On-line Supplementary Material

Genetic Ablation of PDGF-dependent Signaling Pathways Abolishes Vascular Remodeling and Experimental Pulmonary Hypertension

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Materials and Methods

Patient characteristics and immunohistochemistry

Human lung tissues were obtained from patients with idiopathic PAH (IPAH) undergoing lung transplantation. Non-transplanted donor lungs showing no evidence of vascular pathology served as healthy controls. Sample collection and tissue usage were approved by the local Ethics committee, and informed consent was obtained from each patient. Explanted lungs were directly rinsed until blood-free with ice-cold preservation buffer and then fixed in 4% phosphate-buffered paraformaldehyde (Santa Cruz Biotechnology, USA) in PBS. The samples were then dehydrated and paraffin embedded, and 3 µm sections were obtained from fixed tissue blocks. Immunohistochemistry was performed using antibodies directed against the human βPDGFR and targeting phosphorylated tyrosine residues Y1021 and Y751, respectively, with 3,3'-diaminobenzidine as a substrate. Negative controls were performed with the omission of the first antibody. Slides from patients with IPAH and from the control group were always stained together to avoid systematic error.

Animals and exposure to hypoxia

βPDGFR-F3 mice were generated by introducing point mutations into the βPDGFR locus by gene targeting as described [1]. Briefly, point mutations were created that converted the PI3K binding sites of the murine βPDGFR at amino acid residues 739 and 750 from tyrosine to phenylalanine, and the PLCγ binding site at residue 1020 from tyrosine to isoleucine. Male $\beta PDGFR^{F3/F3}$ mice, littermate heterozygous $\beta PDGFR^{F3/+}$ mice and littermate wild-type (WT) controls (C57BL/6J) were kept on a regular diet throughout the experimentation. Exposure of mice to normobaric normoxia (21% O_2) or hypoxia (10% O_2) was performed as described [2]. Briefly, adult male mice (20 – 30 g, 8-12 week) were kept in a ventilated chamber chamber which provides a constant O_2 level of 10% for 21 days. All animal experiments were performed in accordance with institutional guidelines and approved by the local animal committee.

Cells, cell culture and hypoxic exposure of cells

Murine pulmonary arterial smooth muscle cells (mPASMC) were isolated from 6-10 week-old WT or homozygous $\beta PDGFR^{F3/F3}$ mice. Mice were sacrificed and the thorax was dissected. The entire heart-lung complex was removed and placed in a culture dish filled with chilled PBS buffer supplemented with Penicillin-Streptomycin (1%). The pulmonary artery was dissected under a dissection microscope, removed and transferred to an Eppendorf tube containing 1 ml of enzyme solution (collagenase, elastase, trypsin-inhibitor). The artery was

incubated for 15 minutes to detach the adventitia. The residual adventitia was removed using an anatomic forceps. The remaining material was cut into small pieces and incubated in enzyme solution for another 90 minutes at 37°C. After centrifugation, the pellet was resuspended in DMEM culture medium containing 20% FCS and 1% Penicillin-Streptomycin and transferred to a cell culture dish. Following initial expansion, cells were grown in a 5% CO_2 atmosphere at 37 °C in DMEM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 1% nonessential amino acids, and 10% FCS. Human coronary endothelial cells (hcECs) were obtained from Lonza (Basel, Switzerland) and were grown in EBM-2 media in accordance with the manufacturer´s instructions. Experiments were performed with cells from passages 5–10 (functional assays) and 5–15 (biochemical analyses). Cells were grown under normoxic conditions (5% CO2, 95% air, 37°C) in a water-jacketed incubator or exposed to hypoxic conditions (1% O2, 5% CO2, 37°C) in modular incubation chambers (Billups-Rothenberg, Del Mar, CA).

Measurement of pulmonary hemodynamics and right ventricular hypertrophy

Animals were anesthetized with isoflurane and carprofen. They were breathing autonomously through ananaesthesia-gas supplying respiratory mask (compressed air: 0,6l/min, oxygen: 1-1,2l/min and 2% isoflurane). For assessment of the right ventricular systolic pressure (RVP_{syst}), the right jugular vein was dissected and a distal and proximal ligature was placed to prevent retrograde blood flow and to dam the vein. Using a 26Gx1/2 needle the vein was opened and the millar® catheter (Mil-SPR-1000, Föhr Instruments, Heidelberg, Germany) was inserted, the proximal loop was loosened, and the catheter was moved forward and placed in the right ventricle. Correct placement of the catheter was ensured via the characteristic RVP curve. For measurement of the systemic blood pressure, the left carotid artery was dissected and ligated proximal and distal to prevent blood flow. An additional third loose loop, which later fixes the catheter, was placed in between the ligatures. The artery was opened and the catheter was inserted, while the proximal ligature was relaxed. The catheter information was amplified by a PowerLab® amplifier and converted to pressure curves using LabChart7®software (ADinstruments).The system was calibrated before every measurement. After exsanguination of the animals, the lungs and hearts were isolated. The right ventricle was dissected from the left ventricle+septum(LV+S), the samples were weighed to obtain the right to left ventricle plus septum ratio (RV/LV+S) as a measurement of right ventricular hypertrophy [3].

Tissue processing and histology

The PBS-perfused lungs were fixed by immersion in a 3.5% formalin solution. Following paraffin embedding of the tissue, sectioning was performed to obtain 3 µm thick sections. Immunostaining for PCNA was performed with SuperVision 2 (#PD050POL-K, DCS Innovative Diagnostik-Systeme, Hamburg, Germany) and DAB as substrate (#34002, ThermoScientific), counterstaining was performed with methylgreen (#VC-H-3402-L500, Axxora). Double-immunostaining for morphometric analyses was performed with an anti-α-smooth muscle actin (SMA) antibody (dilution 1:900, clone 1A4, Sigma, Saint Louis, Missouri) and anti-human von Willebrand factor antibody (vWF, dilution 1:900, Dako, Hamburg, Germany). The degree of muscularization of small peripheral pulmonary arteries was assessed by using a computerized morphometric analysis system (QWin; Leica, Wetzlar, Germany) as described previously [2]. The QWin system computes the vessel size and categorizes the vessels as nonmuscularized (less than 5 % α-SMA staining), partially muscularized (5 to 70 % α-SMA staining) or fully muscularized (≥ 70 % α-SMA staining). In each mouse, 80 to 100 intra-acinar arteries (20 to 70 µm diameter) were analyzed. Additionally, an elastica van Gieson staining was performed visualizing elastic fibers in order to determine the medial wall thickness. The investigators of hemodynamic and morphometric analyses were blinded for the genotype.

Chemotaxis

PDGF-dependent chemotaxis was assayed utilizing a 48-well modified Boyden chemotaxis chamber (NeuroProbe Inc., Baltimore,MD) and collagen-coated polycarbonate, PVPF, filters (8 µm pore size) (GE Water & Process Technologies, Watertown, MA, USA) as described [4]. Briefly, the lower wells of the chamber were filled with DMEM supplemented with PDGF-BB in the given concentrations or vehicle in the presence or absence of inhibitors as indicated. The filters were coated with 50 mg/ml rat type I collagen (BD Bioscience, Bedford, MA, USA) and fixed atop the bottom wells. SMCs were diluted in DMEM to a final concentration of 4 x 10^5 cells per ml. Where indicated, cells were incubated for 60 min with inhibitors and placed into the upper chamber (50 µl per well). The chamber was kept for 5 h at 37°C in a 5% CO_2 atmosphere and was then disassembled. The cells on the upper surface of the filter were removed, and the cells on the lower surface were fixed and stained with Diff-Quick (Baxter Healthcare, Miami, FL). For quantification, cells on the lower surface of the filter in each well were counted using a 20x magnification raster ocular.

Proliferation

DNA synthesis was analyzed by a 5-bromodeoxyuridine (BrdU)-incorporation assay as described [4]. Briefly, cells were seeded in 96-well-plates and were cultured in 96-well plates in DMEM containing 10% FCS, washed with PBS, starved in DMEM for 24 h, and stimulated with PDGF-BB in the given concentration for 18 h in the presence or absence of inhibitors as indicated. BrdU incorporation was carried out according to the manufacturer´s specifications (#11647229001, Roche) with an incubation time of 5 h.

Apoptosis

Apoptosis was detected by flow cytometry. Cells seeded in 6-well plates were incubated in serum-free media for 16h to induce apoptosis followed by treatment with or without PDGF-BB (50 ng/ml) for an additional 16h. Apoptotic cells were detected by staining with the PE active caspase-3 apoptosis kit (#550914, BD Biosciences) after washing with PBS, fixation and isolation of adherent cells using a rubber policeman according to manufacturer‘s instructions.

Immunoprecipitation of the βPDGFR and Src and Western blotting

Quiescent SMCs were left resting or stimulated with 50 ng/ml PDGF-BB for times indicated. Cells were washed twice with HS (20 mm Hepes, pH 7.4, 150 mm NaCl), and then lysed in EB (10 mm Tris-HCl, pH 7.4, 5 mm EDTA, 50 mm NaCl, 50 mm NaF, 1% Triton X-100, 0.1% bovine serum albumin, 20 µg/ml aprotinin, 2 mm Na_3VO_4, 1 mm phenylmethylsulfonyl fluoride). After centrifugation (20 min, 12,000 × *g*), the supernatants were subjected to Western blot analysis. For immunoprecipitation, Src was precipitated with Src antibody or the βPDGFR was precipitated with a polyclonal βPDGFR antibody (97A) detecting the kinase insert (amino acids 698-797) of the human βPDGFR as described [5]. Immunoprecipitates were resolved on a 7.5% SDS-polyacrylamide gel, and the proteins were transferred to PVDF membrane (BioRad Laboratories, USA) and subjected to Western blot analysis.

Materials

Chemicals were obtained from Sigma-Aldrich Chemie GmbH, (Steinheim, Germany). Rat tail Collagen type I was from BD Biosciences, Elastase from Serva Electrophoresis GmbH,

(Heidelberg, Germany), Collagenase and thrombospondin from Sigma-Aldrich Chemie GmbH, (Steinheim, Germany). PDGF-BB was purchased from PromoCell (Heidelberg, Germany). The antibodies against RasGAP (69.3) and the βPDGFR (97A) were kind gifts from Andrius Kazlauskas, Harvard Medical School, Boston, MA. The phospho-specific p42/44 Erk (#9101), and Akt antibodies (Ser473 #4060), the VEGF receptor (#9698) and the phospho-VEGF receptor (#2478) antibody were from Cell Signaling (Danvers, MA). Antibodies directed against the p85 subunit of PI3K (# 4292), Src (#2123) and PLCγ (#2822) were from Cell Signaling, the actin antibody (#53141), and the SHP-2 antibody (#30169) were from Santa Cruz (Santa Cruz, CA), phospho-specific antibodies directed against tyrosines Y1021 (ab62437) and Y751 (#3161) of the βPDGFR were from Abcam (Cambridge, UK) and Cell Signaling, respectively. For immunohistochemistry, a different Y1021 antibody from Santa Cruz, CA (sc-12909-R) was used. The PCNA antibody (#13110) was from Cell Signaling. The anti-phosphotyrosine antibodies were from Santa Cruz (PY20) and Upstate Biotechnology (Lake Placid, NY) (4G10). LY294002, PD98059 and U73122 were from Merck (Frankfurt, Germany). For immunohistochemistry, the alpha smooth muscle actin antibody (Clone 1A4, #A2547) was from Sigma-Aldrich, the von Willebrand factor antibody (#A0082) was from Dako. Penicillin-Streptomycin, trypsin-inhibitor were obtained from BD Biosciences (Heidelberg, Germany), DMEM (High glucose (4,5g/l) with L-Glutamine) was obtained from PAA Laboratories GmbH (Pasching, Austria), fetal calf serum (FCS) was from Gibco, Invitrogen (Carlsbad CA, USA).

Data analysis

All data are expressed as means ± SEM. Statistical analyses were performed using analysis of variance (ANOVA) followed by Newman-Keuls post-hoc test for multiple comparisons or student t-test, as appropriate. A *P* value <0.05 was considered significant.

References

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