Coagulation factor Xa modulates airway remodeling in a murine model of asthma

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Online Data Supplements

Methods

Animals

All experimental procedures conformed to international standards of animal welfare and were approved by the Laboratory Animal Care and Use Committee of the University of Notre Dame. The male A/J mice (7-10 weeks old) used in this study were maintained under a 12-hr light-dark cycle with free access to water and standard laboratory mouse chow.

Induction of eosinophilic inflammation by intranasal administration of OVA

Mice were first anesthetized by isoflurane (Abbott, North Chicago, IL) inhalation, and then 1 mg/ml OVA was administered intranasally using a micropipette (50 μl). Administration of OVA was performed 3 days per week for 16 weeks. As a control, PBS was administered to the mice (50 μl) intranasally 3 days/week for 16 weeks. At a time of 24 hr after the last administration of antigen, the mice were anesthetized intraperitoneally with a rodent cocktail (0.015 mg xylazine, 0.075 mg ketamine, and 0.0025 mg aceprozamine/g body weight) and the lungs lavaged 3x with sterile phosphate-buffered saline (PBS). BALF cells were centrifuged onto cytospin slides, fixed, and stained with DiffQuick (IMEB, San Marcos, CA). Differential cell counts were

obtained under the light microscope. Total BALF cells were counted using a hemocytometer and supernatants were collected for analyses of FXa activity.

Isolation of macrophages, lymphocytes, and eosinophils

BALF was centrifuged at 300 g and the cell pellet resuspended in 2 ml of PBS and plated in 6-well plates. Cells were incubated at room temperature for 1 hr and then washed with PBS to remove nonadherent cells. Adherent macrophages were lyzed with RLT buffer (Qiagen Inc., Valencia, CA).

Lymphocytes were isolated from the peribronchial lymph nodes by mechanical disruption and then filtered through a nylon mesh (Cell Strainer, Becton Dickinson, Franklin Lakes, NJ). T- and B- cells were then purified using Dynabeads (Dynal Biotech, Oslo, Norway) specific for T- and B-cells. Cells were then lysed with RLT buffer (Qiagen Inc.).

Eosinophils were purified by lectin affinity negative selection, as previously reported (E1). To do so, 10 mg/ml biotinylated Griffonia simplifolia Lectin I (BSL-I: Vector Labs, CA, USA) was first incubated with pooled BALF for 15 min. Lectin-bound cells were allowed to adhere to streptoavidin-conjugated magnetic beads (Dynal Biotech, Oslo, Norway) for 10 min,

and were then selected by using a strong magnetic field (Dynal Biotech, Oslo, Norway). Dynabeads coated with anti-CD4, CD-8, and CD-19 antibodies (Dynal Biotech, Oslo, Norway) were added to remove lymphocytes. The purified cells were 98% eosinophils and viability was greater than 95%.

Quantitative reverse transcriptional-polymerase chain reaction (Q-RT-PCR)

Q-RT-PCR was performed as previously described (E2). Total RNA was isolated from lungs by the RNeasy total RNA purification kit (Qiagen, Valencia, CA). For mRNA levels of amphiregulin and MUC5AC from NCI-H292 cells, the cells were resuspended in RPMI 1640 containing 10% fetal bovine serum (FBS) and antibiotics (100 U penicillin/ml, 100 μg streptomycin/ml, Cambrex Bioscience, Walkersville, MD) and then seeded at a concentration of 3×10^5 cells/ml (2 ml) in 6-well plates. The plates were then incubated for 3 days in a 5% CO_2 incubator, at 37° C. The cells were then washed 2x with PBS and incubated for 3 hr in serum-free RPMI 1640 media containing antibiotics. After 3 hr, FXa (0-30 U/ml) was added to the cells and incubated for 3 hr in a 5% CO_2 incubator at 37° C to detect amphiregulin mRNA. For the anti-amphiregulin studies to detect mRNA levels of MUC5AC, a mouse monoclonal antibody to

human amphiregulin, 10 μg/ml, (R&D Systems, Minneapolis, MN) was pre-incubated for 1 hr, after the 3 hr serum-free incubation, and then FXa (30 U/ml) was added to the cells and incubated for 6 hr. The supernatant was then removed and RNA isolated from the cells using the RNeasy kit (Qiagen). Q-RT-PCR was carried out with the primers and probes listed in Table 1 for FX, PAR1, PAR2, amphiregulin, MUC5AC, and the housekeeping gene RPL19, using an ABI Prism 7700 Sequence Detector. The crossing point (CP) for each reaction of each gene, defined as the PCR cycle at which fluorescence begins to increase above the background and marking the beginning of the log-linear phase of the PCR reaction, was determined.

Assay of FXa activity

FXa activity was determined utilizing a chromogenic assay with S-2222 (Chromogenix, Lexington, MA) as the substrate. Samples were incubated with 20 mM Tris-HCl buffer (pH 7.4) containing 2 mM of S-2222 for 30 min after which the absorbancy was determined at 405 nm. Human FXa (ERL, South Bend, IN) was used as the standard over the concentration range of 0.001-1 U/ml.

Fondaparinux in blood as a function of time

Fondaparinux (FPX) was administered subcutaneously to mice at either a 10 μg or 30 μg dose. Blood was obtained from tail cuts of isoflurane-anesthetized mice. Serum levels of FPX were determined by a FXa chromogenic assay as described above using 10 μl samples in a total reaction volume of 100 μl which also contained antithrombin-III (12.8 μg/ml, Enzyme Research Laboratory). A standard curve was generated using FPX at 0.01-1 μg/ml and FXa at 0.2 U/ml. Percent inhibition was determined as a function of FPX concentration in the standard curve and FPX concentration was correlated to the FXa activity in the serum samples.

Assessment of airway responsiveness

Airway hyperresponsiveness was measured at 24 hr after the last intranasal antigen-challenge as previously reported (E3). The anesthetized and tracheostomized mice were mechanically ventilated and lung resistance (RL) was assessed. The mice were placed inside a whole-body plethysmograph (Buxco Electronic, Troy, NY) and ventilated at a rate of 120 breathes/min with a 0.3 ml tidal volume. Resistance computations were derived from the tracheal pressure and airflow signals using an algorithm of covariance. Methacholine aerosolization (0.25 – 16 mg/ml)

was performed using a nebulizer in line with the respiratory tubing from the ventilator. Responses in total resistance were analyzed over a 3 min period after the last puff.

FPX treatment

In some OVA-challenged mice, from weeks 13 to 16, FPX (GlaxoSmithKline, Triangle Park, NC) was administered subcutaneously since eosinophilic inflammation, increases in airway wall thickness, collagen deposition, and airway hyperresponsiveness plateaued during this period as previously reported (E3). FPX was provided at a dose of 10 μg or 30 μg for 6 days/week to evaluate its effect on the development of airway remodeling. Fluticasone (TOCRIS, Ellisville, MO) was used as a positive control and it was administered intranasally at 10 μg/mouse for 6 days/week.

Collagen deposition, mucus production, and smooth muscle cell hyperplasia in lung tissue

Lungs were obtained 24 hr after the final OVA administration, fixed with periodate-lysine-paraformaldehyde, embedded in paraffin, and sectioned at a thickness of 4 μm. The resulting slides were stained with periodic acid-Schiff (PAS) or Masson's trichrome. Smooth muscle α-

actin (SMA) immunostains were performed utilizing a mouse-anti-human SMA (Sigma) as the primary antibody followed by a HRP-conjugated rabbit-anti-mouse IgG (Serotec, Raleigh, NC) as the secondary antibody. The substrate, 3-amino-9-ethylcarbazole (AEC; Vector Laboratories, Burlingame, CA) was used for detection. Histological analysis was performed using an E600 microscope (Nikon, Tokyo, Japan) attached to a Spot image-analysis system (Diagnostic Instruments Inc, Sterling Heights, MI) on stained lung sections. For each treatment group, 5-9 stained lung sections from each mouse were analyzed (E3).

Measurement of airway wall thickness

Airway wall thickness was determined utilizing Elastica van Gieson stained sections (E4). Cross-sections of airways were used for measuring the airway wall thickness only if the ratio of minimal internal diameter (I.D.) to maximal I.D. was less than 0.5. The following morphometric parameters were measured: the internal perimeter (Pi), the basement membrane perimeter (Pbm), the outer muscle perimeter (Pmo), and the areas enclosed by defined perimeters (Ai, Abm, and Amo). The following two parameters were calculated from the measured values: the mucosal-layer cross-sectional area (Wmuc=Abm-Ai) and the smooth muscle layer cross-sectional area

(Wsm=Amo-Abm). The wall area was normalized with respect to Pbm, to allow comparison among airways of different sizes.

Mucin production from NCI-H292 cells

NCI-H292 cells were seeded at a concentration of 3 x 10^5 cells/ml (0.2 ml) in 96-well plates for 48 hr in RPMI 1640 medium containing 10% FBS and antibiotics (100 U penicillin/ml, 100 μg streptomycin/ml, Cambrex Bioscience) and then cultured in FBS-free medium for 2 days. The cells were then stimulated with various concentrations of FXa, thrombin (ERL), EGF (PeproTech, Rocky Hill, NJ), or activation peptides for PAR1 and PAR2 (TOCRIS), for 48 hr and fixed to detect MUC5AC production. For FPX studies in FXa stimulated cells, 12.8 μg/ml of antithrombin-III was also added. After this, the formalin fixed cells were incubated overnight with mouse-anti-human MUC5AC (45M1, Lab Vision, Fremont, CA). The samples were then washed and incubated with biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA) and detection was achieved utilizing the ABC-AP kit and the substrate p-nitrophenylphosphate (Vector Laboratories). The absorbancy was then measured at 405 nm. Maximal production of MUC5AC (100%) was identified as the absorbance obtained from cells stimulated with 1 μg/ml

EGF.

Table E1. Airway wall thickness

Treatment	WA_{muc}/P_{bm}	WA_{sm}/P_{bm}
OVA (n = 4)	[1,3]24.79 ± 0.35	[2,4]9.73 ± 0.84
PBS (n = 3)	[1]9.81 ± 1.27	[2]4.33 ± 0.97
OVA + FPX (n = 4)	[2]20.17 ± 1.14	[4]7.12 ± 0.16

[1]P = 0.001, [2]P = 0.008, [3]P = 0.001, [4]P = 0.02. WA_{muc}/P_{bm} and WA_{sm}/P_{bm} = $\mu m^2/\mu m$

Table E2. mRNA levels of murine FX, PAR-1, and PAR-2 in lung

Treatment	FX	PAR-1	PAR-2
OVA (n = 6)	[1,2]137.26 ± 16.29	[3,4]5.46 ± 0.67	[5,6]6.36 ± 1.53
PBS (n = 6)	[1]1.03 ± 0.08	[3]1.24 ± 0.22	[5]1.25 ± 0.22
OVA + FPX (n = 5)	[2]59.62 ± 13.08	[4]3.47 ± 0.40	[6]1.62 ± 0.40

The values indicate the fold difference relative to RPL19 mRNA. Data represent the mean ± SEM.
[1]$P = 0.004$, [2]$P < 0.01$, [3]$P < 0.001$, [4]$P = 0.04$, [5]$P = 0.02$, [6]$P = 0.02$. OVA: ovalbumin, PBS: phosphate-buffered saline, FPX: fondaparinux, FX: Factor X, PAR: protease-activated receptor.

References

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