Phospholipase A2-Receptor 1 Promotes Lung-Cell Senescence and Emphysema in Obstructive Lung Disease

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METHODS

Study design

The global objectives of the present study were to demonstrate that PLA2R1 is a major player in the process of lung-cell senescence in COPD, to assess JAK/STAT inhibition as a strategy to counteract PLA2R1 effects on cell senescence, and then to validate these findings in preclinical mouse models. To this end, we first assessed PLA2R1 expression in lungs and cells from 23 patients with COPD and 23 controls and investigated whether PLA2R1 colocalized with senescence and DNA-damage markers. We then evaluated the effects of PLA2R1 on replicative cell senescence in several cell types -- pulmonary endothelial cells (P-ECs) and smooth muscle cells (SMCs) -- from 8 patients with COPD and 10 controls. The cells were infected either by retroviral vectors encoding *PLA2R1*, shRNA against *PLA2R1*, or shRNA against *p53*; or by control vectors. Because we previously demonstrated that the prosenescent effects of PLA2R1 involved JAK2, we then tested whether pharmacological JAK1/2 inhibition (by ruxolitinib) induced in vitro inhibition of cell senescence and of the senescence-associated secretory phenotype (SASP), using cells transduced with the retroviral vector encoding *PLA2R1* gene. Finally, we assessed the lung phenotypes of mice expressing the human *PLA2R1* gene (*PLA2R1*-TG mice) under the control of the CMV promoter (40 mice) and of mice injected intratracheally with a mouse *PLA2R1*-encoding lentiviral vector (40 mice), with both mouse models being subjected to *in vivo* ruxolitinib treatment.

Patients and controls

The study was approved by the institutional review board of the Henri Mondor Teaching Hospital, Créteil, France. All patients and controls signed an informed consent document before study inclusion.

Lung tissue samples were obtained from 46 patients who underwent lung resection surgery at the *Institut Mutualiste Montsouris*, Paris, France. Among them, 23 had COPD and 23 were control smokers matched to the COPD patients on age and sex (Table 1). Inclusion criteria for the patients with COPD were an at least 10 pack-year smoking history and a ratio of forced expiratory volume in 1 second (FEV_1) over forced vital capacity (FVC) <70%. The controls had to have a smoking history greater than 5 pack-years; an FEV_1/FVC ratio greater than 70%; and no evidence of chronic cardiovascular, hepatic, or renal disease. None of the patients had a history of cancer chemotherapy. Lung tissue samples collected during surgery were either immediately immersed in DMEM medium then either used for primary cultures of pulmonary artery smooth muscle cells (PA-SMCs) and pulmonary artery endothelial cells (PA-ECs) or immediately snap-frozen in liquid nitrogen then stored at -80°C until protein extraction. In addition, a fragment of lung tissue was fixed in 4% paraformaldehyde aqueous solution (Sigma Aldrich, St Louis, MI) and used for paraffin embedding. Cells were cultured from a subset of 8 patients and 10 controls.

Mice

Adult male mice (C57Bl/6j) were used according to institutional guidelines, which complied with national and international regulations. All animal experiments were approved by the Institutional Animal Care and Use Committee of the French National Institute of Health and Medical Research (INSERM)–Unit 955, Créteil, France. To study the effects of *PLA2R1* overexpression, we generated mice with constitutive expression of the human *PLA2R1* gene placed under the control of the CMV promoter. Their wild-type littermates served as controls (non transgenic mice, NTG). To investigate the effect of lung *PLA2R1* overexpression, mice were injected intratracheally with a lentiviral vector encoding the murine *PLA2R1* gene under the control of the CMV promoter (LV-PLA2R1, Vector ID:

VB180531-1152skr; VectorBuilder Inc., Chicago, IL) or with a control lentiviral non-coding RNA expression vector (LV ncRNA, Vector ID: VB180531-1150skr, VectorBuilder Inc.). Each mouse was injected with 50 µL of lentivirus solution (10^9 TU/ml diluted in DPBS with 5% of Lipofectamine 2000 (Invitrogen, Carlsbad, CA). In preparatory experiments, we identified lung lentivirus-transduced cells by using a lentiviral vector encoding the mCherry reporter gene under the control of the CMV promoter (LV-mCherry). To assess the *in vivo* effect of the JAK1/2 inhibitor ruxolitinib, mice were treated with 75 mg/kg/day of ruxolitinib or vehicle (5% N,N-Dimethylacetamide, 0.5% methyl cellulose from Sigma-Aldrich) given by gavage. Ruxolitinib used for the animal studies was purchased from Clinisciences (Med Chem Express, Monmouth Junction, NJ).

Animal studies, lung tissue analysis

Mice were anesthetized with an intraperitoneal injection of ketamine (60 mg/kg) and xylazine (10 mg/kg). Right ventricular systolic pressure (RVSP) was measured as described previously. The RV was dissected from the left ventricle (LV) plus septum (LV+S), and these dissected samples were weighed to determine the Fulton index (RV/ [LV+S]). The right lung was quickly removed and divided into two parts, which were immediately snap-frozen in liquid nitrogen then stored at -80°C until total RNA and protein extraction for real-time quantitative polymerase chain reaction (RT-qPCR), Western blot, and ELISA. The left lungs were fixed by intratracheal infusion of 4% paraformaldehyde aqueous solution (Sigma Aldrich) at a transpleural pressure of 30 cmH_2O and processed for paraffin embedding. For morphometry studies, 5 µm-thick sagittal sections along the greatest axis of the left lung were cut in a systematic manner and stained with hematoxylin and eosin. Alveolar and vascular morphometry studies were performed by an observer blinded to treatment and genotype, as described elsewhere.

Lung emphysema was assessed on hematoxylin and eosin sections using the mean-linear-intercept (MLI) method described by Knudsen L et al [1]. Light microscope fields were quantified at an overall magnification of 400, using a 42-point and 21-line grid. We examined 20 fields/animal using a systematic sampling method from a random starting point. To correct area values for shrinkage associated with fixation and paraffin processing, we multiplied them by 1.22, as calculated during a previous study.

Lung fibrosis was evidenced on lung sections stained with Masson's trichrome (HT15-1KT, Sigma-Aldrich) and quantified on lung sections stained with Sirius Red (Picro Sirius Red Stain Kit, ab150681, Abcam, Cambridge, UK) using the modified Ashcroft scale [2]. Briefly, the lungs were scanned microscopically with a 20-fold objective, which allowed the evaluation of fine structures while also providing a sufficiently broad view. The entire section was examined by inspecting each field in a raster pattern. Areas with dominating tracheal or bronchial tissue were omitted. The grades were summarized and divided by the number of fields to obtain a fibrotic index for the lung.

For vascular morphometry, each vessel was categorized as non-muscular (no evidence of vessel wall muscularization), partially muscular, or fully muscular. The percentage of muscular pulmonary vessels was determined by dividing the sum of partially and fully muscular vessels by the total number of vessels in the relevant group of animals, as described previously [3].

To measure the release of cytokines IL-6, MCP-1, IL-1β, and PAI-1 from the lung, we used the Quantikine ELISA kit (R&D systems, Minneapolis, MN) according to the manufacturer's instructions.

Cultured cells from patients and controls

P-ECs and PA-SMCs were collected from the lung samples of 8 patients with COPD and 10 controls and cultured as described previously [4, 5]. The cells were then subjected to repeated passages to determine their replicative senescence threshold and population doubling level (PDL). At each passage, cells at 80%-90% confluence were counted in a hemocytometer using trypan blue. The onset of replicative senescence was defined as cessation of cell division, labeling for senescence-associated *beta*-galactosidase, and cell morphology criteria. PDL was computed as follows: PDL = $(\log_{10}Y - \log_{10}X)/\log_{10}2$, where X was the initial number of seeded cells and Y the final number of cells [5, 6].

To assess the effect of JAK/STAT inhibition by ruxolitinib, cells from patients with COPD and controls were continuously cultured in medium containing either vehicle or ruxolitinib (0.5 μM). The effects of ruxolitinib on cell senescence were evaluated comparatively with those of the vehicle.

We specifically designed experiments to assess the effects of *PLA2R1* inhibition on replicative cell senescence. Cells from patients with COPD and controls were infected with either a retroviral vector encoding short hairpin RNAs targeting *PLA2R1* (shPLA2R1) or a control vector (shControl), as previously described [7]. We then compared the effects of *PLA2R1* knockdown to those of the control vector [8]. The infection protocols were designed to infect nearly all the cells, as demonstrated by fluorescence observed with a GFP-expressing retroviral vector (not shown).

In further specific experiments, cells from patients were infected with a retroviral vector encoding human *PLA2R1* (PLA2R1 virus) or with a control vector as previously described [7, 8]. We then compared the effects of *PLA2R1 overexpression* to those of the control vector. In these experiments, cells were continuously cultured in a medium containing either vehicle or the JAK1/2 inhibitor ruxolitinib (0.5 μM).

For analysis of cultured cells at early and late passages, cells were placed in reduced media containing 0.2% FBS for 24 h for P-ECs or 48 h for PA-SMCs, as previously described [4, 5]. Then, the cells were collected for further RNA and proteins analysis and to determine the percentage of *beta*−galactose-positive cells. The conditioned media were collected and used to quantify IL-8, IL-6, and PAI-1 using Quantikine ELISA kits (R&D systems, Minneapolis, MN) according to the manufacturer's instructions.

Senescence-associated beta-galactosidase staining

At each passage, cells at 60% confluence were fixed with 2% formaldehyde and 0.2% glutaraldehyde for 10 minutes. Then, the cells were washed with PBS and stained in a titrated pH6 solution containing 40 mM citric acid, 150 mM NaCl, 2 mM $MgCl_2$, 5 mM potassium ferrocyanide, and 1 mg/mL X-Gal.

Immunofluorescence

For immunofluorescence, cells were fixed for 10 minutes in methanol. Paraffin-embedded sections of lung were deparaffinized using xylene and a graded series of ethanol dilutions then processed for epitope retrieval using citrate buffer (0.01 M, pH 6; 90°C, 20 min). For PLA2R1 epitope retrieval, we used NeoUltra retrieval solution (NB-23-00178-4, NeoBiotech, Nanterre, France) according to the manufacturer's instructions. For nuclear immunolabeling, tissues were permeabilized with 0.1% Triton X-100 in PBS for 10 minutes.

Saturation was achieved using Dako antibody diluents with 10% goat serum. For double staining, first and second primary antibodies were diluted in Dako antibody diluents with 3% goat serum then incubated for 1 hour at 37°C in a humidified chamber. After PBS washes, the sections were covered with secondary antibody (mixed with mouse, rabbit, rat, or chicken Alexa Fluor® 480, Alexa Fluor® 555, or Alexa Fluor® 660 [Abcam]) for 40 minutes at 37°C in

a humidified chamber. After 5 minutes of staining with DAPI, the sections were protected with coverslips secured with fluorescent mounting medium.

Fluorescence was recorded using an Axio Imager M2 imaging microscope (Zeiss, Oberkochen, Germany) and analysed on digital photographs using Image J software (imagej.nih.gov/ij/).

Immunohistochemistry

For immunohistochemistry on paraffin-embedded sections, after deparaffinization and antigen retrieval, the endogenous peroxidase activity was blocked with 3% H_2O_2 and 10% methanol in PBS for 10 minutes. Permeabilization was done using 0.1% Triton X-100 in PBS for 10 minutes. Saturation was achieved by incubation for 30 minutes at 37°C in antibody diluent (ref.NB-23-00171-1, NeoBiotech) supplemented with 12% of goat serum. The slides were then incubated for 60 minutes at 37°C with primary antibody. The secondary antibody consisted of Sav-HRP conjugates. DAB substrate solution was used to reveal the color of the antibody staining. Nuclear counterstaining was achieved with methyl green.

Protein extraction and immunoblotting

Total proteins were extracted using RIPA lysis buffer (10 mM sodium phosphate pH 8, 150 mM NaCl, 1% sodium deoxycholate, 1% NP40, 0.5% SDS, 1 mM PMSF, 10 mM NaF, 1 mM sodium orthovanadate, and protease inhibitor cocktail [Roche, Meylan, France]). Immunoblots were carried out using the indicated antibodies and detected using an enhanced chemiluminescence detection system (GE Healthcare, Little Chalfont, UK). Densitometric quantification was normalized for the *beta*-actin or GAPDH level using GeneTools software (Ozyme, Montigny le Bretonneux, France).

Real-time quantitative PCR (RT-qPCR)

Total RNA from tissues and cells was extracted with the RNeasy Plus Mini Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. The RNA concentration was assessed using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Les Ulis, France), and RNA integrity was confirmed by electrophoresis. One µg of total RNA was used for cDNA synthesis using SuperScript II Reverse Transcriptase (Thermo Fisher Scientific). The quantitative PCR was performed in triplicate with TaqMan Probes (TaqMan Gene Expression assays, Applied Biosystems, Illkirch, France) and TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific) in the QS6, QuantStudio 6 Flex Real-Time PCR system (Applied Biosystems, Illkirch, France). All data were analysed using QuantStudio Real-Time PCR software (Applied Biosystems). The relative expression level of target genes was calculated by the ΔΔCt method and normalized to the reference genes 18s and SF3A1.
The TaqMan Gene Expression assays used for human and mouse cells are described in supplemental Table S3.

Materials

The JAK1/2 inhibitor ruxolitinib used for animal studies was purchased from Clinisciences (Med Chem Express).

The following primary antibodies were used: mouse monoclonal anti-PLA2R1 antibody (1:200, ab211490, Abcam); rabbit polyclonal anti-PLA2R1 antibody (1:200, ab194256, Abcam); rabbit polyclonal anti-PLA2R1 antibody (1:200, abx129773, ABBEXA); mouse monoclonal anti-CDK2A/p16INK4a (p16, ab54210, Abcam), polyclonal rabbit anti-α-smooth muscle actin (αSMA) antibody (1:400, ab5694, Abcam), polyclonal rabbit anti-CD31 (PECAM) antibody (1:20, ab28364, Abcam), monoclonal rabbit anti-MUC1 (1:200, ab109185, Abcam antibody); polyclonal rabbit anti-53BP1 antibody (1:200, NB100-304,

Novus Biologicals, Littleton, CO); phospho-gamma-H2AX (Ser139) recombinant rabbit monoclonal antibody (RM224) (# MA5-33062, RRID AB_2810155, Thermo Fisher Scientific); polyclonal mouse anti-proliferating cell nuclear antigen antibody, anti-PCNA (1:200, ab29, Abcam); monoclonal mouse anti-JAK1 antibody (1:200; sc-1677, Santa Cruz Biotechnology), polyclonal rabbit anti-JAK2 antibody (1:200, sc-294, Santa Cruz Biotechnology), goat polyclonal anti-phospho STAT3 (Tyr705) antibody 1:200, sc-7993, Santa Cruz Biotechnology); rabbit polyclonal anti-phospho-Jak2 (Tyr1007/1008) antibody (1:200, y #3771, Cell Signaling), rabbit anti-vimentin antibody (1:500, ab92547, Abcam), rabbit anti-fibroblast-specific protein antibody (1:200, ab27957, Abcam), anti-beta-Actin (β-Act, A5316, Sigma); GAPDH (sc-25778); anti-CDK2A/p16INK4a (p16, 92803, Cell Signaling; p16, PA520379, Thermo Scientific); anti-p21 Waf1/Cip1 (p21, 2946, Cell Signaling Technology)

Statistical analysis

Quantitative variables are expressed as individual values and mean or as median (range). Statistical analyses were performed with GraphPad Prism 7 software following the guidelines in GraphPad Prism. One-way analysis of variance (ANOVA) or two-way ANOVA followed by Bonferroni's multiple comparisons test were used to compare the means of more than two independent groups. Values represented as medians (interquartile range) were compared by the nonparametric Kruskal-Wallis test. *P* values less than 0.05 were considered significant.

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