

## Polymer-Enhanced Adenoviral Transduction of CAR-Negative Bladder Cancer Cells

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**Abstract:** The application of adenoviral gene therapy for cancer is limited by immune clearance of the virus as well as poor transduction efficiency, since the protein used for viral entry (CAR) serves physiological functions in adhesion and is frequently decreased among cancer cells. Cationic polymers have been used to enhance adenoviral gene delivery, but novel polymers with low toxicity are needed to realize this approach. We recently identified polymers that were characterized by high transfection efficiency of plasmid DNA and a low toxicity profile. In this study we evaluated the novel cationic polymer EGDE-3,3' for its potential to increase adenoviral transduction of the CAR-negative bladder cancer cell line TCCSUP. The amount of adenovirus required to transduce 50–60% of the cells was reduced 100-fold when Ad.GFP was preincubated with the EGDE-3,3' polymer. Polyethyleneimine (pEI), a positively charged polymer currently used as a standard for enhancing adenoviral transduction, also increased infectivity, but transgene expression was consistently higher with EGDE-3,3'. In addition, EGDE-3,3'-supplemented transduction of an adenovirus expressing an apoptosis inducing transgene, Ad.GFP-TRAIL, significantly enhanced the amount of cell death. Thus, our results indicate that novel biocompatible polymers may be useful in improving the delivery of adenoviral gene therapy.

**Keywords:** Polymer; adenovirus; gene therapy; cancer; bladder cancer

### Introduction

In the United States, bladder cancer is the second most common cancer of the urogenital tract with over 68,000 new cases and 14,000 deaths in 2008.<sup>1</sup> While the majority of lesions are superficial at diagnosis, the risks of recurrence and progression are high and close long-term monitoring is required, which makes bladder cancer one of the costliest

diseases.<sup>2</sup> Despite therapy nearly half of all patients with superficial disease experience relapse or progression within 5 years. Patients who present with advanced disease have, even with multimodal therapy, only 20–40% survival at 5 years. Thus, more effective treatment strategies, aimed at reducing the recurrence and progression of superficial bladder cancer as well as improving therapeutic outcome in patients with advanced disease, are needed.

Gene therapy has garnered significant attention as a therapeutic approach for bladder cancer disease.<sup>3</sup> Viral vectors that have been explored for bladder cancer gene therapy include herpes virus, retrovirus, adeno-associated virus (AAV), and adenovirus. Adenoviral delivery of therapeutic transgenes is particularly attractive since these vectors

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infect a wide range of cells with high efficiency but remain episomal thus minimizing the risk of insertional mutations. Despite the widespread use of adenoviral vectors for transgene expression, in vivo use of adenovirus faces significant hurdles. Neutralization of type 5 adenovirus by pre-existing or induced antibodies results in a significant decrease of target cell infection.<sup>4,5</sup> In addition, following intravenous delivery adenovirus is inactivated by interactions with cells of the innate immune system as well as platelets and erythrocytes;<sup>6,7</sup> these “nonspecific” interactions limit the efficacy of the delivered viral vectors and lead to toxicity.<sup>8</sup> Since adenovirus can be delivered to malignant lesions in the bladder by intravesical instillation, complications associated with systemic delivery may be reduced but cannot be completely avoided. Additionally, challenges such as effective transduction of target cells remain. Adenovirus enters cells via a cell surface protein known as coxsackie and adenovirus receptor (CAR) that physiologically functions as an adhesion protein.<sup>9</sup> In bladder cancer, decreased CAR expression has been associated with increased tumor invasiveness and poor prognosis.<sup>10</sup> Thus the development of strategies to overcome obstacles associated with immune clearance and viral transduction of CAR negative cells is needed.

Cationic lipids and polymers have been employed to enhance adenovirus-mediated gene delivery.<sup>11–18</sup> Cationic lipids and macromolecules can overcome the repulsion

between the negatively charged adenoviral surfaces and anionic glycosaminoglycan coated epithelial cells, leading to enhanced adenoviral infection in cells that downregulate CAR.<sup>19</sup> For example, cationic polymers including poly-L-lysine<sup>11</sup> and poly(ethylene imine) (pEI)<sup>16</sup> have been previously used for enhancing adenoviral infection, but the toxicity of these polymers is a significant limitation to their subsequent use. Thus, while enhancing adenoviral infectivity with cationic polymers remains a promising approach, new polymers that demonstrate high infectivity and low toxicities are needed in order to realize the promise of this strategy. We have recently generated and screened a library of cationic polymers for binding anionic plasmid DNA for cancer cell transfection.<sup>20</sup> A number of candidate polymers demonstrated higher transfection efficacies and lower cytotoxicities compared to the commonly used polymer, pEI. In the current study, we investigated the ability of a candidate polymer from this library to enhance adenoviral transduction in CAR-negative bladder cancer cells. We observed that the candidate polymer, ethylene glycol diglycidyl ether (EGDE)-3,3'-diamino-*N*-methyl dipropylamine (3,3') or EGDE-3,3', demonstrated higher efficacies for enhancing adenoviral transgene expression than pEI. In addition, complexing EGDE-3,3' with an adenovirus expressing TRAIL enhanced bladder cancer cell death. Our results underscore the opportunities presented by novel biocompatible polymers for enhancing adenoviral infection and apoptosis of bladder cancer cells.

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## Materials and Methods

**Materials.** The monomers, ethylene glycol diglycidyl ether (EGDE) and 3,3'-diamino-*N*-methyl dipropylamine (3,3'), were purchased from Sigma-Aldrich. In addition, branched 25 kDa poly(ethylene imine) ( $M_n = 10$  kDa,  $M_w = 25$  kDa, catalog number 408727; henceforth called pEI) was purchased from Sigma-Aldrich.

**Cell Lines and Culture Conditions.** Human bladder cancer cells lines 5637, RT4, UM-UC3, TCCSUP, and SW780 were purchased from the American Type Culture Collection (Manassas, VA) and grown in RPMI containing 10% fetal calf serum, with the exception of RT4 cells, which were grown in McCoy's medium containing 10% fetal calf serum. All medium was supplemented with Gibco 15240 antibiotic-antimycotic mixture (Invitrogen, Grand Island, NY). TCCSUP were used between passages 5 to 15, by splitting nearly confluent cultures up to a 1:10 ratio every 3–4 days.

**Adenovirus.** Replication-deficient adenoviral vectors were generated by the Medical University of South Carolina (MUSC) vector core facility. The transgene cassette for Ad.GFP contains the green fluorescent protein (GFP) reporter under control of the cytomegalovirus (CMV) promoter. The GFP-TRAIL fusion protein was generated by polymerase chain reaction (PCR) amplification of human TNF-related apoptosis inducing ligand (TRAIL) cDNA and inserting it into the plasmid vector pEGFP-C1 (Clontech). The insert was verified by sequencing and subcloned into the RA.D.T. mcs plasmid shuttle vector.<sup>21</sup> The verified plasmid was then provided to the MUSC vector core for adenovirus generation as previously described for an adenovirus expressing Fas Ligand.<sup>21</sup> Ad.GFP-TRAIL is comparable in potency to the Ad.TRAIL-IRES-GFP we previously generated,<sup>22</sup> indicating that the GFP fusion does not interfere with TRAIL function. One difference between these constructs is that transgene expression in Ad.TRAIL-IRES-GFP is under control of the Tet-On system, while Ad.GFP-TRAIL uses the Tet-Off system. A benefit of the GFP-TRAIL fusion protein is that subcellular localization of TRAIL can be studied by fluorescence microscopy to detect GFP.

**Polymer Synthesis.** Polymerizations were carried out as described previously.<sup>20</sup> Briefly, ethylene glycol diglycidyl ether (EGDE; 2.3 mmol) was reacted with equimolar quantities of 3,3'-diamino-*N*-methyl dipropylamine (3,3'), resulting in the formation of the EGDE-3,3' polymer. Neat, as-purchased solutions were employed in the polymerization reaction, which was carried out in 7 mL glass scintillation vials for 16 h. Following completion of the reaction, the

resulting polymer was diluted to a concentration of 10 mg/mL in phosphate-buffered saline (PBS). The solution pH was adjusted to 7.4 using 30% hydrochloric acid in deionized (DI) water in order to compensate for the alkalinity of the cationic polymers. Polymer stock concentrations of 10 mg/mL were used in the experiments.

**Kinetics of Polymerization.** The polymerization reaction involves the reaction of epoxide groups on the diglycidyl ethers with primary and secondary amines of the polyamines. As a result, the disappearance of reactive (primary and secondary) amines with time was employed to follow the kinetics of the diglycidyl ether-polyamine reaction.<sup>20</sup> The ninhydrin assay<sup>23</sup> results in a yellow-orange color in the case of secondary amines and a dark blue/purple color in the case of primary amines and was therefore used to determine the concentration of residual reactive amines using absorbance analyses at 570 nm at each time point as described previously.<sup>20</sup> The assay was carried out every 4 h, and the concentration of reactive amines in the polymerization mixture at a given time point was normalized with the concentration of amines at the beginning of the reaction ( $t = 0$ ) in order to obtain percentage residual reactive amine values.

**Polymer Characterization.** Fourier transform infrared (FT-IR) spectroscopy spectra of the polymer sample were analyzed at two different time points ( $t = 0$  and 16 h) using a Bruker IFS 66 v/S FT-IR spectrometer in order to ascertain the formation of the EGDE-3,3' polymer. The polymer was loaded such that it covered a substantial area around the center of a germanium attenuated total reflectance (GATR) crystal. The sample chamber was maintained at 4 mbar pressure in order to minimize interference from atmospheric water and CO<sub>2</sub>. The absorption spectrum between 650 and 4000 cm<sup>-1</sup> was measured, and the background spectrum of the crystal was subtracted from the sample spectra. Polymer molecular weight and polydispersity were determined using gel permeation chromatography as described previously.<sup>20</sup>

**Adenoviral Transduction.** Cells were plated overnight at a density of  $6 \times 10^4$ /well in a 24-well plate. The next day, Ad.GFP was diluted in growth medium to the appropriate multiplicity of infection (MOI) and growth medium in the wells was replaced with medium containing adenovirus. For experiments with polymers, virus was diluted to the appropriate MOI and preincubated for 10 min at room temperature with 2  $\mu$ L/well polymer (10 mg/mL stock) in a final volume of 67  $\mu$ L/well growth medium. Following the 10 min incubation, 100  $\mu$ L/well growth medium was added to the tube and the growth medium in each well replaced with 169  $\mu$ L of polymer/virus mixture. After 4 h, an additional 400  $\mu$ L growth medium was added to each well to ensure cells had sufficient nutrients for the assay period. Cells were assayed for GFP expression and/or cell death 48 h postinfection.

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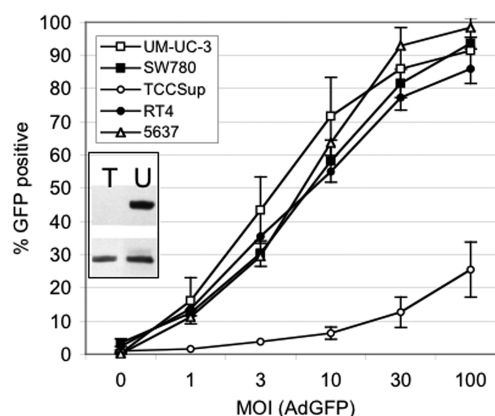


**Western Blot Analysis.** Protein was prepared and analyzed as described previously.<sup>24</sup> The antibody recognizing CAR (M-150) was purchased from SantaCruz Biotechnology (Santa Cruz, CA) and was used at a dilution of 1:1000.

**Flow Cytometry.** All cells (nonadherent and adherent) were included in the analysis. Briefly, 48 h postinfection, media were collected and adherent cells rinsed with PBS prior to adding trypsin. The PBS wash was added to the tubes containing the spent media. After cells had been detached with trypsin, they were pooled with the nonadherent cells. Cells were pelleted at 1500 rpm for 10 min and pellets resuspended in PBS. Viaprobe (20  $\mu$ L), a cell viability detection reagent containing 7-amino-actinomycin D (7-AAD) (BD Bioscience Pharmingen, San Jose, CA), was added where appropriate. Samples were analyzed in the MUSC core facility using a FACSCalibur (BD). A minimum of 10,000 events were evaluated and analyzed with the CellQuest software.

## Results and Discussion

**TCCSUP Bladder Cancer Cells Are Highly Resistant to Adenoviral Transduction Due to Lack of CAR Expression.** The first goal for testing whether cationic polymers from our newly developed library can enhance adenoviral transduction and transgene expression was to identify a cell line that is resistant to adenoviral infection. Adenovirus infects cells via receptor-mediated endocytosis following binding to the cell surface protein CAR (coxsackie and adenovirus receptor). Since CAR expression is frequently decreased in bladder cancer, we screened a panel of bladder cancer cell lines for infectivity and CAR expression. Cells were exposed to increasing concentrations of a replication-deficient adenoviral construct that expresses a CMV-driven GFP reporter gene (Ad.GFP). The percentage of GFP positive cells was assessed by flow cytometry at 48 h postinfection. As shown in Figure 1, transduction rates of 5637, RT4, SW780, and UM-UC-3 cells were similar with 50% infectivity achieved at an MOI of 10. In TCCSUP cells, however, even a 10-fold higher concentration of adenovirus resulted in transduction of only 20%, suggesting these cells may have reduced CAR expression. Analysis of CAR expression by Western blot confirmed that the protein is absent or at least below detectable levels in TCCSUP cells (Figure 1, insert). TCCSUP cells were isolated from a patient with transitional cell carcinoma that later metastasized to the bone.<sup>25</sup> Lack of CAR expression, which decreases cellular adhesion and correlates with bladder cancer aggressiveness, may offer an explanation for this outcome.<sup>10</sup> In addition to downregulated



**Figure 1.** Adenoviral infectivity and CAR expression. Bladder cancer cells were plated overnight and exposed to the indicated amount of Ad.GFP for 4 h. GFP expression was quantified by flow cytometry at 48 h postinfection. Data represent the mean  $\pm$  SD from at least 3 independent experiments, except RT4 cells which were tested only once. Inset: Total CAR expression was assessed by Western blot (U = UM-UC-3, T = TCCSUP). Actin is included as a loading control. Similar results were obtained in at least 2 independent experiments.

CAR, the abundant expression of negatively charged glycosaminoglycans on bladder cancer epithelial cells results in repulsion of adenoviruses due to the anionic nature of their outer surfaces (e.g., hexons).<sup>18,19</sup> Excess cationic charge on the virus surface from the cationic polymer can facilitate electrostatic interactions between the polymer:virus complex (positively charged) and GAGs (negatively charged), facilitating cellular binding and uptake, which in turn results in increased infection. In addition, polymers can mediate viral aggregation toward the surface of targeted cells which also promotes increased transduction.<sup>26</sup>

**Characterization of Cationic Polymers for Adenoviral Transduction.** The observation that TCCSUP cells lack CAR expression and are extremely difficult to transduce with adenovirus allowed us to evaluate the efficacy of polymers on viral uptake and gene expression. Cationic polymers neutralize the anionic charge on plasmid DNA and viral surfaces by means of electrostatic interactions.<sup>27,28</sup> As a result cationic polymers have been employed for the delivery of plasmid DNA and viral based gene expression systems to

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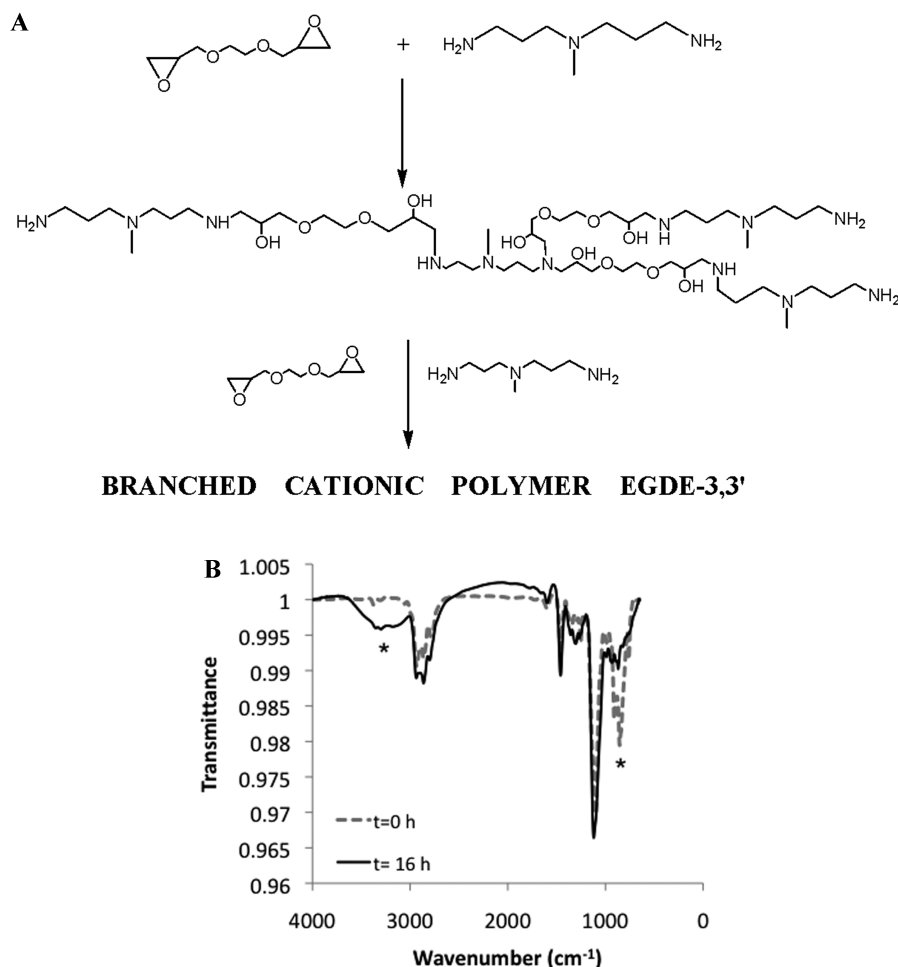
mammalian cells.<sup>11,14,17,26,29–47</sup> Recent results in our laboratory led to the identification of cationic polymers that demonstrated high DNA binding and gene transfection efficacies.<sup>20</sup> We therefore hypothesized that candidate cat-

ionic polymers from this library would be able to neutralize the anionic charge on adenoviral surfaces leading to enhanced transduction of TCCSUP cells that have undetectable CAR expression. The cationic polymer, EGDE-3,3', was among the highest DNA-binding polymers identified in our previous screen.<sup>20</sup> Consequently, we tested the efficacy of both pEI and EGDE-3,3' on adenoviral transduction.

The formation of the EGDE-3,3' polymer was evaluated by the decrease in reactive amines over time using a ninhydrin assay based on absorbance spectroscopy. The amine concentration was determined based on a calibration curve generated using glycine standards. Approximately 70% primary reactive amines had disappeared 12 h after the reaction, after which the concentration of the reactive amines remained unchanged.<sup>20</sup> This indicated that the polymer was formed rapidly and that the reaction rate remained largely invariant after 12 h. The molecular weights of the EGDE-3,3' polymer determined by GPC were  $M_n = 14.52 (\pm 0.26)$  kDa,  $M_w = 72.06 (\pm 0.72)$  kDa, resulting in a polydispersity of 4.96.<sup>20</sup> The formation of the EGDE-3,3' polymer was further analyzed using Fourier transform infrared (FT-IR) spectroscopy following the appearance and disappearance of bands at two different times ( $t = 0$  and 16 h). The reaction time of 16 h was chosen in order to allow the polymerization to reach completion based on the kinetics. At time  $t = 0$  h, peaks in the wavenumber range of 858–918  $\text{cm}^{-1}$ , attributed to the stretching and contraction of C–O groups, can be seen in the monomer mixture indicating the presence of epoxide groups (Figure 2B).<sup>48</sup> However, the reduction in transmittance in this range indicates the disappearance of epoxide groups, which is consistent with the chemistry of the polymerization reaction. The disappearance of the epoxide groups is accompanied by a concomitant increase in hydroxyl (–OH) groups. The appearance of broad bands in the wavenumber range 3382–3402  $\text{cm}^{-1}$  is indicative of the hydroxyl groups generated as a result of the polymerization reaction.<sup>49</sup> Characteristic spectral bands of primary amines were seen at 1100–1128 and 3358–3382  $\text{cm}^{-1}$  due to the presence of C–N and N–H bonds, respectively. FT-IR analysis further confirmed that the diglycidyl ether–polyamine reaction resulted in the formation of the EGDE-3,3' polymer

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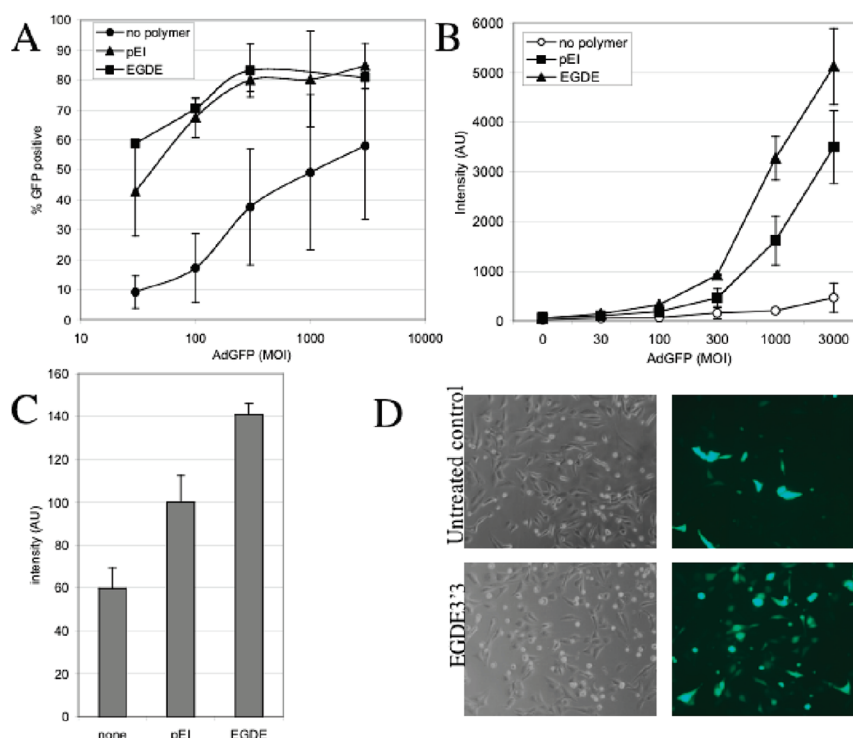
**Figure 2.** (A) Schematic of the polymerization reaction for generating the EGDE-3,3' polymer based on ethylene glycol diglycidyl ether (EGDE) and 3,3'-diamino-*N*-methyl dipropylamine (3,3') monomers. (B) Fourier transform infrared (FT-IR) spectroscopy of EGDE-3,3' polymerization at  $t = 0$  and 16 h. Polymerization reaction can be seen by the emergence of the hydroxyl peak from 3000–4000  $\text{cm}^{-1}$  and the disappearance of the epoxide peak in the 860–950  $\text{cm}^{-1}$  region of the FT-IR spectrum. These regions are marked with asterisks in the figure.

used in the current investigation, and the results are along the lines of those observed previously for such polymerizations.<sup>20</sup>

**Cationic Polymers Facilitate Adenoviral Transduction in TCCSUP Cells.** Having confirmed synthesis of EGDE-3,3' polymer, we next determined the effect of adenovirus-polymer preincubation on viral transduction; pEI was included for comparison in our analysis. In the absence of polymer, even 3000 MOI of Ad.GFP resulted in only 50–60% infectivity. Although the extremely high concentration of adenovirus did not adversely affect TCCSUP cells, the amount is not physiologically relevant. When Ad.GFP was preincubated in the presence of polymers, adenoviral transduction was greatly enhanced and a rate of 50–60% infectivity was now achieved at 30 MOI (Figure 3A). In addition to infectivity, we also evaluated GFP intensity as a measure of transgene expression. Though both polymers enhanced infectivity to a similar extent with respect to the number of cells transduced, the EGDE-3,3' polymer consistently resulted in higher transgene expression in the

individual cells (Figure 3B,C). Brightfield analysis did not indicate any obvious adverse effects of the polymers at the concentrations used, but the long-term effect on viability will be further investigated in the future (Figure 3D). Fluorescent microscopy confirmed the increase in infectivity and reporter transgene expression (Figure 3D). These data indicate that preincubation with polymers decreases the amount of adenovirus required to transduce 50–60% of CAR-negative cells by 100-fold. Both pEI and EGDE-3,3' polymers enhanced viral infectivity, but the latter resulted in improved transgene expression. While the underlying reason for this difference will need to be further investigated, it prompted us to examine EGDE-3,3' in more detail.

**EGDE-3,3' Polymer Enhances Ad.GFP-TRAIL-Mediated Cell Death.** To determine whether polymer-enhanced adenoviral delivery would translate into a biological effect, we quantified cell death achieved by adenoviral delivery of a GFP-TRAIL fusion protein (Ad.GFP-TRAIL). Fusion of the death ligand TRAIL to GFP has in our hands not altered the biological function of TRAIL (data not shown). In Figure



**Figure 3.** Effect of polymers on infectivity and transgene expression. TCCSUP cells were plated overnight and exposed to Ad.GFP either alone or complexed with polymers as described in Materials and Methods. GFP expression was quantified by flow cytometry at 48 h postinfection. (A) % infectivity based on the number of GFP positive cells, (B/C) GFP intensity as measure of transgene expression with increasing MOI (B) and at 30 MOI (C). Data shown are the mean  $\pm$  SD from 3 independent experiments. (D) Representative images under brightfield and fluorescent microscopy of TCCSUP cells exposed to 30 MOI of Ad.GFP without (control) and with (EGDE-3,3') polymer preincubation.

4A, we show that the percentage of GFP positive cells is enhanced when the Ad.GFP-TRAIL is preincubated with EGDE-3,3' polymer, though the effect was less pronounced compared to Ad.GFP. Several possibilities can account for this discrepancy. One possibility is that cells infected with Ad.GFP-TRAIL are undergoing cell death, which may not allow accumulation of GFP fluorescence. The fusion protein also exhibits different subcellular distribution than GFP alone and may not be as brightly fluorescent when TRAIL is added to the GFP sequence. The discrepancy could also be related to the different promoters used in the viral vectors.

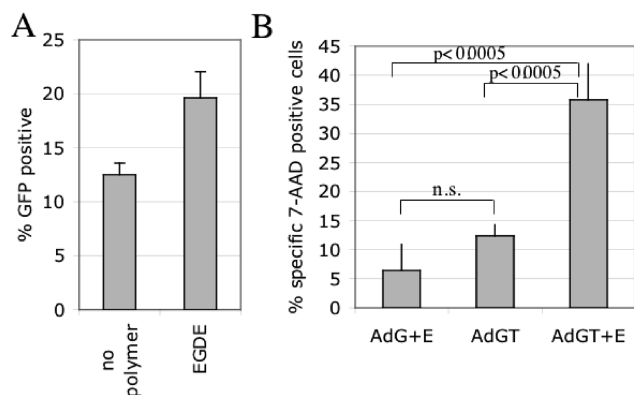
Quantification of cell death was performed by flow cytometry with 7-AAD, which intercalates into the DNA in cells with compromised membrane integrity. The effect of the EGDE-3,3' polymer on cell death was approximately 5%, and the effect of Ad.GFP-TRAIL alone was about 12%. The statistical difference did not reach significance ( $p > 0.05$ ). Preincubation of Ad.GFP-TRAIL resulted in a greater than additive effect reaching 32–40% of cell death. Statistical analysis indicates that cell death was significantly higher when Ad.GFP-TRAIL was precomplexed with EGDE-3,3' (Figure 4, compare AdGT to AdGT+E,  $p < 0.0005$ ). The data indicate that enhanced viral uptake following incubation of adenovirus with EGDE-3,3' translates into a biological response.

**Conclusions and Future Directions.** Our results suggest that the EGDE-3,3' polymer can enhance adenoviral trans-

duction and transgene expression in cells that lack CAR expression. TCCSUP cells were extremely resistant to adenoviral transduction and the EGDE-3,3' polymer reduced the amount of virus required to achieve 50–60% transduction by 100-fold, resulting in the ability to use adenovirus at a physiologically relevant concentration. While the use of polymer–adenovirus complexes restores infectivity of malignant bladder epithelial cells, there is a possibility that these complexes also infect nonmalignant bladder cells. Studies that compare the relative infection efficacy of polymer–adenovirus complexes for normal and transformed bladder cancer cells will be carried out in the future in our laboratories. In addition, the use of targeting antibodies (e.g., anti-EGFR antibody<sup>19,50,51</sup> since EGFR is overexpressed in bladder cancer disease) can further enhance selective targeting of bladder cancer cells over normal bladder epithelial cells. The presence of primary amines on our cationic polymers facilitates easy conjugation of targeting antibodies by means of simple, well-established chemistries (e.g., EDC–NHS coupling). These targeted complexes will also be explored in the future. Future directions will also include extension of our findings to other cells that transduce poorly

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**Figure 4.** Effect of polymers on efficacy of Ad.GFP-TRAIL-induced cell death. (A) Cells were infected with Ad.GFP-TRAIL at 30 MOI (as described for Ad.GFP) in the absence or presence of EGDE-3,3' polymer. Infectivity was quantified by flow cytometry of GFP. (B) Cells were infected with 30 MOI Ad.GFP or Ad.GFP-TRAIL either in the absence or in the presence of the EGDE-3,3' polymer. Membrane integrity was quantified by flow cytometry following staining with 7-AAD. % specific 7-AAD uptake was calculated by subtracting background staining in Ad.GFP infected cells. All data shown are the mean  $\pm$  SD from at least 3 independent experiments. Statistical significance between groups was calculated using the *t* test (n.s. = not significant).

with adenovirus and investigation of other biocompatible polymers from our library for enhancing adenoviral transduction. The use of adenoviral constructs in which therapeutic efficacy relies on local secretion of protein such as cytokines as well as translation into immunocompetent animal models will also be important.

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