SUPPLEMENTAL MATERIAL

Detailed Materials and Methods

Generation of double knockout (*ApoE*$^{-/-}$*Opg*$^{-/-}$) mice

Osteoclastogenesis inhibitory factor (osteoprotegerin, *Opg*) gene-knockout mice on a C57 black 6/J background (*ApoE*$^{+/+}$*Opg*$^{-/-}$) were obtained from CLEA Japan Inc [1]. *Opg*-deficient apolipoprotein E-deficient (*ApoE*$^{-/-}$*Opg*$^{-/-}$) mice were generated by mating the respective null homozygotes with *ApoE*$^{-/-}$*Opg*$^{+/+}$mice (B6.129P2-Apoe tm1Unc/Arc (N10)) sourced from the Animal Resources Centre, Canning Vale, Australia. Resultant double-heterozygous progenies were then inter-crossed and subsequent generations typed to identify and establish homozygous double deficient mouse lines. DNA extracted from ear clippings was amplified on an Applied Biosystems Veriti thermal cycler using forward 5'- GGT CCT CCT TGA TTT TTC TAT GCC-3'and reverse 5'- TGC CCT GAC CAC TCT TAT ACG GAC-3' primers to identify the wild type allele and forward 5' GCT GCA TAC GCT TGA TCC GGC-3'and reverse 5'- TAA AGC ACG AGG AAG CGG TCA-3' primers to detect the *Opg*-null allele (both primer pairs amplifying at 94°C 30 sec, 62°C 30 sec and 72°C 30 sec). *ApoE*-null allele was identified using the forward 5'-CGA AGC CAG CTT GAG TTA CAG AA-3' and reverse 5'-AGA GCC GGA GGT GAC AGA TCA G-3' primer set (amplifying at 96°C 60 sec, 60°C 60 sec and 72°C 3 min). 5µl of the PCR products were loaded on a 1% agarose gel pre-stained with Gel-Red (Biotium, CA) and electrophoresis performed for 45min at 100V. PCR products were visualised by ultraviolet transillumination (Supplementary Figure I).

Mouse model of AAA and *in vivo* studies

Approval for animal studies was obtained from the local ethics committee and experimental work performed in accordance with the institutional and ethical guidelines of James Cook University, Australia, and conforming to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, USA). Mice were housed in an individually-ventilated, temperature/humidity-controlled cage system (Aero IVC Green Line; Tecniplast) on a 12-hour light/dark cycle, and maintained on normal laboratory chow and water *ad libitum*. The angiotensin II (Ang2)-infusion model was implemented as previously described [2]. Briefly, an osmotic micro-pump (ALZET Model 1004, Durect Corporation, USA) containing Ang2 (Sigma-Aldrich) dissolved in sterile water was inserted into the subcutaneous space left of the dorsal midline under anaesthesia (4% isoflurane inhalation) to administer Ang2 or H_20 vehicle at a rate of 1.0 µg/kg/min over the experimental period. Three separate animal studies were performed:

1) Ang2-induced aortic dilatation in *Opg*-deficient C57BL/6 (*ApoE*$^{+/+}$*Opg*$^{-/-}$) mice. Thirteen week old male *ApoE*$^{+/+}$*Opg*$^{+/+}$ and *ApoE*$^{+/+}$*Opg*$^{-/-}$ mice (n=30 per group), were infused with Ang2 over 28 days, after which regional aortic diameters (arch, thoracic aorta (TA), suprarenal aorta (SRA), infrarenal aorta (IRA)) were determined by morphometric analysis following dissection.
2) Effect of Opg deficiency on aortic dilatation in Ang2-infused ApoE$^{-/-}$ mice. Six month old male *ApoE*$^{-/-}$*Opg*$^{+/+}$ (n=18) and *ApoE*$^{-/-}$*Opg*$^{-/-}$ mice (n=17) were infused with Ang2 for 28 days. In vivo dilatation of the SRA was monitored by ultrasound at 14-day intervals, while maximum aortic arch, TA, SRA, and IRA diameters were determined at harvest or fatality by morphometric analysis.
3) Effect of Opg deficiency on early event mechanisms within the aorta of Ang2-infused ApoE$^{-/-}$ mice. Six month old male *ApoE*$^{-/-}$*Opg*$^{+/+}$ and *ApoE*$^{-/-}$*Opg*$^{-/-}$ mice (n=6 per group) were infused with Ang2 for seven days, after which whole aortas were processed for mRNA and protein analysis.

Measurement of mouse aortic diameters

Maximum diameters of aortic arch, TA, SRA, and IRA (Supplementary Figure II) were determined by morphometric analysis (Study 1 & 2) while *in vivo* dilatation of the SRA was monitored at 14 day intervals by ultrasound (Study 2) as previously described [2]. Necropsy was performed within 24 hours of sudden mouse fatality to confirm aortic rupture as cause of death. Phosphate buffered saline-perfused aortas were harvested from mice completing the study protocol or that died during the study and were placed on a graduated template and digitally photographed (Coolpix 4500, Nikon). Maximum diameter of the arch, TA, SRA, and IRA were determined from the images using computer-aided analysis (Adobe® Photoshop® CS5 Extended version 12, Adobe Systems Incorporated). Ultrasound measurements of the SRA were obtained prior to Ang2 infusion (base-line) and at days 14 and 28 post pump insertion. Scans were performed in sedated mice (i.p., 40 mg/kg ketamine, 4 mg/kg xylazine) using a MyLabTM 70 VETXV platform (Esaote, Italy) with a 40 mm linear transducer at an operating frequency of 10 MHz (LA435; Esaote, Italy) to provide a sagittal image of the SRA. Maximum SRA diameter was measured at peak systole using the calliper measurement feature. Good inter-observer reproducibility of morphometric and ultrasound analysis has been demonstrated previously in our laboratory [3,4]

Cytokine/chemokine assay of mouse serum

Serum samples collected after seven days of Ang2 infusion (Study 3) were assessed using a multiplex quantitative ELISA-based assay (Q-Plex™, Quansys Biosciences). Q-Plex™ technology is based on placement of immobilized capture antibody in 350–500 μm spots at the bottom of polypropylene 96-well plates to capture target proteins (for more details see http://www.quansysbio.com/assay-development/). Each spot is printed with a different analyte capture antibody, in this case against IL-1α, IL-1β, Il-2, IL-3, IL-4, IL-6, IL-10, IL-12, IL-17, MCP-1, TNFα, MIP-1α, GMCSF, and RANTES. Two internal control assays are designed within the assay to ensure integrity of results from each well. The Q-Plex™ multiplex array system has been previously validated as an alternative to individual immunoassays, with acceptable inter assay variability and very sensitive levels of detection [5,6,7]. Analyte concentration was quantified against known standards by densitometry using the ChemiDoc™ imaging system (Bio-Rad Laboratories) supported by QuantityOne™ 1-D Analysis Software (Bio-Rad Laboratories), and expressed as pg/ml.

Preparation of mouse aortic samples

Simultaneous isolation of nucleic acids and protein from whole aortas was performed using TRIzol® Reagent (Sigma-Aldrich) as per the manufacturer's instructions. Total RNA and protein samples were prepared for real time PCR and protein analysis (TaqMan® Protein Assay and zymography), respectively.

Real time PCR

QuantiTect® Primer Assays were used to determine gene expression for *Mmp2* (QT00116116), *Mmp9* (QT00108815), *Ctss* (QT00102116), *Pparg* (QT00100296), *Nfκb* (QT00154091), *Bax* (QT00102536), *Bcl2* (QT00156282), *Cnn1* (QT00105420), Mapk1 (QT00133840), *Mapk3* (QT00103355), and *Mapk14* (QT00161945) in mouse aortas using quantitative real time (qPCR) as previously described [8]. The relative expression of these genes in aortas of experimental and control animals was calculated by using the concentration-Ct-standard curve method and normalized using the average expression of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*; QT01658692) for each sample using the Rotor-Gene Q operating software version 2.0.24. *Gapdh* was chosen as the 'housekeeping' gene since analyses showed its expression to be similar in categories assessed. The QuantiTect SYBR® Green one-step RT-PCR Kit (Qiagen) was used according to the

manufacturer's instructions with 40ng of total RNA as template. All reactions were independently repeated in duplicate to ensure the reproducibility of the results.

TaqMan® Protein Assay

CTSS protein in mouse aortic tissue was quantified using the TaqMan® Protein Assay (TPA, Applied Biosystems) [9]. Polyclonal antibodies to cathepsin S (ab18822, Abcam) and β-Actin (4967, Cell Signaling Technology) were biotinylated using the Lightning-Link™ Biotin Conjugation Kit (Innova Biosciences) prior to oligonucleotide labelling with the 'TaqMan® Protein Assay Open Kit' (Applied Biosystems) as per manufacturer's instructions. The protein assay was performed using the 'TaqMan® Protein Assay Core Reagents Kit' with 'Master Mix' (Applied Biosystems) according to manufacturer's instructions. Real-time quantitative PCR was performed using the Rotor-Gene Q operating software version 2.0.24. Relative protein expression between control and experimental samples was calculated by using the concentration-Ct standard curve method and normalized using the average expression of β-actin for each sample. Each assay included a no-protein control (NPC) to calculate ΔCt values (Ct value (sample) - Ct value (NPC)), a linear range generated for each sample, and a ΔCt threshold was designated. The fold change between samples was calculated between the crossover points of each linear trend line at the ΔCt threshold.

Zymography

Measurement of MMP2 and MMP9 in mouse aortic tissue using gelatin zymography was performed as previously described [10]. Extracted protein from mouse aortas was separated at room temperature on a 10% acrylamide-SDS gel containing 0.5% gelatin. Following several washes in 2.5% (vol/vol) Triton X-100, gels was incubated for 12 hours at 37°C in 50 m MTris (pH 8) containing 5 mM CaCl2. Bands were visualized in a 10% ethanol/10% acetic acid solution after staining with 0.5% Coomassie blue (R-250), with enzyme activity semi-quantified with densitometric analysis using the ChemiDoc™ imaging system (Bio-Rad Laboratories) and QuantityOne™ 1-D Analysis Software (Bio-Rad Laboratories).

Cathepsin S activity assay

Measurement of CTSS activity within mouse aorta was performed using a commercial assay (K144-100; Biovision) as per manufacturer's instructions. The fluorescence-based activity assay utilized the preferred CTSS substrate sequence Val-Val-Arg (VVR) labelled with amino-4-trifluoromethyl coumarin (AFC). Free AFC cleaved from the synthetic Z-VVR-AFC substrate was detected using a POLARStar Omega™ fluorescence plate reader (BMG Labtech) at 400 nm excitation and 505 nm emission and used as a quantitative measure of CTSS activity expressed as change in fluorescence units (ΔFU) per minute per μg protein.

Cell culture

Healthy human aortic vascular smooth muscle cells (AoSMC; Clonetics® human aortic SMC, Lonza) and AASMC isolated from human AAA biopsies (n=6) by combined collagenase and elastase digestion [11] were seeded at $1x10^5$ cells/ml into separate cultures and maintained in DMEM+10% FBS at 37°C, 5% CO_2. Culture supernatants and cell lysates were harvested at confluency or after a maximum of 96 hours and assayed by ELISA for secreted and cellular CTSS, respectively. In a separate study, AASMC and AoSMC were seeded separately at $1x10^5$ cells/ml and maintained in DMEM+10% FBS at 37°C, 5% CO_2 to ~80% confluency. Following 24 hours in low-serum (1%) medium, cells were refreshed with phenol red-free DMEM+10% FBS and incubated at 37°C, 5% CO_2 in the presence of recombinant human (rh)Opg (Enzo Life Sciences; 50 nM, n=6), rhOpg + Ctss inhibitor (Z-FL-COCHO, EMD Millipore; 20 nM), or media alone (control). Cells were harvested after 36 hours and assayed for elastase activity.

Assessment of elastase activity

Elastase activity in cell lysate was assessed using a commercial assay (EnzChek® Elastase Assay; Molecular Probes) as per manufacturer's instructions. The fluorescence-based activity assay utilized soluble bovine neck ligament (DQ™) elastin labelled with BODIPY® FL dye. Fluorescent fragments released upon elastolytic digestion were detected using a POLARStar Omega™ fluorescence plate reader (BMG Labtech) at 505 nm excitation and 515 nm emission and used as a quantitative measure of elastase activity expressed as fluorescence units per minute per μg protein.

ELISA for CTSS

Cell culture samples were assayed using the Total CTSS DuoSet ELISA systems (DY1183; R&D Systems) as per manufacturer's instructions. Use of these assays previously in our laboratory have demonstrated excellent recovery and intra- and inter-assay reproducibility [12,13]. Sample optical density was measured using a Sunrise™ absorbance plate reader (Tecan) at 450 nm. Assay detection limits for CTSS was 16-1000 pg/ml. Concentrations were expressed as pg/mg protein.

Statistics

Data were analysed using GraphPad Prism (version 6) and TIBCO Spotfire S+ (version 8.2). Parametric or non-parametric tests were applied appropriate to distribution of data. Comparison of cell culture end-point data was performed using Mann-Whitney *U* test. For mouse studies, aortic end-point data for maximum diameter, MMP2/9/CTSS protein, and mRNA expressions, were compared between *ApoE*$^{-/-}$*Opg*$^{-/-}$ and *ApoE*$^{-/-}$*Opg*$^{+/+}$ mice by Mann-Whitney *U* test. Comparison between baseline and end-point serum cytokine concentrations was performed within each mouse group using paired-t test, while median fold-increase in concentrations of individual cytokines was compared between *ApoE*$^{-/-}$*Opg*$^{-/-}$ and *ApoE*$^{-/-}$*Opg*$^{+/+}$ mice by Mann-Whitney *U* test. Mouse data obtained as a function of time, i.e. blood pressure and ultrasound of SRA diameter, was compared within each group by repeat measures one-way ANOVA, and between *ApoE*$^{-/-}$*Opg*$^{-/-}$ and *ApoE*$^{-/-}$*Opg*$^{+/+}$ mice by mixed-effects linear regression. Kaplan-Meier survival curves were analysed using log-rank (Mantel-Cox) test. In all cases P values less than 0.05 were considered significant.

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