Supplementary Digital Content Data 1

Lung cancer-associated myofibroblasts reveal distinctive ultrastructure and function

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Methods

Study subjects

5 of the patients were life-long non-smokers. 18 patients were smokers of whom 15 were current smokers and 3 ex-smokers. Those ex-smokers had smoked 15-30 pack-years; two of them had quit smoking 20 years ago and one 4 months before the lung cancer operation. The lung cancer staging categories of the patients included stages I (n=9), II (n=8), and III (n=4).

Supplementary digital content Figure 1 displays the flow chart of different methods (dash lined), which were employed in the investigations of lung cancer-associated fibroblasts (CAF) and lung fibroblastic cells by Western blotting and collagen gel contraction, and lung cancer associated myofibroblasts (CAM) and myofibroblasts from tumor-free peripheral and central lung as individual cells by TEM and IEM.

Cell culture

The tissue samples from lung resections were prepared and cells were cultured as described previously. Briefly, pieces of tissues were washed with ice-cold PBS, cut into approximately 1 mm³ pieces and incubated with collagenase I (500 U/ml) - DNAase I (20 U/ml) at +37º C / 5 % CO₂ for 3 h. The pieces were placed in culture medium (Minimum essential medium Eagle, α-modification ( Sigma, Steinheim, Germany) containing 13 % fetal bovine serum (FBS-500, PromoCell, Heidelberg, Germany), 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES, Sigma), 100 U/ml penicillin (Sigma), 100 µg/ml streptomycin...
(10 mg/ml, Sigma), 2.5 µg/ml amphotericin B (Sigma) and 2 mM L-glutamine (Sigma)) and allowed to attach for one day. The remaining tissue pieces were washed away. Cells were allowed to grow to 80 % of confluence, passaged (5 mg/ml trypsin - 2 mg/ml EDTA (Sigma)) and plated at a density of 2 500 cells per cm². Cells in passages 2, 3 or 4 were used in the experiments.

TEM

The specimens were prepared as previously described¹. The samples of every patient were analysed. Cells were fixed in 1 % glutaraldehyde and 4 % PFA mixture in 0.01 M phosphate buffer (PBS) for 10 min. The cells were scraped off, pelleted and fixation was continued for 1 h. The pellet was immersed in 2 % Agarose (in dH₂O) and postfixed in 1 % osmium tetroxide in dH₂O for 30 min. The specimens were dehydrated in acetone and immersed in Epon LX112 (Ladd Research Industries, Vermont, USA)-acetone. Thin sections (80 nm) were cut with Leica Ultracut UCT ultramicrotome (Leica Microsystems, Vienna, Austria) and stained with uranyl acetate and lead citrate and examined in a Philips CM 100 transmission electron microscope (FEI company, Eindhoven, The Netherlands) equipped with a Morada CCD camera (Olympus Soft Imaging System Solutions GMBH, Münster, Germany).

Quantitation of TEM

The quantitation of TEM features was done as previously described¹. The TEM findings of each sample were semi-quantified systematically as follows: amount of actin filaments as low (+), moderate (++ or strong (+++); frequency of extracellular component of FNX as low (+), moderate (++) or strong (+++); amount of extracellular matrix (ECM) surrounding cells as
low (+), moderate (++) or strong (+++); type of FNX as tandem, plaque or track-like; subtypes of track-like FNX as 1) straight and rigid, 2) curved and 3) fragmentary and scanty (Figure 2), and focal densities, dilated rough endoplasmic reticulum (rER), adherens-type junctions as numbers of positive cases (in parentheses, percentage value). TEM findings of each sample were analyzed from a 500 µm² area blindly without any knowledge of the diagnosis. The frequency of the extracellular component of FNX was estimated as follows: negative, no FNX was found; low, FNX was found in 10 % of the cells; moderate, FNX was found in 30 % of the cells; strong, FNX was found in 60 % of the cells.

Antibodies

Details on antibodies used in the study are listed in supplementary digital content Table 1.

IEM

The specimen preparation has been previously described1,2. Cells were fixed in 4 % PFA in 0.1 M PBS with 2.5 % sucrose. After pelleting, the cells were immersed in 12 % gelatin in PBS and kept on ice for 30 min and then immersed in 2.3 M sucrose in PBS for 24 h. The ultrathin sections were cut with a Leica EM UC6 cryoultramicrotome. In the immunolabelings, ultrathin sections were incubated in 0.1 % glycine + PBS followed by incubation in 1% bovine serum albumin (BSA) in PBS. All washes and antibody dilutions were performed in PBS + 1 % BSA. With the monoclonal antibody, the secondary antibody rabbit anti-mouse IgG was applied (supplementary digital content Table 1). The samples were incubated with Protein A-gold conjugates (10 nm for single labeling and for double labeling 10 nm and 5 nm particles were applied)3. Sections were embedded in methylcellulose and studied as described above.
Quantitation of the expression of α-SMA by IEM

Four rectangles which had areas of 0.26 µm² were diagonally marked from the upper left corner to the lower right corner of the image and the numbers of gold particles were counted in each rectangle as described previously. The total area of quantitation was 10.4 µm² in each patient sample. Values of CAM were compared with those of myofibroblasts cultured from tumor-free lung tissue. The results were reported as relative protein expression in IEM.

Western blotting

The blotting was done as previously described. Cells were lysed in lysis buffer (50 mM Tris pH 7.6, 0.1 % Triton X-100, 0.9 % NaCl, 0.2 % sodium azide (Sigma), 0.1 % deoxycholic acid (DOC, Sigma), 1x Protease Inhibitor Cocktail Tablet (Roche, Mannheim, Germany)) and centrifuged (10 000 rpm, 10 min, +4 °C). The protein concentration of samples was determined by the microplate assay (DC Protein Assay Kit, Bio Rad, UK) according to the manufacturer’s instructions. 20-µg aliquots in sample buffer (0.1 M Tris-HCl pH 6.8, 0.2 M dithiothreitol, 25 % glycerol, 1mg/ml bromophenol blue, 4 % SDS) were loaded on 12 % SDS-PAGE with running buffer (25 mM Tris, 190 mM glycine, 0.1 % SDS) at 200 V. After the electrophoresis, the proteins were transferred to nitrocellulose membrane (pore size 0.45 µm, Protran® Nitrocellulose Transfer Membrane, Schleicher & Schuell, BioScience, Dassel, Germany) with blotting buffer (30 mM Tris, 0.24 M glycine, 20 % ethanol) overnight and blocked in 5 % milk powder-PBST (PBS-0.1 % Tween 20) for 1 h. After washing with PBST, the membrane was incubated with the primary antibody against human α-SMA and reference protein GAPDH followed by the secondary antibody (supplementary digital content Table 1). The intensity of the protein band was analysed with an Odyssey infrared imager (LI-COR...
Biosciences). Equal loading and transfer were confirmed by staining some membranes with 0.1 % Ponceau S in 5 % acetic acid or by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) detection. The data were reported as relative protein expression such that intensities detected for each sample were normalized against the intensity of the control sample on the same membrane. The same control sample was used on every gel/membrane to minimize the variation between different membranes. Cells derived from a patient with normal peripheral lung were used as a control sample.

Collagen gel contraction assay

The mixture of collagen (extracted from rat tail, 0.75 mg/ml) gel and cell suspension (300 000 cells per ml) in serum-free medium was cast into the 24-well plate and allowed to set for 15 min in the cell culture incubator. Gels were detached from the walls of wells with a spatula and 1 ml of serum-free medium was added into each well. The areas of the gels were measured every day (days 0-3) by MCID™ Core Image Analysis System software. The results are represented as percentages compared to original size of collagen gel as previously described.

Immunocytochemistry

For the phenotyping of cultured cells, previously characterized immunocytochemical stainings for smooth muscle cells, fibroblasts and myofibroblasts were visualized by light microscopy. Cells were stained for α-SMA (myofibroblast), EDA-fibronectin (EDA-Fn) (myofibroblast, fibroblast), vimentin (fibroblast, myofibroblast), desmin (smooth muscle cell, myofibroblast) and E-cadherin (epithelial cell). The cells were cultured on glass slides at
passages 2, 3 or 4, fixed with 3 % paraformaldehyde and stained with Dako REAL™ EnVision™ Detection System, Peroxidase/DAB+, Rabbit/Mouse – Kit (Dako, Glostrup, Denmark) according to the manufacturer’s instructions. The primary antibodies used are listed in the supplementary digital content Table 1 and they were incubated for 1h at room temperature.

References


