

Altered matrix production in the distal airways of asthmatic individuals

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Online data supplement

Subjects and sampling of tissue

Central fibroblasts were sampled from central airways (segmental or subsegmental carina in the right lower and upper lobes) and distal fibroblasts were sampled from parenchymal compartments (alveoli) (transbronchial biopsies \approx peripheral right lobe at a distance of \geq inch from pleura). Occasionally small airways were detected in the biopsies, but the predominant numbers of biopsies only tissue had alveoli.

Cell culture and morphological characterization

The bronchial and distal biopsies were cut and cultured in 25 cm² tissue culture flasks (Nunc, Roskilde, Denmark) with DMEM supplemented with 10% Fetal Clone III (Hyclone, Logan, UT, US), 1% L-glutamine, 0.5% gentamicin, and 5 μ g/ml amphotericin. The cell cultures were kept in a humidified 37°C cell incubator with an atmosphere of 5% CO₂ and were trypsinized when they reached confluence. Centrally-derived fibroblasts were cultured from centrally-derived biopsies taken from 15 of these 23 individuals: 8 asthma patients (8/11, 72%) and 7 controls (7/12, 58%). Distally-derived biopsies were taken from 19 of 23 individuals. Distally fibroblasts cultures were established from all these 19 individuals (100 % of total, Table 1).

For the morphological studies, the cells were used at passage number 3–5, fixed in 1% glutaraldehyde (BDH Chemicals Ltd, City, England) for 30 minutes and stained with 0.5% crystal violet (Merck) for 2 hours. On 250 cells/subject cellular extensions were counted. The areas of cells were randomly selected and done in a double- blinded manner to avoid bias when counting the cells. This was done using a computerized image analysis package (Leica Q500IW software; Leica, Wetzlar, Germany). To characterize the fibroblasts further, cells were seeded in

4-well chambers (7,000 cells/well) for 24 hours. Paraformaldehyde (4%) fixed cells were then washed in PBS (3 x 5 min) and subsequently blocked for 1 hour with 0.5% BSA in TBS. This was followed by incubation with primary antibodies to vimentin (1:100; Santa-Cruz Biotechnology, Santa-Cruz, CA), prolyl-4-hydroxylase (P-4-OH) (1:100; Acris antibodies, Hiddenhauser, Germany), or SM22 (1:100; Abcam, Cambridge, UK) for 1 hour. Secondary antibodies against the relevant isotypes of the primary were applied for 1 hr followed by buffer wash and nuclear staining (DAPI, molecular probes). Secondary antibodies were all purchased from Molecular Probes (Invitrogen, Carlsberg, CA). Control experiments were performed with and without primary antibody, and with and without secondary antibody. Control sections were included in all experiments to correct for background fluorescence. Isotype matched controls were used in combination and for each type of primary antibody to reduce the risk of detecting unspecific staining. All images were captured with an inverted microscope (Nikon TE200E) equipped with a high-resolution camera (Nikon DXM 1200C) and merged with NIS elements software (Nikon).

Trichrome staining in transbronchial biopsies

Periodate-paraformaldehyde-lysine (PLP)-fixed transbronchial biopsies from patients with asthma and controls were stained with trichrome staining for measurement of collagen expression. The biopsies collected did not show any differences in size, vascularity, or muscle content. From each individual at least 3 sections (each 5 μ m) separated by at least 40 μ m were selected. Total area analyzed from asthma sections was 4.2mm² (0.298 (0.063-0.7073)) vs. 3.089mm² (0.236 (0.031-0.860)) for controls tissue. Sections were photographed using a TE2000E fluorescence microscope (Nikon, Tokyo, Japan) equipped with a bright field DXM1200C

camera (Nikon, Tokyo, Japan). Pictures from each biopsy were analyzed with ImageJ software (NIH) where a plug in software application for spectral analysis was used to identify, separate, and measure blue collagen staining. The same program was also used to calculate the total tissue area. Collagen areas are presented as % of total tissue area. Middle-size vessels were excluded in the analysis in all biopsies.

Proliferation assay

Cells were plated on 96-well plates (5,000 cells/well) and cultured in 10% serum for 6, 24, and 48 hours. To remove non-adherent cells, medium was removed after 6 hours and fresh medium was added for the remaining 24 hours. Cells were then fixed in 1% glutaraldehyde for 30 min and stained thereafter for 30 min in 0.1% crystal violet. The excess crystal violet was then gently removed by washing with water. The cells were then lysed overnight with 1% Triton-X100 (Sigma-Aldrich) and absorbance was measured at 595 nm in a linearity way.[1] Normalization of the data is unique to every patient since data in every experiment are normalized to 6 hours incubation (6 hours = Time after harvested the cells and started the experiments). After 6 hours, the cells have attached to the surface but not yet started to proliferate. Every sample thus has its own normalization point. Cell viability has been tested with trypan blue and less than 0.3% of the cells were stained. The crystal violet method has also been validated and compared with other proliferation assays such as using a cell counter and the linearity has shown to be the same. For more information see reference.[2]

Proteoglycan measurement

Cells were cultured in ^{35}S -supplemented (50 $\mu\text{Ci/ml}$) sulfate-poor medium for 24 hours. Labeled ^{35}S -proteoglycans were separated on a DEAE-52 cellulose anion exchange resin column as previously described.[3] Unincorporated ^{35}S was efficiently removed by extensive washing of the DEAE-52 column with 0.05 and 0.5M acetate in 6M Urea, pH 5.8. The PGs were then finally eluted by using 4 M Guanidine, 0.01M NEM, 0.01M EDTA, 1mM of PMSF and 0.05M acetate, pH5.8. Unspecific binding and free ^{35}S were further checked by using gel chromatography on Sephacryl S-500 HR on this fraction in 4M GuHCl which showed only traces of ^{35}S in the total volume. Furthermore, unspecific binding of ^{35}S was further also controlled by using specific degradation with protease or lyases followed by gel chromatography where less than 0.1% of free sulphate was noted. Thus it can be eliminated that nonspecific binding of ^{35}S does not occur.

Total ^{35}S -labeled proteoglycan was quantified using a scintillation counter (Beckam LS-6500TM). Individual proteoglycans (versican, biglycan, perlecan, and decorin) were separated on a 3–12% polyacrylamide gel electrophoresis and analyzed using Fuji BAS 2000TM and adjusted for total cell protein (BCA protein assay kit; Pierce, Thermo Fisher Scientific, MA). For selection of proteoglycans size of the macromolecules and size of the corresponding protein core after specific degradation (with heparin and/or chondroitin ABC lyases) of the polysaccharide give reliable information concerning versican, biglycan decorin and perlecan. The position of this proteoglycans have earlier also been documented with western blot and mass spectrometer identification (MALDI-TOF-MS).[3-4] Other proteoglycans such as syndecans and glypicans can be neglected since there is only 2-3 % of those in the culture medium related to total amount of proteoglycans secreted from the fibroblasts.[5]

Reference List

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