AGR2 is induced in asthma and promotes allergen-induced mucin overproduction

Bradley W. Schroeder, MD, PhD, Catherine Verhaeghe, PhD, Sung-Woo Park, MD, PhD, Louis T. Nguyenvu, MBA, Xiaozhu Huang, MD, Guohua Zhen, MD, PhD, and David J. Erle, MD

ONLINE DATA SUPPLEMENT

METHODS

Antibodies and antisera

Antibodies used were anti-human AGR2 (EPR3278, Epitomics); anti-mouse AGR2 (Ab22208, Abcam); anti-GRP78/GRP94 (AB12223, Abcam); anti-MUC5AC monoclonal antibodies 19M1, 21M1, 62M1, 166M1 (all generously provided by Dr. Jacques Bara, INSERM, France (1, 2)), CLH2 (Millipore), 1-13M1 (Thermo Scientific), and 45M1 (Santa Cruz Biotechnology); anti-MUC5B rabbit antiserum (kindly provided by Dr. C. William Davis, University of North Carolina) (3); anti-MUC5B (H300, Santa Cruz Biotechnology); anti-Giantin (AB24586, Abcam). Normal rabbit IgG (Santa Cruz Biotechnology) and normal mouse IgG (Invitrogen) were used as controls.

Immunofluorescence

5 μm sections of paraformaldehyde-fixed, paraffin-embedded lung were deparaffinized, hydrated, microwaved, incubated in 100 mM glycine, and washed in Tris-buffered saline (TBS) with 0.05% Tween-20. After blocking in 10% normal goat serum plus 2% bovine serum albumin (BSA), sections were incubated with primary antibodies diluted in 2% BSA/TBST, washed, incubated with secondary antibodies, washed, treated with Sudan black (4) and washed in TBS prior to mounting. 4',6-diamidino-2-phenylindole (DAPI, 1 μg/ml) was included in the secondary antibody solution where indicated. For experiments using 2 antibodies from the same species, slides were microwaved between incubations with the first secondary and second primary antibodies (5). All samples for a given experiment were processed in parallel using antibody master mixes.

Immunoprecipitation and immunoblotting

For analysis of human AGR2-MUC5AC complexes, A549 lung carcinoma and MG63 osteosarcoma cell lines (ATCC) were lysed in 25 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.5% Tween20 2× mini-Complete protease inhibitor (Roche), 1 mM PMSF (Sigma), and 25 mM N-ethyl maleimide. AGR2 was immunoprecipitated from lysates

with anti-human AGR2 (EPR3278, Epitomics) and Dynabead-protein G slurry (Invitrogen).

For analysis of mucin levels in mouse lung, proteins for mucin immunoblotting were isolated by homogenization of whole lung and proximal airways in 100mM Tris, 5mM EDTA, 6-M guanidine HCl, 2X mini-complete proteinase inhibitor (Roche), 1mM PMSF, and 25mM N-ethyl Maleimide. Lysates were dialyzed against 6-M Urea/Tris/EDTA overnight at 4C, then aliquotted, quantified by nanodrop and BCA, then stored at -20C or -80C. 100ug of lung protein were loaded in each well.

Proteins were separated using non-reducing 0.7% agarose/3% acrylamide gels containing 6 M urea, 50 mM Tris, 40 mM glycine, and 0.35% SDS for electrophoresis. Proteins were transferred to PVDF (Immobilon FL, Millipore), blocked in 5% milk/TBST or 3% BSA/TBST, and incubated in primary antibody followed by HRP-conjugated secondary antibodies or HRP-conjugated streptavidin (Invitrogen). After incubation in 1.25 mM luminol, 200 μM p-Coumaric acid, and 0.1% hydrogen peroxide in 100 mM Tris, pH 8.5, luminescence was detected using a FluorchemM imager (ProteinSimple) and densitometry performed using ImageJ (v1.43u, NIH).

Quantitative morphometry

For mucous cell measurements, 5 μm vertical sections of paraformaldehyde-fixed, paraffin-embedded *Agr2*$^{-/-}$ and *Agr2*$^{+/+}$ allergen-challenged mouse lung were deparaffinized and stained with periodic acid-Schiff (PAS) reagent and counterstained with hematoxylin, as previously described. Airway epithelium was delimited under low-power magnification and the Computer Assisted Stereology Toolbox (CAST) grid system (Olympus Denmark, Albertslund, Denmark) was used to randomly survey non-overlapping high-power fields (HPF). The numbers of mucous cells were counted and the volume of stored mucin was measured using the rotator method. A minimum of 25 high-power fields per slide were scored (n=5-6 mice per group). For AGR2

immunofluorescent cell counts, stained cells were identified in Z-stacks of large airway epithelium (trachea to second-generation bronchi) acquired at 100 × magnification (≥ 12 Z stacks/mouse, 3 mice/group). All cell counts were performed by a blinded observer.

Airway Epithelial Brushing

An airway brush made of polyethylene tubing scored with fine grit sandpaper was inserted into an incision between the first and second tracheal rings, and advanced 2 cm into the left and then the right mainstem bronchi. Brushes were immediately immersed in Buffer RLT and RNA was extracted using the RNAeasy Micro kit (Qiagen).

References

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