Recombinant E1-Deleted Adenoviral Vectors Induce Apoptosis in a Rat Airway Epithelial Mucous Goblet Cell Line

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ABSTRACT—Replication-defective adenoviruses (Ad) are used as vectors for delivering therapeutic genes to human airway cells. We examined whether E1-deleted Ad vectors (Ad5-CMV-LacZ) had effects on cell kinetics in SPOC1 cells, which is a rat airway epithelial mucous goblet cell line. There was a vector multiplicity of infection (moi)-dependent increase of the transduction efficiency of the LacZ reporter gene in SPOC cells. Cell proliferation was inhibited in the vector-infected cells compared with that in vehicle-exposed cells. However, increased cell death was observed in the vector-infected cells with a higher moi. The morphology of vector-exposed cells revealed apoptotic features including nuclear condensation and a fragmented nucleus. These results indicate that higher moi of vectors allows the cells to achieve higher gene transfer, but also induce apoptosis of infected cells. Minimizing the induction of apoptosis of vector-infected cells may be an important strategy for the prolongation of transduction efficiency of Ad vectors in airway epithelial mucous goblet cells.

Keywords: SPOC1 cell, E1-deleted adenoviral vectors, LacZ gene, Apoptosis, Mucous goblet cell

Because the adenoviral vectors (Ad vectors) efficiently transduce foreign genes into a wide variety of cells types and into proliferating as well as non-proliferating cells, Ad vectors are candidates for gene therapy for various airway diseases (1–3). However, in airway cells, several limitations should be considered for controlling pulmonary diseases with gene therapy in vivo using Ad vectors (1). Ad vectors are known to cause extensive host immune and inflammatory responses, and the immune response may be responsible for the loss of transgene expression (4, 5). We have found that Ad vectors perturb cell proliferation in human airway epithelial cells and lung adenocarcinoma cells, reflecting apoptosis and slowing of the cell cycle (6, 7). Although submucosal glands may be the target for gene therapy of human airway diseases (8, 9), cell kinetics following Ad vector infection in airway epithelial mucous goblet cells has not been elucidated.

The SPOC1 cell line is a novel goblet cell line derived from rat trachea (10, 11). Cultures of SPOC1 cells are reported to secrete mucin on exposure to phorbol 12-myristate 13-acetate and ionomycin (12). Thus SPOC1 cells may be a useful tool for studying airway secretory differentiation and function (10, 11).

The present study was designed to examine whether E1-deleted Ad vector (Ad5-CMV-lacZ) had effects on cell kinetics of a rat airway epithelial mucous goblet cell line, such as SPOC1 cells.

MATERIALS AND METHODS

Ad vector

Replication defective adenoviral vector based on human Ad 5 serotype (hAd5) was used for the study. In the hAd5-CMV-LacZ, E1 and E3 sequences were deleted and replaced with a mini-gene containing the CMV promoter, and a cytoplasmic LacZ gene inserted at the site of the E1 deletion of Ad (13–15). The Ad vector was propagated in 293 cells, purified by CsCl gradient ultracentrifugation and stored at -70°C until used for infection of rat airway epithelial mucous goblet cells. Ad vector's titer (transducing unit (TU/ml)) was determined by the number of LacZ gene expressing 293 cells per milliliter of vectors by histochemical X-gal staining (13–15). The experiments used the vector produced with titers of $1-10 \times 10^{11}$ (TU/ml). The ratio of TU to viral particle number (as measured by OD at 260 nm) was approximately 1:20–50.

Cell cultures

In the present study, a rat airway epithelial mucous goblet cell line, SPOC1 cells, were kindly given by Randel SH (Chapel Hill, NC, USA) (11). All experiments using SPOC1 cells were performed on a single clone, between passage 14 to 15. The cells were fed daily with Dulbecco's modified Eagle's medium-Ham's F-12-base culture medium (DMEM-F-12) (Gibco, Life Technologies, Inc., Grand Island, NY, USA) with supplements (10–12). The medium was supplemented with 30 mM *N*-2-hydroxyethylpiperazine-*N'*-2 ethanesulfonic acid, 6.5 mM L-glutamate, 10 μ g/ml insulin, 0.1 μ g/ml hydrocortisone, 0.1 μ g/ml cholera toxin, 5 μ g/ml transferrin, 50 μ M phosphoethanolamine, 80 μ M ethanolamine, 25 ng/ml epidermal growth factor, 1% (vol/vol) bovine pituitary extract, 1 mg/ml bovine serum albumin (essentially globulin-free, no. A7638; Sigma Chemical Co., St. Louis, MO, USA), 50 U/ml penicillin, and 50 μ g/ml streptomycin. The medium was also supplemented with 10 nM retinoic acid.

Ad vector infection of SPOC1 cells

SPOC1 cells were plated at 1×10^5 cells per dish in 6-well supports (Costar, Cambridge, MA, USA), allowed to attach for 12 h, and then the non-adherent cells were removed by gentle washing with phosphate-buffered saline (PBS). One day after plating, the total cell number/dish equaled approximately $1-2 \times 10^5$ ($n=5$). Dishes were then randomly assigned to one of six experimental groups: multiplicity of infection (moi) equal to 1 (10^0), 10 (10^1), 100 (10^2), 1000 (10^3), 10000 (10^4) or vehicle control. The moi was determined as one transducing unit per cell in 293 cells (13–15). Ad vector was added to the dishes in 1.5 ml of DMEM-F-12 and exposed to the cells for 1 h at 37°C in air plus 5% CO₂. Then the cells were washed with PBS and fed with fresh DMEM-F-12. The control group was exposed to 1.5 ml of vehicle (DMEM-F-12) alone for comparable time periods and washed as above.

Assessment of transduction efficiency of Ad vectors in SPOC1 cells

One day after infection, the transduction efficiency was quantitated as the percentage of LacZ expressing cells using X-gal staining (13–15). In brief, cultured cells were washed with PBS, fixed for 8 min in 0.5% glutaraldehyde in PBS, then washed with PBS containing 1 mM MgCl₂ and left at 4°C for 15 min. The cells were overlaid with a freshly prepared histochemical reaction mixture containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-*b*-D-galactopyranoside (X-gal), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 1 mM MgCl₂ in PBS for 4 h at 37°C. In all experiments, at least three wells for each group were stained for LacZ gene expression and

more than 500 cells/well were counted to determine the percentage of LacZ expressing cells.

Cell proliferation and viability after Ad vector infection in SPOC1 cells

The cell proliferation rate in SPOC1 cells was determined by counting cells every other day for 5 days after exposure of Ad vector to the cells. The cell numbers of five dishes for each group were calculated using a standard hemacytometer following the detachment by 0.1% trypsin plus 1 mM ethylenedinitrilo-tetraacetic acid (EDTA) in PBS (trypsin-EDTA solution). Cell viability was assessed by trypan blue exclusion. SPOC1 cells were plated at 5×10^4 cells per dish in 12-well supports (Costar), allowed to attach for 12 h, and then non-adherent cells were removed by gentle washing with PBS. One day after plating, the total cell number/dish equaled approximately $5-6 \times 10^4$ ($n=5$). Dishes were then randomly assigned to one of four experimental groups: moi equal to 10^0 , 10^2 , 10^4 or vehicle control. Ad vector was added to the dishes in 1.5 ml of DMEM-F-12 and exposed to the cells for 1 h at 37°C in air plus 5% CO₂. The control group was exposed to 1.5 ml of vehicle (DMEM-F-12) alone for comparable time periods.

Detection of apoptosis after Ad vector infection in SPOC1 cells

Morphologic characteristics of cells after Ad vector infection in SPOC1 cells: Morphologic characterization of cells was performed by transmission electron microscopy (TEM). One day after seeding SPOC1 cells (10^6) on two 60-mm dishes, cells in one dish were exposed to Ad vector (moi of 10^4 , 1 h) and the other dish was exposed to vehicle (1 h). At 24 h after Ad vector exposure, the detached cells in the culture medium of Ad vector-exposed dish were harvested by centrifugation at 800 r.p.m. (200 $\times$ g). The detached and adherent cells of the vehicle-exposed dish were collected using by EDTA solution. The harvested cells were immediately fixed with 2% glutaraldehyde and 2% PFA in 0.1 M phosphate buffer (pH 7.2) and then postfixed with 1% buffered osmium tetroxide (OsO₄) in 0.1 M phosphate buffer at 4°C for 1 h (16). After alcohol dehydration, the cells were embedded in Epon Resin according to the standard method and sectioned using a LKB-Huxley ultramicrotome to final thickness of 60–90 μ m. Sections were stained with uranyl acetate and lead acetate and then examined in a Hitachi H-7100 transmission electron microscope (Hitachi, Tokyo) (16).

Quantification of apoptosis using TUNEL staining in culture dish: To determine the ratio of apoptotic cells to the total cell in a culture dish, modified terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) was performed using Apoptag®

(Oncor Co., Gaithersburg, MD, USA) (16). SPOC1 cells were plated at 3×10^5 cells per dish in 6-well culture dishes (Costar). One day after seeding cells, the cells in each of nine wells were exposed to Ad vectors for 1 h at moi of 10^0 , 10^2 , 10^4 or vehicle, respectively. At 24, 48 and 72 h after Ad vector infection, the cells harvested using trypsin-EDTA solution were stained by the modified TUNEL method. In brief, the cells harvested from the culture dish using trypsin-EDTA were fixed by 2% paraformaldehyde on ice for 15 min. The cells were washed with PBS and pelleted ($200 \times g$ for 5 min). After centrifugation, the supernatant was removed, and cells were resuspended in 32 μ l of equilibration buffer. After centrifugation at $200 \times g$ for 5 min, the supernatant was removed, and cells were incubated with 8 μ l of TdT enzyme with 19 μ l of reaction buffer at 37°C for 1 h. The reaction was terminated by addition of 20 μ l of stop buffer with 680 μ l of distilled water. After centrifugation, the supernatant was removed and resuspended in 20 μ l of anti-digoxigenin-fluorescein with 38 μ l of blocking solution, then incubated at room temperature for 30 min. The cell suspension was added with 10 μ l of 0.1% triton X-100 in PBS. After centrifugation, the supernatant was removed, the cells were resuspended in 1 μ g of propidium iodide (PI) (Sigma) with 10 μ g of RNase (Boehringer Mannheim Co, Tokyo.) in 200 μ l of PBS for 15 min, and then washed with PBS. The anti-digoxigenin-fluorescein—positive cells (apoptotic cells) were counted using a hemacytometer under a fluorescent microscope (Microphot, EPI-FL3; Nikon, Tokyo). Total cell numbers were determined by counting cells with PI staining under the microscope. The ratio of apoptotic cells to total cells in each sample (minimum of 500 cells counted in each sample) was calculated.

Statistical analyses

Data were presented as the mean $\pm$ S.D. Statistical analyses were performed using analysis of variance (ANOVA) with Fisher's protected least significant difference method or Student's *t*-test using by Stat View 4.0 (Abacus Concepts, Inc., Berkeley, CA, USA). A $P < 0.05$ was considered to be statistically significant.

RESULTS

Gene transduction efficiency of Ad vectors in SPOC1 cells

The efficiency of LacZ reporter gene transfer to SPOC1 cells is shown in Fig. 1. There was a dose-dependent relationship between the transduction efficiency of Ad vectors in SPOC1 cells and the vector moi. More than 80% of cells were transduced with Ad vectors at a moi of 10^3 and 10^4 .

Fig. 1. The transduction efficiency of hAd5-CMV-LacZ vectors in SPOC1 cells.

Growth curves of SPOC1 cells with or without Ad vector infection

The kinetics of cell proliferation after exposure to Ad vector or vehicle in SPOC1 cells are shown in Fig. 2. The growth curves of Ad vector infected cells were shifted to the right in a moi-dependent manner. Furthermore, the viability of cells as indexed by trypan blue exclusion was lower in vector-exposed cells than in vehicle-exposed cells

Fig. 2. Kinetics of cell proliferation after exposure to vector (hAd-CMV-LacZ) or vehicle in SPOC1 cells. The value of cell numbers in each day was expressed as the mean $\pm$ S.D. value of the cell populations of three dishes in each group. The * indicates statistically different from the value with vehicle exposure ($P < 0.05$). $\square$ vehicle, $\circ$ Ad (moi = 10^0), $\blacksquare$ Ad (moi = 10^2), $\bullet$ Ad (moi = 10^4).

Fig. 3. Viability of cells as indexed by trypan blue exclusion as a function of time after vector and vehicle exposure to SPOC1 cells. The * indicates statistically different from the value with vehicle exposure ($P < 0.05$). Each value presented as the mean $\pm$ S.D. ($n=3$). □ vehicle, ▨ Ad (moi=10⁰), ▩ Ad (moi=10²), ■ Ad (moi=10⁴).

(Fig. 3). Lower viability of Ad-vector-infected cells lasted at least 48 h after Ad vector infection compared with the viability of vehicle-exposed cells.

Detection of apoptosis in Ad-vector-infected cells

Micrographs of transmission electron microscopy of vehicle-exposed cells and Ad-vector-exposed cells are shown in Fig. 4. Compared with vehicle-exposed cells, Ad-vector-infected cells exhibited features of apoptosis such as cell shrinkage, nuclear condensation and nuclear fragmentation. Morphologic features of apoptosis was also found in Ad-vector-infected cells at a moi of 10⁰ and

Fig. 5. Effect of Ad vector (hAd-CMV-LacZ) infection on induction of apoptosis in SPOC1 cells. The ratio of apoptotic cells (TUNEL-positive cells) to total cells was measured in SPOC1 cells with exposure to Ad vectors. Each value is presented as the mean $\pm$ S.D. ($n=3$). The * indicates statistically different from the value with vehicle exposure ($*P < 0.05$). □ vehicle, ▨ Ad (moi=10⁰), ▩ Ad (moi=10²), ■ Ad (moi=10⁴).

10². However, the number of apoptotic cells to total cells was smaller in Ad vector infected cells at a moi of 10⁰ and 10² than in the cells at a moi of 10⁴. Furthermore, the apoptotic cells exhibiting the morphologic features of nuclear condensation/fragmentation were decreased with increasing days after Ad vector infection in each moi of Ad vectors. The percentage of apoptotic cells was quantitated by DNA strand breaks detected by digoxigenin-conjugated dUTP labeling (TUNEL) (Fig. 5). The ratio of apoptotic cells (TUNEL-positive cells) to total cells

Fig. 4. Transmission electron microscopy of detached and adherent SPOC1 cells the exposed to vehicle harvested from the culture dish using EDTA solution (A) or detached SPOC1 cells exposed to Ad vector (hAd-CMV-LacZ) at a moi of 10⁴ (B). Scale bar = 7 μ m.

was increased with an increase of vector moi. However, the apoptotic cells after Ad vector infection decreased with time in culture in accordance with the decreased number of morphologic nuclear alterations.

DISCUSSION

The present study demonstrated that E1-deleted Ad vector efficiently transduced the foreign gene to cultured SPOC1 cells. A vector moi-dependent increase of the transduction efficiency of LacZ reporter gene was observed in SPOC1 cells. Although replication defective Ad vectors are known to infect various types of cells, the efficiency of Ad-mediated gene transfer is not the same in the different types of airway cells (1–3, 17). However, the current data of the efficiency of Ad-mediated gene transfer to SPOC1 cells, which is an rat airway epithelial mucous goblet cell line, are identical to the data of other airway cells (2, 6, 7, 13).

The present study also demonstrated that the vector infection inhibited the growth of the SPOC1 cells. Further experiments revealed that Ad vector caused an increased apoptotic cell death in the infected cells. Thus the Ad vector-induced apoptosis may be at least in part responsible for the slower growth of SPOC1 cells after Ad vector infection. Although there was a vector-moi-dependent relationship between vector moi and the frequency of apoptosis of SPOC1 cells, the viability of the Ad-infected SPOC1 cells was restored within 72 h. This suggests that Ad-vector-induced apoptosis predominantly may occur and may be completed within 24 to 48 h after Ad vector is exposed to the cells. Apoptosis is an active mode of programmed cell death, and the process is mostly accomplished within 72 h. In airway mucous cells, the process may be completed within 48 h. Consequently, the viability of the Ad-infected SPOC1 cells are increased with increasing days after the Ad vector exposure to the cells. However the cell growth is affected by both the viability of the cells and the cell density in culture; log phase growth was retarded in Ad vector infected cells in a higher moi.

Because the transient gene expression is one of major limitations of Ad-mediated gene therapy for pulmonary diseases, the loss of Ad-vector-infected cells due to apoptosis may not be advantage for the efficacy of gene transduction of Ad vectors in gland cells. Unfortunately, we did not determine the mechanism of the Ad-vector-induced apoptosis in SPOC1 cells. However, several possibilities are considered. First, the greater amount of vector protein at a higher moi seems to be harmful to cells in culture. Second, other Ad genes retained in E1-, E3-deleted Ad vectors may induce apoptotic cell death in airway cells. Although many Ad early genes including

E1a and E3 are known to induce apoptosis and/or inhibit cytolysis in the infected cells (18–20), these genes have been deleted from Ad vectors utilized for gene therapy of human airway disease. However, it has been reported that adenovirus type 5 E4 open reading frame 4 (orf 4) induces apoptosis in transformed cells in vitro (21, 22). In addition, E1-, E4-deleted adenovirus vector has been reported to achieve the persistence of transgene expression in the liver and lung in mice (23). These observations suggest that Ad E4 genes retained in the current E1-, E3-deleted adenovirus vectors may be responsible for the vector-induced apoptosis in a rat airway epithelial mucous goblet cell line.

SPOC1 cell is the first mucin-secreting cell line from the airways, and it was developed from spontaneous immortalization in secondary cultures of rat tracheal epithelial cells (10). Subsequently, the cells were demonstrated to differentiate into goblet-like cells under appropriate culture conditions (11) and to secrete mucin. However, the cells in the current study are not confluent. The previous data suggest that the cultured cells 6–10 days after confluence are useful for examining the ability of mucous secretion with stimulants (11, 12). Thus, further studies are needed to elucidate the transduction efficiency of Ad vectors and Ad-vector-induced apoptosis in mucin-rich goblet cells using the cultured cells several days after confluence.

In summary, Ad-mediated gene transfer to rat tracheal epithelial gland cells was very efficient in a moi-dependent fashion. However, the higher moi of Ad vectors could induce apoptotic cell death in Ad-vector-infected cells and subsequently reduce the transduction efficiency of Ad vectors with increasing days. Because a higher moi of Ad vectors may be necessary to transduce a foreign gene to target cells in vivo, Ad-vector-induced apoptosis may decline transgene expression of Ad vectors in airway epithelial glands. These effects of Ad vectors on cell kinetics are disadvantageous for efficacy of gene therapy of airway disease by reducing the transgene expression of Ad vectors. To minimize the induction of apoptosis of vector-infected cells may be an important strategy for the prolongation of transduction efficiency of Ad vectors in airway epithelial mucous goblet cells.

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