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Original Article

Impact of azithromycin treatment on macrophage gene expression in subjects with cystic fibrosis

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Abstract

Background: Azithromycin treatment improves clinical parameters in patients with CF, and alters macrophage activation from a pro-inflammatory (M1) phenotype to a pro-fibrotic, alternatively activated (M2) phenotype. The transcriptional profile of cells from patients receiving azithromycin is unknown.
Methods: Gene expression in association with macrophage polarization, inflammation, and tissue remodeling was assessed from sputum samples collected from patients with CF. Transcriptional profiles and clinical characteristics, including azithromycin therapy, were compared.
Results: Expression of NOS2 and TNFα was decreased in subjects receiving azithromycin, whereas expression of M2-associated genes was unaffected. Principal component analysis revealed gene expression profiles consistent with M1- (MMP9, NOS2, and TLR4) or M2-polarization (CCL18, fibronectin, and MR1) in select subject groups. These expression signatures did not significantly correlate with clinical characteristics.
Conclusions: Pro-inflammatory gene expression was low in subjects receiving AZM. Genes were stratified into groupings characteristic of M1- or M2-polarization, suggesting that overall polarization status is distinct among patient groups.
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Keywords: Macrophage phenotype; Cystic fibrosis; Azithromycin

1. Introduction

Patients with cystic fibrosis (CF) endure a progressive pulmonary pathology governed by a chronic, exaggerated inflammatory response [1]. Defects in the cystic fibrosis transmembrane regulator (CFTR) gene result in lungs with thickened mucus that are culture positive for a wide variety of bacteria, most commonly *Pseudomonas aeruginosa*. Progression of the disease is characterized by repeated acute bacterial flares during which an influx of neutrophils and macrophages occurs [2], causing a decline in pulmonary function over time [1]. Azithromycin (AZM) is a macrolide antimicrobial agent commonly used in this patient population for its anti-inflammatory properties. Chronic AZM therapy improves clinical outcome measures in CF patients including delayed lung function decline, time to acute pulmonary exacerbation, and requirement for antimicrobial treatment [3–7]. Our previous work demonstrated that AZM polarizes macrophages

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away from the pro-inflammatory classically activated (M1) phenotype and toward the anti-inflammatory, pro-fibrotic alternatively activated (M2) phenotype [8]. This results in decreased neutrophil influx and blunted pulmonary injury in mice infected with *P. aeruginosa* [9]. In a previous study of 48 CF patients that evaluated M1 and M2 polarization biomarkers, we showed heightened expression of the M2 protein mannose receptor (MR) on alveolar macrophages as well as an inverse correlation between both MR expression and M2-effector protein arginase expression as pulmonary function declines [10]. These results suggest that M2 function has a role in the pathophysiology of CF as the disease progresses and the protective effect of AZM may be related to its ability to induce M2 polarization.

Here we investigated the impact of chronic AZM therapy and other clinical parameters including positive bacterial culture and other antibiotics received on gene expression associated with macrophage polarization and fibrosis development in cells isolated from sputum samples from patients with CF. We utilized principal component analysis (PCA) to group gene expression values in order to describe the macrophage gene expression signature in these patients.

2. Methods

2.1. Clinical design

The study was approved by the Institutional Review Board at the University of Kentucky Medical Center. Patients were recruited from the pediatric and adult CF clinics during routine visits. Patients diagnosed with CF who were not acutely ill as determined by the treating pulmonologist were included, although if a subject had completed a course of therapy for an acute exacerbation, they were eligible for inclusion. Patients were ineligible if they had a decline in pulmonary function measured as the forced expiratory volume in one second (FEV1)% predicted compared to baseline measurements or if acute antibiotic therapy was required. Additional exclusion criteria included other active infectious processes (both pulmonary and systemic), malnutrition, HIV infection, and cancer.

2.2. Sample collection

Subjects provided a spontaneous sputum sample during routine clinic visits. Samples were subjected to a digestion process with the addition of 0.1% DL-dithiothreitol (Promega, Madison, WI) plus 50 units/ml deoxyribonuclease I (Sigma Aldrich, St. Louis, MO) to decrease viscosity. The process was repeated once if the sample was deemed to be overly viscous. The entire sample was utilized for later analysis, as no enrichment for macrophages was performed. Cells were placed in RNAlater (Ambion, Austin, TX) and frozen at −80 °C.

2.3. Generation of cDNA

RNA was isolated using commercially available RNeasy Mini Kits (Qiagen, Valencia, CA) according to a standard protocol and quantified using a NanoDrop 2000 (Thermo Scientific, Wilmington, DE). Reverse transcription was performed using materials from Applied Biosystems (Foster City, CA). A set amount of isolated mRNA was incubated with a reverse transcriptase cocktail with random hexamers.

2.4. Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed on the samples for a group of genes that are associated with macrophage polarization, inflammatory responses, and fibrosis using TaqMan gene probes (Applied Biosystems, Foster City, CA). The genes for inducible nitric oxide synthase (NOS2), Toll-like receptor 4 (TLR4), tumor necrosis factor alpha (TNFα), interleukin (IL)-1α, IL12α, and IL12β were selected as genes produced by the M1 phenotype in the context of inflammation. M2 and anti-inflammatory genes selected included arginase I (ARG1), arginase II (ARG2), chemokine (C–C motif) ligand 18 (CCL18), MR1, IL10, and transforming growth factor beta (TGFβ). Matrix metalloprotease 9 (MMP9), collagen 1A1 (COL1A1), and fibronectin were chosen as representative genes for tissue remodeling. It is important to note that many of the inflammatory and fibrotic genes are expressed in a variety of the immune cells found in the lungs. Thus, this analysis provides a general picture of the inflammatory and fibrotic status of the lungs of these individuals. Samples were run in triplicate and analyzed using an ABI 7900HT system (Applied Biosystems, Foster City, CA). Gene expression was calculated as the relative expression between the gene of interest and the housekeeping gene GAPDH.

2.5. Statistical analyses

PCA was performed as a method of data reduction utilizing the statistical software package SPSS (IBM, Chicago, IL). PCA creates a new set of uncorrelated variables comprised of combinations of the original variables [11]. The resulting new variables were weighted using the correlations between the various genes. The first generated component explains the highest amount of the total variance between the initial genes; the second explains the next highest amount of total variance, and so forth. Components with an eigenvalue greater than 1 were deemed to explain a significant amount of the total variance. Additional statistical analysis was performed utilizing GraphPad Prism (GraphPad Software, La Jolla, CA). Comparisons between groups were made using t-tests or the Kruskal–Wallis test for non-parametric distributions for continuous variables and by Fisher's exact test for nominal data.

3. Results

3.1. Subject recruitment

Thirty patients were recruited for the study, with a total of 65 samples collected from September 2009 to December 2010. Of these, 30 were original samples, and 18, 12, 4, and 1 were first, second, third, and fourth follow-up samples, respectively, collected during subsequent routine visits. While the median age of the recruited population was 16 years (range 8–48), a majority were under the age of 18 years (66%). A wide range of

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