,11]. However, in this study, relative efficiencies of the AAV vectors in MLE-12 cells were AAV6 > > AAV2 > > AAV5. AAV5 performed particularly poorly with efficiencies of less than 10% in each cell type. Studies with AAV5 and AAV6 in lung cells to date have been limited. Our results may represent differences in cell types or model systems compared with previous studies and may suggest that the transduction capability of AAV5 is more limited than previous studies indicate. Lipofectamine 2000 achieved high gene transfer efficiencies in MLE-12 cells but not in MAECs. This may reflect differences in cell cycle rates as cell division within the primary cell cultures is slower than that of the cell lines and lipofection requires breakdown of the nuclear membrane for entry to the nucleus.

High transfection efficiencies can be offset by high toxicity with some vectors. This was not the case in this study, however, as no significant reduction in cell viability occurred with the best performing viral and nonviral vectors. Notably, AAV2 appeared to have a growth-promoting effect on MLE-12 cells by 48 h, which may also be undesirable, however, in the context of antitumor therapy.

The physical gene delivery method of gene gun bombardment was also evaluated. With the system used in this study, the target cells are bombarded under vacuum using a high-pressure helium burst to launch the particles at the target cells. The experimental procedure is relatively harsh on the cells and parameters require optimization to minimize cell damage. Cells exposed to vacuum for prolonged periods become stressed and may die. The helium blast used to transfer the particles also has a blast effect on the cells. The spread of particles before impact must also be accounted for. When the microparticles are launched at the target site, they have to cover a distance of at least 3 cm before impact with the cells making it difficult to specifically target areas on the culture dish and only a proportion of the dish can be targeted.

Despite these difficulties, both cell types were successfully transfected using the gene gun in this study. Accurate evaluation of transfection efficiency and direct comparison with the viral and nonviral methods was not possible, however, because only a subpopulation of the total population harvested from the dishes for analysis after bombardment was actually targeted. However, the viral and nonviral methods are likely to be more efficient methods of transfecting MLE-12 cells. Although cell damage was minimal with MLE-12 cells, the procedure was lethal in about 50% of MAECs. Further optimization of experimental parameters may reduce the extent of cell damage. Nonetheless, within the area targeted, approximately 25% of MAECs were transfected. It can therefore be concluded that bombardment is a more efficient gene delivery method for primary mouse cells than AAV5, Lipofectamine 2000 and SuperFect. If bombardment was combined with a method for selecting transfected cells, such as fluorescence-activated cell sorting, this would provide the means of transfecting primary lung cells *in vitro* without the disadvantages associated with viral and nonviral vectors.

In conclusion, all gene delivery methods examined here indicate that lung tumour cells are transfected more

easily than normal lung cells. AAV6 and VSV-G may be useful for gene therapy strategies that require targeting of both normal and tumour cells whereas Lipofectamine 2000 is potentially highly selective for lung tumour cells.

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