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Suppl. Methods

Preparation, culture and stimulation of precision cut lung slices (PCLS)

PCLS were prepared from tumor free lung explants from patients who underwent lung resection for cancer at KRH Hospital Siloah-Oststadt-Heidehaus or the Hanover Medical School (both Hannover, Germany). All patients gave written consent and all investigations using human tissue were approved by the ethics committee of the Hanover Medical School and are in accordance with The Code of Ethics of the World Medical Association. Tissue was processed immediately on the day of resection as described before (1, 2). Briefly, human lung lobes were cannulated with a flexible catheter and the selected lung segments were inflated with warm (37°C) low melting agarose (1.5% in Dulbecco’s Modified Eagle’s Medium Nutrient Mixture F-12 Ham (DMEM) supplemented with L-glutamine, 15mM HEPES without phenol red, pH 7.2–7.4 (Sigma-Aldrich, Hamburg, Germany), 100U/mL penicillin, and 100µg/mL streptomycin (both from Biochrom, Berlin, Germany). After polymerization of the agarose solution on ice, tissue cores of a diameter of 8mm were prepared using a sharp rotating metal tube. Subsequently, the cores were sliced into approx. 300µm thin slices in Earle’s Balanced Salt Solution (EBSS; Sigma-Aldrich) with a Krumdieck tissue slicer (Alabama Research and Development, Munford, AL). PCLS were washed 3× for 30min in DMEM and used for experiments. Two PCLS/well were incubated with 6µg/ml FXII, 6µg/ml FXIIa (both from SEKISUI Diagnostic, Pfungstadt, Germany), 10µg/ml PPK, 10µg/ml PK, 15µg/ml HK (all from Haemochrom Diagnostica GmbH, Essen Germany), or 15µg/ml HKa
(Enzyme Research Laboratories, South Bend, IN) in 500µl DMEM under normal cell culture conditions (37°C, 5% CO₂) for up to 48h. In some experiments 100µg/ml soybean trypsin inhibitor (SBTI; Sigma-Aldrich), 10µg/ml anti-FXII antibody (Zytomed Systems, Berlin, Germany), or 10µg/ml IgG isotype control (Sigma-Aldrich) were included. Supernatants and tissue samples were subsequently collected. Tissue was lysed using 1% Triton X-100 in PBS (0.1M sodium phosphate, 0.15M NaCl, without Ca²⁺ and Mg²⁺, pH 7.4) for 1h at 4°C. Supernatants and cell lysates were supplemented with 0.2% protease inhibitor cocktail (Sigma-Aldrich). Alternatively, tissue was snap frozen in liquid nitrogen for protein and RNA isolation. Viability of the tissue was assessed by a LDH Cytotoxicity Detection Kit (Roche, Mannheim, Germany) according to manufacturer’s instruction. Endotoxin was not detected in the FXII and FXIIa preparation as determined by a Pierce® LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific, Erlangen, Germany).

RNA isolation and cDNA synthesis

Isolation of RNA from PCLS was performed using a peqGOLD Total RNA Kit (Peqlab, Erlangen, Germany) according to the manufacturer's instruction. One µg of RNA was reverse-transcribed as described previously (3).

Real-time PCR

For the real-time PCR (qPCR) the following oligonucleotide primers were used: C1INH forward 5´-CTCCGATACCCGCCTTGTC-3´, C1INH reverse 5´-GCACCTTTTATAACTG-AGTTTTTGAAATGTA-3´; IL-1β forward 5´-CTGAATCTCCCTCCTCCAACA-3´, IL-1β reverse 5´-CCAAAGCCACAGGTATTTTG-3´; IL-8 forward 5´-GCAGAGGGTTTTGGAAGAATG-3´, IL-8 reverse 5´-GCCAGGCACAGCTTATTTTG-3´; IL-6 forward 5´-CTTTTCAGCCATCTTGGAGAAGGGAAGGTG-3´, IL-6 reverse 5´-TTTTCTTACAGCCACGCAAGG-3´; CXCL5 forward 5´-TGTTTTACAGCCACGCAAGG-3´, CXCL5 reverse 5´-GGAGGCTAC-
CAC TTCCACCT-3’; LIF forward 5’-GCATCTGAGGTTTCCTCAA-3’, LIF reverse 5’-TTCCAGTGCAGAACAACAG-3’, TNF-α forward 5’-GACAAGCTGTAGCCCCATGT-3’, TNF-α reverse 5’-TCT CAGCTCCACGGCATT-3’, β-actin forward 5’-CAGAGCCTCGCCCTTTGCC, β-actin reverse 5’-GATGGAGGGGAAGACGGC-3’, PBGD forward 5’-CCCACGCGAATCCTCAT-3’, PBGD reverse 5’-TGTCTGGTAACGGCAATGCG-3.

β-actin and PBGD served as reference genes. Cycling conditions were 95 °C for 6min, followed by 40 cycles of 95 °C for 20s, 58 °C for 30s, and 73 °C for 30s. All changes in the target gene mRNA levels are presented as delta Ct (ΔCt) which was calculated by subtracting the Ct value of the target gene from the Ct value of the reference gene.

**Immuoassays**

FXII and BK were quantified by AssayMax FXII ELISA Kit (Assaypro, Saint Charles, MO) and Human Bradykinin ELISA Kit (Cusabio Biotech Co., Wuhan, China), respectively, according to the manufacturer’s instruction. C1INH was measured by Human Plasma Protease C1 Inhibitor ELISA Kit (CUSABIO and CusAb, College Park, MD) as described by the supplier. TNF-α, IL-6, LIF, CXCL5, IL-1β, and IL-8 concentrations in PCLS culture supernatants and lysates were determined using Human TNF-α Quantikine ELISA kit, Duo-Set Human IL-6, DuoSet Human LIF, DuoSet Human CXCL5 (all from R&S System, Wiesbaden, Germany), Mini TMB EDK Human IL-1β, and Mini TMB EDK Human IL-8 (both from PeproTech, Hamburg, Germany), respectively, according to the manufacturer’s instruction. Human FXIIa antigen levels were quantified using a B7 nanobody capture ELISA as previously described (4).

**Western blot analysis**

Twenty µl BALF, 10µl PCLS culture medium or 10µg cell/PCLS lysates were separated on a SDS polyacrylamide gel, followed by electrotransfer to a PVDF membrane. After blocking
with 5% non-fat dry milk in TBST buffer (25mM Tris-Cl, 150mM NaCl, 0.1% Tween 20, pH 7.5), the membrane was incubated overnight at 4°C with one of the following antibodies: goat anti-FXII (cat. no. 206-0056; Zytomed Systems), rabbit anti-HK (cat. no. ab35105), rabbit anti-FXI (cat. no. ab200643; both from Abcam, Cambridge, MA), goat anti-PPK (cat. no. AF2498; R&D Systems), mouse anti-phospho-Erk1/2(Thr202/Tyr204) (cat. no. 9106), rabbit anti-phospho-Akt(Ser473) (cat. no. 4058; both from Cell Signaling Technology, Frankfurt am Main, Germany), mouse anti-ICAM-1 (cat. no. sc8439; Santa Cruz Biotechnology, Santa Cruz, CA). Afterwards, the membranes were incubated with peroxidase-labelled secondary antibodies (all from Dako, Glostrup, Denmark). Final detection of proteins was performed using an ECL Plus Kit (Amersham Biosciences, Freiburg, Germany). As a loading control β-actin (detected with a mouse anti-β-actin antibody, cat. no. A1978; Sigma-Aldrich), total Erk1/2 (detected with a rabbit anti-Erk1/2, cat. no. 4695), and total Akt (detected with a rabbit anti-Akt, cat. no. 2966, both from Cell Signaling Technology) were used.

**Cell culture and stimulation**

Human lung microvascular endothelial cells (HMVEC; Lonza, Hessisch Oldendorf, Germany) were cultured in EGMTM-2MV BulletKitTM (Lonza) at 37°C in humidified atmosphere with 5% CO₂. HMVEC seeded into 96-well tissue culture plates, were serum-starved overnight and then stimulated for 4h with PCLS culture supernatant either supplemented or not supplemented with 10µg/ml a neutralizing human TNF-α monoclonal antibody (R&D Systems, cat. no. AF-210-NA) or isotype-matched control (Sigma-Aldrich). Finally, the cells were lysed in 1× Laemmli sample buffer containing 10% β-mercaptoethanol.

**PK-like activity in BALF**

BALF PK-like activity was measured using the chromogenic substrate S-2302 (HD-Pro-Phe-Arg-pNA·2HCl; Chromogenix, Mölndal, Sweden). Briefly, BALF (diluted 1:2) in 20mM Na-
citrate, 150mM NaCl, 1% bovine serum albumin and 1% Tween 20 was mixed with S-2302 (final concentration 0.6mM) in duplicate and then incubated for 1h at 37°C. Thereafter, the hydrolysis of S-2302 was measured spectrophotometrically at 405nm in microtiter plate reader (Molecular Devices, Biberach, Germany). Parallel samples were supplemented with C1 esterase inhibitor (C1INH; 250µg/ml; CSL Behring, Marburg, Germany), soybean trypsin inhibitor (SBTI; 30µM; Sigma-Aldrich), anti-FXII/FXIIa antibody (10 µg/ml; Zytomed Systems), or IgG isotype control (10 µg/ml; Sigma-Aldrich).

C1INH activity

C1INH activity in BALF samples was measured by MicroVue Complement C1-Inhibitor Plus EIA (Quidel Corporation, San Diego, CA) according to the manufacturer’s instruction. Concentrations greater than or equal to 68% mean control are considered normal. As a control plasma pooled from 100 healthy subjects was used.

Suppl. References


Suppl. Figure 1. BK levels are increased in ARDS BALF. (a) Quantification of BK in BALF from patients with ARDS obtained within 24h of the diagnosis (n=54; indirect ARDS n=48, direct ARDS n=6) and control subjects (cardiogenic lung edema (CLE; n=10), healthy control subjects (n=43)) as assessed by immunoassay. **p<0.01, ***p<0.001 vs healthy control subjects; +p<0.05 vs CLE. (b) Quantification of BK in BALF from patients with ARDS (day 1, n= 54; day 3-4, n=9; day 6-8, n=8) and healthy subjects (n=43) as assessed by immunoassay. ***p<0.001 vs healthy subjects. BK, bradykinin; BALF, bronchoalveolar lavage fluid.
Suppl. Figure 2. FXII/FXIIa induces activation of Erk1/2 and Akt in PCLS. (a) Activation, as assessed by phosphorylation, of Erk1/2 and Akt in PCLS exposed to either FXII or FXIIa. Total Erk1/2 and total Akt were used as loading controls. Representative western blots are demonstrated. (b) Densitometric analysis of (a). n=6-8. *p<0.05, ***p<0.001 vs untreated. FXII, factor XII; PCLS, precision cut lung slices.
Suppl. Figure 3. C1INH is expressed in PCLS. (a) Expression of C1INH in PCLS as assessed by qPCR. n=9. Data are expressed as ΔCt value. PBGD was used as a reference gene. Liver was used as a positive control. (b) Quantification of C1INH in culture media obtained from PCLS stimulated either with FXII or FXIIa for 48h. n=4. *p<0.05 vs untreated. (c) Expression of C1INH in PCLS exposed either to FXII or FXIIa for 48h as assessed by qPCR. Data are expressed as ΔCt value. PBGD was used as a reference gene. n=4-5. C1INH, C1 esterase inhibitor; FXII, factor XII; PCLS, precision cut lung slices.
Suppl. Figure 4. SBTI reduces PK-like activity in PCLS culture media. PK-like activity in culture supernatants of PCLS exposed to FXII in the absence or presence of soybean trypsin inhibitor (SBTI). n=5. ***p<0.001 vs untreated without SBTI; +++p<0.001 vs FXII-treated without SBTI. FXII, factor XII; PCLS, precision cut lung slices.