Molecular Immunology 63 (2015) 550–558

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

The effect of the decoy molecule PA401 on CXCL8 levels in bronchoalveolar lavage fluid of patients with cystic fibrosis

Oliver J. McElvaney[a,1], Niamh O'Reilly[a,1], Michelle White[a], Noreen Lacey[a], Kerstin Pohl[a], Tanja Gerlza[b], David A. Bergin[a], Hilary Kerr[a], Cormac McCarthy[a], M. Emmet O'Brien[a], Tiziana Adage[b], Andreas J. Kungl[b], Emer P. Reeves[a,*], Noel G. McElvaney[a]

[a] Respiratory Research Division, Royal College of Surgeons in Ireland, ERC Beaumont Hospital, Dublin 9, Ireland
[b] ProtAffin Biotechnologie AG, Impulszentrum Graz-West, Reininghausstraße 13a, 8020 Graz, Austria

ARTICLE INFO

Article history:
Received 19 June 2014
Received in revised form 1 October 2014
Accepted 18 October 2014
Available online 2 November 2014

Keywords:
Cystic fibrosis
Bronchoalveolar lavage fluid
Glycosaminoglycan
Interleukin-8/CXCL8
Antagonist decoy
Neutrophils

ABSTRACT

Background: The chemokine interleukin-8 (CXCL8) is a key mediator of inflammation in airways of patients with cystic fibrosis (CF). Glycosaminoglycans (GAGs) possess the ability to influence the chemokine profile of the CF lung by binding CXCL8 and protecting it from proteolytic degradation. CXCL8 is maintained in an active state by this glycan interaction thus increasing infiltration of immune cells such as neutrophils into the lungs. As the CXCL8-based decoy PA401 displays no chemotactic activity, yet demonstrates glycan binding affinity, the aim of this study was to investigate the anti-inflammatory effect of PA401 on CXCL8 levels, and activity, in CF airway samples *in vitro*.
Methods: Bronchoalveolar lavage fluid (BALF) was collected from patients with CF homozygous for the *ΔF508* mutation ($n = 13$). CXCL8 in CF BALF pre and post exposure to PA401 was quantified by ELISA. Western blot analysis was used to determine PA401 degradation in CF BALF. The *ex vivo* chemotactic activity of purified neutrophils in response to CF airway secretions was evaluated post exposure to PA401 by use of a Boyden chamber-based motility assay.
Results: Exposure of CF BALF to increasing concentrations of PA401 (50–1000 pg/ml) over a time course of 2–12 h *in vitro*, significantly reduced the level of detectable CXCL8 ($P < 0.05$). Interestingly, PA401 engendered release of CXCL8 from GAGs exposing the chemokine susceptible to proteolysis. Subsequently, a loss of PA401 was observed ($P < 0.05$) due to proteolytic degradation by elastase like proteases. A 25% decrease in neutrophil chemotactic efficiency towards CF BALF samples incubated with PA401 was also observed ($P < 0.05$).
Conclusion: PA401 can disrupt CXCL8:GAG complexes, rendering the chemokine susceptible to proteolytic degradation. Clinical application of a CXCL8 decoy, such as PA401, may serve to decrease the inflammatory burden in the CF lung *in vivo*.

1. Introduction

Cystic fibrosis (CF) is the most common lethal genetic disease in Caucasians that is caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR) chloride channel (Riordan et al., 1989). CF patients suffer from persistent pulmonary infections accompanied by chronic neutrophil-dominated inflammation that results in severe progressive lung injury. Mechanisms for how reduced CFTR function leads to chronic lung disease in CF include altered ion transport across the airway epithelium and dehydration of the airway surface layer (Matsui et al., 1998). The dehydrated airways are lined with thick mucus which contains elevated levels of anionic glycosaminoglycans (GAGs) produced on the bronchial epithelial cell surface (Reeves et al., 2011a). GAGs are unbranched, O-linked linear polysaccharide chains normally linked to a core protein (to form proteoglycans) located on the cell surface of almost all eukaryotic cell types (Adage et al., 2012a; Rek et al., 2009). The immobilisation of chemokines, specifically CXCL8 by GAGs plays a major role in the establishment of haptotactic gradients that contribute to the recruitment of leukocytes through the

Abbreviations: CF, cystic fibrosis; BALF, bronchoalveolar lavage fluid; GAGs, glycosaminoglycans; FEV1, forced expiratory volume in 1 s.

* Corresponding author at: Respiratory Research Division, Department of Medicine, Royal College of Surgeons in Ireland, Beaumont Hospital, Dublin 9, Ireland.
E-mail address: emerreeves@rcsi.ie (E.P. Reeves).

[1] Share joint first authorship.

http://dx.doi.org/10.1016/j.molimm.2014.10.013
0161-5890/

endothelium during inflammatory exacerbations (Proudfoot et al., 2003). GAGs in the lung are located on bronchial cells in the interstitial space between the capillary endothelium and the alveolar epithelium and the most abundant include heparan sulphate (HS) and chondroitin sulphate (CS) (Solic et al., 2005; Suki et al., 2005). In lung tissue CXCL8 has been demonstrated to bind specific regions depending on the presence of HS and CS and disruption of the GAG binding domain of CXCL8 or the removal of HS and CS leads to a significant reduction in the detection of this chemokine (Frevert et al., 2003). Moreover, increased concentrations of GAGs have been found in BALF from children with CF (Bhaskar et al., 1998; Hilliard et al., 2007) and secretion of HS (Solic et al., 2005), CS (Khatri et al., 2003; Rahmoune et al., 1991) and hyaluronic acid (Sahu, 1980; Wyatt et al., 2002) is markedly increased in bronchial cells and CF tissues. GAGs have also been shown to influence the chemokine profile of the CF lung by binding and stabilizing CXCL8 in its active form thus promoting the activation and infiltration of neutrophils (Reeves et al., 2011b; Schlorke et al., 2012).

PA401 is a recombinant therapeutic protein developed by ProtAffin Biotechnologie AG using its patented CellJammer® technology, an engineering platform used to introduce heightened GAG binding affinity into wild type GAG binding proteins and to disrupt receptor binding function (Adage et al., 2012a,b). CXCL8 was chosen as a target for this engineering strategy due to its association with HS and its involvement in numerous neutrophil driven inflammatory diseases including CF, chronic obstructive pulmonary disorder (COPD), rheumatoid arthritis, psoriasis and Crohn's disease (Yang et al., 1999). The recombinant CXCL8 decoy molecule is engineered with a deletion of the first six amino acids including the ELR motif which is essential for receptor binding of human CXCL8. Therefore, disruption of the protein sequence in this area inhibits the chemokine's interaction with its specific G protein-coupled receptors CXCR1 and CXCR2 on leukocytes. In addition, non-crucial amino acids in the GAG binding site were replaced with basic amino acid residues to increase the GAG binding affinity of the chemokine (Adage et al., 2012a). With these engineered mutations the novel CXCL8 decoy is capable of rapid binding to endothelial GAGs without binding to GPCRs, thus displacing wild type CXCL8 (Bedke et al., 2010). The aim of the present study was to investigate the ability of PA401 to disrupt the interaction between native CXCL8 and protective GAGs in BALF samples of people with CF, thus rendering the chemokine susceptible to clearance. Some of the results of this study have been previously reported in abstract form (McElvaney et al., 2013).

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents were purchased from Sigma–Aldrich Chemical Co. Ltd., Dublin, Ireland unless indicated otherwise. PA401 (CXCL8 Δ6 F17K F21K E70K N71K) was generated by ProtAffin Biotechnologie AG, Graz, Austria, according to published protocols (Falsone et al., 2013).

2.2. Patient recruitment and sample collection

Patients with CF homozygous for the *ΔF508* mutation ($n = 13$; age 23.6 ± 4.13 mean ± SD; % forced expiratory volume in 1 second (FEV1) 56.3 ± 21.65 predicted) were recruited to this study. BALF samples were collected from patients attending Beaumont Hospital and full informed consent was obtained pre-procedure according to a protocol approved by Beaumont Hospital Ethics Committee. BAL was performed by instilling and aspirating 1 ml/kg of sterile normal saline into the lingula and the right middle lobe. Obtained BALF was then centrifuged at 500 × *g* for 10 min at 4 °C and the cell free supernatant was aliquoted and stored at −80 °C.

2.3. Native PAGE and Western blot analyses

BALF (20 μg protein) or 2 ng carrier free recombinant human CXCL8 (RhCXCL8; Biovision Inc. Milpitas, CA, USA) as a positive control were subjected to native gel electrophoresis in NativePAGE Novex 4–16% (w/v) Bis–Tris gels (Innovagen, Lund, Sweden). Gels were run at 130 V for 1.5 h and were stained with Alcian Blue Solution (Merck Millipore) or Azure A chloride stain overnight (Powell et al., 2010) and then destained with ultra pure water.

For Western blot analysis, proteins were transferred onto polyvinylidene difluoride (PVDF) membrane at 30 V for 1.5 h using a wet blotter. RhCXCL8 was loaded in order to visualise migration of the chemokine. Membranes were blocked for 1 h in 3% (w/v) dry milk and 1% (w/v) bovine serum albumin (BSA) in phosphate buffered saline (PBS) containing 0.05% (v/v) Tween-20 and then incubated overnight in 1 μg/ml monoclonal mouse anti-CXCL8 specific Ab (MAB208; R&D Systems, Abingdon, UK) or 0.3 μg/ml polyclonal rabbit hCAP-18 specific Ab (Innovagen, Lund, Sweden). The secondary Ab were HRP-linked anti-mouse IgG or HRP-linked anti-rabbit IgG (Cell Signalling Technology, Danvers, MA, USA) incubated at room temperature for 1 h. Immuno-reactive protein bands were visualised employing Immobilon Western chemiluminescent horseradish peroxidase (HRP) substrate (Millipore, MA, USA) on the Syngene G:Box chemi XL gel documentation system (Synoptics, Cambridge, UK). Alternatively, immuno-reactive protein bands were visualised employing SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) after exposure to Kodak® X Omat LS Film.

2.4. Surface plasmon resonance (SPR) affinity measurements

K_d values for PA401 and CXCL8 binding to immobilised HS were determined by SPR measurements at 25 °C on a BiacoreX100 system (GE Healthcare, Uppsala, Sweden) as recently described (Gerlza et al., 2014). In short, PBS plus 0.005% (w/v) Tween (Merck, Darmstadt, Germany) was used as a running buffer. Biotinylated GAGs were immobilised on C1 sensor chips (GE Healthcare) which were activated with EDC/NHS and coated with neutravidin (0.2 mg/ml in acetate buffer pH 4). For each binding experiment, 7 different concentrations of the respective PA401 or CXCL8 were measured in quadruplicates. Contact times for all injections and dissociations were 120 s at 30 μl/min. The regeneration solution (1 M NaCl) was enclosed directly after each dissociation time with 30 μl/min and 60 s contact time after each cycle. The maximum response signals of protein binding to the GAG surface, corresponding to the plateaus of the respective sensorgrams, were used for Scatchard plot analysis and the calculation of equilibrium K_d values.

2.5. Enzyme linked immunosorbent assay (ELISA) measurements in pooled CF BALF

The concentration of CXCL8 or PA401 was measured by ELISA conducted in accordance with the manufacturer's instructions (R&D Systems and ProtAffin, respectively). In brief, 96-well plates (Nunc, Naperville, IL) were coated with 100 μl mouse IgG1 anti-human CXCL8/IL-8 (Cat # MAB208, R&D Systems, diluted to 2 μg/ml)) or monoclonal rat anti-PA401 (# 8A12, ProtAffin, diluted to 7.5 μg/ml) in Voller's Solution (15 mM $NaCO_3$, 35 mM $NaHCO_3$, 3 mM NaN_3, pH 9.8)). The plate was covered and incubated at 4 °C overnight. The plate was washed with 200 μl of Washing Buffer (PBS containing 0.1% (v/v) Tween 20) three times and blocked for 1 h by adding 200 μl of Blocking Buffer (PBS containing 1% (w/v) BSA) to block non-specific binding sites in the coated wells. Standards