Detailed Materials and Methods

Janus Kinase 3, a Novel Regulator for Smooth Muscle Proliferation and Vascular Remodeling

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Reagents and Cell Culture

Rat aortic smooth muscle cells (SMCs) were cultured by enzymatic digestion method from rat thoracic aorta as described previously.[1, 2] SMCs were maintained in Dulbecco's modification of Eagle's medium (DMEM, Invitrogen) containing 10% fetal bovine serum (FBS, Hyclone) and 5% L-glutamine (Corning) at 37°C in a humidified atmosphere with 5% CO_2. Phenotype of primary cultured SMCs were confirmed by the expression of smooth muscle α-actin and SM22α. Chemicals were obtained from the following sources: rhPDGF-BB (R&D Systems, 220-BB), Janex-1 (Santa Cruz, sc-205354), Sp600125 (Sigma Aldrich, S5567), S3I-201 (Sigma Aldrich, SML0330), SB203580 (Sigma Aldrich, S8307), LY294002 (EMD Millipore, 440202), U0126 (Sigma Aldrich, U120). Antibodies were obtained from the following sources: JAK3 (Cell Signaling, #8863), phospho-JAK3 (Santa Cruz, sc-16567), STAT3 (Cell Signaling, #9139), phospho-STAT3 (Cell Signaling, #9145), SAPK/JNK (Cell Signaling, #9252), phospho-SPAK/JNK (Cell Signaling, #9251), PCNA (Santa Cruz, sc-56), α-SMA (Abcam, ab5694), Cyclin D1 (Santa Cruz, sc-8396), Bcl-2 (Santa Cruz, sc-492), Bax (Cell Signaling, #2772), cleaved Caspase 3 (Cell Signaling, #9661), α-Tubulin (Cell Signaling, #2125).

Construction of Adenovirus

cDNA fragment encoding the full length of human JAK3 was amplified from JAX3 plasmid (DNASU, HsCD00038537) by PCR, and then inserted into the pShuttle-IRES-hrGPF-1 vector (Agilent) through XhoI site. The resultant recombinant JAK3 plasmid was verified by sequencing. Rat JAK3 short hairpin RNA (shJAK3) was constructed into pRNAT-H1.1/Adeno vector (Genscript) through MluI and HindIII site. Adenoviral vector of JAK3 and shJAK3 was constructed using AdEasy system described previously.[3] Adenovirus was purified by gradient density

ultracentrifugation of cesium chloride followed by dialyzing in dialysis buffer (135 mmol/L NaCl, 1 mmol/L MgCl2, 10 mmol/L Tris-HCl, pH 7.5, 10% glycerol). JAK3 shRNA cDNA sequences were: 5'- CGC GTC TCT ACT TGC AGT CCA GAA TGC CAG CTT CAA GAG AGC TGG CAT TCT GGA CTG CAA GTA GAT TTT TTC CAA A -3' (top strand) and 5'- AGC TTT TGG AAA AAA TCT ACT TGC AGT CCA GAA TGC CAG CTC TCT TGA AGC TGG CAT TCT GGA CTG CAA GTA GAG A -3' (bottom strand). Control scramble shRNA (shScr) sequences were: 5'-CGC GTC GAT CGA TGA TTC GCC CGG CGT CTT CAT AAT TCA AGA GAT TAT GAA GAC GCC GGG CGA ATC ATC GAT CTT TTT TCC AAA-3' (top strand) and 5'-AGC TTT TGG AAA AAA GAT CGA TGA TTC GCC CGG CGT CTT CAT AAT CTC TTG AAT TAT GAA GAC GCC GGG CGA ATC ATC GAT CGA-3' (bottom strand).

EdU Cell Proliferation Assay

Equal numbers (5 x 10^4) of SMCs were seeded into 12-well cell culture plates. Cells were starved (DMEM containing no FBS and 5% L-glutamine) for 24 hours, and then treated with PDGF-BB or other factors as indicated for 48 hours. Cells were then incubated with 5-Ethynyl-deoxyuridine (5-EdU) by following the manufacturer's recommendation (EMD Millipore). EdU-positive cells were counted from 10 different microscopic fields (10x). Proliferation rate was assessed by the following formula: [Cell numbers at 48 hours / Cell numbers at 0 hour]. The experiments were repeated for three times with three replicates for each treatment.

Real-time Quantitative PCR (qPCR)

Total RNA of cultured cells was extracted using Trizol Reagent (Invitrogen), and then reverse transcribed to cDNA using iScript cDNA Synthesis kit (Bio-Rad). Real time qPCR was performed with Stratagene Mx3005P qPCR instrument using SYBR Green master mix (Agilent Technologies). Each sample was amplified in triplicate.[5] JAK3 primer sequences were 5′-CCT GCC TGT TTA TCA TTC GCT -3′ (forward) and 5′-AAG ACT TGA GTG TCC ACG TCC -3′ (reverse).

Western Blot Analysis

Rat SMCs were starved in DMEM containing no FBS and 5% L-glutamine for 24 hour, and then treated with PDGF-BB or other factors as indicated for 24 hours. Cells were washed with PBS twice, followed by protein extraction using RIPA buffer (50 mmol/liters Tris-HCl, pH 7.4, 1% Triton X-100, 0.25% w/v sodium deoxycholate, 150 mmol/ liter NaCl, 1 mmol/liter EGTA, protease inhibitors (Thermo Scientific), phosphatase inhibitors (Thermo Scientific), and 0.1% SDS). Protein

concentration was measured using BCA Protein Assay Reagent (Thermo Scientific). Equal amounts of proteins were resolved on SDS-PAGE gels and then transferred to PVDF (Bio-Rad) or nitrocellulose membranes (Bio-Rad). Nonspecific bindings were blocked with 5% BSA, and then incubated with primary antibodies in blocking buffer at 4°C for 16 hour, followed by incubation with HRP-conjugated secondary antibody (Sigma) at Room temperature for 1 hour. The protein levels were detected with enhanced chemiluminescence (Millipore). [4]

Rat Carotid Artery Injury Model and Adenoviral Gene Transfer

Rat carotid artery balloon injury was performed using 2F Fogarty arterial embolectomy balloon catheter (Baxter Edwards Healthcare) as described previously.[1, 6] Adenovirus expressing green fluorescent protein (Ad-GFP) or shJAK3 (Ad-JAK3) was introduced into balloon-injured carotid artery by incubation of 100 μl adenovirus (5 x 10^9 pfu) for 20 minutes as described previously.[7] Balloon-injured artery segment was collected at 3, 7, and/or 14 days after the surgery. The segments were perfused with saline, fixed with 4% paraformaldehyde, and then embedded in paraffin for further sectioning and subsequent morphometric analyses in a double-blinded manner.

Histomorphometric Analysis, Immunohistochemistry (IHC), and Immunofluorescent Staining (IF)

Balloon-injured artery sections (5 μm) used for analyses among different groups were evenly distributed in the vessel segment collected.[8] The sections were stained with modified hematoxylin and eosin (HE) or Elastica van Gieson (VG) reagents, and the cross-sectional images were captured using Eclipse 90i Nikon microscope. The circumference of lumen, internal elastic lamina, and external elastic lamina were measured by Image-pro Plus Software. Sections for IHC and IF were rehydrated, blocked with 10% goat serum or donkey serum, permeabilized with 0.01% Triton X-100 in PBS, and then incubated with JAK3, phospho-JAK3, PCNA, α-SMA primary antibody at 4°C overnight followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody for IHC or with FITC- or TRITC-conjugated secondary antibodies for IF. The sections were counterstained with hematoxylin for IHC or DAPI for IF. Negative control of IHC and IF was performed by incubating with Immunoglobulin G (IgG) antibody. Image J software was used to measure the intensity of IHC positive staining by following the previous publication.[9] Mean value of the staining intensity for each group was acquired from 10 artery sections. To quantify the protein level, the mean value of IHC postive signal of each group less the background (negative control) signal was caliberated to the mean vavlue of the staininig intensity in uninjured vessels, in which the background signal was also substracted. The protein level relative to the control

group was shown as a fold increase of the signal intensity that was assessed by the following formula: [(Mean value of IHC staining intensity – Mean value of negative control staining intensity) / (Mean value of IHC staining intensity of uninjured vessels – Mean value of negative control staining intensity)]. The subsequent statistical anaylses were performed as described below.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay

The artery samples were prepared from the serial sectioning of balloon-injured artery, and the in vivo cell apoptosis was evaluated by detecting DNA fragmentation using a TUNEL assay kit (R&D System) by following the manufacturer's instruction.

Statistical Analysis

Results were presented as mean ± S.D. Comparison between two groups was evaluated with two-tailed independent Student's t-test. Comparison among more than two groups was evaluated by one-way ANOVA followed by Fisher's least significant difference (LSD) test. P value < 0.05 was considered as statistically significant.

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