Doxycycline improves clinical outcomes during cystic fibrosis exacerbations

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Methods

Sputum and Serum Processing. Sputum was obtained by spontaneous expectoration (i.e., no sputum induction). Once sputum was collected, the sputum was diluted 1:1 with 0.9% sodium chloride and centrifuged at 3,000 rpm for 10 min with separation of pellet from supernatant. The supernatant was collected, protein concentration was measured, and then separate aliquots were saved for measurements (−80°C). For serum processing, whole blood was collected and processed in serum tubes, as previously described [1].

MMP-9 and TIMP Assays. MMP-9-specific ELISA-based assays were used to quantify MMP-9 levels and activity (R&D Systems, Minneapolis, MN) as previously shown [1]. Briefly, samples were diluted to fit manufacturer's sensitivity for individual kits (0–16 ng/ml for MMP-9 kits). Both samples and recombinant enzyme standards were prepared and incubated for 2 h at room temperature in 96-well plates coated with monoclonal antibodies for the MMP(s) (MAB 911 for MMP-9) of interest. After incubation, samples and standards were activated with 1 mM aminophenylmercuric acetate (APMA), a chemical activator of MMPs, and further incubated for 2 h at 37°C. After incubation, a fluorogenic substrate (Fluor-Pro-Leu-Gly-Leu-Ala-Arg-NH_2) was placed in each well, and the plate was incubated at 37°C for 18 h. The plate was then read on a spectrophotometer (SpectraMax Gemini, Molecular Devices, Sunnyvale, CA; excitation and emission wavelength of 320 and 405 nm, respectively), and data were quantified using standard curves provided with the kits. These results provided both baseline active MMP-9 levels and total MMP-9 levels.

For the studies of TIMP-1 (R&D Systems, Minneapolis, MN), samples were diluted to fit manufacturer's sensitivity for ELISA (0–10 ng/ml) as previously done [1]. Both samples and recombinant TIMP-1 standards were prepared and incubated for 2 h at room temperature in 96-well plates coated with TIMP-1 monoclonal antibodies. Bound TIMP-1 was then conjugated with

a horseradish peroxidase-based secondary antibody for 1 h. A colorimetric substrate (hydrogen peroxide and chromagen) was placed in each well, and color change was assessed after 30 min on a colorimeter (Bio-Rad, Hercules, CA) via standard curves provided with the kits.

Neutrophil Elastase Assays. Neutrophil elastase-specific ELISA kits were used to measure concentrations in clinical samples (Assaypro, St. Charles, MO). Briefly, samples were diluted to fit manufacturer's kit specificity (0–10 ng/ml). Both samples and recombinant neutrophil elastase standards were prepared and incubated for 2 h at room temperature in 96-well plates coated with a polyclonal antibody for neutrophil elastase. A biotin-labeled monoclonal antibody to neutrophil elastase substrate was then added and incubated for 1 h. Thereafter, streptavidin was added for 30 min and a chromagen substrate was added thereafter. Finally, the plate was read on a spectrophotometer (SpectraMax Gemini, Molecular Devices) at 450 nm, and data were quantified using standard curves provided with the kits.

Cytokine analyses. CF sputum supernatants were analyzed for cytokine content using a MAGPIX System (Luminex) employing xMAP technology as has been previously described. Briefly, 25 µL of sputum supernatant was incubated overnight at 4°C with commercially available magnetic beads coated with specific antibodies (EMD Millipore). Beads were incubated next with biotinylated detection antibodies, and then with Streptavidin-Phycoerythrin, with wash steps in between and after. Finally, beads were resuspended in MAGPIX Drive fluid and analyzed on the MAGPIX using xPONENT v4.2 (Luminex). A standard curve and control samples were included. Using MILLIPLEX Analyst v3.5.5.0 (Vigene Tech), sample values were extrapolated using a 5-parameter logistic curve.

Electrospray ionization-liquid chromatography tandem mass spectrometry (ESI-LC MS/MS) for Ac-PGP detection. Ac-PGP was measured in sputum samples using a MDS Sciex (Applied Biosystems) API-4000 spectrometer equipped with a Shimadzu HPLC as previously demonstrated [2]. HPLC was done using a 2.1 × 150-mm Develosi C30 column (with

buffer A: 0.1% formic acid and buffer B: acetonitrile plus 0.1% formic acid: 0–0.6 min 20% buffer B/80% buffer A, then increased over 0.6–5 min to 100% buffer B/0% buffer A). Background was removed by flushing with 100% isopropanol plus 0.1% formic acid. Positive electrospray mass transitions were at 312-140 and 312-112 of Ac-PGP.

Reference:

1. Gaggar A, Li Y, Weathington N, et al. Matrix metalloprotease-9 dysregulation in lower airway secretions of cystic fibrosis patients. *Am J Physiol Lung Cell Mol Physiol.* 2007; **293**(1): L96-L104.
2. Gaggar A, Jackson PL, Noerager BD, et al. A novel proteolytic cascade generates an extracellular matrix-derived chemoattractant in chronic neutrophilic inflammation. *J Immunol.* 2008; **180**(8): 5662-9.