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Effect of the adenovirus E1A gene on nitric oxide production in alveolar epithelial cells

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ABSTRACT

This study determined the effect of the adenovirus E1A gene on nitric oxide (NO) production in alveolar epithelial (A549) cells. E1A-positive A549 cells (E1A transfectants), E1A-negative A549 cells (control transfectants) and untransfected A549 cells were placed in 96-well tissue culture plates. After stimulation with lipopolysaccharide (LPS) or cytokine mixture (CM), the biochemical reaction products of NO (nitrite and nitrate) in the culture medium were measured by chemiluminescence. The inducible (iNOS) and the endothelial (eNOS) isoforms of nitric oxide synthase (NOS) protein expression were examined by Western blotting. iNOS mRNA expression was examined by Northern blotting and RT-PCR. CM-induced NO production by E1A-positive A549 cells was significantly lower than that of E1A-negative cells ($p < 0.0001$). LPS stimulation failed to enhance NO production in both cell types. CM induced iNOS protein expression in E1A-negative A549 cells, but not in E1A-positive cells. eNOS protein expression was constitutive and was not affected by CM stimulation, LPS stimulation or E1A. CM induced iNOS mRNA expression in E1A-negative A549 cells, but not in E1A-positive cells. In conclusion, the adenovirus E1A gene suppressed NO production through transcriptional control of the iNOS gene in A549 cells. This inhibition of NO production may enable the virus to persist in human tissue, since NO is an antiviral effector of the innate immune system.

Keywords Adenovirus E1A gene, bronchial epithelial cells, inducible nitric oxide synthase, nitric oxide production, pulmonary epithelial cells

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INTRODUCTION

Adenoviruses are a group of DNA viruses that infect the epithelial cells of the respiratory, gastrointestinal and genitourinary tracts. Infection is usually self-limiting, but latent virus infection is implicated as a risk-factor for obstructive lung diseases [1,2]. Adenovirus has been detected at high frequency in children with steroid-resistant asthma [2]. Adenovirus E1A protein and mRNA are detected frequently in the lungs of patients with chronic obstructive pulmonary disease (COPD) [1,3]. Studies of cultured human alveolar epithelial cells have also shown that transfection with this virus gene upregulates the expression of intercellular adhesion molecule-1 (ICAM-1) and interleukin-8 when these cells are challenged with endotoxin or noxious particles [4–7]. These studies suggest that latent adenovirus infections, and the persistence of the E1A gene in the alveolar epithelial cells, may have a role in the pathogenesis of obstructive airway disease by amplifying the inflammation [8].

Nitric oxide (NO) is an antiviral effector of the innate immune system, and inhibits replication of a wide variety of DNA and RNA viruses in cell cultures and animals. Cao *et al.* [9] showed that adenovirus E1A suppressed inducible NO synthase (iNOS) expression in the mouse macrophage cell line RAW264.7. Viral inhibition of NO may affect the host innate immune system and enable viruses to survive in the lung. Therefore, the aim of this study was to determine whether

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NO production by human alveolar epithelial cells is affected by the adenovirus E1A gene. The effect of E1A on iNOS and endothelial NO synthase (eNOS) expression in these cells was also studied.

MATERIALS AND METHODS

Reagents

Lipopolysaccharide (LPS) from *Escherichia coli* strain 0111:B4 (Sigma, St Louis, MO, USA) was dissolved in sterile distilled water. The final concentration of LPS was 10 μg/L. Recombinant human tumour necrosis factor-α, interferon-γ (IFN-γ) and interleukin-1β were purchased from Roche Diagnostics (Basel, Switzerland) and were used to produce a cytokine mixture (CM: tumour necrosis factor-α 10 ng/mL; interleukin-1β 500 U/mL; IFN-γ 100 U/mL).

Cell culture

The cells used in this study have been described previously [4,5,10]. In brief, A549 cells (ATCC, Rockville, MD, USA) were transfected with the E1A gene of adenovirus 5. The E1A transfectants E4 and E11 investigated in this study were A549 cell clones that were transfected stably with a plasmid carrying the adenovirus 5 E1A gene, driven by its own promoter, while control transfectants C4 and C8 were clones transfected with a control plasmid lacking the E1A gene. Analysis by Western and Northern blots showed that all E1A transfectants produced relatively high levels of E1A mRNA and proteins [4]. Wild-type and transfected A549 cells were grown in Eagle's minimum essential medium supplemented with fetal bovine serum 10% v/v (Hyclone, Logan, UT, USA). Transfectants were maintained under constant selection with active G418 (Gibco BRL, Rockville, MD, USA) 280 mg/L.

Measurements of nitrite and nitrate

To measure NO production by A549 cells, the biochemical reaction products of NO (nitrite and nitrate) were measured in the culture medium. Cells were placed in 96-well tissue culture plates at 4×10^4 cells/well on the day before stimulation with LPS or CM. The culture medium was collected after incubation for 24 h with or without stimulation. The nitrate (NO_3^-) in culture medium was reduced to nitrite (NO_2^-) with *Aspergillus* nitrate reductase (DOJINDO, Kumamoto, Japan). The resulting nitrite content of the incubation medium was measured by a chemiluminescence technique involving a glass purge–reflux vessel connected to an NO analyser (NOA 280; Sievers Instruments, Boulder, CO, USA). Aliquots (10 μL) of the culture medium that had been treated with the converting enzyme were injected into the purge–reflux vessel, where nitrite was reduced to NO by potassium iodide 1% v/v in glacial acetic acid. NO was then detected by the NO analyser.

Western blot analysis

Cells were lysed with the Mammalian Cell Lysis Kit (Sigma). The protein concentration was measured by the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Protein (30 μg) from A549 cells was separated by electrophoresis through SDS-PAGE 10% w/v gels and transferred to nitrocellulose (Hybond-ECL; Amersham, Arlington Heights, IL, USA). Membranes were blocked with Tris-buffered saline containing skimmed milk 5% w/v and Tween-20 0.1% w/v, and then incubated with the primary antibodies, anti-iNOS (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-eNOS (1:100; Santa Cruz Biotechnology), anti-ICAM-1 (1:200; Santa Cruz Biotechnology), anti-β-actin (1:1000; Sigma), anti-adenovirus 5 E1A (1:2000; Sigma) in Tris-buffered saline containing skimmed milk 5% w/v and Tween-20 0.1% w/v at room temperature for 1 h. The blots were washed extensively and then incubated for 1 h with anti-rabbit IgG conjugated to horseradish peroxidase (1:25 000; Amersham) or anti-mouse IgG conjugated to horseradish peroxidase (1:50 000; Amersham). Binding of secondary antibody was detected using an enhanced chemiluminescence detection system (ECL plus; Amersham).

iNOS mRNA expression

iNOS mRNA expression was examined by Northern blotting and RT-PCR. Northern blotting was performed as described previously [11]. A549 cells, grown until they were confluent in 10-cm dishes, were left in media or exposed to LPS 10 μg/L or CM for 6 h, after which total RNA was extracted with Isogen (Wako, Osaka, Japan). This RNA, at 20 μg/lane, was separated on a formaldehyde–agarose 1% w/v gel, transferred to a Hybond-N membrane (Amersham) and fixed to the membrane under vacuum at 80°C. Next, the membrane was prehybridised, and then hybridised for 20 h with the DNA probes, which were labelled with [α-^{32}P]-dCTP (Amersham) using random primers. RT-PCR was performed as described previously [12]. Total RNA (1 μg) was reverse transcribed to cDNA using a Takara RNA-PCR kit (Takara, Kyoto, Japan) according to the manufacturer's recommendations. Briefly, total RNA, random hexadeoxyribonucleotides and avian myeloblastosis virus reverse transcriptase were used for cDNA synthesis. The cDNA was amplified by PCR, using specific primers for iNOS (5′-CAAGTGGAAGTTCACCAACAGC-3′ and 5′-GATATCTTCGTGATAGCGCTTCTGGC-3′ [13,14]) and a control mRNA, GAPDH (5′-ATTCCATGGCACCGTCAAGGCT-3′ and 5′-TCAGGTCCACTGACACGT-3′). Amplification was performed with cycles of 95°C for 1 min, 55°C for 30 s and 72°C for 1 min in a thermal cycler (Perkin-Elmer/Applied BioSystems, Foster City, CA, USA). In preliminary experiments, the appropriate PCR cycle number was selected from cycle numbers that showed a linear relationship to the signal intensity from the PCR product on ethidium bromide-stained agarose gels. For semiquantitative evaluation of iNOS and GAPDH mRNAs, 30 and 26 cycles were chosen, respectively. The PCR products were analysed on an agarose 1% w/v gel, and the intensity of the ethidium bromide fluorescence was evaluated by NIH Image v. 1.62 (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Data were expressed as a group mean ± SEM, with *n* referring to the number of wells studied in each group. Group comparisons were made through an analysis of variance (ANOVA) with Bonferroni's correction for multiple comparisons. Values of $p < 0.05$ were considered significant.