Latent Adenovirus Infection in COPD*

Shizu Hayashi, PhD

We have concentrated on the adenovirus as the source of the heightened inflammatory response of the lungs of patients with COPD. We have concentrated in particular on the responses to agents such as lipopolysaccharides and environmental particulates that contaminate the air we breathe, and we have accumulated evidence that the E1A gene of this virus could be the key player in this process. As other intracellular pathogens such as *Chlamydia pneumoniae* have recently been implicated in the pathogenesis of COPD, our studies on the adenovirus E1A could serve as the model for investigating the interaction between host and extrinsic factors in the chronic progression of this debilitating lung disease.
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Key words: adenovirus; COPD; latent infection; viral DNA

Abbreviations: CAT = chloramphenicol acetyltransferase; ICAM = intercellular adhesion molecule; HBE = human bronchial epithelial; IκB = inhibitor of κB; IL = interleukin; LPS = lipopolysaccharide; NF-κB = nuclear factor-κB; TLR = toll-like receptor

Epidemiologic studies suggest that childhood infections are an independent risk factor for the development of COPD in adulthood.[1–3] We are interested in adenovirus infections because they are a common cause of bronchiolitis in children and young adults[4–9] that could predispose them to the development of COPD. In particular, group C adenovirus is endemic, and exposure to it during childhood could lead to latent infections that persist in the lung,[10] tonsils,[11–13] and peripheral blood lymphocytes.[14] COPD is characterized by cough and sputum production, peripheral airways obstruction, and emphysematous destruction of the lung surface area. All three of these components have been related to an inflammatory process located in the conducting airways and lung parenchyma.[15–18] Although cigarette smoking is the major risk factor for the development of COPD, only 15 to 20% of all smokers develop airways obstruction.[19–21] Therefore, we postulate that adenovirus that remains from a previous infection of the lungs in a minority of smokers enhances the inflammatory process in their lungs.

There are 47 different serotypes of human adenovirus, and 9 of these are documented respiratory pathogens. The genome of adenovirus is a double-stranded linear DNA of approximately 36,000 base pairs.[22] It consists of inverted terminal repeats that are required for the replication of the viral DNA, and between these termini are the transcription units that specify the viral proteins required to allow the viral genes to be expressed and viral replication to proceed. There are five early transcription units (Fig 1). These are the first viral genes expressed during an active infection, and they initiate three processes. One process is to induce the host cell to enter the S phase of the cell cycle and thereby to provide a replicating environment. The second process is to provide protection from the host immune system, and the third process is to produce the viral proteins needed for viral DNA replication. All three goals require transcriptional activation of viral genes, and the principal activating proteins are those encoded by the E1A gene (Fig 1), which itself is controlled by its own constitutively active promoter.

We targeted the E1A gene as the viral gene responsible for enhancing host inflammatory mediator expression because its gene products are well-characterized transactivators that function by interacting with numerous cellular transcription factors,[23] transcriptional coactivators,[24,25] and cell cycle regulatory proteins.[26] These studies on E1A have focused on this viral protein as a model with which to study the regulation of transcription in mammalian cells, but this regulation has not been the target of studies on pathologic processes. The modulation of the activity of nuclear factor-κB (NF-κB), which is a key transcription factor regulating the expression of inflammatory mediator genes,[27] by E1A[28–30] as well as the indirect effects of E1A on NF-κB that are mediated by coactivators cyclic adenosine-3′,5′-monophosphate responsive element binding
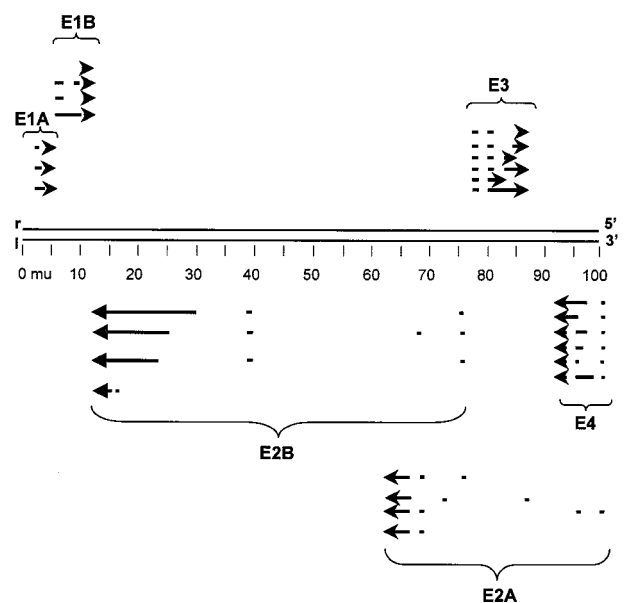

FIGURE 1. Transcription map of adenovirus early genes. There are six early transcription units (*ie*, E1A, E1B, E2A, E2B, E3, and E4), and each produces multiple transcripts. Arrows indicate the sequences transcribed and the direction of transcription. The gaps in the arrows indicate the position of introns. mu = map units.

protein and a related p300 protein,[30,31] have been demonstrated. These results are in keeping with the concept that cyclic adenosine-3′,5′-monophosphate responsive element binding protein/p300 can interact with a wide variety of factors that regulate transcription, including DNA binding proteins, the proteins of the basal transcriptional machinery, as well as viral proteins such as E1A, and some of these can bind to each other.[32] The vast array of signals that bombard a cell could be integrated in this fashion and could result in the formation of specific multiprotein complexes bound to DNA, which ultimately controls the activity of a gene. Within this schema, E1A could exert its influence, whether it be activating or repressing, through its direct interaction with different components of this multiprotein complex. The regulation of the genes of inflammatory mediators either directly or indirectly by such a mechanism is of particular interest to us because this could drive the inflammatory process that is present in the airways of all smokers to produce airways obstruction in the 15 to 20% of those with latent adenoviral infections.

STUDIES ON HUMAN TISSUE

Several years ago, we postulated that the persistence of adenoviral DNA in lung tissue might contribute to the pathogenesis of COPD. We demonstrated that lung tissue from patients with COPD carries more group C adenoviral DNA, specifically its E1A gene, than that of nonobstructed control subjects who were matched for age, gender, and smoking history.[10] Although this could have been explained by a greater susceptibility to infection by adenovirus in smokers or patients with respiratory disorders, it seemed unlikely for two reasons. First, a comparison of group C adenoviral E1A DNA in the lungs of patients with bronchiectasis[33] and a variety of other chronic lung disorders[34,35] showed that smokers with COPD carry the heaviest load of adenoviral E1A DNA. Second, in the acute exacerbations that characterize COPD, rhinovirus, influenza virus, parainfluenza virus, and coronavirus were implicated in these acute respiratory illnesses, but not adenovirus.[36] This strongly suggests that the presence of adenovirus in the lungs of COPD patients is not due to an increased susceptibility to these infections because of smoking or exacerbations.

Early studies showed that acute adenovirus infections target the lung epithelial cells,[37] and subsequently we demonstrated that adenoviral E1A protein was expressed in the epithelial cells lining the airways, the alveoli, and the submucosal glands of COPD patients.[38] Together with the presence of viral E1A DNA, this localization of the E1A proteins in the epithelium of lungs suggests that adenovirus E1A can persist in human lungs and that this viral transactivating protein may be an important etiologic agent in the pathogenesis of COPD. More recent studies have shown that levels of all inflammatory cell types found in the lung except B cells were increased in lungs of patients with severe emphysema and that this increased inflammation was associated with a fivefold increase in the number of alveolar epithelial cells expressing E1A protein.[39] Furthermore, the E1A gene was found to be integrated at a specific site on chromosome 19 in the lung cells of patients with COPD.[40]

STUDIES IN INTACT ANIMALS

Our studies in a guinea pig model of latent adenovirus infection of the lung supported the hypothesis that adenovirus plays a role in the pathogenesis of COPD. After the initial acute infection, which resembles adenovirus infections in humans, had subsided and the replicating virus was no longer detected, adenovirus E1A DNA and protein continued to be found in a few alveolar and airways epithelial cells.[41] Moreover, bronchiolitis persisted. In this model, latent adenovirus infection caused a lung inflammatory response to a single short-term exposure to cigarette smoke with a significant increase in the numbers of macrophages and CD4+ lymphocytes over the increase caused by cigarette smoke alone.[42] More recently, studies on chronic cigarette smoke exposure in this model supported the role of adenovirus in enhancing lung inflammation due to cigarette smoke, because the combined effect of smoke exposure and latent adenovirus infection led to an increase in lung volume, airspace volume, and lung weight and to a decrease in the surface/volume ratio compared to smoking alone.[43,44]

STUDIES ON LUNG EPITHELIAL CELLS IN VITRO

The demonstration of adenoviral E1A expression in human lung epithelial cells[38] provided a basis for studying the interaction between E1A and host regulatory mechanisms that could stimulate the expression of inflammatory mediators. A549 human pulmonary epithelial cells stably expressing adenoviral E1A were developed.[45] The quantification of candidate inflammatory mediators by enzyme-linked immunosorbent assays showed that intercellular adhesion molecule (ICAM)-1 and interleukin (IL)-8 were markedly increased by lipopolysaccharide (LPS) stimulation, but only in E1A-positive cells.[45,46] Both LPS-mediated ICAM-1 and IL-8 inductions were serum-dependent, but an LPS receptor, CD14, was not detected on the surface of these cells. Northern blot analysis confirmed that this regulation of both ICAM-1 and IL-8 was primarily at the messenger RNA level. Other inflammatory mediators, such as monocyte chemoattractant protein-1, IL-1β, IL-6, granulocyte macrophage colony-stimulating factor, and granulocyte colony-stimulating factor, were not affected.[46] Taken together, these results indicate that adenovirus E1A regulates specific inflammatory mediators in response to stimulation by LPS and suggest a common mechanism by which this regulation is achieved.

The increased number of neutrophils in the BAL lavage fluid from patients with COPD compared to control subjects[47] is reflected in higher concentrations of IL-8 in induced sputum from these patients.[48] IL-8 is a chemokine that mainly acts as a potent neutrophil chemoattractant and activator. Our findings of not only increased expression of IL-8 but also of ICAM-1, a cell-surface glycoprotein that serves as a ligand for adhesion receptors including the leukocyte β₂ integrins, suggest that the migration and activation of inflammatory cells in response to cigarette smoke or other noxious stimuli might be

regulated by cytokines that are secreted by lung epithelial cells and that a dysregulation of this process by adenovirus E1A could lead to the emphysematous destruction that is associated with COPD.

To elucidate the mechanism by which adenoviral E1A enhances the expression of ICAM-1 and IL-8 in response to LPS stimulation, we investigated transcription factors that bind to the 5′ regulatory region of both genes,[49,50] particularly those known to be affected by E1A or LPS. One of these, NF-κB, is usually found in the cytoplasm bound to the inhibitor of κB (IκB), which masks the nuclear localization signal of NF-κB. On appropriate stimulation, signals transduced through the cell culminate in the phosphorylation of IκB, which marks the degradation of this inhibitor.[27] The released NF-κB translocates to the nucleus where it binds to its recognition sequence in the promoters of genes activated by that stimulus. While LPS is a well-known stimulus of NF-κB, CD14, its receptor, is not intrinsically connected to the transmembrane signal transduction apparatus. It has been shown that LPS also binds to the transmembrane toll-like receptors (TLR) that initiate signal cascades that are shared with the IL-1 receptor, resulting in the phosphorylation of IκB.[51] Of the nine TLR genes identified to date in mammals, controversy exists between TLR2 and TLR4 as specific receptors of bacterial endotoxins, although evidence favors the latter for endotoxins from Gram-negative bacteria[51] such as the LPS used in our own experiments. In this regard, it is interesting to note that in cases of acute respiratory exacerbations, particularly in patients with severe COPD, Gram-negative bacteria have been isolated.[52,53]

Electrophoretic mobility shift assays of transcription factors demonstrated that LPS treatment activated NF-κB binding complexes that consisted of RelA and p50; however, this occurred only in the nuclei of E1A-positive cells.[54] This finding was confirmed when nuclear translocation of NF-κB, as detected by immunofluorescence staining, was observed after LPS stimulation, again occurring only in E1A-positive cells.[54] The same treatment did not increase the binding activity of activator protein-1, another transcription factor the activity of which is documented to be affected by E1A,[28,55,56] in either E1A-positive or E1A-negative cells.

That E1A enhances the transcription of the ICAM-1 and IL-8 genes, particularly through the modulation of NF-κB activity, was further supported by results from a biological assay of promoter activity based on the chloramphenicol acetyltransferase (CAT) reporter gene. For this, pBS-CAT-p (obtained from Dr. S.W. Caughman; Emory University; Atlanta, GA),[57] an expression vector carrying the ICAM-1 promoter including the NF-κB binding site linked to the CAT gene, was used to transfect the stable E1A-expressing A549 cells and control transfectants.[58] Again, ICAM-1 promoter activity in response to LPS stimulation was increased by 2.8 times in E1A-positive cells compared to controls.

The above studies are centered on the bronchoalveolar carcinoma cell line A549. Because of their ease in culturing and their capacity to form stable E1A transfectants, we have gained valuable insight into the regulation of the expression of host cell inflammatory mediators by the adenoviral E1A. An acknowledged limitation of this model system is that these are mutated cancer cells and are most likely type II alveolar epithelial cells in origin.[59] As such, they are more relevant to the pathogenesis of the emphysematous changes than to the airways obstruction component of COPD. Therefore, we developed primary human bronchial epithelial (HBE) cells expressing E1A,[60] which are more relevant to the pathogenesis of the airways disease.

As reported,[60] we compared these HBE cells stably expressing E1A either to primary untransfected HBE cells from the same patient or, in one case, to primary cells and a cell line from the same patient transfected with the adenovirus E1A expression plasmid that did not express the E1A messenger RNA or protein. As with the A549 cells, these E1A-positive HBE cells responded to LPS stimulation with enhanced ICAM-1 and IL-8 messenger RNA expression, the activation of NF-κB, and increased ICAM-1 promoter activity compared to the controls. While neither E1A-positive cells nor control HBE cells expressed the CD14 LPS receptor, both expressed low levels of TLR2 messenger RNA (Fig 2) with no change after LPS stimulation. These results demonstrate that adenovirus E1A enhances the expression of specific inflammatory mediators in both A549 and HBE cells and does so, most likely, through the transcription factor NF-κB.

Although these two types of pulmonary epithelial cells respond to LPS stimulation in a parallel fashion, their expression of LPS receptor types is different. E1A induces TLR2 messenger RNA expression in A549 cells, and this expression is not affected by LPS stimulation. On the other hand, as described above, no difference in TLR2 messenger RNA expression was observed between E1A-positive HBE cells and control cells, and, again, LPS did not affect the expression. More recently, TLR4 messenger
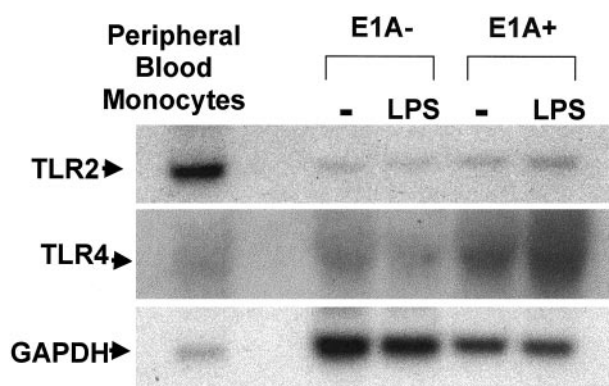

FIGURE 2. Northern blot analysis comparing TLR2, TLR4, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messenger RNA expression in control HBE cells (E1A−) and adenovirus E1A-expressing HBE cells (E1A+), which either were left untreated (−) or were stimulated with 10 μg/mL LPS for 6 h (LPS), and in unstimulated human peripheral blood monocytes. The complimentary DNA probes for the TLR2 and TLR4 messenger RNA were from Genentech Inc (South San Francisco, CA) and Tularik Inc (South San Francisco, CA), respectively.

RNA expression, while present at low levels in control HBE cells, was found to be significantly increased in the E1A-positive counterparts, and LPS stimulation did not alter this expression (Fig 2). Whether these differences in TLR2 or TLR4 expression caused by E1A in A549 and HBE cells, respectively, relate to the enhanced expression of IL-8 and ICAM-1 in response to LPS in these cells requires further investigation.

Other investigations using HBE cells have demonstrated that they are unresponsive to an extract made from cigarette smoke, even in the presence of E1A (Y. Higashimoto; unpublished data). However, as the E1A-positive A549 cells enhance the expression of IL-8 in response to the PM_{10} particles,[61,62] the particulate components of cigarette smoke may be more relevant in the regulation of IL-8 expression than its soluble constituents.

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