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Original Article

Osteopontin is increased in cystic fibrosis and can skew the functional balance between ELR-positive and ELR-negative CXC-chemokines☆

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Abstract

Background: The glycoprotein osteopontin plays important roles in several states of disease associated with inflammation, for example by recruiting neutrophils but its expression and possible roles in cystic fibrosis (CF) have not been investigated.
Methods: Immunohistochemistry and ELISA were used to detect osteopontin in clinical samples. In addition, osteopontin-binding and functional interference with antibacterial (ELR-negative) and neutrophil-recruiting (ELR-positive) CXC-chemokines were investigated using *in vitro* assays.
Results: Increased osteopontin-expression was found in the airways of CF patients compared with controls. Interestingly, osteopontin bound to ELR-negative CXC-chemokines, reducing their antibacterial and receptor-activating properties while no binding or interference with the function of ELR-positive chemokines was found.
Conclusions: High expression of osteopontin is likely part of the dysregulated inflammation seen in CF, impairing the activities of ELR-negative chemokines that both serve as innate antibiotics and recruit NK and cytotoxic T cells, instead promoting an excessive influx of neutrophils, and may thus contribute to disease progress.

Keywords: Cystic fibrosis; Host defense; Osteopontin; Chemokines; Neutrophils

1. Introduction

Cystic fibrosis (CF) is an autosomal recessive disease due to mutations in the *cystic fibrosis transmembrane conductance regulator* (*CFTR*) gene. The effect of this mutation is defects in ion transport across the epithelium of many organs including the airways where thick sputum and chronic bacterial infections, *Pseudomonas aeruginosa* being a major pathogen, present major clinical challenges [1]. In CF, there is also a high degree of Th1 profiled inflammation, including a massive influx of neutrophils, having tissue-damaging properties through release of proteases and reactive oxygen species (ROS) [1,2].

Osteopontin (OPN) is a highly anionic phosphorylated glycoprotein, initially identified as a bone matrix protein but later found to be expressed by a broad range of tissues and cells [3,4]. The molecule is involved in many processes including inflammation where it can act as a cytokine, for example as a chemoattractant for macrophages and neutrophils [5]. OPN

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expression increases in response to inflammation, cellular stress and injury [6,7]. In addition, OPN itself strongly up-regulates the expression of neutrophil-recruiting ELR-positive chemokines (i.e. chemokines having the amino acids glutamic acid–leucine–arginine preceding the CXC-motif), e.g. GRO-α/CXCL1, GRO-β/CXCL2, GRO-γ/CXCL3, ENA-78/CXCL5, GCP-2/CXCL6, and IL-8/CXCL8 and also activate NF-κB, i.e. typical features of the inflammation seen in CF [8,9]. High expression of several ELR-positive CXC chemokines has been demonstrated in CF and these recruit and activate neutrophils through interaction with the surface-expressed CXCR1 and CXCR2 receptors [10]. Furthermore, ELR-negative CXC chemokines are present in CF (i.e. MIG/CXCL9, IP-10/CXCL10, and I-TAC/CXCL11) and their expression is dependent on the prototypic Th1 cytokine IFN-γ, which is also up-regulated in CF airways [11]. These chemokines signal through the CXCR3 receptor, resulting in recruitment and activation of NK cells, cytotoxic T cells, and mast cells to sites of inflammation [10,11]. Defensin-like bactericidal properties of ELR-negative chemokines have been demonstrated in several studies and, in particular MIG/CXCL9, exhibit broad activity against both Gram-positive and Gram-negative bacteria [12–14]. Recently, we showed that MIG/CXCL9 is expressed in CF and has antibacterial activity against *P. aeruginosa*. In addition, we could demonstrate that there is a decreased presence of CXCR3-bearing cells (i.e. NK cells and cytotoxic T cells) in CF lung tissue compared with controls [15].

In this study, we investigated OPN expression in the airways during CF and how this molecule affects the functional balance between ELR-positive and ELR-negative chemokines. Interestingly, OPN binds and reduces both the bactericidal and receptor-activating properties of the ELR-negative chemokines while no effect on the receptor-activating properties of ELR-positive chemokines was observed. Together with the neutrophil-recruiting properties of OPN itself, interactions with chemokines may promote the imbalanced cellular influx of immune cells to the airways in CF and impair host defense.

2. Material and methods

2.1. Special reagents

Recombinant human GRO-β/CXCL2, GCP-2/CXCL6, IL-8/CXCL8, MIG/CXCL9, IP-10/CXCL10, I-TAC/CXCL11 and OPN were from PeproTech (London, UK). Rabbit anti-serum against OPN was generously provided by Dr. Dick Heinegård, Lund. Goat anti-rabbit antibody conjugated to horseradish peroxidase was from Bio-Rad (Hercules, CA). *P. aeruginosa* elastase was from Sigma-Aldrich (St. Louis, MI) and human neutrophil elastase was from Calbiochem (Darmstadt, Germany). G418, Fluo-4 AM and Fura-red were from Invitrogen (Stockholm, Sweden).

2.2. Immunohistochemistry

CF lung tissue (end stage) explants were obtained during transplantation and lung tissue from patients undergoing thoracic surgery for lung cancer (patient characteristics described in Supplemental Table 1). All subjects gave their written informed consent to participate in the study, which was approved by the regional ethics committee in Lund, Sweden, LU412-03. Samples were submerged in 4% buffered formaldehyde. After dehydration and paraffin embedding, 3 μm sections were generated from the tissue blocks. After rehydration and antigen retrieval, rabbit antibodies against OPN or pre-immune serum (diluted 1:1000) were added, and bound antibodies were detected using horseradish peroxidase-conjugated secondary goat anti-rabbit (diluted 1:2500) and visualized using 3,3-diaminobenzidine as chromogen.

2.3. Sputum samples

Sputum samples were obtained from 26 CF patients which were dissolved in three times the volume in PBS on a shaker, overnight at 4 °C, and then spun down. Induced sputum from three healthy individuals was obtained after inhalation of 5 mL nebulized sodium chloride (30 mg/mL) (patient characteristics described in Supplemental Table 2). All subjects gave their written informed consent to participate in the study, which was approved by the Regional Ethics Committee in Lund, Sweden (2011/434).

2.4. ELISA

OPN concentrations were quantified in sputum using ELISA as per the instructions provided by the manufacturer (R&D Systems, Minneapolis, MN).

2.5. SDS-PAGE and Western blotting

Recombinant human OPN, sputum from CF patients and healthy controls were separated on Tris–tricine gels. Coomassie Brilliant Blue R-350 Sigma-Aldrich (St. Louis, MI) was used to visualize the proteins. For Western blotting, the proteins were first transferred onto an Immobilion-P membrane (EMD Millipore, Darmstadt, Germany) using a blotting system (Bio-Rad, Hercules, CA). The membrane was blocked in 5% dry milk diluted in PBS with 0.05% Tween (PBST) for 1 h at room temperature and incubated with polyclonal antibodies against OPN (diluted 1:1000) overnight at 4 °C. After washings in PBST, incubation with goat anti-rabbit IgG conjugated with horseradish peroxidase (diluted 1:2500) was performed. Finally, the membrane was washed in PBST and bound antibodies were visualized using Supersignal West Pico Chemiluminescent Substrate (Pierce/Thermo Scientific, Göteborg, Sweden) and a ChemiDoc imaging system (Bio-Rad).

2.6. Mass spectrometry and N-terminal sequencing

Mass spectrometric analysis was performed using MALDI MS or nano HPLC Orbitrap as previously described [16]. Automated N-terminal sequencing was performed using a