Activation of Wnt/β-catenin signaling in abdominal aortic aneurysm: A potential therapeutic opportunity?

Methods

Human aortic samples

Human samples were obtained from patients undergoing open surgical repair of AAA at the Hospital de la Santa Creu i Sant Pau (HSCSP; Barcelona, Spain). Additionally, healthy aortas from multi-organ donors were included, as previously described.[1-3] Control samples showed no evidence of AAA, atherosclerotic lesions or other abnormalities that could affect the study. Abdominal aorta samples were collected following strict ethical guidelines. The use of human discarded tissue was approved by the HSCSP Ethics Committee (IIBSP-NET-2019-40) and conducted according to the Declaration of Helsinki. Further, a written informed consent was obtained from patients and control individuals or from their legal representatives. Aortic samples were rapidly stored at -80 °C for subsequent RNA extraction or processed adequately for immunohistochemical analysis.

In vivo studies

The study was carried out in 12 weeks-old male apolipoprotein-E-deficient mice (*Apoe*$^{-/-}$; B6.129P2-Apoe$^{tm1Unc/J}$) that were housed at the Animal Experimentation Unit (Institut de Recerca HSCSP, Barcelona, Spain). To induce AAA, Angiotensin II (AngII) was subcutaneously infused (1000 ng/kg body weight [BW]/min; Sigma-Aldrich, St Louis, MO, USA) via osmotic micropumps (model 1004, Alzet; Durect Corporation, Cupertino, CA, USA) for 28 days, as described.[1-4] Mice were randomized distributed in four groups: AngII-infused mice, AngII-infused mice treated daily by oral gavage with the porcupine inhibitor LGK974 (Xcess Biosciences Inc., San Diego, CA, USA) at 5 mg/kg/day, AngII-infused mice receiving a daily intraperitoneal injection of PRI-724 at 15 mg/Kg/day and saline-infused control mice receiving vehicle. Dosage regimens were chosen based on previous studies.[5,6] Drugs administration started 24 h before micropump implantation. After four weeks, mice were deeply anesthetized via intraperitoneal injection with ketamine (150 mg/kg) and medetomidine (1 mg/kg) and euthanized by thoracotomy. Harvested aortas were examined to establish the presence and severity of AAA (see below) and appropriately processed for further analysis.

Non-invasive measurement of systolic blood pressure

Systolic blood pressure was non-invasively determined in conscious mice prior to and weekly throughout the experimental period using the tail-cuff plethysmography method

(CODA® tail-cuff blood pressure system; Kent Scientific Corporation; Torrington, CT, USA) as reported.[1-4]

Ultrasound assessment of abdominal aortic diameter

Abdominal aortic diameter was measured by ultrasound imaging before AngII infusion and weekly until the end of the experimental protocol using a Vevo 2100 ultrasound with a 30 MHz transducer applied to the abdominal wall (VisualSonics, Toronto, ON, Canada), as previously described.[1-4] The severity of the aneurysm was established from both ultrasonographic information and visual examination, based on the Manning scale[7]: type 0, no aneurysm; type I, dilated lumen in the suprarenal region of the aorta with no thrombus; type II, remodeled tissue in the suprarenal region that frequently contained thrombus; type III, a pronounced bulbous form of type II that contained thrombus, and type IV, a form in which there are multiple AAAs containing thrombus.

Basic Measurements of cardiac function by echocardiography (M-mode and Doppler)

Anesthetized mice (2% isoflurane inhalation) were subjected to a transthoracic echocardiography, using a Vevo 2100 ultrasound with a 30 MHz transducer (VisualSonics).[2,8] Two-dimensional and M-mode images were obtained in parasternal long-axis and short-axis views, respectively. The following parameters were recorded: heart rate (HR), end-diastolic interventricular septum thickness (IVSd), left ventricular (LV), LV end-diastolic posterior wall thickness (LVPWd), LV anterior wall thickness at diastole (LVAWd), LV end-diastolic internal diameter (LVEDD) and LV end-systolic internal diameter (LVESD). LV mass, LV ejection fraction (LVEF) and LV fractional shortening (LVFS) were determined. LV stroke volume and cardiac output (CO) were calculated:

$$\text{LV stroke volume } (\mu L) = \text{LV end-diastolic volume} - \text{LV end-systolic volume}$$

$$\text{CO } (mL/\ min) = (\text{LV stroke volume} \times HR) / 1000$$

Real-time PCR

Total RNA was isolated from both mouse and human aortas using TriPure Isolation Reagent (Roche Diagnostics, Mannheim, GE) according to the manufacturer's instructions. A total of 500 ng of RNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantification of mRNA levels was carried out by real-time PCR using an ABI PRISM 7900HT sequence detection system (Applied Biosystems). Specific primers

and probes provided by the Assay-on-Demand system (Applied Biosystems) or Integrated DNA Technologies (Coralville, IA, USA) were used. Relative mRNA levels were determined using the $2^{-\Delta\Delta Ct}$ method.

Western blotting

Lysates were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Immobilon, Merck-Millipore; Burlington MA, USA, IPVH00010). Membranes were incubated overnight with an antibody against non-phospho (Active) β-Catenin (Ser33/37/Thr41) (D13A1; Cell Signalling Tech, Danvers, MA, USA) or an anti-β-actin antibody (A5441, Sigma-Aldrich). Bound antibodies were detected using the appropriate horseradish peroxidase-conjugated secondary antibodies (Dako Products, Agilent, Santa Clara, CA, USA) and the Luminata™ Western HRP Substrate (Immobilon, Merck-Millipore).

Histological and immunohistochemical analysis

Harvested arteries were fixed in 4% paraformaldehyde/0.1 M PBS (pH 7.4) for 24 hours, embedded in paraffin and cut. Tissue sections (5 µm-thick) were deparaffinized, rehydrated and blocked. Inmunohistochemistry was carried out with an anti-active-β-catenin antibody (clone 8E7; Sigma-Aldrich). Color was developed by incubation with 3,3'-diaminobenzidine (DAB) and then sections were counterstained with haematoxylin. Samples in which the primary antibody was omitted were used as negative controls. The histological characterization of aortic samples was carried out by haematoxylin-eosin staining.

Statistical Analysis

Results are shown as mean ± standard error of the mean (SEM) or boxplots. In this latter case, the upper and lower boundaries of each box display 75% and 25% percentile of data, respectively; the median is indicated by a horizontal line and the minimum and maximum values as whiskers. Significant differences were analysed using one way ANOVA, two-way ANOVA with repeated measures or two-way ANOVA followed by Bonferroni's post-hoc tests. When the distribution of data failed the D'Agostino-Pearson omnibus normality test, the Mann-Whitney U test with the Tukey's post-hoc test or the Kruskal-Wallis test with Dunn's multiple comparison post-hoc test were applied. Differences in AAA incidence were analyzed by the $\chi 2$ test. Data were analyzed with the GraphPad Prism version 6.01. Differences were considered significant at $p < 0.05$.

References

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