

ONLINE SUPPLEMENT

Acidinium inhibits human lung fibroblast to myofibroblast transition

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MATERIAL AND METHODS

Isolation and cultivation of human fibroblasts

Human lung tissue was obtained from patients who were undergoing surgery for lung carcinoma and who gave informed consent. Bronchial healthy areas of surgically resected lung tissue were used to obtain human bronchial fibroblasts.

Data presented throughout the study was from human bronchial fibroblasts obtained from smoker patients. For comparison, bronchial fibroblasts were also isolated from COPD patients and only used to explore the effect of acclidinium on carbachol or TGF- β 1-induced myofibroblast transition. Clinical data of patients is showed in supplementary table 1.

The protocol for obtaining human tissue was approved by the local ethical review board for human studies (General Hospital of Valencia, Spain). Bronchial tissue was: cut into small pieces; treated with pronase (1 mg/mL; Calbiochem[®], Novabiochem[®], San Diego, CA, USA) at 37°C for 30 min; placed in cell culture plates and incubated in Dulbecco's Modified Eagle's Medium (DMEM); and supplemented with 10% foetal calf serum (Sigma, St. Louis, MO, USA), 100 U/mL penicillin/streptomycin and 2% fungizone (GIBCO, Grand Island, NY, USA). After approximately 2 weeks, fibroblasts had grown from the tissue and were passaged by standard trypsinisation. Cells from passages 3–10 were used in all experiments described in the present study.

Supplementary table 1. Clinical features. FEV1: forced expiratory volume in one second; FVC: forced vital capacity; TLC: total lung capacity; PaO₂: oxygen tension in arterial blood; PaCO₂: carbon dioxide tension in arterial blood; Pack-yr = 1 year smoking 20 cigarettes-day.

| | Smokers (n=8) | COPD (n= 3) |
|---------|------------------|----------------|
| Age, yr | 68±7 | 65±6 |

| | | |
|------------------------------|------|------|
| Tobacco consumption, pack-yr | 20±3 | 40±8 |
| FEV1, % pred | 94±6 | 69±6 |
| FVC, % pred | 93±8 | 88±7 |
| FEV1/FVC % | 89±7 | 72±5 |
| TLC %pred | 87±4 | 96±5 |
| PaO ₂ , mmHg | 92±7 | 87±7 |
| PaCO ₂ mmHg | 36±3 | 38±4 |

Stimulation of human fibroblast

Carbachol was selected as a cholinergic agonist as it is widely used in the literature and is resistant to degradation by cholinesterases present in human lung fibroblasts.^{1,2} In this study, we used carbachol 10⁻⁵M concentration as we observed that it produced near maximal response, in agreement with other studies of human lung fibroblast cell culture models using cholinomimetics.^{3,4}

Real time RT-PCR

Total RNA was isolated from cultured human bronchial fibroblasts by using TriPure[®] Isolation Reagent (Roche, Indianapolis, USA). Integrity of the extracted RNA was confirmed with Bioanalyzer (Agilent, Palo Alto, CA, USA). The reverse transcription was performed in 300 ng of total RNA with the TaqMan reverse transcription reagents kit (Applied Biosystems, Perkin-Elmer Corporation, CA, USA). cDNA was amplified using assays-on-demand specific primers pre-designed by Applied Biosystems for muscarinic acetylcholine receptors (mAChR) M1, M2 and M3, α_1 (I)-collagen (col type I), α -smooth muscle actin (α -SMA) and TGF- β 1 (catalogue nos. Hs00912795_m1, Hs00265208_s1, Hs00327458_m1, Hs00164004_m1, Hs00559403_m1 and Hs00171257_m1) in a 7900HT Fast Real-Time PCR System (Applied Biosystems)

using Universal Master Mix (Applied Biosystems). Relative quantification of these different transcripts was determined with the $2^{-\Delta\Delta C_t}$ method using glyceraldehyde phosphate dehydrogenase (GAPDH) as endogenous control (Applied Biosystems; 4352339E) and normalised to control group.

Transfection of siRNAs

Small interfering RNA (siRNA), including the scrambled siRNA control, were purchased from Ambion (Huntingdon, Cambridge, UK). M1, M2 and M3 muscarinic receptor gene-targeted siRNAs (identification nos. s3024, s3026 and s230642, respectively) were designed by Ambion. The human bronchial fibroblasts were transfected with siRNA (50 nM) in serum and antibiotic-free medium. After a period of 6 h, the medium was aspirated and replaced with medium containing serum for a further 42 h before carbachol stimulation. The transfection reagent used was lipofectamine-2000 (Invitrogen, Paisley, UK) at a final concentration of 2 μ l/mL. The mRNA expression for M1, M2 and M3 transcripts was determined by real-time RT-PCR (as described above) after 48 h post-silencing and compared with siRNA control at the respective time to determine silencing efficiency. Furthermore, M1, M2 and M3 protein expression was measured by western blot after 48 h of silencing, as described in the western blotting section.

Western blotting

Western blot analysis was used to detect changes in col type I (138 kD), α -SMA, TGF- β 1 (40–60 kD), p-ERK1/2 (42–44 kD), RhoA-GTP (22 kD), M1 (52 kD), M2 (70 kD), M3 (75 kD) and ChAT (65 kD). Cells were scraped from a confluent 25-cm² flask and lysed on ice with a lysis buffer consisting of a complete inhibitor cocktail plus 1 mM

ethylenediaminetetraacetic acid (Roche Diagnostics Ltd, West Sussex, UK) with 20 mM Tris base, 0.9% NaCl, 0.1% Triton X-100, 1 mM dithiothreitol and $1 \mu\text{g mL}^{-1}$ pepstatin A. The Bio-Rad assay (Bio-Rad Laboratories Ltd., Herts, UK) was used (following manufacturer's instructions) to quantify the level of protein in each sample to ensure equal protein loading. Sodium dodecyl sulphate polyacrylamide gel electrophoresis was used to separate the proteins according to their molecular weight. Briefly, 20 μg proteins (denatured) along with a molecular weight protein marker, Bio-Rad Kaleidoscope marker (Bio-Rad Laboratories), were loaded onto an acrylamide gel consisting of a 5% acrylamide stacking gel stacked on top of a 10% acrylamide resolving gel and run through the gel by application of 100 V for 1 h. Proteins were transferred from the gel to a polyvinylidene difluoride membrane using a wet blotting method. The membrane was blocked with 5% Marvel in PBS containing 0.1% Tween20 (PBS-T) and then probed with a rabbit anti-human col type I (1:1,000) antibody (polyclonal antibody; Affinity Bioreagents, Golden, USA; catalogue no. PA1-26204), mouse anti-human anti- α -SMA (1:1,000) antibody (monoclonal antibody; Sigma; catalogue no. A5228), goat anti-human TGF- β 1 (1:1,000) antibody (monoclonal antibody; R&D Systems; catalogue no. AB-246-NA), rabbit anti-human M1, M2 and M3 (1:1,000) antibodies (polyclonal antibodies; Santa Cruz Biotechnology, Santa Cruz, CA, USA; catalogue nos. sc-9106, sc-9107 and sc-9108, respectively) and rabbit anti-human ChAT (1:1,000) antibody (monoclonal antibody; Millipore Bioscience Research Reagents, Temecula, CA, USA; catalogue no. AB143) which were normalised to mouse anti-human β -actin (1:10,000) antibody (monoclonal antibody; Sigma; catalogue no. A1978). p-ERK1/2 expression was determined with the rabbit anti-human p-ERK1/2 (1:1,000) antibody (monoclonal antibody; Cell Signalling, Boston, Massachusetts, USA; catalogue no. 4376S) and was normalised to total rabbit anti-human ERK1/2

(1:1,000) antibody (monoclonal antibody; Cell Signalling, Boston, Massachusetts, USA; catalogue no. 4695). The expression of RhoA-GTP was determined with the RhoA IP/WB activation assay kit (NewEast Bioscience, Malvern, PA, USA; catalogue no. 80601) according to the manufacturer's instructions. The enhanced chemiluminescence method of protein detection using enhanced chemiluminescence reagents, ECL plus (Amersham GE Healthcare, Buckinghamshire, UK), was used to detect labelled proteins. Densitometry of films was performed using the Image J 1.42q software (available at <http://rsb.info.nih.gov/ij/>, USA). Results were expressed as ratios of the endogenous controls β -actin or total RhoA as appropriate, and normalised to control group.

Immunofluorescence

Fibroblasts were seeded into 12-well plates, each containing a glass coverslip, and cultured for 24 h in supplemented DMEM. Then they were serum-deprived for 24 h. Quiescent fibroblasts were stimulated with the indicated substances for 48 h. Cells were washed with ice-cold PBS and fixed in 4% paraformaldehyde for 30 min at room temperature, and immunostained as previously outlined.⁶ Briefly, cells were permeabilised (20 mM HEPES pH 7.6, 300 mM sucrose, 50 mM NaCl, 3 mM MgCl₂, 0.5% Triton X-100), blocked (10% goat serum in PBS), and incubated with the primary antibody (mouse anti-human anti- α -SMA [1:200] antibody) overnight at 4°C followed by secondary antibody anti-mouse-FITC (1:100; Molecular Probes, Leiden, The Netherlands). Cells were then washed 3xPBS and fixed with a Mowiol mounting medium. Staining was examined by epifluorescence microscopy (\times 400 and \times 1000; Nikon eclipse TE200 inverted microscope, Tokyo, Japan), and positive cells were

counted in a total of 6 fields per condition and were referred to the percentage of control.

Enzyme-linked immunosorbent assays

Quantitative ELISAs for TGF- β 1 and acetylcholine (ACh) were done with supernatants of subconfluent human bronchial fibroblasts on a 6-well plate following 48 h of stimulation with quantikine human TGF- β 1 immunoassay (R&D Systems; catalogue no. 891124) and ACh assay kit (Abcam, UK; catalogue no. ab65345), respectively. To measure latent complexes of TGF- β 1, activation was accomplished by acid treatment. Therefore, 0.5 mL of cell culture supernatants were treated with 0.1 mL of 1 mol/L HCl, incubated for 10 min, and then neutralised with 0.1 mL of 1.2 mol/L NaOH/0.5 mol/L HEPES. The cell content of cAMP was measured as previously described.⁷ Cells were placed in DMEM with 1% FCS for 24 h before measurements to arrest growth. The experimental protocol consisted of incubation of cells with acridinium for 30 min followed by addition of carbachol (10^{-5} M) for 10 min and isoprenaline (10^{-6} M) for another 10 min. These concentrations and times of incubation were selected from the literature.⁸ Total cAMP content was determined using a commercially available biotrack enzyme immunoassay kit (ref RPN2251; Amersham, Bucks, UK). Absorbance was read at 450 nm. The lower limit of sensitivity of the enzyme immunoassay was 12.5 fmols cAMP well⁻¹ and results were expressed as fmol well⁻¹.

Cell proliferation assay

Human bronchial fibroblast proliferation was measured as previously outlined⁹ by colorimetric immunoassay based on BrdU incorporation during DNA synthesis using a cell proliferation enzyme-linked immunosorbent assay BrdU kit (Roche, Mannheim,

Germany; catalogue no. 11647229001) according to the manufacturer's protocol. Cells were seeded at a density of 3×10^3 cells/well on 96-well plates and incubated for 24 h. Cells were then exposed to different experimental conditions. The 490 nm absorbance was quantified using a microplate spectrophotometer (Victor 1420 Multilabel Counter, PerkinElmer). Proliferation data refer to the absorbance values of BrdU-labeled cellular DNA content per well. Stimulation is expressed as x-fold proliferation over basal growth of the untreated control set as unity.

Wound closure assays

Human bronchial fibroblast closure studies were carried out to measure the migration capacity of fibroblasts as previously outlined.¹⁰ Prior to plating the cells, the large end of a sterile p-200 pipette tip was placed in the central area of a 6-well culture plate to prevent access of cells. Following this, 1.0 ml of supplemented DMEM containing 1×10^6 cells/ml was carefully placed in the well. Cells grew around the pipette tip until 100% of confluence (~3 days). After 48 h of carbachol or vehicle (control) exposure in the presence or absence of acridinium (10^{-9} - 10^{-7} M), Y27632 (10 μ M), dbcAMP (1 mM) or PD98059 (10 μ M), circular wound-edge was created in the center of well by removing the pipette tip. At this stage, cells were washed twice with culture media to eliminate floating and dead cells and wound closure was monitored immediately after creation of circular wound-edge using a 5x phase contrast objective lens and was digitally captured at regular time intervals after wounding until fully repaired. Wound areas were analysed using Image J 1.42q software (available at <http://rsb.info.nih.gov/ij/>, USA); the extent of repair was calculated and expressed as a percentage of the original wound area.

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