Induction of mesenchymal cell phenotypes in lung epithelial cells by adenovirus E1A

Ali R. Behzad, Kiyoshi Morimoto, John Gosselink, Jennifer Green, James C. Hogg and Shizu Hayashi

James Hogg iCapture Center for Cardiovascular and Pulmonary Research, St. Paul's Hospital, Vancouver, British Columbia, Canada V6Z 1Y6

Address correspondence to:

Shizu Hayashi

University of British Columbia James Hogg iCapture Center for Cardiovascular and Pulmonary Research

St. Paul's Hospital, 1081 Burrard Street

Vancouver, British Columbia, Canada V6Z 1Y6

Tel: (604) 806-8346

Fax: (604) 806-8351

Email: shayashi@mrl.ubc.ca

Short title for manuscript: adenovirus E1A lung cell transformation
Abstract

Epithelial-mesenchymal transformation is now recognized as an important feature of tissue remodeling. The present report concerns the role of adenovirus infection in inducing this transformation in an animal model of COPD.

Guinea pig primary peripheral lung epithelial cells (PLE) transfected with adenovirus E1A (E1A-PLE) were compared to normal guinea pig lung fibroblasts (NLF) transfected with E1A (E1A-NLF). These cells were characterized by PCR, immunocytochemistry, electron microscopy, and Western and Northern analyses. Electrophoretic mobility shifts were performed to examine NF-κB and AP-1 binding activities.

E1A-PLE and E1A-NLF positive for E1A DNA, mRNA and protein expressed cytokeratin and vimentin but not α-smooth muscle actin. Both had cuboidal morphology and junctional complexes but did not contain lamellar bodies or express surfactant A, B or C mRNAs. These two cell types differed, however, in their NF-κB and AP binding after LPS stimulation, possibly due to differences in the expression of the subunits that comprise these transcriptional complexes.

E1A transfection results in the transformation of PLE and NLF to a phenotype intermediate between that of the two primary cells. We postulate that this intermediate phenotype may have a major role in the remodeling of the airways in COPD associated with persistence of E1A DNA.
Introduction

Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and mortality worldwide (1). The loss of lung elastic recoil caused by emphysema and the remodeling process that obstructs the small airways cause the gradual decline in forced expiratory volume in one second (FEV₁) that characterizes this condition (2). Recent studies based on the NIH sponsored national emphysema treatment trial have shown that the progression of COPD is associated with thickening of the walls of the small conducting airways by a repair and remodeling process (2). Although the inhalation of toxic gases and particles is the major risk factor for COPD (3) viral infections, particularly childhood infection add to the risk of developing this condition (1). The adenoviral DNA is retained by peripheral epithelial cells of the lung in humans where the viral E1A gene is expressed (4) without replication of a complete virus (5). Case control studies have shown that the E1A DNA from group C adenoviruses is present in greater amounts in lungs of patients with COPD than in controls with normal lung function matched for age, sex and smoking history (5), and that the excess inflammation in the lungs of smokers with severe emphysema is associated with increased numbers of alveolar epithelial cells expressing E1A (6).
These human observations are supported by experiments in guinea pigs where, in the absence of actively replicating virus but presence of E1A DNA, adenovirus infection resulted in excess lung inflammation following a single exposure to cigarette smoke (7) and increased parenchymal and airway wall inflammation with greater emphysematous destruction after chronic exposure (8). Furthermore, E1A DNA and protein persisted in epithelial cells in lungs of these animals (9).

Reports from several laboratories suggest that a phenomenon termed epithelial-mesenchymal transformation, where epithelial cells transform to fibroblasts capable of producing connective tissue matrix, is an important feature of tissue remodeling (10, 11). In the present study the hypothesis that adenoviral infection followed by persistent expression of its E1A gene is capable of producing epithelial-mesenchymal transformation was examined by investigating guinea pig peripheral lung epithelial cells (PLE) that express adenoviral E1A. We also compared the changes that occur during adenoviral E1A transformation of primary guinea pig PLE to the transformation of fibroblasts that frequently contaminate primary epithelial cultures and present evidence that both cell types are transformed to an intermediate phenotype.

Materials & methods:

Animals and cell culture reagents

Female pathogen free guinea pigs (Cam Hartley strain) (*Cavia Porcellus*, 200 to 250 g) were obtained from Charles River Canada (St. Constant, Quebec). These studies were approved by the University of British Columbia Committee on Animal Care.
G293 cells were from the American Type Culture Collection (Manassas, VA). Tissue culture-treated polycarbonate filters (Nucleopore filters, Transwell™) were from Costar (Cambridge, MA); DMEM, gentamycin, Fungizone and G418 from Gibco BRL Inc. (Gaithersburg, MD); FBS from HyClone Laboratories (Logan, UT); BSA, Matrigel, and Dispase from Collaborative Biomedical Products (Bedford, MA); epidermal growth factor (EGF), keratinocyte growth factor (KGF), hydrocortisone, ITS (insulin-transferrin-selenous acid) and penicillin/streptomycin from Sigma Chemical Co. (St. Louis, MO).

**Isolation and culture of PLE**

PLE were isolated from guinea pig lungs as described previously (12) with one modification to improve purity. Instead of IgG panning, panning crude cell suspensions on uncoated 75 cm² culture flasks for 30 minutes and collecting nonadherent cells provided 3.0 ± 0.5 x 10⁷ PLE cells per guinea pig (n=14) where 93 ± 5% of cells excluded trypan blue dye.

Purified PLE were plated at 3-5 x 10⁶ cells/10 cm² onto uncoated 6-well plates or 24 mm Transwell plates with polycarbonate inserts coated with 1 ml of Matrigel/DMEM 2:1 (vol/vol) in DMEM, 10% FBS, 1% penicillin/streptomycin, 10 µg/ml gentamycin, and 2.5 µg/ml Fungizone. On the following day adherent cells were fed with DMEM; 5 ng/ml KGF to stimulate type-II cell proliferation (13); penicillin/streptomycin, gentamycin and Fungizone as above; 2% FBS; 1x ITS; 10 ng/ml EGF and 100 nM hydrocortisone.

**Assessment of cell purity in primary culture**

PLE from two guinea pigs were separately purified and plated in culture dishes containing three glass coverslips. The cells from one guinea pig were analyzed after 3
days and the other after 4 days of culture. Following cold methanol fixation cells were examined by light microscopy. The 400 cells identified on each coverslip (1,200 cells per animal) were classified as either PLE (cobble-stone morphology), normal lung fibroblasts (NLF) (spindle-shaped morphology) or unclassified.

**Immortalization of primary PLE cells**

The plasmid pE1Aneo (from Dr. Frank Graham, McMaster University) carries the entire E1A gene with its promoter (nucleotides 25 to 1770 of adenovirus 5) and the neomycin resistance gene driven by the SV-40 large T antigen promoter.

Primary PLE from eight guinea pigs were cultured at 3-5 x 10^6 cells/10 cm² for 1 to 4 days on uncoated 6-well plates or in Matrigel-coated Transwell plates before exposing 6 h to pE1Aneo complexed to Lipofectamine reagent (Gibco BRL Inc.) according to manufacturer’s directions. After three days cells were selected with 250 µg/ml of G418. Cells grown on uncoated 6-well plates that were resistant after two weeks were replated onto new 6-well plates, while those grown on a Matrigel were first harvested as described (14) and replated onto uncoated 6-well plates. After selection of G418 resistant single cell clones, all subsequent analysis of a selected number of these clones (see Results section) were carried out on cells from passages 3 to 4. The same restriction on passage number was applied to the E1A-NLF below.

**Culturing and immortalization of normal guinea pig lung fibroblasts**

NLF isolated as described (15) from two guinea pigs were plated onto separate uncoated 6-well plates at a density of 2 x 10^5 cells/10 cm² in DMEM containing 10% FBS and 1% penicillin/streptomycin. Cells were cultured for 2 to 3 days before
transfecting with the pE1Aneo-liposome complex. G418 resistant cells were replated on 12-well plates.

Detection of E1A DNA, mRNA and protein

E1A DNA in DNA extracted from transfected and untransfected cells was determined by PCR as described (7). RNA from these cells was reverse transcribed then amplified using forward and reverse primers, 5'-AGTGACGACGAGGATGAAGAGG-3' and 5'-TCTCAGGAGGTGTGTTAGAAGG-3', equivalent to nucleotides 953 to 974 and 1381 to 1402, respectively, of the adenovirus type 5 sequence (Genbank gi: 58484). The expected size of the PCR products are 194 and 334 bp for the 12 and 13S E1A mRNAs, respectively. E1A protein was detected by immunofluorescence staining (4) and Western blotting.

Characterization of primary and transfected cells

Electron microscopy

Approximately 3.0 x 10^7 freshly isolated PLE were processed into five Epon blocks for transmission EM (16). One ultrathin section from each block was stained with uranyl acetate and lead citrate for examination on a Philips 400 transmission EM. The number of each cell type was expressed as percentage of the total number of cells examined. The morphology of these cells was compared to that of purified PLE and PLE transfectants cultured for four days on polycarbonate inserts coated with Matrigel/DMEM 2:1 (vol/vol) in 24 mm Transwell plates. Untransfected and transfected NLF, confluent after culturing four days on uncoated polycarbonate inserts inside 24 mm Transwell plates, were examined in a similar manner.

Immunohistochemistry
Immunohistochemistry was performed (17) using mouse monoclonal antihuman antibodies (Dako, Golstrup, Denmark) against cytokeratins (acidic and basic) and vimentin at 4.6 µg/ml and α-smooth muscle actin at 5.0 µg/ml. Respective concentrations of an isotype matched mouse IgG (Sigma Chemical Co.) served as controls. Staining intensity of 30 cells in random fields of view was scored as -, + or ++ by visual assessment.

**Northern analysis of surfactant mRNAs**

RNA was extracted from freshly purified PLE, primary PLE cultured on 100 mm plastic dishes or on polycarbonate inserts coated with Matrigel inside 24 mm Transwell plates and the E1A transfectants using TRIzol (Gibco BRL Inc.) while RNeasy Maxi kits (Qiagen Inc., Mississauga, ON) were used for guinea pig lung. Northern blot analysis followed using cDNAs listed below.

Human surfactant A, B and C and 18S ribosomal cDNA probes cross-hybridize with the respective guinea pig RNAs. Probes were labeled with α-32P-dCTP (Amersham, NJ, USA) by random priming.

**Electrophoretic mobility shift assay (EMSA)**

Cells grown on 75 cm² flasks were stimulated with 10 µg/ml LPS for 2 hrs. Nuclear extracts were prepared from these and untreated cells and EMSA for NF-κB and AP-1, as well as the respective cold competition and super-shift assays, were performed as described (18).

**Results**

**Assessment of primary PLE purity**
Light microscopic examination of 1,200 primary PLE cultured for 3 days showed 50.3±6.7% had a cobble-stone morphology typical of PLE, 4.6±0.7% were spindle-shaped fibroblasts and 45±7.3% were rounded cells of undetermined origin. Another 1,200 cells examined after 4 days of culture were 45.8±6.9% PLE, 15.8±1.7% fibroblasts and 38.4±5.3% of undetermined origin.

Immortalization of primary PLE and NLF

Primary PLE grown on uncoated plates and transfected with pE1Aneo produced G418 resistant colonies in 3 of the 12 wells. Nine resistant single cell clones (E1A-PLE clones 1 to 9) were expanded from one of these colonies and all nine were maintained under constant selection with 100 µg/ml G418. Similarly, primary PLE grown on Matrigel produced G418 resistant colonies in 2 of the 12 wells from which six resistant single cell clones (E1A-PLE clones 10 to 15) were established. Cells in all 15 PLE clones had a cobble-stone morphology and continued to divide after reaching confluency. Clones 1, 2, 10 and 11 were used in the current studies.

Primary NLF transfected with pE1Aneo produced G418 resistant colonies in 4 of the 12 wells. One clone from a single resistant cell was obtained from each (E1A-NLF clones 1 to 4). All four showed cobble-stone morphology, continued to divide after reaching confluency and were maintained under selection with G418. E1A-NLF clones 1 and 2 were used in the current studies.
**E1A DNA, mRNA and protein in transfected cell lines**

E1A DNA was detected in E1A-PLE clones 1, 2, 10 and 11 and E1A-NLF clones 1 to 4 (Fig. 1A). RT-PCR of RNA from E1A-PLE and E1A-NLF clones resulted in the amplification of two bands of size expected from the 12 and 13S E1A mRNAs, respectively, which were also present in RNA from A549 cells infected with adenovirus 5 (Fig. 1G). No bands were seen in RNA from primary NFL and RT-PCR of the 18S ribosomal RNA confirmed equal RNA loading (data not shown). Immunohistochemistry showed that the E1A-PLE and NLF transfected clones had strong nuclear staining for E1A (Fig. 1B and C, respectively) while no staining was observed with the isotype matched control IgG (Fig. 1D and E). On Western blots, the same two E1A monoclonal antibody binding proteins are detected in E1A-PLE and E1A-NFL and these are similar in size to the two largest E1A proteins detected in G293 cells (Fig. 1F)(19). The size markers indicate that these proteins are 40 to 50 kDa and larger than that predicted by their amino acid sequence. Phosphorylation could account for part of this discrepancy, but because of this discrepancy, it is difficult to assign the two to the 12 and 13S isoforms. While mRNA abundance does not necessarily predict protein abundance, mRNAs representing both isoforms are expressed by these two cells (see above).

**Electron microscopy**

In a total of 324 cells from a freshly isolated PLE preparation 28% were alveolar macrophages, 25% had characteristic features of Clara cells including laminated vesicular inclusions and a heterogeneous population of electron dense granules (Fig.
2A), 25% were type-II cells with characteristic lamellar bodies (Fig. 2B), 12% were alveolar type-I cells, 6% were ciliated bronchiolar epithelial cells and 4% were eosinophils. No fibroblasts were identified in these EM studies.

Primary PLE grown on Matrigel-coated polycarbonate inserts formed three-dimensional aggregates composed of polarized cuboidal cells facing a central lumen with numerous microvilli at the cell apices, lamellar inclusions in the cytoplasm and intercellular junctional complexes (Fig. 3A). In contrast, E1A-PLE clones grown in the same manner formed three-dimensional aggregates composed of 3 to 4 cuboidal cells with junctional complexes but lacking lamellar bodies or microvilli (Fig. 3B). When grown on uncoated polycarbonate filters these cells formed monolayers of polarized cuboidal cells with junctional complexes (Fig. 3C).

NLF grown on uncoated polycarbonate filters formed two types of clusters in the same dish. Some clusters were composed of monolayers of spindle-shaped cells with long cytoplasmic extensions (Fig. 4A) while others, of multiple layers of cells with irregular morphology (Fig. 4B). No junctional complexes were observed in either type. E1A-NLF clones, on the other hand, formed monolayers of polarized cuboidal cells with junctional complexes (Figs. 4C and 4D).

**Immunohistochemistry**

Table 1 summarizes the immunohistochemical results shown in figure 5. Primary PLE (Fig. 5A), E1A-PLE clones (Fig. 5B) and E1A-NLF clones (Fig. 5C) all showed cytoplasmic staining for cytokeratin, whereas primary NLF did not (Fig. 5D). Staining
intensity was similar in E1A-PLE and E1A-NLF clones, but significantly less than in primary PLE cells (table 1).

All four cell types showed positive cytoplasmic staining for vimentin (Fig. 5E-H) with not significant difference in staining intensity between the cells (table 1).

Primary PLE showed weak perinuclear staining for α-smooth muscle actin (Fig. 5I) while NLF showed strong cytoplasmic staining (Fig. 5L). In contrast, no staining was observed in E1A-PLE (Fig. 5J) or E1A-NLF clones (Fig. 5K).

Contaminating lung fibroblasts found around PLE in primary cultures were negative for cytokeratin (Fig. 5A) but stained strongly for vimentin (Fig. 5E) and α-smooth muscle actin (data not shown).

No staining was observed when cells were stained with isotype matched control antibodies (Fig. 5M-P).

Surfactant mRNA expression

Northern hybridization (Fig. 6) showed that guinea pig lung and freshly purified PLE express surfactant A, B and C mRNAs (lanes a and b, respectively). Primary cultures of PLE grown on Matrigel-coated polycarbonate filters expressed only surfactant B mRNA (lane c) and those grown on plastic dishes did not express surfactant mRNAs (lane d). E1A-PLE clones grown on either Matrigel-coated polycarbonate filters (lanes e and f) or plastic dishes (data not shown) did not express surfactant mRNAs. 18S ribosomal RNA used as an internal control was similar in these cells (bottom panel).
**EMSA**

LPS stimulation induced NF-κB binding activity in nuclear extracts of E1A-NLF and E1A-PLE but not NLF (Fig. 7A). In E1A-PLE the specific binding complexes that were detected are numbered I to III in order of their migration through the gel from fastest to slowest with complex II having the greatest intensity (lane f). Binding activity in E1A-NLF was represented by complexes that generally migrate faster than those in E1A-PLE with a possible overlap with complex I (lane d) but a lower intensity of binding than those in E1A-PLE.

EMSA of AP-1 demonstrated constitutive binding in nuclear extracts of NLF that was not altered by LPS stimulation, while in E1A-NLF and E1A-PLE, LPS increased AP-1 binding (Fig. 7C). The bands representing AP-1 binding in E1A-NLF migrated faster than that of the single NLF or double E1A-PLE bands.

In E1A-PLE supershift assays using antibodies to p50 and p65 for NF-κB and JunB, c-jun and c-fos for AP-1 and competition assays using excess unlabeled specific or unrelated oligonucleotides confirmed the specificity of the proteins bound to the respective oligonucleotides (Fig. 7B and D). With respect to NF-κB, the antibody to p65 mainly supershifted complex III and II while the antibody to p50 shifted complex I and II (Fig. 7B). This suggests that p50 and p65 are part of complex II. With respect to AP-1 both c-Jun and c-Fos antibodies produced supershifted bands while JunB did not (Fig. 7D). E1A-NLF supershifted bands parallel to those in E1A-PLE were found, both for NF-κB and AP-1 (data not shown), except the supershifted bands originated from the smaller complexes formed by these transcription factors in E1A-NLF.
Discussion

Our results show that adenovirus E1A DNA can be used to immortalize guinea pig PLE and fibroblasts. Clonally derived E1A-expressing PLE show a limited number of type-II cell phenotypes but failed to show more specific markers of this differentiation such as laminated vesicular inclusions and microvilli or expression of surfactant mRNAs. When grown on uncoated plastic dishes they showed some epithelial characteristics such as weak cytokeratin expression and cobble-stone morphology, and when grown on uncoated polycarbonate filters they showed cuboidal morphology and junctional complexes. These features are all consistent with previous reports characterizing E1A-immortalized cell lines established from primary cultures of rat type-II pneumocytes grown on uncoated plastic (20-22). These rat cells had epithelial phenotypes of cytokeratin expression, junctional complexes and cuboidal morphology and also lacked the differentiation markers of type-II cells. Despite our attempt to improve the retention of primary differentiation by culturing the primary PLE on matrix, before and during the transfection procedure, the transformed cells failed to maintain the specific features of type-II cells.

With respect to fibroblasts, our findings suggest that E1A causes a mesenchymal-to-epithelial transformation. While NLF grow as monolayers of cells with spindle shape morphology interspersed with multilayered foci of cells with irregular shapes to give the culture a “peak and valley” appearance, E1A-expressing NLF switch this parent phenotype to that reminiscent of PLE transfectants. This epithelialization, including cuboidal morphology, junctional complexes and cytokeratin production, was further supported by downregulation of α-smooth muscle actin, a mesenchymal marker
that was strongly positive in the parent NLF. These results are supported by a report by Frisch of adenovirus E1A-induced mesenchymal-to-epithelial transformation in human tumor cells of mesenchymal origin (23). The E1A-expressing tumor cells, like our transfected NLF, expressed junctional complexes and cytokeratin. Frisch suggested that conversion to an epithelial phenotype requires that mesenchymal transactivators which repress epithelial gene transcription are either not expressed or inactivated by E1A, particularly by the 12S form of the E1A protein that could be expressed by the E1A-NLF in our study. Based on this hypothesis, the introduction of E1A into mesenchymal cells in our case could inactivate these repressors and result in the induction of epithelial genes. Indirect evidence for the lack of such a potential repressor in our transfected NLF comes from the results of the EMSA analysis of AP-1 where, compared to NLF, transfected NLF showed an AP-1 complex of greater mobility, that is a smaller complex, suggesting the possible absence of a repressor that was part of the AP-1 complex in the untransfected NLF.

Alcorn and coworkers (24) showed that fibroblasts are a major source of contamination in primary cultures of type II cells. In our case spindle shaped fibroblast-like cells that are negative for cytokeratin but positive for vimentin and α-smooth muscle actin comprise 5 and 15% of the primary PLE preparation after 3 and 4 days of culturing, respectively. This 3-fold increase in fibroblasts compared to PLE whose numbers remained virtually unchanged is indicative of the higher mitotic activity of contaminating fibroblasts. Consequently, the E1A transfected cells that we initially assumed were PLE, could have arisen from contaminating fibroblasts with subsequent mesenchymal-to-epithelial transformation as suggested by Frisch (23).
Contrary to the above explanation, evidence has been presented that strongly suggests that the two types of E1A transfected cells are different. Initially, differences in DNA binding activity of the transcription factors NF-κB and AP-1 by NLF, E1A-NLF and E1A-PLE and changes in this activity in response to LPS stimulation were presented. Nuclear extracts from NLF have very weak NF-κB binding activity and this basal activity is not increased upon stimulation with LPS. E1A transfected NLF also have low basal binding activity, but in contrast to NLF, E1A transfected cells respond to LPS with increased binding. Transfected PLE likewise have low basal activity and, like transfected NLF, respond to LPS but in this case respond more strongly. Besides this difference in response strength, additional complexes, larger in size than that in transfected NLF, are present. With respect to AP-1, NLF show constitutive binding activity that is not affected by LPS. Transfected NLF not only have a lower basal AP-1 binding activity but form a smaller complex that is now responsive to LPS. Transfected PLE also respond to LPS but the complexes formed more closely resembles that found in untransfected NLF. While E1A enhanced NF-κB and AP-1 binding activity in response to LPS in both NLF and PLE, the differences in the transcriptional complexes formed in each cell demonstrate that these cells are inherently different despite other phenotypic similarities induced by E1A.

The active DNA-binding forms of both NF-κB and AP-1 are dimers composed of up to five of the NF-κB/Rel family for NF-κB (25) and, for AP-1, of members of the Fos and Jun families consisting of five and three members, respectively (26). To identify the subunits that comprise the complexes formed by the two transcription factors and thus determine the source of the difference between the complexes formed in the transfected
PLE and NLF the supershift assay was used. Interestingly, our results showed that some of the subunits that contribute to the respective complexes in E1A-PLE and E1A–NFL are the same. Since the overall size of some of the binding complexes in the two cell types differs, this suggests that the other subunit forming the respective dimer might be different. In addition to this or alternatively, proteins that associate with these transcription factors in the process of building the multi-protein transcriptional complex may have cell type-specific expression (reviewed in 27) and thus contribute to the size differences in the sequence-specific DNA binding complexes identified in the two cell types. These possibilities of cell type-specific expression of transcription factor subunits and/or associated factors further support our argument that E1A transfected PLE did not originate from transfection of contaminating fibroblasts. Therefore we conclude that transfection of PLE and NLF results in E1A-expressing cell lines with intermediate epithelial-mesenchymal features that may be related to dedifferentiation induced by E1A. This conclusion is in agreement with Cajal and colleagues (28) who transformed epithelial and fibroblastic tumor cells with adenovirus E1A and found that both converged to similar phenotypes regardless of cell origin.

E1A induced a more mesenchymal phenotype in guinea pig PLE, that is, loss of surfactant and reduction in cyokeratin expression, markers of epithelial cells, but retention of vimentin expression. Epithelial-mesenchymal transformation has been documented in fibroblasts originating from epithelial cells in progressive kidney disease (10), idiopathic pulmonary fibrosis (11) and liver cirrhosis where hepatic stellate cells converted to myofibroblasts (29). Since adenovirus E1A inhibits the transcriptional activity of many promoters (30-32), E1A repression of differentiation markers in PLE
may induce a process where PLE first lose their differentiation markers to move into an intermediate phenotype and then, possibly by transactivation of mesenchymal genes by its 13S isoform (reviewed in 33), reach the mesenchymal phenotype.

The epithelial-mesenchymal transformation induced by adenovirus E1A in guinea pig PLE is particularly interesting because remodeling of the peripheral airways with increased thickening of their walls was recently shown to be associated with COPD severity (2). We suspect that mesenchymal transformation of epithelial cells might contribute to the remodeling process observed in the peripheral lung in COPD by deregulating matrix deposition in the airway wall. The E1A induced transformation of PLE, therefore, provides support for a role of adenovirus infection in the pathogenesis of the airway remodeling process. These guinea pig cells also provide a model to study the underlying mechanisms leading to the expression of a more mesenchymal phenotype as well as related changes that may affect airways remodeling. Furthermore our model of persistent E1A expression after adenoviral infection in the guinea pig could provide a useful way to study this aspect of the pathogenesis of COPD in vivo because it can amplify the effects of chronic cigarette smoke exposure and increase polymorphonuclear leukocyte, macrophages and CD4 and CD8 lymphocytes in the peripheral airways (8) that is not apparently observed in mice. Also, this guinea pig model, where only a minority of lung epithelial cells retain E1A (9), might better reflect the situation in human lungs (4) compared to a transgenic mouse model of lung specific E1A expression (33) where all alveolar and bronchiolar cells (34) have the potential to express these viral proteins.
Acknowledgements

The authors would like to thank Frank Graham, McMaster University, for the pE1Aneo plasmid and Stuart Greene and Dean English for the technical assistance with digital images. This work was supported by CIHR grant 7246.
References


Table 1. Characteristics of peripheral lung epithelial cells (PLE), normal lung fibroblasts (NLF) and their E1A transfectants

<table>
<thead>
<tr>
<th>Cells</th>
<th>Cuboidal morphology</th>
<th>Immunostaining*</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cytokeratin</td>
<td>Vimentin</td>
<td>α-Smooth</td>
<td>Surfactant mRNA</td>
</tr>
<tr>
<td>PLE</td>
<td>+</td>
<td>++(^{a,b})</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E1A PLE</td>
<td>+</td>
<td>+(^{b})</td>
<td>++</td>
<td>-(^{b})</td>
<td>-</td>
</tr>
<tr>
<td>E1A NLF</td>
<td>+</td>
<td>+(^{b})</td>
<td>++</td>
<td>-(^{b})</td>
<td>nd</td>
</tr>
<tr>
<td>NLF</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>nd</td>
</tr>
</tbody>
</table>

*Staining scored by two observers (AB and SH) as -, +, or ++ and interobserver variability \(R^2/R_{max} = 0.8691\) where \(R^2\) is the Pearson coefficient of mean square contingency and \(R_{max}\) is the maximal possible value of the Pearson \(\chi^2\) coefficient. The averages of the combined scoring from the two observers were analyzed by the Fisher Exact test with Bonferroni corrections for multiple comparisons.

\(^{a}\) \(p < 0.05\), significantly different from E1A PLE and E1A NLF

\(^{b}\) \(p < 0.05\), significantly different from NLF

nd: not done
Figure Legends

Figure1. Detection of adenovirus E1A DNA by PCR, mRNA by RT-PCR and protein by immunofluorescence staining and Western blotting. (A) Ethidium bromide stained agarose gels of E1A PCR products (486 bp) from E1A-PLE clones 1, 2, 10 and 11 (left panel, lanes b-e, respectively) and E1A-NLF clones 1 to 4 (right panel, lanes g-j, respectively). Untransfected PLE (left panel, lane a) and untransfected NLF (right panel, lane f) were used as negative controls. Monoclonal anti-E1A antibody applied to (B) E1A-PLE clone 1 and (C) E1A-NLF clone 1 and isotypic control antibody applied to them in (D) and (E), respectively. Scale bar equals 10 µm. The same E1A antibody applied to a Western blot (F, top panel) detected two E1A isoforms in E1A-PLE clones 1 and 2 (lanes a, b) and in E1A-NFL clones 1 and 2 (lanes c, d) that were equivalent to two of largest E1A polypeptides detected in G293 cells (lane e). An antibody to β-tubulin was used to control for protein loading in the corresponding lanes (F, bottom panel). The corresponding size markers are shown in lane f. (G) Ethidium bromide stained agarose gel of RT-PCR products of E1A mRNA from cells with the same lane designation as in (F) except adenovirus 5 infected A549 cells replaced the G293 cells as a positive control in lane e.
Fig. 1
Behzad A.R., Morimoto, K., Gosselink, J., Green, J., Hogg, J.C., Hayashi S.

A

486 bp

B

D

C

E

F

E1A

β-tubulin

G

13S E1A

12S E1A
Figure 2. Electron micrographs of freshly purified peripheral lung epithelial cells. (A) Clara cell with laminated inclusions (arrowheads) and a heterogeneous population of electron dense granules. (B) Type-II cell with characteristic lamellar bodies (arrowheads). Scale bar equals 2 µm.

Fig. 2
Behzad A.R., Morimoto, K., Gosselink, J., Green, J., Hogg J.C., Hayashi S.

Figure 3. Electron micrographs of purified PLE in primary culture and E1A-PLE clone. (A) primary cultured PLE grown on Matrigel-coated polycarbonate filter forming three-dimensional aggregates of cuboidal cells with lamellar bodies (arrow), junctional complexes (arrowheads) and apical microvilli (*) that line a central lumen (L). (B) E1A-PLE clone 1 grown on Matrigel-coated polycarbonate filter with junctional complex (arrowhead) between cells. Inset in (B) is a higher magnification of that junctional complex. (C) E1A-PLE clone 1 grown on an uncoated polycarbonate filter forms a
monolayer of cells with polarized cuboidal morphology and junctional complexes (arrowhead). Scale bar equals 2 µm.
Fig. 3
Behzad A.R., Morimoto, K., Gosselin, J., Green, J., Hogg J.C., Hayashi S.
Figure 4. Electron micrographs of NLF and E1A-NLF clone 1 grown on uncoated polycarbonate filters. NLF form areas of monolayer (A) and multilayer (B) growth. (C) E1A-NLF clone 1 grows as a monolayer of cells with cuboidal morphology and junctional complexes (arrow). (D) Higher magnification of an apical junctional complex (arrow) between two adjacent E1A-NLF cells. Scale bar equals 2 µm.
Fig. 4
Behzad A.R., Morimoto, K., Gosselink, J., Green, J., Hogg J.C., Hayashi S.
Figure 5. Immunostaining for cytokeratin, vimentin and α-smooth muscle actin. Primary culture of PLE (first row), E1A-PLE clone 1 (second row), E1A-NLF clone 1 (third row) and NLF (fourth row) grown on coverslips and stained with mAbs to cytokeratin (A-D), vimentin (E-H), α-smooth muscle actin (I-L) or isotype matched IgG (control antibody, M-P). Contaminating fibroblasts are seen around epithelial colonies (A and E). Scale bar equals 10 µm.
Figure 6. Northern blot analysis for surfactant A, B and C mRNAs. Surfactant A, B, or C mRNA expression (upper, upper middle, bottom middle panels, respectively) in total cellular RNA extracted from a guinea pig lung (lane a), freshly purified PLE (lane b), primary culture of purified PLE grown on Matrigel-coated polycarbonate filter (lane c), the same cells grown on plastic (lane d), and E1A-PLE clones 1 and 10 grown on Matrigel-coated polycarbonate filter (lanes e and f). 18S ribosomal RNA (bottom panel) was used as an internal control.

Fig. 6
Behzad A.R., Morimoto, K., Gosselink, J., Green, J., Hogg J.C., Hayashi S.

Figure 7. Detection of NF-κB and AP-1 binding activity in NLF, E1A-NLF clone-1 and E1A-PLE clone-1. (A) Electrophoretic mobility shift assay (EMSA) using the immunoglobulin κ NF-κB probe, 5’-CGC TTG ATG AGT CA GCG GAA-3’ (NF-κB binding sequence of the human immunoglobulin κ chain gene underlined) was performed on nuclear extracts from untreated cells (a,c,e) and cells stimulated with 10 µg/ml LPS for 2 hr (b,d,f). Arrows indicate the positions of specific protein-DNA complexes (I to III), non-specific complex and unbound probes. (B) NF-κB binding in
nuclear extracts from E1A-PLE stimulated as in (A) in the presence of excess cold AP-1 (a) or NF-κB (b) oligonucleotides and antibodies against p65 (c), p50 (d) or control IgG antibody (e). Arrows indicate complexes I to III and bands supershifted by p65 and p50 antibodies. (C) EMSA using the AP-1 probe, 5'-CGC TTG AGT CA G CCG GAA-3' (AP-1 binding sequence of human collagenase gene underlined) was performed on nuclear extracts from the same cells as in (A) with or without LPS stimulation (same lane designation as in A). (C) AP-1 binding in nuclear extracts from E1A-PLE stimulated as in (A) in the presence of excess cold NF-κB (a) or AP-1 (b) oligonucleotides and antibodies against JunB (c), c-Jun (d), c-Fos (e) or control IgG antibody (f). Arrows indicate bands supershifted by c-Jun and c-Fos antibodies. Similar results were obtained for both NF-κB and AP-1 binding activity when a different pair of cells, namely E1A-NLF clone-2 and E1A-PLE clone-2, were compared.
Fig. 7
Behzad A.R., Morimoto, K., Gosselink, J., Green, J., Hogg J.C., Hayashi S.