TGFβ1 inhibits pseudoaneurysm formation after aortic patch angioplasty

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Methods

Synthesis and characterization of nanoparticles conjugated patches

Pericardial patches were covalently modified using NP loaded with TGFβ1 and SB431542 as previously described. [1] TGFβ1 (4 μg) and SB431542 (5 mg) were dissolved in PBS (200 μL) and added to chloroform (2 mL) containing carboxylated poly(lactic-coglycolic acid) (PLGA) (100 mg). The mixture was then added drop-wise to 5% polyvinyl alcohol (PVA) (4 mL) and sonicated three times to form NP. The NP were dispersed in 0.2% PVA solution (200 mL) to evaporate the solvent for 2 h while stirring (40 ng TGFβ1/mg NP, 100ng SB431542/mg NP, encapsulation efficiency 10%). Patches (7 mm × 5 mm) were conjugated with the NP using ethyl (dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide (EDC/NHS) chemistry. EDC (20 μ mol/mL) and NHS (10 μ mol/mL) were dissolved in MES solution (0.1 M; pH 5) for the conjugation. Carboxyl groups on the NP were activated using EDC (1 mL) for 30 min and substituted with NHS by adding NHS (1 mL) for 30 min. The solution pH was raised from 5 to 8 using NaOH (1 M). Patches were then placed into the activated NP suspension and incubated at 37 °C overnight. To assess TGFβ1 release from NP patches, the patches were incubated in PBS at 37 °C. The

supernatant was collected at the desired time points and used to quantify TGFβ1 using a Micro BCA Protein Assay Kit (Thermo Fisher Scientific). From the study, sustained release of TGFβ1 was observed for 2-3 weeks.

Mouse lung endothelial cell culture

Mouse lung endothelial cells (MLEC, passage 3-6) were cultured in endothelial basal medium 2 (EBM-2) with endothelial cell growth media-2 MV SingleQuot Kit Supplement & Growth Factors (Lonza), consisting of 20% FBS (Gibco) and 1% penicillin/streptomycin (Gibco), 2 mM L-glutamine (Corning Life Sciences). When MLEC reached approximately 80% confluence, changed the medium to FBS free blank EBM-2 medium for 12 h, then NP and NP- TGFβ1 (5 μmol/ml) and SB431542 (5μmol/ml) were added to the cells in the 6-well plate for 1h at room temperature. After treatment, washed the MLECs by cold PBS twice, then extracted cell lysate for Western blot.

Animal Model

Male Wistar rats (6–8 week old) were used for patch implantation (n=92). Microsurgical procedures were performed as previously described.[2] Briefly, anesthesia was given via isoflurane inhalation, a midline abdominal incision was made, and the infrarenal aorta was exposed. The site of patch implantation was approximately 2 mm below the level of the origin of the renal arteries; the infrarenal aorta was clamped and a longitudinal 3 mm arteriotomy was made on the anterior wall of the aorta. The arteriotomy was closed with a pericardial patch (3 mm × 1 mm × 0.6 mm; Xenosure;

LeMaitre Vascular, Burlington, MA) using interrupted 10-0 nylon sutures. Rats were sacrificed on postoperative 0 h, 1 h, 6 h, 24 h, day 7, or day 30 and the patches and organs explanted for analysis. No immunosuppressive agents, antibiotics or heparin were given at any time. Pseudoaneurysms were noted by gross appearance during specimen harvest and confirmed on histological analysis. Normal aortas were harvested from male Wistar rats (6–8 week old); the normally healing patches, small pseudoaneurysms and large pseudoaneuryms were carefully harvested along with the complete infrarenal aorta.

Histology

Rats were anesthetized with isoflurane inhalation, and tissues were fixed by transcardial perfusion of phosphate buffered saline (PBS) followed by 10% formalin. Tissue was removed and fixed overnight in 10% formalin followed by a 24-hour immersion in 70 percent alcohol. Tissue was then embedded in paraffin and sectioned (5 µm thickness). Tissue sections were de-paraffinized and stained with hematoxylin and eosin. A pseudoaneurysm was defined to be present if there was dilation of the aortic lumen with degeneration of the site of anastomosis of the pericardial patch to the native aorta wall with tissue deposition forming a “pseudo-vessel” wall; this area, in small and large pseudoaneurysms, was compared to the area immediately adjacent to the patch among normally healing aortae. Elastin breaks were compared within the residual native aortic wall among all groups.

Immunohistochemistry

Tissue sections were de-paraffinized using xylene and a graded series of alcohols. For antigen-retrieval, sections were heated in citric acid buffer (pH=6.0) at 100°C for 10 minutes. Non-specific background staining of endogenous peroxidase was treated with 0.3% hydrogen peroxide for 30 minutes, and sections were blocked with 5% normal Bovine serum albumin (BSA) in PBS (pH=7.4) for 1 hour at room temperature. Sections were then incubated using primary antibodies or PBS (for negative controls) overnight at 4°C. After overnight incubation, the sections were incubated with Dako EnVision reagents for 1 h at room temperature and treated with Dako Liquid DAB Substrate Chromogen System (Dako). Finally, the sections were counterstained with Dako Mayer's Hematoxylin. Proliferation and apoptosis indices were determined by counting the number of PCNA- or cleaved caspase-3- positive cells, respectively, and dividing by the total number of cells counted in the high-power field. For cell counts, in normal healing aortae and small pseudoaneurysms, two or three high power fields were assessed within the vessel or pseudoaneurysm wall immediately adjacent to the patch; in large pseudoaneurysms, up to four high power fields were assessed in random sites within the pseudoaneurysm wall. In some of the microphotographs (Figures 1 and 3), dotted lines showing the media-adventitia interface indicate the outer border of the elastin fibers. Image J image processing software was used for analysis of lumen areas as well as α-actin, collagen and TGFβ density (National Institutes of Health; Bethesda, MD).

Immunofluorescence

Tissue sections with rhodamine were de-paraffinized and examined under the immunofluorescence microscope directly. Otherwise, tissue sections were de-paraffinized then heated in citric acid buffer (pH=6.0) at 100°C for 10 minutes; tissues were blocked with 5% bovine serum albumin (BSA) and then incubated with primary antibodies or PBS (for negative controls) overnight at 4°C. To visualize and quantify cells, sections were stained with the fluorescent dye 4', 6-diamidino-2-phenylindole (DAPI; Invitrogen) to mark cellular nuclei.

Western blot

Cells were crushed and mixed with buffer including protease inhibitors (Roche, Complete Mini 12108700) prior to sonication (5 sec) and centrifugation (135000 rpm, 15 min). Equal amounts of protein from each experimental group were loaded for SDS-PAGE, followed by Western blot analysis with signals detected using the ECL detection reagent. Control aortae, normal healing aortae, small pseudoaneurysms, and large pseudoaneurysms were harvested from the animals, with each sample analyzed individually without combination of samples.

Primary and secondary antibodies

Primary antibodies included: anti-α-actin (Abcam, ab5694; IHC and IF, 1:100); anti-cleaved caspase-3 (Cell Signaling #9661; IHC, 1:50; WB, 1:1000); anti-CD68 (ED1; Abcam, ab31630; IHC, 1:200); collagen 1 (Abcam, ab21286; IF and IHC, 1:100), anti-

GAPDH (Cell Signaling, 14C10; WB, 1:2000); anti-IL-10 (Abcam, ab9969; IF, 1:100); vWF (Abcam, ab11713; IF, 1:100), iNOS (Abcam, ab15323; IF, 1:50), TGM2 (Abcam, ab421; IF, 1:100), IL-10 (Abcam, ab9969; IF:1:100), anti-proliferating cell nuclear antigen (PCNA) (Dako monoclonal mouse Anti-PCNA; IF, 1:100), phosphor-smad2 (Millipore, AB3849; IF and IHC, 1:50; WB, 1:500), phopho-TAK1 (Abcam, ab109404; WB, 1:500), TAK (ThermoFisher, PA5-20083; IF, 1:50; WB, 1:500), TGFβR1 (Santa Cruz, SC-9048; IF, 1:50; WB, 1:1000), anti-transglutaminase 2 (TGM2; #37557; IF, 1:100); TGF β1 (Santa Cruz, SC-31609; IF, 1:50).

Secondary antibodies used for IF were: donkey anti-goat Alexa-Fluor-488, donkey anti-rabbit Alexa-Fluor-488, donkey anti-rabbit Alexa-Fluor-568, donkey anti-mouse Alexa-Fluor-568 and chicken anti-mouse Alexa-Fluor-488 conjugated antibodies from Invitrogen (1:1000). For IHC, sections were incubated with EnVision reagents for 1 h at room temperature and treated with Dako Liquid DAB+ Substrate Chromogen System (Dako). Finally, the sections were counterstained with Mayer's hematoxylin.

Statistical analysis

Data are expressed as the mean ± SEM. Values conformed to Gaussian distributions as determined by the Shapiro-Wilk normality test. Statistical significance for data analyses was then determined by t-test or ANOVA with post-hoc testing performed by the Tukey's multiple comparisons test. P-values less than 0.05 were considered significant. Data were analyzed using Prism 7.0 software (GraphPad Software; La Jolla, CA).

Study Approval

All experiments were approved by the Institutional Animal Care and Use Committee at the Yale University School of Medicine. All experiments were carried out in accordance with the approved guidelines.

References

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2. Bai H, Li X, Hashimoto T, Hu H, Foster TR, Hanisch JJ, Santana JM, Dardik A. Patch angioplasty in the rat aorta or inferior vena cava. *J Vis Exp*. 2017;120:e55253-e55258.