Supplemental Material

Intramyocardial VEGF-B_{167} gene delivery delays the progression towards congestive failure in dogs with pacing-induced dilated cardiomyopathy

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Short title: VEGF-B_{167} in dilated cardiomyopathy

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Methods

Surgical instrumentation

Dogs were sedated with acepromazine maleate (1 mg/kg im), anesthetized with pentobarbital sodium (25 mg/kg iv) and ventilated with room air. A thoractotomy was performed in the fifth intercostals space. One fluid-filled Tygon catheter was inserted into the descending thoracic aorta and one in the left atrial appendage, while a silicon catheter was placed in the coronary sinus. A solid-state pressure gauge (Konigsberg Instruments, Pasadena, CA) was inserted into the LV through the apex. A Doppler flow transducer (Craig Hartley, Houston, TX) was placed around the left circumflex coronary artery, and two myocardial pacing leads were attached to the LV free wall. Two pairs of piezoelectric crystals were implanted in the mid-myocardium of the LV free wall, orthogonal to the ventricular long axis, 10-15 mm apart, to assess regional circumferential shortening. The viral vector was injected by the surgeon in a blind fashion. Wires and catheters were run subcutaneously to the intrascapular region and the chest was closed in layers. Antibiotics were given after surgery, and the dogs were allowed to fully recover for 7–10 days, then trained to lay quietly on the laboratory table.

Hemodynamics, LV regional shortening and echocardiographic recordings

Hemodynamic parameters were recorded on paper and also stored in computer memory at a sampling rate of 250 Hz. The piezoelectric crystals were connected to a sonomicrometer to measure cyclic changes in the segmental length. The best functioning pair of crystal was identified at baseline, before starting the pacing protocol, and then utilized for all the measurements from that point on. The segmental length was utilized to calculate percent segmental shortening and the area of the LV pressure-segment length loop, a surrogate of regional stroke work. These values served as indices of regional contractile function. Two-dimensional and M-mode echocardiography was performed (Sequoia C256; Acuson, Mountain View, CA) to measure ejection fraction, LV dimensions and wall thickness. Images were obtained from a right parasternal approach at the midpapillary muscle level, according to the criteria of the American Society of Echocardiography. Hemodynamics and echo were recorded and analyzed in a non blinded fashion. However, hemodynamics is

unequivocally quantifiable and objectively verifiable. On the other hand, in our Department, we have at least three experienced echocardiographists, and they customarily cross-evaluate their respective measurements.

Myocardial blood flow and oxygen consumption

Left circumflex coronary blood flow was measured with a pulsed Doppler flowmeter (Model 100, Triton Technology).

Absolute myocardial flow was measured by injecting $5x10^6$ of stable isotope-labeled 15 μm microspheres (Biopal, Worcester, MA) through the left atrial catheter, both at spontaneous heart rate and during pacing, while withdrawing blood reference samples from the aortic catheter. The neutron-activated isotopes contained in those microspheres allowed us to measure myocardial blood flow in selected regions of the ventricular wall, a method previously used by us (1)

Blood gases were measured in a blood gas analyzer (Instruments Laboratory, Bedford, MA). PO_2 was multiplied by 0.003 and added to O_2 content measured by a hemoglobin analyzer (CO-Oximeter; Instrumentation Laboratory, Bedford, MA) to obtain total oxygen content (vol/vol). Myocardial oxygen consumption (MVO_2) was calculated by multiplying the arterial-coronary sinus difference in total oxygen content by mean coronary blood flow.

Histology and immunofluorescence

To determine alterations in microvascular density, endothelial cells were detected using FITC-conjugated Lycopersicum esculentum lectin (Vector Laboratories) and vascular smooth muscle cells using a Cy3-conjugated anti α-SMA mouse monoclonal antibody (1A4, Sigma). Nuclei were counterstained with DAPI. All microvessels were normalized by the number of cardiac fibers.

Apoptotic cells were visualized by the TdT-mediated dUTP nick end labeling (TUNEL) assay on frozen sections (5 μm thick), using the in situ cell death detection kit, TMR red (Roche Diagnostics) according to the manufacturer's instructions. Anti-α-sarcomeric actinin monoclonal antibody (EA-53) 1:100 (Abcam, Cambridge MA, USA) was used to identify cardiomyocytes. The number of apoptotic cardiomyocytes was calculated as the percentage of the TUNEL positive per 1000 total cardiomyocyte nuclei. Images were analyzed using MetaView 4.6 quantitative analysis software (MDS Analytical Technologies, Toronto, Canada).

Finally, cardiomyocyte cross-sectional area was quantified from photomicrograph of FITC-conjugated Lycopersicum esculentum lectin stained paraffin histological sections, using ImageJ software (NIH). We have used these methods previously (2).

PCR, ELISA and Western blotting

Total DNA was extracted from LV tissue by proteinase-K digestion and phenol/chloroforme extraction. Real-time PCR amplification of AAV genomic DNA was performed on 100 ng of total DNA using primers and TaqMan probe designed to specifically recognize sequences on the CMV promoter present in all the AAV vectors used in this study.

Total RNA was extracted from LV tissue using TRIzol reagent (Invitrogen) according to manufacturer instructions, DNAse I digested and reverse transcribed using hexameric random primers. The cDNA was then used as template for real-time PCR amplification of the murine VEGF-B_{167} (AAV-carried transgene) and dog VEGF-B_{167}, VEGF-A_{165}, VEGFR-1, VEGFR-2 and Neuropilin-1 trancripts. Both TaqMan

technology and Syber Green incorporation were used in real time reactions. Gene expression was normalized by hypoxanthine phosphoribosyl - transferase (HPRT). The sequences of the primers used are:

CMV DNA:	Forward: 5'-TGGGCGGTAGGCGTGTA -3', Reverse: 5'-GATCTGACGGTTCACTAAACGAG-3'
Mouse VEGF-B_{167}:	Forward: 5'-TTGCACTGCTGCAGCTGGCTC-3', Reverse: 5'-GCTGGGCACTAGTTGTTTGA-3'
Canine VEGF-B_{167}:	Forward: 5'-AGAAGAAAGTGGTGCCATGG-3', Reverse: 5'-GCTGGGCACTAGTTGTTTGA-3'
Canine VEGF-A_{165}:	Forward: 5'- AACCCTGGAGCGTTCCCTGT -3', Reverse: 5'- ACCGCCTGGGCTTGTCACAT-3'
Canine VEGFR-1:	Forward: 5'- AAATCTGCCTGTGGAAGGAATG -3' Reverse: 5'- GCCTGAGCCATGTTCAAGGT -3' Probe: FAM-AACAGTTCTGCAGTA
Canine VEGFR-2:	Forward: 5'- GGAACCGGAACCTCACCAT -3' Reverse: 5'- GGCAGGTGTAGAGGCCTTCA -3' Probe: FAM- CGTAGGGTGAGGAAG
Canine neuropilin-1:	Forward: 5'- GTCAGAGATTATCCTGGAATTTGAAAG-3' Reverse: 5'- GGCAGGTGTAGAGGCCTTCA -3' Probe: FAM- CGTAGGGTGAGGAAG
Canine Hprt:	Forward: 5'- TCATTACGCTGAGGATTTGG -3' Reverse: 5'- AGAGGGCTACGATGTGATGG -3'

Dog serum samples were analyzed by ELISA for the presence of mouse VEGF-B_{167} secreted in the circulation from the transduced heart tissue. A specific commercially available polyclonal antibody (R&D) against mouse VEGFB167 was diluted at the concentration of 100 ng/ml and added to 96-well plates coated overnight at 4°C with 50 μl of undiluted or 1:50 diluted dog serum. Serial concentration, from 0,01 to 50 ng/ml, of recombinant mouse VEGFB167 was used as control standard. Plates were blocked for 2 h at RT with PBS containing 5% BSA. After overnight incubation and extensive washing with PBS containing 0.05% Tween 20 plates were incubated for 2hr at room temperature with an anti-goat HRP-labeled detection antibody. Following addition of 3, 3′,5 ,5′-Tetramethylbenzidine (TMB) substrate (Fisher Scientific), absorbance was measured at 450 nm using a fluorescence reader.

Western blot was utilized to quantify the activated/cleaved caspase-3 and to determine the activation state of Akt, and of its two targets GSK-3β and FoxO3a. The constitutive form of heat shock protein of 70 kDa (HSC70) was used for loading control. Eighty μg of proteins were resolved on SDS-PAGE minigels and transferred to nitrocellulose membranes (GE Healthcare), which were incubated with the following primary antibodies (dilution 1:1000) overnight at 4°C: rat monoclonal

HSC70 (SPA-815' StressGen Assay Designs), rabbit polyclonal anti-Akt, rabbit polyclonal anti-pAkt (Thr 308), rabbit polyclonal anti-pGSK-3β (Ser 9), rabbit polyclonal anti cleaved-caspase 3, rabbit polyclonal anti-pFoxO3a (Thr32), rabbit polyclonal anti-Foxo3a (all from Cell Signaling) and rabbit polyclonal anti-GSK-3β (Calbiochem).

Cultured cardiac myocytes

Neonatal cardiomyocytes were prepared as follows. 1-3 day neonatal rat ventricles were disaggregated by mechanical dissection followed by repeated digestions in HBSS using 0.06% Collagenase (IV) (Worthington Biochemical, Lakewood NJ) and 0.1 % trypsin. Following repeated digestion cells were centrifuged at 300 x g for 10 min and differentially plated to remove fibroblasts. Myocytes were plated at a density equivalent to 6-8 x 10^{-6} cells/100-mm plate in DMEM/F12 (10%FBS, P/S, 0.1mM 5'-bromo-2'deoxyuridine). On the day following preparation the media was removed and fresh media added DMEM/F12, (5% HS, P/S) (3-4).

Apoptosis was determined by flow cytometry (Millipore/Guava Easycyte Mini) using the Guava TUNEL Assay (Millipore) according to the manufacturer's guidelines. Acspase-3 and -9 activities were measured using a Colorimetric Activity Assay kit, according to the manufacturer's instructions (Chemicon International). Optical Density values were normalized to the sample protein concentration (5-6)

Ang II-induced mitochondrial $O_2^{\cdot-}$ production was assessed by flow cytometry using MitoSOX Red (Invitrogen, Carlsbad CA), a mitochondrion-specific hydroethidine-derivative fluorescent dye, as previously reported (5-7). Cell debris (low forward and side scatter), dead cells (Sytox Green and annexin V positive) and apoptotic cells (annexin V positive) were gated out for analysis. The data is presented as fold change in the median intensity of MitoSOX fluorescence when compared with the respective controls. To demonstrate the localization of MitoSox signal to mitochondria, the cells were counterstained with MitoTracker green and visualized by confocal microscopy. In separate experiments, the effects of VEGF-B_{167} pretreatment on cardiomyocyte peroxide production was measured fluorometrically using the C-H_2DCFDA fluorescence assay, as reported (5). Changes in mitochondrial membrane potential were detected by flow cytometry using the cationic carbocyanine dye 5,5',6,6'tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide (JC-1, Invitrogen) according to the manufacturer's guidelines.

References

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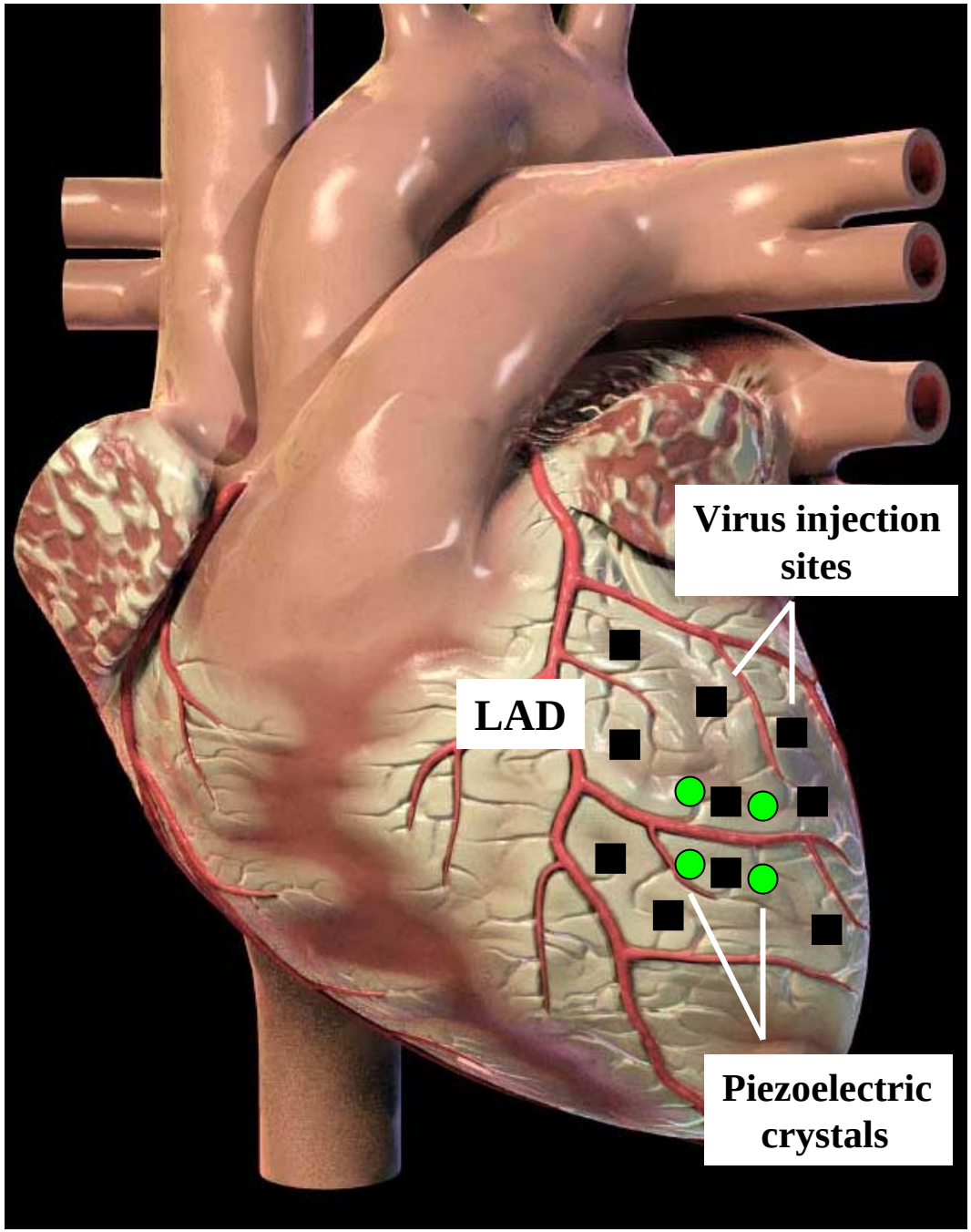
Online Figures

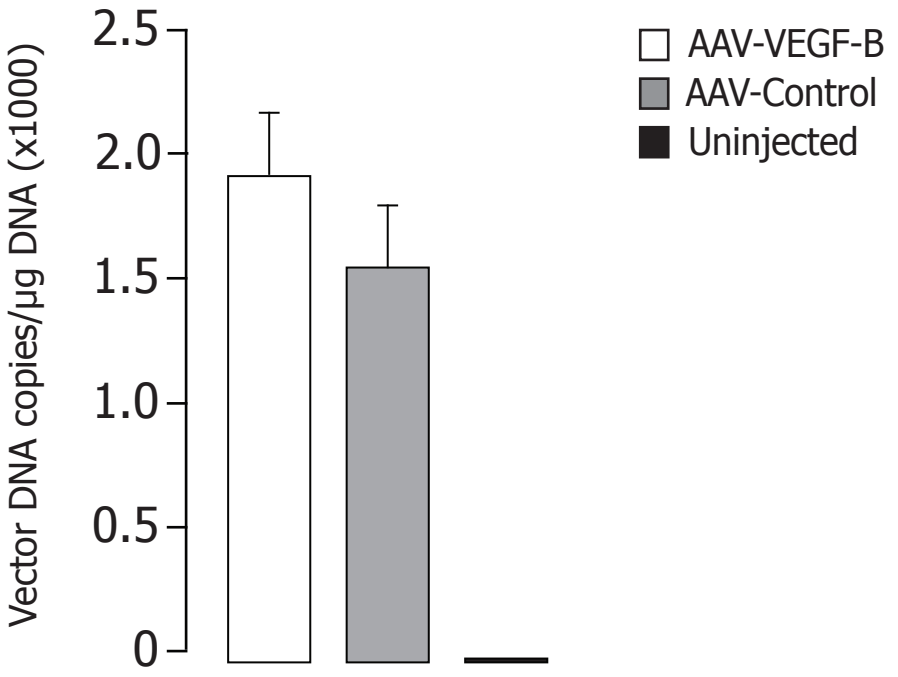
Figure I. Predefined map for AAV intramyocardial injections.
Three injections were performed along the first diagonal of the left anterior descending (LAD) coronary artery and 7 along the second diagonal. The vector was never injected distal to the third diagonal. Two sites of injection corresponded to the space between the implanted piezoelectric crystals.

Figure II. Levels of AAV genomes and AAV-mediated transgene expression in myocardium.
Upper panel: the amount of AAV genome was not significantly different between AAV-VEGF-B and AAV-Control LV myocardium at the injection site. n= 5 per group.
Lower panel. The murine VEGF-B167 transgene transcript was clearly expressed in the injection site and detectable also in the LV remote site, although the level was approximately 85% lower. n= 5 per group.

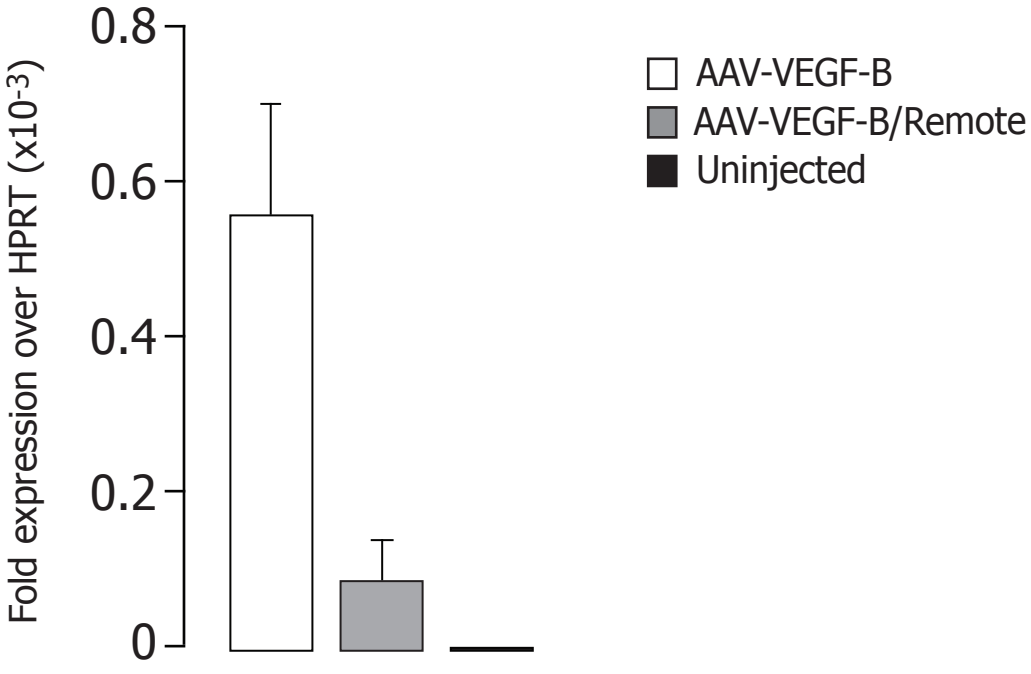


Online Figure I

AAV genomes



AAV transgene mRNA



Online Figure II