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Research paper

Cathelicidins display conserved direct antiviral activity towards rhinovirus

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ABSTRACT

Human rhinoviruses (HRVs) are the most common cause of viral respiratory tract infections, and are associated with significant morbidity and mortality in immunocompromised individuals and patients with pre-existing pulmonary conditions. The therapeutic options available are extremely limited and therefore novel therapeutics for HRV infections are of significant interest. Cathelicidins have been shown to have potent antiviral activity against a range of pathogens and are known to be key immunomodulatory mediators during infection. We therefore assessed the antiviral potential of cathelicidins from humans and other mammalian species against HRV, together with the potential for the human cathelicidin to modulate apoptotic pathways and alter cell viability during HRV infection. We demonstrate that LL-37, the porcine cathelicidin Protegrin-1, and the ovine cathelicidin SMAP-29 display potent antiviral activity towards HRV and that this activity is visible when either the virus is exposed to the peptides prior to cell infection or after cells have been infected. We further demonstrate that, in contrast to established findings with bacterial infection models, LL-37 does not induce apoptosis or necrosis in HRV-infected lung epithelial cells at physiological or superphysiological concentrations, but does reduce the metabolic activity of infected cells compared to uninfected cells treated with similar peptide concentrations. Collectively, the findings from this study demonstrate that the mechanism of action of cathelicidins against rhinovirus is by directly affecting the virus and we propose that the delivery of exogenous cathelicidins, or novel synthetic analogues, represent an exciting and novel therapeutic strategy for rhinovirus infection.

1. Introduction

Human rhinoviruses (HRVs) are the most common cause of viral respiratory tract infections and are associated with significant morbidity and mortality in young children, in the elderly, in immunocompromised individuals, and in patients with pre-existing pulmonary conditions [1]. HRVs have historically been associated with relatively benign upper respiratory tract infections (*i.e.* the common cold). However, studies have recently provided clear evidence of the major role that rhinoviruses can play in triggering exacerbations of asthma and chronic obstructive pulmonary disease [2,3], in causing severe bronchiolitis in infants and children [4,5], as well as pneumonia [6]. There are no current specific effective treatments for HRV infection, and prevention by vaccination has thus far been shown to be almost impossible due to the fact that there have been more than 100 circulating serotypes of HRV identified [7]. The therapeutic options available are predominantly supportive and are only applicable for mild upper respiratory infections, however, for serious respiratory infections in immunocompromised individuals there are no efficient treatments available. The development of specific effective therapies for HRV infections is therefore an urgent requirement.

Cationic Host Defense Peptides (HDPs) constitute a major component of the innate immune system and can be found in a variety of lifeforms, contributing to the first-line of defense against invading pathogens. The sole human cathelicidin, a cationic antimicrobial peptide of 18 kDa called hCAP-18, is encoded by the CAMP gene and is expressed in a variety of cell types including neutrophils, macrophages and epithelial cells. The precursor peptide hCAP-18 is cleaved in to its active form, LL-37, by extracellular proteinase-3 [8] and while concentrations of approximately 5 μg/ml have been detected in bronchoalveolar lavage (BAL) fluid in healthy infants [9], concentrations of LL-37 can rise to ~30 μg/ml in the BAL fluid of patients with respiratory infection, or with cystic fibrosis (CF) [10]. Cathelicidins have been shown to modulate innate and adaptive immunity, predominantly mediated through their interaction with cells, such as monocytes, dendritic cells and T cells [11–13]. Cathelicidin knockout mouse-models show an increased

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susceptibility to bacterial infections of the skin, lung, urinary tract, and gastrointestinal tract which confirms the crucial role of cathelicidins in the immune response to bacterial infections [14,15].

We have previously shown that LL-37 displays potent antiviral activity against the respiratory pathogen, influenza A virus (IAV) both *in vitro* and *in vivo* [16], and several other studies have revealed a role for LL-37 in defence against a variety of other viral pathogens including Respiratory Syncytial Virus (RSV), dengue virus, Human Immunodeficiency Virus-1 (HIV-1), Herpes Simplex Virus (HSV), vaccinia virus and Adenovirus [17–24]. In addition, we and others have shown that cathelicidins from other mammalian sources such as murine (mCRAMP), porcine (Protegrin-1), ovine (SMAP-29) and bovine (BMAP-28, indolicidin) have direct antiviral activity against these human pathogens and other emerging pathogens, including Dengue virus [16,20,22,25–28] (Reviewed in detail in [29]). It has also recently been demonstrated that vitamin D-mediated upregulation of cathelicidin in human bronchial cells from CF patients resulted in reduced HRV load *in vitro*, and that treating cystic fibrosis bronchial epithelial cells with LL-37 (20 μg/ml) post-infection resulted in a significant reduction in viral replication, although the underlying mechanism remains unclear [30].

In addition to their direct antimicrobial and antiviral activity, cathelicidins have also been shown to have a potent capacity for altering inflammatory cytokine responses and apoptotic pathways in healthy and infected cells [31–35], at physiological, and inflammatory, concentrations. We have previously shown that LL-37 is capable of enhancing the apoptosis of infected airway epithelial cells, together with inducing rapid secondary necrosis in apoptotic neutrophils. Additionally, the porcine cathelicidin, Protegrin-1, has been shown to induce necrosis in U937 tumor cells, and Ca^{2+}-dependent necrotic cell death in human K562 erythroleukemia cells [36,37]. While the underlying mechanisms for the cathelicidin-mediated induction of necrotic cell death or apoptosis in a number of cell types remains to be fully established, it is a clear indicator of the immunomodulatory potential of cathelicidins, the role of which in viral infection remain to be understood.

Due to the potent antiviral activity of cathelicidins and their ability to modulate the immune system, along with the urgent need to develop specific effective antiviral treatments against rhinovirus, this study investigates the antiviral activity of the human cathelicidin, LL-37, together with cathelicidins from ovine (SMAP-29) and porcine (Protegrin-1) sources against HRV in *in vitro* models of infection. In addition, we also investigate the underlying antiviral and cell-based mechanisms of the anti-HRV activity displayed by LL-37 in order to more clearly understand the direct and indirect antiviral capacity of cathelicidins in HRV infection. We show that the human cathelicidin, LL-37, together with the porcine and ovine cathelicidins Protegrin-1 and SMAP-29, display potent antiviral activity against HRV, through a mechanism distinct from the induction of apoptosis.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM), Eagle's minimal essential medium (E-MEM), L-glutamine, Foetal bovine serum (FBS), non-essential amino acids (NEAA), PBS, trypsin/EDTA, BSA, and Fibronectin were all purchased from Sigma-Aldrich, UK. Vectashield Hardset mounting medium with 49,6-diamidino- 2-phenylindole (DAPI) was supplied by Vector Laboratories, UK. CellTiter96 Aqueous Non-Radioactive Cell Proliferation Assay (MTS) and CytoTox 96 Non-Radioactive Cytotoxicity Assay (LDH) were supplied by Promega, UK. The Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate Nick-End Labelling (TUNEL) Assay was supplied by Roche Applied Science. Reconstituted peptide masses were characterised by MALDI-TOF analysis (Proteomics Facility Moredun Research Institute, UK).

2.2. Peptide synthesis

The peptides were assembled using the Fmoc/tBu solid-phase peptide synthesis approach [38] using either model 433A (Applied Biosystems, CA, USA) or model Liberty (CEM Corporation, NC, USA) automated peptide synthesizers followed by cleavage in the trifluoroacetic acid (TFA)/phenol/thioanisole/ethanedithiol/water (10:0.75:0.5:0.25:0.5, w/w) mixture at 25 °C for 90 min followed by precipitation with cold diethyl ether. The crude peptides were purified by preparative reversed-phase high-pressure liquid chromatography (RP-HPLC). The peptide purity (> 98%) was confirmed by analytical RP-HPLC, and the masses were confirmed by mass spectrometry. Following lyophilization, the purified peptides were obtained in the form of their TFA salts; namely: LL-37 (LLGDFFRKSKEKIGKEFKRIV QRIKDFLRNLVPRTES), LL-37 analog having "scrambled" sequence (RSLEGTDRFPFVRLKNSRKLEFKDIKGIKREQFVKIL), termed sLL-37 (control peptide). Since porcine Protegrin-1 (PG-1; RG-GRLCYCRRRFCVCVGR −amide) was obtained in its reduced form, its two disulfide bridges, connecting Cys-6 and Cys-15, and Cys-8 and Cys-13, were formed by air oxidation of the HPLC purified, all-reduced peptide. SMAP-29 was synthesized in the same way as described above for LL-37. All peptides were dissolved in endotoxin-free ultrapure water (Sigma-Aldrich, UK) and stored at −20 °C until use.

2.3. Respiratory epithelial cell culture

The WI-38 human fetal embryonic lung fibroblast cell line was purchased from the Public Health England (PHE) Culture Collection, Salisbury, UK, and cultured in Eagle's minimum essential medium (E-MEM), supplemented with 1% L-glutamine (v/v), 1% non-essential amino acids (v/v), 1% streptomycin/penicillin (v/v) and 10% fetal bovine serum (v/v). The A549 adenocarcinoma human alveolar basal epithelial cells were provided by Edinburgh Napier University. Cells were cultured in DMEM, supplemented with 1% L-glutamine (v/v), 1% non-essential amino acids (v/v), 1% streptomycin/penicillin (v/v) and 10% fetal bovine serum (v/v). WI-38 cells and A549 were cultured at 37 °C in a humidified incubator with 5% CO_2.

2.4. Virus propagation

Human Rhinovirus strain 1B (RV1B) was purchased from the National Collection of Pathogenic Viruses (NCPV) of Public Health England Virus Collection, Salisbury, UK. HRV was propagated in the Hela and WI-38 cell line by infecting cells in serum-free DMEM for 2 h at 33 °C. The inoculum was then removed and cells were incubated for 24–48 h in low serum media (5% FBS supplemented DMEM). WI-38 cells were then frozen at −20 °C for 3 h and virus was harvested by freezing and thawing the cells and subsequently centrifuging at 14,000 × *g* and recovering the supernatant, which was subsequently frozen and stored in aliquots at −80 °C until use.

2.5. Viral titer assessment ($TCID_{50}$)

A $TCID_{50}$ assay was used to determine the infectious titer of rhinovirus. In order to determine the virus titer of the virus stocks, WI-38 cells were seeded in a 96-well plate and cultured overnight. Ten-fold serial dilutions of the original virus stock were made using serum-free media and subsequently added to the cells which were then incubated for 1 h at 33 °C. Following incubation, the inoculum was removed and cells were cultured in 5% media (v/v) for a period of 5 days before analysis. $TCID_{50}$ was subsequently determined using the Reed-Muench method [39]. The same procedure was repeated in A549 epithelial cells.

For experimental assessment of the antiviral effects of cathelicidins (human LL-37, scrambled LL-37, ovine SMAP-29, or porcine PG-1), WI-