ONLINE SUPPLEMENT

TITLE

Sphingosine-1-phosphate is increased in patients with idiopathic pulmonary fibrosis and mediates epithelial to mesenchymal transition.

AUTHORS

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MATERIAL AND METHODS

Cell culture and stimulation

Alveolar macrophages were isolated from BAL fluid by means of adherence properties on a culture cell plate. Macrophage cell purity was 99.2 ± 4.9% as assessed by May-Grunwald staining of cytospin preparations.

Human alveolar type II cells (ATII) were isolated from human lung tissue as described previously, with modifications.[1] The protocol for purification was as described previously with modifications.[2] Briefly, to isolate alveolar type II cells, lung

parenchyma tissue was cut in approximately 1 mm thick sections and lavaged with saline. The lung sections were digested with 0.25% trypsin (T8003; Sigma, St. Louis, MO) dissolved in saline (100 ml) and suspended in 0.9% NaCl at 37ºC for 30 minutes. After digestion, the lung sections were treated with DNase dissolved in saline (7,500 U/100 ml), and filtered through nylon meshes ranging in pore size from 150 to 30 mm. The resulting cell suspension was centrifuged (250 x g, 20 min at 10ºC) through a sterile Percoll gradient and the alveolar type II cell–rich band was removed. A second DNase treatment (2,000 U/100 ml) was administered and the cells were recovered as a pellet by centrifugation at 250 x g for 20 minutes. These cells were resuspended in 5 ml of DCCM-1 (Biological Industries, Kibbutz Beit Haemek, Israel) supplemented with a 2% (wt/vol) L-glutamine and subjected to differential attachment on a plastic Petri dish. No adherent alveolar type II cells were collected after 2 hours and cells were counted to establish the final yield of freshly purified cells. Alveolar type II cell viability was assessed with trypan blue (Sigma), showing greater than 95% viability. Cell purity was routinely assessed by epithelial cell morphology and immunofluorescence analysis with pan-cytokeratin and pro-surfactant protein C (both positive) as well as α-SMA and CD45 (both negative) of cytocentrifuge preparations of ATII cells. ATII cells used throughout this study demonstrated 95% ± 3% purity. Finally, ATII cells were suspended in DMEM plus 10% FCS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 g/ml streptomycin and cultured for 24 hours to allow attachment. Phenotypic characterization was done after this time period. After media change, cells were cultured for a maximum of 3 days in a humidified atmosphere of 5% CO_2 at 37°C.

The A549 cells were purchased from American Type Culture Collection (Rockville, MD, USA) and were cultured in supplemented Roswell Park Memorial Institute (RPMI) 1640 medium at 37ºC in a humidified atmosphere of 5% CO_2 in air, as outlined.[3] Cells

at 60–70% confluence were serum-deprived by incubation for 12–18 h in RPMI 1640 medium containing 0.1% (v/v) foetal bovine serum prior to stimulation with S1P or other agents. The human type II alveolar cell line A549 has been broadly used as a model of EMT because it retains features and metabolic properties characteristic of type II cells.[4-6] Thus, A549 cells were considered to be an appropriate model in which to study the effect of S1P on EMT. Different drug modulators were added 30 min before S1P. Stimulators and pharmacological modulators were replaced each 24 h. S1P (10^{-8}–10^{-5} M), TGF-β1 (5 ng/ml), Rho kinase inhibitor Y27632 (10 μM), Smad3 inhibitor SIS3 (10 μM), and the antioxidant N-acetyl-□-cysteine (1 mM; NAC) were purchased from Sigma Chemical Co. (UK), and the SPHK1 inhibitor N,N-dimethylsphingosine (5 μM, DMS) was purchased from Cayman Chemical (Ann Arbor, MI, USA). W146 (1 μM; Avanti Polar Lipids, Inc., USA), JTE013 (1 μM; Cayman Chemical, USA), and CAY10444 (10 μM; Cayman Chemical) were used to selectively inhibit $S1P_1$, $S1P_2$, and $S1P_3$, respectively, as previously outlined.[7-9] Monoclonal anti-human TGF-β1 antibody (4 μg/mL; anti-TGF-β1; catalogue no. AB-246-NA; R&D Systems, Madrid, Spain) was added 30 min before a stimulus to block the active form of TGF-β1 in the culture supernatant, as previously described.[10]

Detection of RhoA-GTP, S1P, total soluble collagen, and supernatant TGF-β1

A commercially available enzyme-linked immunosorbent assay (ELISA)-based RhoA-GTP activity assay (G-LISA; Cytoskeleton, Denver, CO, USA) was used to measure the relative RhoA-GTP activity of serum-starved A549 cells after experimental treatments, as previously outlined.[11] S1P levels in human serum and BAL fluid were analysed using an S1P competitive ELISA kit (Echelon Biosciences Inc., Salt Lake City, UT, USA) according to the manufacturer's instructions. Total soluble collagen was measured in

A549 culture supernatants by the Sircol assay (Biocolor, Belfast, Ireland), according to the manufacturer's instructions. Quantitative ELISAs for TGF-β1 were performed with supernatants of subconfluent A549 cells on a six-well plate following 72 h of S1P (1 μM) stimulation, using a Quantikine Human TGF-β1 Immunoassay (catalogue no. 891124; R&D Systems) according to the manufacturer's instructions.

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 Bioanalizer (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 SPHK1 (cat. nº: Hs00184211_m1), α-SMA (cat. nº: Hs00909449_m1) vimentin (cat. nº: Hs00185584_m1) col type I (cat. nº: Hs01028970_m1), E-cadherin (cat. nº: Hs01023894_m1), ZO-1 (cat. nº: Hs01551876_m1), TGF-β1 (cat. nº: Hs00171257_m1) and GAPDH (cat. nº: 4352339E) as a housekeeping. Relative quantification of these different transcripts was determined with the $2^{-\Delta\Delta Ct}$ method using glyceraldehyde phosphate dehydrogenase (GAPDH) as endogenous control (Applied Biosystems; 4352339E) and normalised to control group.

Protein array

Lung tissues were first homogenized with ultraturrax T-25 (ICT SL, Spain), lysated with CeLyA Lysis Buffer CLB1 (Zeptosens), incubated during 30 minutes at room

temperature and centrifuged (5 min at 15,000xg) in order to remove debris. The supernatants were collected, frozen in liquid nitrogen and stored at -80ºC.

Protein concentration was determined using a Bradford-Coomassie Plus Assay Kit (Pierce). Protein content was adjusted to 2 mg/mL and samples were subsequently diluted using 90% spotting buffer (PBS + 10% DMSO + 5% Glicerol) and 10% CLB1 (Zeptosen) to obtain four different protein concentrations corresponding to 100, 75, 50 and 25% (0.2 mg/mL, 0.15 mg/mL, 0.1 mg/mL and 0.05 mg/mL) of the primary spotting solution. The Nano-Plotter (GeSiM) impregnated the chips (Zeptosens) with drops (400 pL) of each dilution and these chips were blocked by nebulization with Blocking Buffer BB1 (Zeptosens) using the ZeptoFOG Blocking Station (Zeptosens).

For each of these four dilutions, duplicate spots were arrayed onto ZeptoMARK chips (Zeptosens) as single sample droplets of about 400 pL, using a Micro Pipetting System Nano-plotter^{TM} (NP2.1, GeSiM, Großerkmannsdorf, Germany).

After spotting, the chips were dried for 1 h at 37ºC and blocked in an ultrasonic nebulizer (ZeptoFOG, Zeptosens) with CeLyA Blocking Buffer (BB1, Zeptosens). Blocked chips were rinsed with water (Milli-Q quality), dried and stored at 4ºC in the dark until further use. Antibody incubations were done in CeLyA Assay Buffer CAB1 based on BSA according to standard protocols (Zeptosens). The chips were assembled with chip fluidic structures in a ChipCARRIER (Zeptosens) and were incubated with primary antibodies (1:500 dilution in CAB1) overnight at room temperature. After rinsing the system with assay buffer, the chips were incubated with secondary fluorescence-labeled anti-species antibodies (Zenon Alexa Fluor 647, Molecular Probes) (1:500 dilution in CAB1) for 1h at room temperature. After rinsing the system with assay buffer to remove the excess secondary antibody, the fluorescence readout was performed with the ZeptoREADER instrument (Zeptosens).

at an extinction wavelength of 635 nm and an emission wavelength of 670 nm. The fluorescence signal was integrated over a period of 1–10 s, depending on the signal intensity. Array images were stored as 16-bit TIFF files and analyzed with the ZeptoView Pro software package (version 2.0, Zeptosens). Relative intensities were obtained by plotting net spot intensities against protein concentrations of the spotted samples determined by a Bradford assay as described above. Briefly, the eight datapoints for each sample were fitted using a weighted linear least squares fit.[12] The relative intensity was then interpolated at the median protein concentration. The SD calculated from the fit is indicative for the linearity of the dilution series. Subsequently the data were renormalized to correct for small variations in protein content using β-actin as internal standard. Primary antibodies used were rabbit anti-human SPHK1 antibody (cat. nº: HPA022829, Sigma), mouse anti-human α-SMA (cat. nº: A5228, Sigma), rabbit anti-human Col Type I antibody (cat. nº: PA1-26204, Affinity Bioreagents, Golden, USA;), mouse anti-human vimentin (cat. nº: V6389, Sigma).

Western blot

Western blot analysis was used to detect SPHK1, TGF-β1, α-SMA, vimentin, E-cadherin and p-Smad3 proteins in A549 cells. Cells were scraped from 25 cm^2 plates and lysed on ice with a lysis buffer consisting of 20mM Tris, 1 mM ethylenediaminetetraacectic acid (EDTA), 0.9% NaCl, 0.1% Triton X-100, 1 mM dithiothreitol and 1 μg ml^{-1} pepstatin A supplemented by a complete protease inhibitor cocktail. The Bio-Rad assay (Bio-Rad Laboratories Ltd., Herts, UK) was used 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 of protein (denatured) mixed with

2x loading buffer (comprising 160mM Tris HCl (pH 6.8), 4% SDS, 20% glycerol, 1.4mM ß-mercaptoethanol, 0.04% bromophenol blue) along with a molecular weight protein marker, Bio-Rad Kaleidoscope marker (Bio-Rad Laboratories), was loaded onto an acrylamide gel consisting of a 5% acrylamide stacking gel on top of a 12% acrylamide resolving gel and run through the gel by application of 100 V for 1 hour. Proteins were transferred to a polyvinylidene difluoride (PVDF) 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 rabbit anti-human SPHK1 antibody (cat. nº: HPA022829, Sigma), goat anti-human TGF-β1 (1:1,000) antibody (monoclonal antibody; R&D Systems; catalogue no. AB-246-NA), human anti-mouse α-SMA (1:1000: cat. nº: A5228, Sigma), human anti-mouse E-cadherin (1:1000: cat. nº: CM1681, ECM BioSciences), human anti-mouse vimentin (1:1000: cat. nº: V6389, Sigma), p-Smad3 (1:1000: cat. nº: PS1023, Calbiochem) and a rabbit anti-human α-actin antibody (1:1000; Sigma, UK) or total Smad3 (1:1000: cat nº. 566414, Calbiochem) as house-keeping reference, followed by the corresponding peroxidase-conjugated secondary (1:10,000) antibody. The enhanced chemiluminescence method of protein detection using ECL-plus (GE Healthcare, Amersham Biosciences, UK) was used to detect labelled proteins. Quantification of protein expression was performed by densitometry relative to β-actin expression or total Smad3 expression using the software GeneSnap version 6.08.

Immunohistochemistry

For SPHK1 immunohistochemical analysis of human pulmonary tissue, specimens were fixed, embedded in paraffin, cut into sections (4–6 μm), and stained with haematoxylin, as reported previously.[13] The sections were incubated with rabbit anti-

human SPHK1 antibody (1:100; Sigma) for 24 h at 4°C. Anti-rabbit secondary antibody (1:100; Vector Laboratories, Burlingame, CA, USA) conjugated with avidin-biotin complex/horseradish peroxidase was used for detection. Non-immune IgG isotype was used as a negative control.

DCFDA fluorescence measurement of reactive oxygen species.

A549 cells were treated with different S1P receptor antagonists at the same time that DCFDA. At the end of the incubation period (30 min) cells were stimulated with S1P or with the positive control H_2O_2 and intracellular fluorescence derived from DFC formation was monitored each 5 min. Results were expressed as DFC fluorescence in relative fluorescence units (RFU).

Statistics

All of the data analysis from human samples and clinical data were performed by non-parametric tests and described as median and interquartile range [IQR]. When the comparisons concerned only 2 groups, between-group differences were analyzed by the Mann Whitney test. Correlations between S1P levels and SPHK1 expression with the clinical features as well as correlations of α–SMA, vimentin and col type I with SPHK1 lung tissue expression were analyzed using the Spearman correlation analysis. $p < 0.05$ was considered statistically significant.

For in vitro cell experiments performed in A549 cell line, results were expressed as mean (SE) of n experiments since Gaussian distribution for each data set was confirmed by histogram analyses and Kolmogorov–Smirnov test. In this case, statistical analysis

was carried out by parametric analysis of variance followed by appropriate post hoc tests including Bonferroni correction. Significance was accepted as $p < 0.05$.

Discussion

Study limitations:

In the present study, abundant S1P levels were found in the BAL fluid from patients with IPF, and high SPHK1 expression levels were identified in the lung tissues and alveolar macrophages. However, some important differences between our control and IPF groups need to be discussed. The proportion of smokers in the control group was higher than that in the IPF group. Although there is currently no evidence of an effect of cigarette smoke on S1P levels or SPHK expression, this represents a limitation of our work and warrants future research. Nevertheless, when smoker controls and smoker IPF patients were excluded in a post-hoc analysis, S1P serum and BAL levels as well as SPHK1 alveolar macrophage expression remained statistically higher in IPF patients discounting cigarette smoking as a confounding factor. Furthermore, we measured SPHK1 expression in alveolar macrophages because these are the main leukocytes recovered by BAL. In this sense, the high number of neutrophils in the BAL fluid of patients with IPF may be thought to have contributed to the elevated S1P levels observed in the BAL fluid. However, neutrophils have low amounts of S1P, negating this explanation.[14] Another possible confusing factor is related to the higher proportion of patients with IPF treated with steroids. Currently, there is no evidence of an effect of steroids on serum and BAL levels of S1P. However, lower levels of S1P are expected in steroid-treated patients because S1P is considered to be a pro-inflammatory mediator in asthma,[15] and steroids are immunosuppressors. In the present study, a post-hoc analysis

did not detect a statistical difference in the S1P level between untreated and steroid-treated patients.

As limitation of the in vitro data presented in the mechanistic experiments, it could be considered lacking in statistical power because the relatively small sample (3 to 4 independent experiments per condition). However, the homogeneous population of A549 cell line allow getting statistical significance with this number of experiments as we previously reported.[3]

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