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Brief exposure to cigarette smoke impairs airway epithelial cell innate anti-viral defence

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ABSTRACT

Background: Human rhinovirus (hRV) infections commonly cause acute upper respiratory infections and asthma exacerbations. Environmental cigarette smoke exposure is associated with a significant increase in the risk for these infections in children.
Objective: To determine the impact of short-term exposure to cigarette smoke on innate immune responses of airway epithelial cells infected with hRV.
Methods: A human bronchial epithelial cell line (HBEC-3KT) was exposed to cigarette smoke extract (CSE) for 30 min and subsequently infected with hRV serotype 1B. Viral-induced cytokine release was measured with AlphaLISA and viral replication quantified by shed viral titer and intracellular viral copy number 24 h post-infection.
Results: CSE induced a concentration-dependent decrease in CXCL10 ($p < 0.001$) and IFN-β ($p < 0.001$), with a 79% reduction at the highest dose with an associated 3-fold increase in shed virus. These effects were maintained when infection was delayed up to 24 h post CSE exposure. Exogenous IFN-β treatment at $t = 0$ after infection blunts the effects of CSE on viral replication ($p < 0.05$).
Conclusion: A single exposure of 30 min to cigarette smoke has a lasting impact on epithelial innate defence providing a plausible mechanism for the increase in respiratory infections seen in children exposed to second-hand tobacco smoke.

1. Introduction

The adverse health effects of exposure to cigarette smoke have been extensively studied with cigarette smoke now recognised as a key risk factor for development of cancer, heart disease, stroke and chronic obstructive pulmonary disease (CDC, 2010). Smokers, as well as those exposed to sidestream smoke, are more susceptible to viral infections of the respiratory tract, as well as infection by various bacterial pathogens (Feldman and Anderson, 2013). Cigarette smoke increases incidence, severity and treatment resistance in respiratory diseases as such as asthma by causing deficiencies in the innate immune system (Baena-Cagnani et al., 2009). Tobacco smoke compromises the immune system by a variety of mechanisms (Goncalves et al., 2011) including deleterious effects on the functions of the lung epithelium (Mehta et al., 2008). The respiratory epithelium is the site of interface between the environment and the lung. Beyond their function as a barrier, epithelial cells play a central role in host-defence by orchestrating the initiation, maintenance, and regulation of both innate and adaptive immune responses in the airway (Vareille et al., 2011). Tobacco smoke induces inflammation in the respiratory epithelium while also having immunosuppressive effects and increasing susceptibility to infection (Bauer et al., 2013; Stampfli and Anderson, 2009).

Epithelial cells are the major target for hRV infections in the airway (Bedke et al., 2009). As such, the epithelial cells and the antiviral responses they exhibit are the most essential to consider in an infection. IFN-β plays a crucial role in epithelial cell defence against respiratory viruses by inducing antiviral activities, controlling of spread of infection and viral replication and initiating immune cell recruitment. During a hRV infection IFN-β is primarily produced by the epithelial cells and exerts its effects on surrounding cells through induction of the expression of a variety of IFN responsive genes (ISGs) (Stetson and Medzhitov, 2006). Expression of key inflammatory and antiviral effector molecules, such as IFN-β, during hRV infection is initiated through pattern recognition receptors including Toll-like receptors (TLR) 3, 7 and 8 and the RNA helicases Retinoic Acid Inducible Gene (RIG)-I and Melanoma Differentiation-Associated Protein (MDA)-5 (Chen et al., 2006; Slater et al.,

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2010; Wang et al., 2009) activating signalling pathways converging on transcription factors Interferon Regulatory Factor (IRF)-3 and NF-κB (Nuclear Factor kappa-light-chain-enhancer of activated B cells). Secreted IFN-β binds to the IFN-α/β receptor on epithelial cells and stimulates the JAK/STAT pathway leading to the assembly of a regulatory complex (ISGF3) composed of STAT1-STAT2 dimers and IRF9. ISGF3 binds to IFN-stimulated response elements (ISRE) in the promoters of IFN-stimulated genes. There have now been numerous reports that cigarette smoke impairs the RIG-I and JAK/STAT pathway, resulting in a reduced type 1 IFN response following infection with hRV, RSV or influenza in bronchial epithelial cells (Proud et al., 2012; Modestou et al., 2010; Wu et al., 2011). STAT-1 activation particularly has been found to be suppressed by CSE exposure, which would be expected to limit downstream induction host anti-viral proteins. This effect was attributable to the free radicals and highly reactive species contained in cigarette smoke and were reversed by use of antioxidants such as NAC, a GSH precursor. The discovery of an IFN-β deficiency in asthmatic bronchial epithelial cells (Wark et al., 2005) has led to the notion that IFN-β may provide a novel method for treatment of viral-induced asthma exacerbations. It has also led to the idea that exposures such as cigarette smoke may play a direct role in the development of this deficiency in asthmatic bronchial epithelial cells.

While most studies have focused on adults and active smokers, tobacco smoke has a significant effect on children's health. Approximately 38% of children are exposed to environmental tobacco smoke (ETS) in the home, whereas 23.8% were exposed by maternal smoking during pregnancy, and a significant proportion of children are active smokers (Baena-Cagnani et al., 2009). The American Academy of Pediatrics lists the documented effects of second-hand tobacco smoke on children's health as including: otitis media, upper respiratory infections, bronchitis, pneumonia, poor lung growth and asthma inception and exacerbation (American Academy of Pediatrics, 2011). Many of these effects appear to be linked to increasing the likelihood and/or severity of respiratory viral infection. It is becoming increasingly clear that viral infections are of significant importance in the induction and exacerbations of wheeze-related illnesses in both children and adults of all ages. During infancy up to 30% of children will suffer from a wheezing illness, with over 50% of cases having a viral basis (Patel et al., 2008). Rhinoviruses are the cause of a relatively mild respiratory illness, the common cold, in older children and adults. However in early life, rhinoviruses seem to be important lower airway pathogens that contribute to the initiation of asthma and subsequent acute illnesses and exacerbations of asthma (Gavala et al., 2011; Gern, 2009).

More studies are needed to gain insight in the relationship between tobacco smoking, ETS and the immune response and inflammatory lower and upper respiratory illnesses and, in particular, in the molecular mechanisms of the effect of tobacco exposure on viral infection of the airway epithelium. A number of recent studies (Bauer et al., 2008; Castro et al., 2008; Eddleston et al., 2011; Groskreutz et al., 2009; Hudy et al., 2010; Proud et al., 2012) have probed these mechanisms using respiratory syncytial virus, hRV or poly I:C to mimic viral stimulation of pattern-recognition receptors in a variety of cells (A549, BEAS-2B cell lines and primary cells) with aqueous smoke extracts (CSE) or particulate matter. Most of these studies applied the cigarette smoke component for long periods (12–24 h). Given the importance of both viral infections and cigarette smoke exposure in the development of respiratory disease, particularly in children, we investigated the effects of a single, very short period of exposure time – 30 min to model intermittent exposure. Following initial demonstration of the effect on cytokine expression and viral release of this short exposure time model, we aimed to determine whether the effects of smoke were transient, and finally whether they could be reversed with treatment of exogenous IFN-β.

2. Methods

2.1. Materials

The Human Bronchial Epithelial Cell (HBEC-3KT) cell line from Ramirez et al. (2004) was kindly provided by Dr. Keith Chappell, University of Queensland. All cell culture medium and supplements were purchased from Invitrogen Life Technologies.

2.2. Culture of human bronchial epithelial cell line

HBEC-3KTs were maintained in Keratinocyte Serum-Free Media (KSFM) supplemented with heat-inactivated foetal calf serum (FCS, 10%), L-glutamine (2 mmol/ml), epidermal growth factor (2.5 μg), bovine pituitary extract (25 mg) and Pen/Strep/Fungizone (PSFZ, 100 unit pen/10,000 μg strep/25 μg/mL fungizone). Cells were seeded at 50,000 cells per well of 24 well flat bottom tissue culture plate (BD falcon) and cultured in KSFM (10% FCS) until 90% confluent, at 37 °C and 5% CO_2. Upon reaching 90% confluence, cells were starved with KSFM (2% FCS) for 24 h.

2.3. Preparation of cigarette smoke extract and treatment of cells

CSE was prepared following adaptation of the method of Laan et al. (2004). In brief, two filtered Winfield Red (1.2 mg of nicotine, 16 mg of tar, 15 mg of CO; Phillip Morris, Moorabbin, VIC, Australia) cigarettes were bubbled through 25 mL of KSFM (2% FCS) with a modified syringe-driven apparatus. A 30 mL volume of smoke was drawn over 10 s, followed by a 20 s break. This process was repeated ten times per cigarette. The resulting suspension was filtered through a 0.2 μm filter (Millipore, Billerica, MA, USA). This filtered CSE was deemed to have a concentration of 2. All experiments were carried out using undiluted extract unless otherwise stated. In the case of a dose response, dilutions were made in fresh KSFM (2% FCS) and applied to HBEC-3KT cultures within 30 min of preparation. Fresh CSE was prepared before each experiment. In preliminary experiments HBEK-3KT were exposed to CSE at doses up to two cigarettes/25 ml and for periods up to 24 h exposure (see Fig. E1 online supplement). As there was no reduction in cell viability under any of the study conditions we chose to perform all future experiments with 30 min exposure to CSE.

2.4. Preparation of and infection with HRV-1B

hRV-1B (ATCC) was propagated in Ohio-HeLa cells. HeLa-rhinovirus cultures were maintained in Opti-MEM Reduced Serum Medium supplemented with FCS (2%), L-glutamine (2 mmol/ml), $MgCl_2$ (30 mM; Sigma), Tryptose (4%; Sigma) and PSFZ (100 unit pen/10,000 μg strep/25 μg/mL fungizone). Infected HeLa cells were harvested after 24 h and stocks prepared as HeLa lysates. Viral titer was assessed using a TCID50 assay (50% tissue culture infective dose). Preliminary studies were conducted using a range of infectious doses, with multiplicity of infection (MOI) of 1, 3 and 5. A dose-dependent increase in IFN-β and CXCL10 was seen following infection with hRV1B, but not with vehicle control or heat-inactivated virus (Fig. E2, online supplement). Subsequent experiments used an infective dose of 3 MOI unless otherwise stated.

hRV1B from the lysate was adsorbed onto cells aided by rocking of the plate every 15 min, for 90 min at 33 °C, with a total volume of 250 μl of hRV1B in KSFM (2% FCS) for each treatment.

2.5. Cytokine measurement

Specific AlphaLISA kits (Perkin Elmer) were used for quantitative determination of human IFN-β and CXCL10 from the superna-