



# Polycations increase the efficiency of adenovirus-mediated gene transfer to epithelial and endothelial cells *in vitro*

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Recombinant adenoviruses are being developed for gene therapy for cystic fibrosis and other lung diseases, and for prevention and treatment of vascular thrombosis. A major limitation to the clinical utility of adenoviruses is the low efficiency of gene transfer achieved *in vivo*. In addition, little is known about the initial interactions between adenoviruses and the target cell. To address the hypothesis that the negative charge presented by membrane glycoproteins reduces the efficiency of adenovirus-mediated gene transfer, primary cultures of human airway, Madin–Darby canine kidney cells, an immortalized cystic fibrosis airway epithelial cell line, and primary cultures of sheep pulmonary artery endothelium were infected with recombinant adeno-

virus containing the *E. coli lacZ* reporter gene (Ad2 $\beta$ gal2) in the presence of various polyions. For each cell type, adsorption of Ad2 $\beta$ gal2 in the presence of the polycations polybrene, protamine, DEAE-dextran, and poly-L-lysine significantly increased the percentage of cells that express *lacZ*. The polyanion heparin did not significantly alter gene transfer efficiency, but completely abrogated the effects of polycations. These data provide evidence that negatively charged moieties on the cell surface reduce the efficiency of adenovirus-mediated gene transfer, and that alteration of the charge interaction between adenoviruses and the cell surface may improve the potential clinical application of these vectors.

**Keywords:** gene therapy; polycations; glycoconjugates; vascular thrombosis; cystic fibrosis

## Introduction

Gene therapy for treatment of several hereditary and acquired diseases has gained increasing attention in the past decade.<sup>1–4</sup> Despite remarkable progress in the development of both viral and nonviral vectors and the initiation of clinical gene therapy trials, the low efficiency of gene transfer is one of the major obstacles to the clinical application of many of these vectors. Recombinant adenoviruses have been evaluated extensively because of their relatively high efficiency and ability to drive expression of a foreign gene in nondividing cells. Although efficient adenovirus-mediated gene transfer to cultured epithelial<sup>5</sup> and endothelial<sup>6,7</sup> cells, and to various *in vivo* airway<sup>8</sup> and vascular models<sup>9,10</sup> has been reported, differentiated epithelial and endothelial cells are relatively resistant to adenovirus infection, requiring high multiplicities of infection (MOI) and a relatively long physical contact time between adenovirus and target cell for significant gene transfer.<sup>11–16</sup>

The mechanisms for this relative inefficiency are poorly understood, but potentially include secreted mucins, inflammatory mediators, cell surface sialoglycoconjugates, tight junctions, polarized expression of viral binding or internalization receptors, and differential rates of

endocytosis at the apical and basolateral cell membranes. Apical membrane sialoglycoconjugates, either by steric hindrance or charge interactions related to the abundant negatively charged sialic acid residues, may interfere with adenovirus binding to the target cell. The importance of sialic acid and cell surface charge in virus–cell interactions has been demonstrated for several enveloped and nonenveloped viruses, such as retrovirus,<sup>17,18</sup> murine sarcoma and leukemia viruses,<sup>19–21</sup> avian sarcoma virus,<sup>22</sup> respiratory syncytial virus,<sup>23</sup> reovirus,<sup>24</sup> and rotaviruses.<sup>25,26</sup> For example, Kahn *et al*<sup>18</sup> studied the effects of various concentrations of polybrene and DEAE-dextran on transduction of bovine aortic endothelial cells using retroviral vectors, and found a significantly enhanced transduction efficiency with the use of these polycations.

Despite the relatively well understood phases of adenovirus infection,<sup>27</sup> little is known about the potential implications of charge interactions for cell binding and entry of adenoviruses. Our recent work has revealed that removal of negatively charged sialic acid residues from cultured polarized epithelial cells significantly improves the efficiency of adenovirus-mediated gene transfer to these cells.<sup>28</sup> In the current study, we hypothesized that the addition of positively charged polymers during adsorption of recombinant adenovirus would also increase the efficiency of gene transfer. Primary cultures of human bronchial epithelial (HBE), sheep pulmonary artery endothelial cells (SPAEC), type I Madin–Darby canine kidney (MDCK) cells, and immortalized bronchial

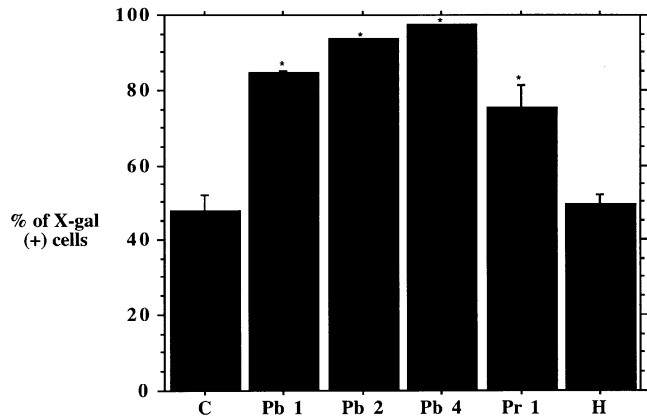
epithelial (IB3) cells were used to study the effects of several polyions on adenovirus-mediated gene transfer. The data demonstrate for the first time that a number of different polycations increase the efficiency of gene transfer to epithelial and endothelial cells by recombinant adenovirus. These findings have significant implications for the clinical application of current adenovirus vectors and, more importantly, for the development of more efficient vectors.

## Results

### *Incubation of primary and immortalized human airway epithelial cells and MDCK cells with recombinant adenovirus in the presence of polycations increases the efficiency of gene transfer*

To evaluate the effects of polycations on the efficiency of adenovirus-mediated gene transfer, primary human bronchial epithelial (HBE), and type I MDCK cells were incubated with Ad2 $\beta$ gal2 for 1 h at 37°C using several different MOI in the presence or absence of the polycationic polymers polybrene and protamine.  $\beta$ -Galactosidase expression was evaluated by X-gal staining after 48 h.

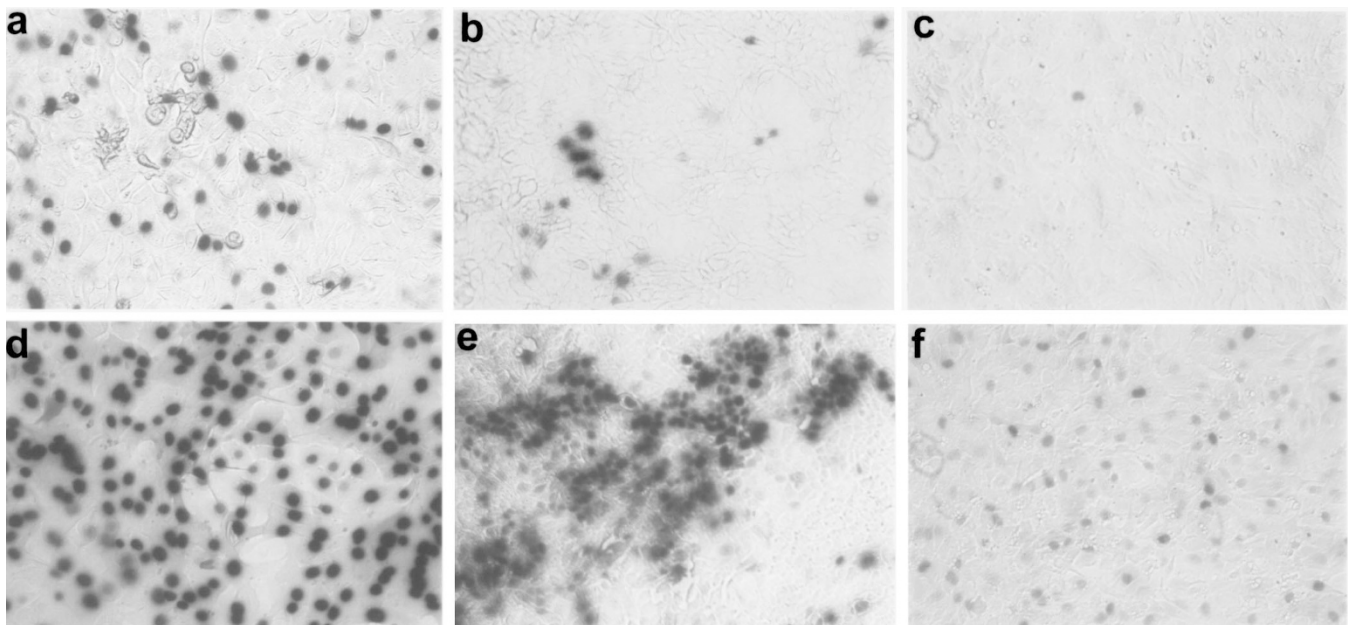
**HBE cells:** As shown in Figures 1 and 2, Ad2 $\beta$ gal2 infection of primary HBE cells at an MOI of 25 resulted in *lacZ* expression in  $48 \pm 4\%$  of the cells, whereas adsorption in the presence of polybrene at concentrations of 1, 2, and 4  $\mu\text{g}/\text{ml}$  increased the percentage of cells expressing the transgene to  $85 \pm 1\%$ ,  $94 \pm 1\%$ , and  $97 \pm 1\%$  of HBE cells, respectively ( $P < 0.0001$  by ANOVA). Similarly, viral adsorption in the presence of 1  $\mu\text{g}/\text{ml}$  of protamine resulted in transgene expression in  $75 \pm 6\%$  of the cells ( $P = 0.0002$  by ANOVA). At an MOI of 10, the increase in gene transfer efficiency with polybrene was more



**Figure 2** Efficiency of gene transfer by Ad2 $\beta$ gal2 to primary HBE cells with and without polyions. Ad2 $\beta$ gal2 was adsorbed to HBE cells at an MOI of 25 in the absence or presence of polybrene and protamine at various concentrations and heparin at 50 U/ml for 1 h. X-gal staining done 48 h later revealed a significantly increased percentage of *lacZ*-expressing cells in wells that were incubated with polybrene and protamine ( $P \leq 0.0002$ ), but no difference from control when heparin was added to the adsorption media. Note that there is a dose response to polybrene. Pb denotes polybrene; Pr, protamine; H, heparin. Numbers represent different concentrations of polybrene and protamine in  $\mu\text{g}/\text{ml}$ . (\*) denotes  $P < 0.0001$  compared with control.

marked. The percentage of *lacZ*-expressing cells increased more than two-fold, from  $19 \pm 1\%$  to  $46 \pm 2\%$  ( $P < 0.0001$  by ANOVA) when adsorption of Ad2 $\beta$ gal2 was performed in the presence of 4  $\mu\text{g}/\text{ml}$  of polybrene (data not shown).

As has been shown for the effects of polybrene on the efficiency of retrovirus-mediated gene transfer,<sup>18</sup> there is a limited dose response relationship for the polycation effect. At polybrene concentrations of 4  $\mu\text{g}/\text{ml}$  and above



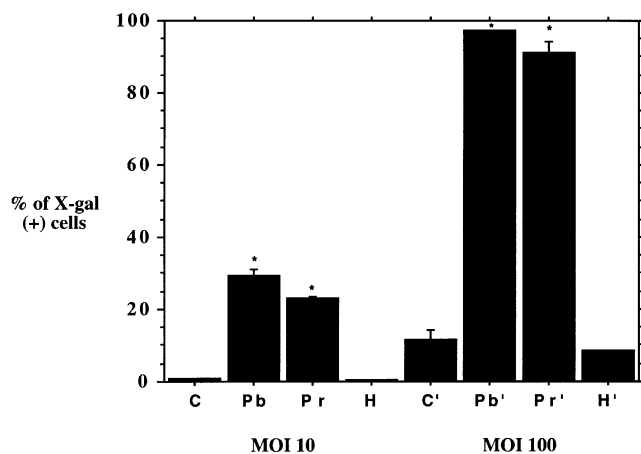
**Figure 1** *LacZ* expression in cultured HBE, MDCK and SPAEC cells following Ad2 $\beta$ gal2 infection. HBE (a, d), MDCK (b, e), and SPAEC (c, f) were incubated with Ad2 $\beta$ gal2 for 1 h in the absence (a, b, c) or presence (d, e, f) of polybrene at 4  $\mu\text{g}/\text{ml}$  and X-gal stained 48 h later to detect transgene expression. Note the significant increase in *lacZ* expression as indicated by increased numbers of blue nuclei in cultures when polybrene was present during viral adsorption.

(see Figure 2), and protamine concentrations of 5  $\mu\text{g}/\text{ml}$  and above (data not shown), the gene transfer efficiency reached a plateau insofar as further increases in polycation concentration resulted in minimal changes in the percentage of cells expressing the transgene. Therefore subsequent experiments were performed with polybrene and protamine concentrations of 4 and 5  $\mu\text{g}/\text{ml}$ , respectively.

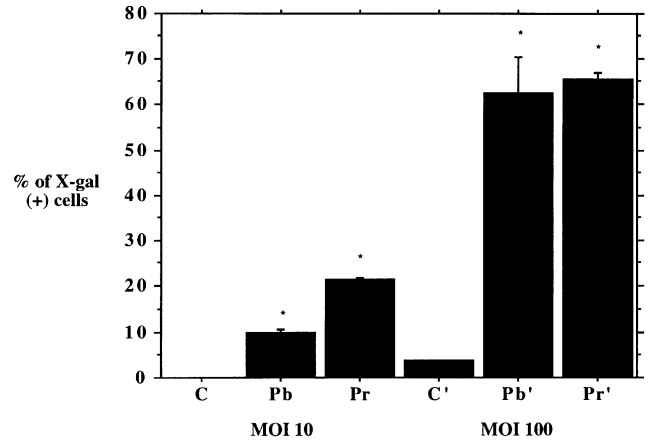
**MDCK cells:** Ad2 $\beta\text{gal}2$  infection of MDCK type I cells at MOI of 10, 25 and 100 resulted in transgene expression in  $1 \pm 0.1\%$ ,  $5 \pm 0.4\%$  and  $12 \pm 3.0\%$  of cells, respectively. At each MOI the percentage of transgene-expressing cells increased significantly when viral adsorption was performed in the presence of a polycation. As shown in Figure 3, the effect of polycations was most pronounced at lower MOIs, with an approximately 30-fold increase in the percentage of transgene expressing cells at an MOI of 10 for polybrene ( $P < 0.0001$  by ANOVA), and a 23-fold increase at the same MOI for protamine ( $P < 0.0001$ ). Although the relative increase in  $\beta$ -galactosidase expression following Ad2 $\beta\text{gal}2$  infection at an MOI of 100 was of lesser relative magnitude than at an MOI of 10, the difference was very significant insofar as nearly 100% of the cells expressed *lacZ* when polycations were present during viral adsorption (see Figure 3). The effect at an MOI of 25 was similar (data not shown). When polybrene was added to the media for 1 h after adenoviral adsorption, there was no significant increase in *lacZ* expression ( $12 \pm 1\%$  versus  $16 \pm 1\%$ ,  $P > 0.05$ ), suggesting that polybrene is affecting viral binding and/or internalization.

**Incubation of primary sheep pulmonary artery endothelial cells (SPAEC) with recombinant adenovirus in the presence of polycations increases the efficiency of gene transfer**

As shown in Figure 4, infection of SPAEC with Ad2 $\beta\text{gal}2$  at an MOI of 10 and 100 resulted in transgene expression in  $<0.5\%$  and  $4 \pm 0.2\%$  of the cells, respectively. Viral



**Figure 3** Efficiency of gene transfer by Ad2 $\beta\text{gal}2$  to MDCK cells with and without polycations. MDCK cells were infected with Ad2 $\beta\text{gal}2$  and X-gal stained 48 h later as described in Materials and methods. Two different MOIs (10 and 100) are shown. Note that *lacZ* expression following Ad2 $\beta\text{gal}2$  infection is observed in a significantly higher percentage of cells when 4  $\mu\text{g}/\text{ml}$  of polybrene or 5  $\mu\text{g}/\text{ml}$  protamine were present during viral adsorption; the percentage with 50 U/ml of heparin was not different from adenovirus alone. (\*) denotes  $P < 0.0001$  compared with control.

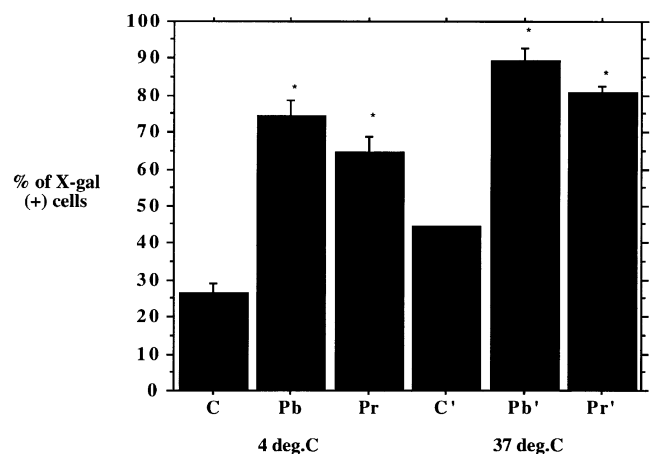


**Figure 4** Efficiency of gene transfer by Ad2 $\beta\text{gal}2$  to SPAEC cells with and without polycations. Effects of polybrene and protamine at two different MOIs (10 and 100) are shown. Note that adsorption of adenovirus in the presence of polycations significantly increases the percentage of *lacZ*-expressing SPAEC cells. (\*) denotes  $P < 0.0001$  compared with control at the same MOI.

adsorption in the presence of polybrene increased transgene expression more than 20-fold at an MOI of 10 ( $P < 0.0001$ ) and 16-fold at an MOI of 100 ( $P < 0.0001$ ). Similar results were obtained with protamine, as  $\beta$ -galactosidase expression increased more than 40-fold at an MOI of 10, and 17-fold at an MOI of 100 ( $<0.5\%$  versus  $22 \pm 0.3\%$ , and  $4 \pm 0.2\%$  versus  $66 \pm 2\%$ , respectively;  $P < 0.0001$  for both MOI).

**Incubation of immortalized human airway epithelial (IB3) cells with recombinant adenovirus in the presence of polycations increases the efficiency of gene transfer**

IB3 cells were incubated with Ad2 $\beta\text{gal}2$  at an MOI of 5 and 100 in the presence of polybrene or protamine. As demonstrated in Figure 5, at an MOI of 5, the percentage of cells that expressed *lacZ* was  $44 \pm 0.4\%$ ; when Ad2 $\beta\text{gal}2$  adsorption was performed in the presence of



**Figure 5** Efficiency of gene transfer by Ad2 $\beta\text{gal}2$  to the IB3 airway epithelial cell line with and without polycations. IB3 cells were infected with recombinant adenovirus at 4° or 37°C in the absence or presence of polybrene and protamine. Note that, at both adsorption temperatures, there is a two- to three-fold increase in *lacZ* expression when adenoviral adsorption is performed in the presence of polycations. (\*) denotes  $P < 0.0033$  compared with adenovirus alone at the same temperatures.

polycations, the percentage of positive cells increased approximately two-fold to  $89 \pm 4\%$  with polybrene ( $P < 0.0001$ ), and  $81 \pm 2\%$  with protamine ( $P = 0.0002$ ). At an MOI of 100, transgene expression was observed in over 90% of cells in the presence or absence of polycations (data not shown).

*The effects of polycations are observed with adsorption of virus at either 4°C or 37°C, consistent with a polycation-mediated increase in adenoviral binding*

To begin to determine whether the observed increase in gene transfer efficiency is due to enhanced viral attachment or internalization, viral adsorption to IB3 cells was performed at either 37°C or 4°C. The magnitude of increase in the percentage of cells that express *lacZ* was similar under both conditions, as shown in Figure 5. When adsorption was performed at 37°C, the percentage of positive cells increased approximately three-fold with polybrene and two-fold with protamine ( $P = 0.0001$  for both conditions). When Ad2βgal2 adsorption was performed at 4°C in the presence of polybrene or protamine, the percentage of positive cells was slightly lower, but the magnitude of the polycation effect was nearly identical.

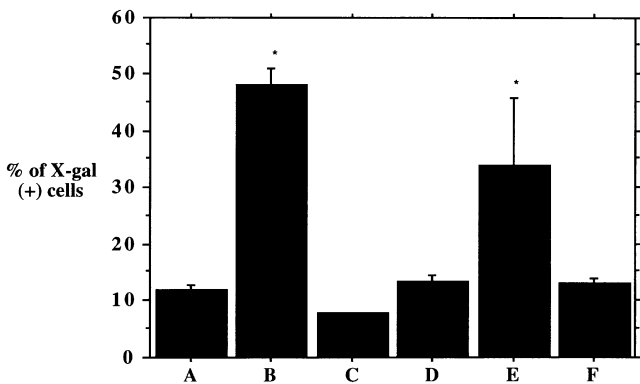
*The effects of polycations are abrogated by co-incubation with heparin*

Initial experiments with heparin alone were done to determine the effect of polyanions on recombinant adenovirus infection. In each of the cell lines, viral adsorption in the presence of heparin at up to 50 U/ml did not result in statistically significant differences in the percentage of cells expressing *lacZ* (see Figures 2, 3 and 6). To determine whether co-incubation of heparin and polycations during viral adsorption would alter transgene expression, Ad2βgal2 was adsorbed to HBE cells at 37°C for 1 h in the presence of both polybrene and hep-

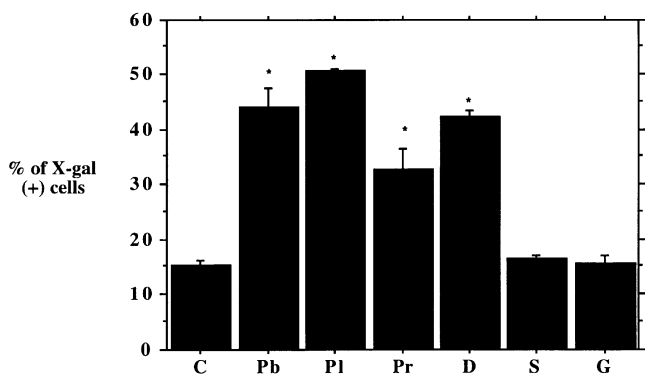
arin. As shown in Figure 6, at an MOI of 10, transgene expression increased four-fold (from  $12 \pm 1\%$  to  $48 \pm 3\%$  of the cells) with polybrene incubation, but was not different from the control when adsorption was performed with heparin alone. Moreover, Ad2βgal2 adsorption in the presence of both polybrene and heparin resulted in *lacZ* expression in  $13.2 \pm 1\%$  of cells, which is not significantly different from adenovirus alone. These data demonstrate that polyanions such as heparin have no effect on gene transfer efficiency, but that polyanions inhibit the polybrene-mediated increase in gene transfer efficiency. In addition, as shown in Figure 6, similar effects on gene transfer were observed when adenoviral adsorption was performed after a 2-min wash of cells with polybrene or both polybrene and heparin immediately before adenoviral incubation. Polybrene wash increased gene transfer efficiency (from  $12 \pm 1\%$  to  $34 \pm 12\%$ ,  $P = 0.0018$ ), indicating that the polycation need not be present during viral adsorption. Combined polybrene and heparin wash did not result in a significant change in the percentage of *lacZ*-expressing cells ( $12 \pm 1\%$  versus  $13 \pm 1\%$ ,  $P = 0.864$ ).

*The effects of polycations are observed with polymers ranging in size from 3000 to over 500 000 Da*

To determine whether the size of the polycation was critical to its effects on gene transfer efficiency, Ad2βgal2 at an MOI of 25 was adsorbed in the presence of the following polycations that vary in molecular weight: polybrene (3000 Da), protamine (4000 Da), DEAE-dextran (500 000 Da), and poly-L-lysine (70 000–150 000 Da). As shown in Figure 7, each of the polycations significantly increased the efficiency of gene transfer ( $15 \pm 1\%$  with adenovirus alone versus  $44 \pm 4\%$  with polybrene,  $51 \pm 1\%$  with poly-L-lysine,  $33 \pm 4\%$  with protamine, and  $42 \pm 1\%$  with DEAE-dextran,  $P < 0.0001$  for each polycation by ANOVA). As demonstrated in Figure 7, the percentage of *lacZ*-expressing cells was unaltered by the addition of free sialic acid ( $P = 0.74$ ) or glycophorin A ( $P = 0.91$ ) during adsorption of Ad2βgal2, however, both free sialic acid or glycophorin A abrogated the effects of polycations (data not shown), suggesting that the effect is due to charge interactions rather than interaction with specific oligosaccharide moieties, and is independent of chain length.



**Figure 6** Rinsing the cells with media containing polycations before viral adsorption increases gene transfer efficiency. The effects of washing HBE cells with polyanions before viral adsorption was compared to co-incubation of virus and polyanion. (A) Adenovirus; (B) adenovirus adsorbed with media containing 4 μg/ml polybrene; (C) adenovirus adsorbed with medium containing 50 U/ml heparin; (D) adenovirus adsorbed with medium containing 4 μg/ml polybrene and 50 U/ml heparin; (E) cells rinsed with medium containing 4 μg/ml polybrene before adenovirus adsorption; (F) cells rinsed with media containing 50 U/ml of heparin before adenoviral adsorption. Note that a three- to four-fold increase in the percentage of *lacZ*-expressing cells is observed when virus is adsorbed in the presence of polybrene or when cells are washed with polybrene before adsorption of adenovirus. In contrast, rinsing the cells or adsorbing virus in the presence of heparin does not alter transgene expression, but completely abrogates the effects of polybrene. (\*) denotes  $P < 0.0001$  compared with (A).



**Figure 7** Effects of multiple polyanions on Ad2βgal2-mediated gene transfer to primary HBE cells. HBE cells were incubated with Ad2βgal2 in the presence of each of the polyanions for 1 h, and X-gal staining was performed 48 h later. Note that polycations of various sizes each significantly increased the percentage of *lacZ*-expressing cells compared to the adenovirus control; polyanions had no effect. C, adenovirus control; Pb, polybrene; Pl, poly-L-lysine; Pr, protamine; D, DEAE-dextran; S, free sialic acid; and G, glycophorin A. (\*) denotes  $P < 0.0001$  compared with control.

## Discussion

Previous studies have shown that charge interactions are important for the cellular binding and entry of a number of viruses.<sup>17–24</sup> For example, influenza virus and bovine coronaviruses require sialic acid residues for cell binding.<sup>29,30</sup> In contrast to these enveloped viruses, the molecular mechanisms of cell entry employed by adenoviruses are only partially understood,<sup>26,31</sup> and the importance of cell surface charge in viral attachment and entry has not been evaluated to date. Our recent work has revealed that enzymatic removal of negatively charged sialic acid residues from epithelial cell surface glycoconjugates significantly increases adenovirus-mediated gene transfer,<sup>28</sup> suggesting that the negative charge of sialic acid residues on cell surface glycoconjugates may impair the binding of adenoviruses to epithelial cells.

In the current study, we extend these observations and show that the presence of positively charged polymers of different sizes during or immediately before adenovirus adsorption markedly increases the efficiency of adenovirus-mediated gene transfer to both epithelial and endothelial cells. This effect occurs when viral adsorption is performed at either 37°C or 4°C, suggesting that polycations enhance the attachment of adenovirus to the cell. Moreover, negatively charged moieties such as heparin do not significantly alter gene transfer efficiency. However, heparin completely abolishes the effect of increased gene transfer caused by polycations. These data demonstrate for the first time that charge interactions influence the efficiency of adenovirus-mediated gene transfer to both epithelial and endothelial cells, and suggest that modifying these interactions may be a useful strategy to improve the efficiency of recombinant adenovirus-mediated gene transfer to these cell types.

Recent work suggests that adenoviruses enter cells in two stages. The initial binding of the knob of fiber protein to an unknown receptor is followed by internalization that is mediated in part by  $\alpha v\beta 3$  and  $\alpha v\beta 5$  integrin receptors.<sup>31</sup> The fiber protein is a trimer of the 581 (serotype 5) or 582 (serotype 2) amino acid fiber polypeptides,<sup>32</sup> and contains at least one glucosamine residue.<sup>33</sup> Some evidence suggests that the interactions of fiber with the cell involve amino acids rather than the carbohydrate moieties present on both the putative receptors and fiber.<sup>34</sup> Although the amino acid sequence of the fiber protein in adenovirus serotype 2 has been found to contain six negatively charged amino acids in the C-terminus, the exact conformation and net charge of this protein, and the precise binding site within the fiber knob remain unclear.

Epithelial and endothelial cell membranes contain glycoconjugates that are sialylated and/or sulfated, and thereby confer a negative charge to the cell surface. In airway epithelia, glycoproteins, such as the transmembrane mucin MUC1, and glycolipids, such as the gangliosides GM1 and GM2, are sialylated and expressed abundantly in the apical membrane.<sup>35</sup> Structurally similar glycolipids and glycoproteins, such as the endothelial mucins GlyCAM-1 and CD34, are expressed by endothelial cells.<sup>36</sup> Exposed on the luminal surface, these anionic glycoconjugates are ideally situated to present a charge barrier to lumenally delivered gene transfer vectors. Thus, epithelial and endothelial cells have similarities in cell structure that may constitute barriers to

gene transfer in both cell types and contribute to the low efficiency of gene transfer with recombinant adenoviruses.

We speculate that the effect of polycations could occur via a number of mechanisms, including neutralization of cell surface negative charge, formation of a bridge between the negatively charged cell surface and adenovirus particle, or an increase in the permeability of cell monolayers. In the first instance, one need not implicate additional or alternative receptors. Binding and internalization would occur via fiber and penton base, albeit more efficiently after negation of the charge barrier. In contrast, a bridging effect of the polycations could occur through enhancement of fiber or penton base binding, or through bridging of an alternative viral protein to a cell receptor. Lastly, we cannot rule out an effect of polycations on epithelial permeability, since Peterson and colleagues<sup>37</sup> have demonstrated that polycations such as protamine decrease the transepithelial electrical resistance and increase the permeability of MDCK monolayers to mannitol. However, two observations argue against increased epithelial permeability as the primary mechanism for a polycation-mediated increase in gene transfer. First, in published studies to date, the reduction in transepithelial electrical resistance becomes evident after 10 min of exposure at 37°C.<sup>37,38</sup> However, when protamine at concentrations up to 100  $\mu\text{g/ml}$  was added at 4°C there is no increase in permeability.<sup>39</sup> In our studies, an increase in gene transfer efficiency of similar magnitude is observed after exposure of cell monolayers to polycations for 2 min at 4°C before adenoviral adsorption. The time course and temperature independence of the polycation effect on gene transfer efficiency therefore speaks against increased permeability as a mechanism. Second and more importantly, the effects of polycations are also observed in subconfluent cell cultures in which a tight monolayer and cell polarization are not present. This argues against altered epithelial permeability as a mechanism for increased gene transfer.

In summary, adenovirus-mediated gene transfer to epithelial and endothelial cells is modulated by the presence of negatively charged cell surface molecules that appear to alter viral binding to cells. The addition of cationic polymers before or during viral adsorption significantly increases the efficiency of gene transfer. This finding has important implications for the use of recombinant adenoviruses for gene therapy. Administration of a polycation with recombinant adenovirus may permit a decrease in the required quantity of virus, which may in turn impact on the host inflammatory response.<sup>1,3</sup> In addition, by determining and altering the binding sequence and/or the charge of the fiber protein, adenovirus-mediated gene transfer may potentially be made more efficient, thereby improving the prospects for successful clinical application.

## Materials and methods

### Production of recombinant adenoviruses

Serotype 2, E1-deleted recombinant adenovirus containing the *E. coli lacZ* reporter gene driven by the cytomegalovirus promoter (Ad2 $\beta$ gal2) was constructed, purified, and assayed for the number of plaque forming units (p.f.u.) per ml as previously described.<sup>40,41</sup> Viral stocks

were stored in 5% sucrose and kept frozen at  $-80^{\circ}\text{C}$  until use.

#### *Isolation and primary culture of human bronchial epithelial (HBE) cells*

HBE cells were isolated from native lungs of transplant recipients, as previously described.<sup>42</sup> In brief, airways were dissected from surrounding adventitium, and placed in ice-cold HEPES-buffered minimum essential medium containing penicillin, streptomycin and amphotericin B. After incubation for 12–16 h at  $4^{\circ}\text{C}$  in 0.1% Protease XIV (Sigma, St Louis, MO, USA), airway epithelial cells were obtained by gently scraping the epithelium with the blunt end of a forceps. Supernatant from the washed tissue was spun, and the cell pellet was plated on type IV human placental collagen (Sigma) coated tissue culture flasks in bronchial epithelial growth medium (BEGM; Clonetics, San Diego, CA, USA). Exempt approval for the use of human lung tissue was obtained from the University of Pittsburgh Investigational Review Board.

#### *Cell lines*

Type I Madin–Darby canine kidney (MDCK) cells were obtained from ATCC and grown in Dulbecco's minimum essential medium (DMEM)/Ham's F12 supplemented with 3% FBS. Sheep pulmonary artery endothelial cells (SPAEC) were obtained from collagenase-digested pulmonary arteries, enriched by fluorescence activated cell sorting of di-I-acetylated low density lipoprotein uptake<sup>43</sup> and used from passage 12–15. Immortalized bronchial epithelial cells (IB3) were kindly provided by Dr Pamela Zeitlin (Johns Hopkins University, Baltimore, MD, USA) and grown in Ham's F12 supplemented with 10% FBS. The growth medium was changed every 3 days. MDCK cells from passage 5 to 15, SPAEC from passage 12 to 15, and IB3 cells from passage 30 to 40 were grown in 24- or 48-well culture plates. Except as noted, experiments were performed after cells reached confluency.

#### *Polyions*

Protamine sulfate was obtained from Eli Lilly (Indianapolis, IN, USA). Polybrene, poly-L-lysine, DEAE-dextran, sialic acid, heparin, and glycofhorin A were obtained from Sigma.

#### *Incubation of cells with recombinant adenovirus*

The number of cells in one well of a 24-well plate was determined and Ad2 $\beta$ gal2 dilutions were prepared in the growth medium appropriate for the cell type to obtain MOIs of 10, 25 or 100. Wells were washed with HBSS once, and viral adsorption was performed for 1 h at  $4$  or  $37^{\circ}\text{C}$  in a minimal volume to maximize contact of viral particles with cells. Plates were rocked every 15 min to assure even distribution of viral solution.

Viral adsorption was done in the presence of polyions at various concentrations, and in subsequent experiments, concentrations that were found to be maximally effective were used. After determination of the effects of polyions, viral adsorption was performed in the presence of both a polycation and a polyanion. Also, to determine whether brief exposure of cells to polyions alters gene transfer, cells were rinsed with medium containing polybrene or both polybrene and heparin for 2 min immediately before adenoviral adsorption in growth

medium. In addition, adenovirus adsorption was performed before incubation with medium containing polybrene to evaluate whether the effect of polybrene is specific to the viral adsorption phase.

In each experiment, wells that received Ad2 $\beta$ gal2 alone were used to determine the baseline efficiency of gene transfer by recombinant adenovirus. Negative control experiments were done by incubating wells with media alone, and with media containing polyions without virus. In each experiment, duplicate wells were used for each condition, and each experiment was performed at least twice for confirmation of results. As there was some variation from experiment to experiment due to minor differences in cell number, representative experiments are shown.

#### *Detection of lacZ expression*

Forty-eight hours after viral adsorption, expression of the  $\beta$ -galactosidase transgene was determined by staining the cells with 1 mg/ml of 5-bromo-4-chloro-3-indoyl- $\beta$ -galactopyranoside (X-gal; Boehringer Mannheim, Indianapolis, IN, USA) solution for 4 to 6 h.<sup>42</sup> The percentage of transgene expressing (blue) cells in three representative fields ( $>1000$  cells per field) was determined by counting under inverted phase microscopy.

#### *Statistical analyses*

The mean percentage of X-gal positive cells in duplicate wells under various conditions was analyzed for statistical difference using ANOVA with Statview software (Abacus Concepts, Berkeley, CA, USA). When statistical differences were found, individual comparisons were made using Fisher's PLSD post-hoc analysis.

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