JOURNAL OF THE AMERICAN COLLEGE OF CARDIOLOGY
© 2015 BY THE AMERICAN COLLEGE OF CARDIOLOGY FOUNDATION
PUBLISHED BY ELSEVIER INC.
VOL. 66, NO. 2, 2015
ISSN 0735-1097/$36.00
http://dx.doi.org/10.1016/j.jacc.2015.04.071

Intracoronary Cytoprotective Gene Therapy

A Study of VEGF-B_{167} in a Pre-Clinical Animal Model of Dilated Cardiomyopathy

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ABSTRACT

BACKGROUND Vascular endothelial growth factor (VEGF)-B activates cytoprotective/antiapoptotic and minimally angiogenic mechanisms via VEGF receptors. Therefore, VEGF-B might be an ideal candidate for the treatment of dilated cardiomyopathy, which displays modest microvascular rarefaction and increased rate of apoptosis.

OBJECTIVES This study evaluated VEGF-B gene therapy in a canine model of tachypacing-induced dilated cardiomyopathy.

METHODS Chronically instrumented dogs underwent cardiac tachypacing for 28 days. Adeno-associated virus serotype 9 viral vectors carrying VEGF-B_{167} genes were infused intracoronarily at the beginning of the pacing protocol or during compensated heart failure. Moreover, we tested a novel VEGF-B_{167} transgene controlled by the atrial natriuretic factor promoter.

RESULTS Compared with control subjects, VEGF-B_{167} markedly preserved diastolic and contractile function and attenuated ventricular chamber remodeling, halting the progression from compensated to decompensated heart failure. Atrial natriuretic factor–VEGF-B_{167} expression was low in normally functioning hearts and stimulated by cardiac pacing; it thus functioned as an ideal therapeutic transgene, active only under pathological conditions.

CONCLUSIONS Our results, obtained with a standard technique of interventional cardiology in a clinically relevant animal model, support VEGF-B_{167} gene transfer as an affordable and effective new therapy for nonischemic heart failure. (J Am Coll Cardiol 2015;66:139–53) © 2015 by the American College of Cardiology Foundation.

Gene transfer meets the need for novel molecular therapies targeting known molecular alterations that occur specifically in cardiac cells and cannot be reversed by conventional pharmacological agents. Therefore, despite initial hurdles, gene therapy remains an attractive, highly promising option to treat various pathological conditions, including heart failure (HF), especially as better-suited viral vectors have become available (1–3). One example is a recent Phase II clinical trial demonstrating the potential of cardiac gene therapy for HF with reduced ejection fraction (4).

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Manuscript received January 18, 2015; revised manuscript received April 24, 2015, accepted April 28, 2015.

ABBREVIATIONS AND ACRONYMS

AAV = adeno-associated virus serotype 9

ANF = atrial natriuretic factor/protein

CMV = cytomegalovirus

DCM = dilated cardiomyopathy

GFP = green fluorescent protein

HF = heart failure

LV = left ventricular

LVEDP = left ventricular end-diastolic pressure

NOX2 = nicotinamide adenine dinucleotide phosphate oxidase

VEGF = vascular endothelial growth factor

VEGFR-1 = vascular endothelial growth factor receptor-1

VEGFR-2 = vascular endothelial growth factor receptor-2

Investigators have proposed various cardiac gene therapy strategies, depending on the target enzyme or structural protein they deem to be critically involved in compensatory or maladaptive cellular alterations. Over the past 5 years, we and others have shown the beneficial effects of vascular endothelial growth factor (VEGF)-B gene transfer in experimental models of cardiac injury (5–7).

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VEGF-B, 1 of the 5 members of the mammalian VEGF family, is a major pro-survival (rather than pro-angiogenic) factor (8). It selectively binds vascular endothelial growth factor receptor (VEGFR)-1, whereas the more extensively studied pro-angiogenic VEGF-A binds both VEGFR-1 and vascular endothelial growth factor receptor-2 (VEGFR-2). The marked cytoprotective/antiapoptotic (9) and minimally angiogenic action of VEGF-B renders it particularly well suited for gene therapy of nonischemic dilated cardiomyopathy (DCM), a severe pathological condition not caused by coronary artery disease in which the increased rate of apoptosis seems to play a major role (10–12). Unfortunately, no specific antiapoptotic pharmacological agents are currently available to clinicians.

Although DCM occurs much less frequently than ischemic disease, it remains largely untreatable and is responsible for most U.S. cardiac transplants (13). VEGF-B-based cytoprotective therapy might prove successful in the fight against this severe pathological condition. Therefore, the goal of the present study was to: 1) validate a clinically applicable cardioselective VEGF-B gene therapy in a large animal model of DCM; 2) test the efficacy of a safer approach on the basis of inducible VEGF-B transgenes turned on and off in response to, respectively, the occurrence or remission of the pathological condition; and 3) test the hypothesis that VEGFR-1 is the principal mediator of the cytoprotective action exerted by VEGFs. We delivered VEGF-B_{167}, the prevalent VEGF-B isoform (14), in canine tachypacing-induced HF. This is the best-characterized model of DCM, reproducing numerous pathophysiological and molecular alterations of the human disease (7,15–17). Parallel experiments were conducted in cultured cardiomyocytes.

METHODS

Fifty-three adult male, mongrel dogs (22 to 25 kg body weight) were chronically instrumented as previously described (Online Appendix) (7,17,18). The dogs were randomly divided into 5 experimental groups (Figure 1). Transgenes were encapsidated into adeno-associated virus serotype 9 (AAV9, henceforth indicated as AAV) and infused in the left coronary artery (anterior descending + circumflex branches) in 4 groups. Ten chronically instrumented dogs did not receive AAV. Intracoronary AAV delivery was performed 10 to 15 days after the surgical procedure or after 2 weeks of pacing. Dogs were lightly anesthetized (10 to 20 mg/kg of pentobarbital intravenously); after local anesthesia, a 5-F sheath was inserted percutaneously into the right femoral artery for coronary catheterization. Left circumflex and anterior descending coronary arteries were selectively and alternatively catheterized by using a 2.5-F microinfusion catheter to infuse 20 ml of AAV suspension (1×10^{13} to 5×10^{13} viral particles) in normal phosphate-buffered solution containing 3 ng/kg of adenosine and 5 ng/kg of substance P. The AAV suspension was administered slowly over 20 min followed by 10 min of intracoronary infusion of physiological saline solution. Simultaneously with AAV intracoronary delivery, 1 μg/kg/min of nitroglycerin was infused intravenously. Adenosine, substance P, and nitroglycerin were used to increase permeability in myocardial capillaries. Hemodynamic parameters were recorded during this procedure until full post-anesthesia recovery.

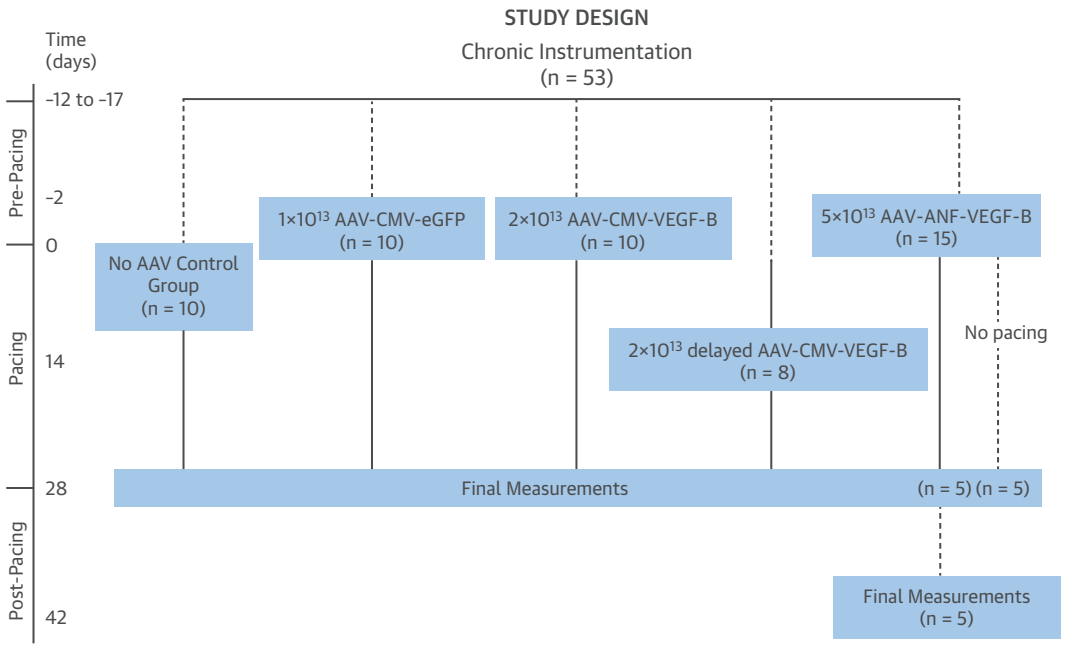
To induce HF, dogs were subjected to left ventricular (LV) pacing with an external pacemaker set at 210 beats/min for 3 weeks; the pacing rate was increased to 240 beats/min for an additional week. On the basis of our previous studies (17,18), this pacing protocol causes DCM and compensated HF during the first 3 weeks, culminating in severe HF at 27 to 30 days. All of the dogs were killed at 28 days to compare in vivo and ex vivo data at a fixed time point.

The protocol was approved by the Institutional Animal Care and Use Committee of Temple University, and it conformed to the guiding principles for the care and use of laboratory animals published by the National Institutes of Health.

Histological and polymerase chain reaction analysis of cardiac tissue was performed as previously described by us (Online Appendix) (6,7,19,20). To determine cytoprotective effects of VEGF-B_{167}, neonatal rat cardiomyocytes were isolated and cultured with production of reactive oxygen species measured as previously described (19,21,22). They were exposed to VEGF-B_{167}, VEGF-A, VEGF-E, and placental growth factor in the absence or in the presence of angiotensin II or norepinephrine ($50\ M^{-6}$).

STATISTICAL ANALYSIS. Data are presented as mean ± SEM. Statistical analysis was performed with

FIGURE 1 **Experimental Groupings**



Transgenes were encapsidated into adeno-associated virus serotype 9 (AAV) and infused in the left coronary artery (anterior descending + circumflex branches) in randomized groups of dogs. ANF = atrial natriuretic factor promoter; CMV = cytomegalovirus promoter; eGFP = enhanced green fluorescent protein; VEGF = vascular endothelial growth factor.

commercially available software (IBM SPSS Statistics, IBM Corporation, Armonk, New York). Hemodynamic, cardiac functional, histological, and molecular changes at different time points were compared by using 1-way analysis of variance for repeated measures and comparisons between groups by 2-way analysis of variance, in both cases followed by the Student-Newman-Keuls post-hoc test. When samples were not normally distributed, a nonparametric test was used, and data are presented as box plots. For all statistical analyses, significance was accepted at $p < 0.05$.

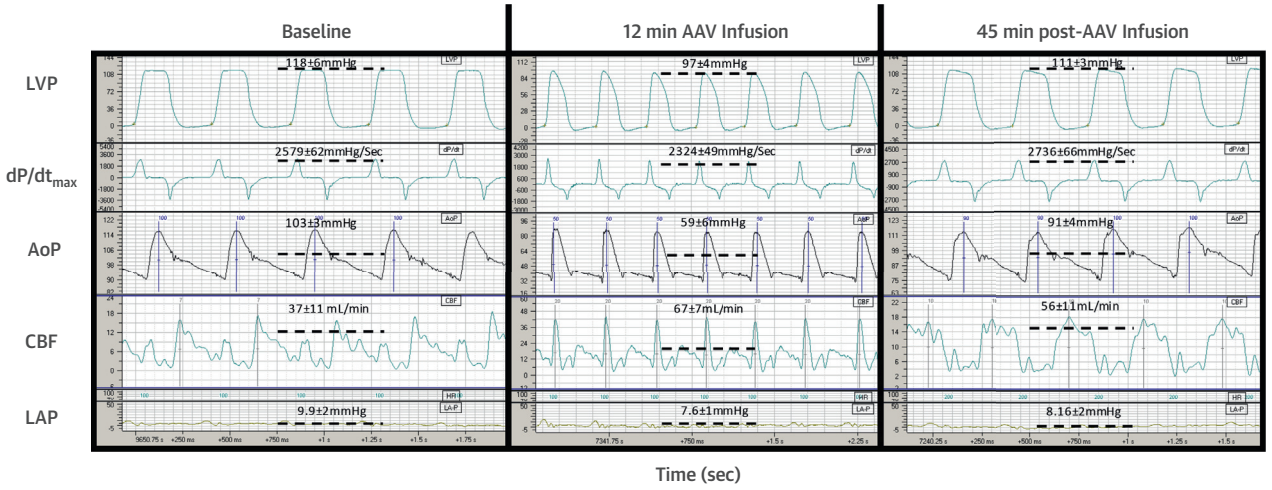
RESULTS

Intracoronary gene delivery was hemodynamically well tolerated. Co-infusion of vasodilators such as adenosine, nitroglycerin, and substance P during intracoronary AAV delivery caused the following reversible changes (**Figure 2**): increased coronary blood flow, decreased systolic pressure (~20 mm Hg), and altered LV pressure waveform shape during the diastolic phase, reflected by decreased dP/dt_{min} (minimal first derivate of LV pressure). Left atrial pressure was not affected.

Mouse VEGF-B_{167} (henceforth indicated as VEGF-B) gene or the green fluorescent protein (GFP) reporter gene, both controlled by the constitutively active cytomegalovirus (CMV) promoter, were delivered to 2 groups (**Figure 1**). One group received 1×10^{13} AAV-CMV-GFP (used as control group), and the other group received 2×10^{13} AAV-CMV-VEGF-B. AAV administered 2 days before starting the pacing protocol allowed time for transgene expression, which typically takes ~10 days when carried by this type of viral vector (3). To eliminate the possibility that early gene transfer could have exerted a preemptive action, 1 group of dogs received 2×10^{13} AAV-CMV-VEGF-B in the left coronary artery after 2 weeks of pacing, at a stage of compensated HF, thus simulating a more realistic clinical scenario. All the functional measurements were acquired at a spontaneous heart rate, with the pacemaker turned off.

Dogs transduced with GFP displayed the typical progressive deterioration of hemodynamic parameters over 4 weeks (**Figure 3**), as previously described (17,18); this time course was not significantly different compared with that in nontransduced dogs undergoing cardiac pacing (Online Figure 1). At 28 days, left ventricular end-diastolic pressure (LVEDP) increased to ~25 mm Hg, indicating congestive, decompensated HF. Conversely, cardiac transduction

FIGURE 2 Minor Hemodynamic Changes During Intracoronary AAV Delivery



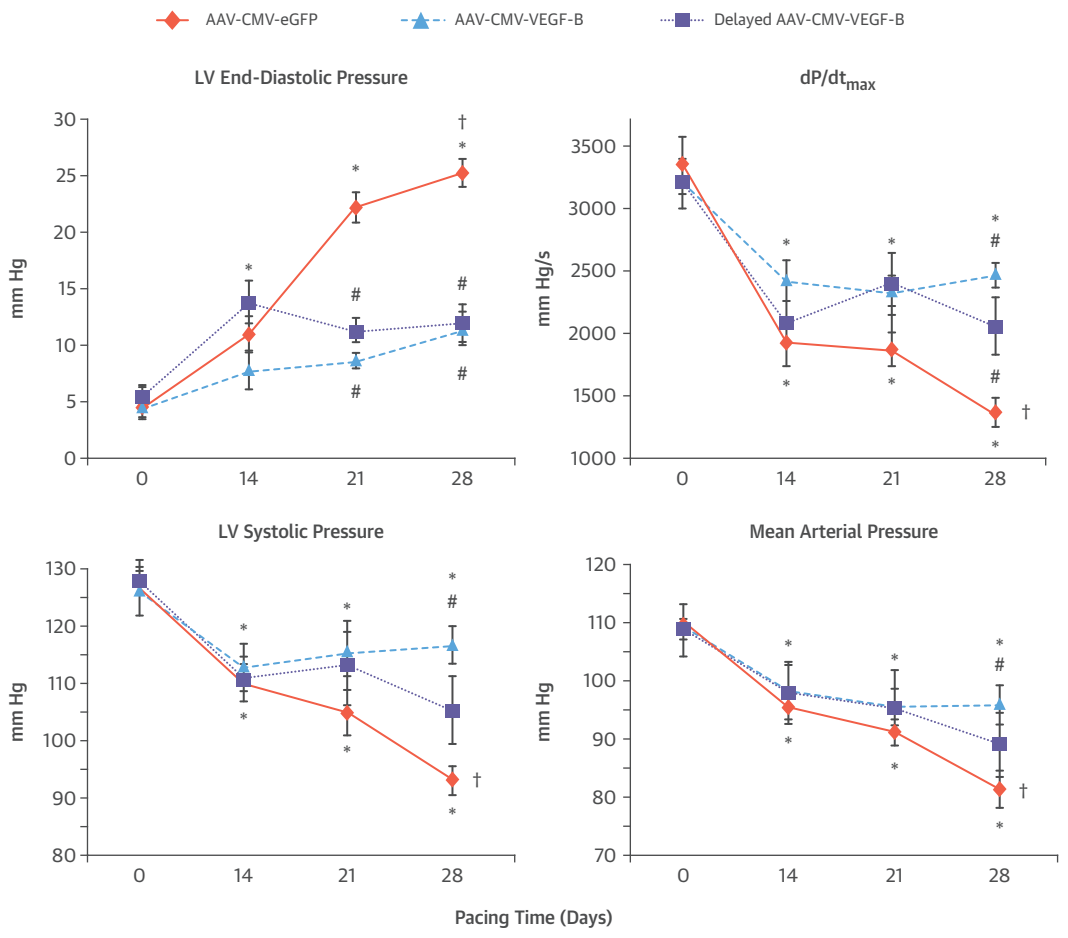
Representative hemodynamic tracings simultaneously recorded through chronically implanted probes and catheters before, during, and after AAV intracoronary delivery. The scale was automatically adjusted by the acquisition system and differs in the 3 panels. AAV = adeno-associated virus serotype 9; AoP = aortic pressure (with pressure average value); CBF = blood flow in the circumflex coronary artery (with flow average value); dP/dt = first derivative of left ventricular pressure (with dP/dt_{max} average value); LAP = left atrial pressure (with pressure average value); LVP = left ventricular pressure (with systolic pressure average value).

with VEGF-B markedly attenuated the hemodynamic derangement. The most notable effect was no significant change during the entire pacing period in LVEDP in those dogs transduced early; in the delayed transduction group, further increases were prevented after the second week. LV systolic pressure, mean arterial pressure, and dP/dt_{max} (maximal first derivative of LV pressure), although significantly decreased after 2 weeks of pacing compared with baseline, displayed no further significant decrease in the 2 groups transduced with VEGF-B. Finally, coronary blood flow did not change significantly over time in any of the groups (Online Figure 2). Therefore, compared with the control HF group, VEGF-B gene delivery halted the transition from compensated to decompensated HF, even in hearts with significant functional impairment.

A desirable strategy in gene therapy would be on the basis of inducible transgenes turned on and off in response to, respectively, the occurrence or remission of the pathological condition. We therefore generated a construct consisting of the 5'-flanking region -638/62 of the rat atrial natriuretic factor (ANF), which includes most of the ANF promoter and enhancer, linked to the VEGF-B gene. Our goal was to obtain VEGF-B expression only in response to intracellular ANF inducers, adopting a previously validated strategy (23,24). ANF is expressed in failing but not normal ventricles (25,26); therefore, VEGF-B would be expressed only during the development of HF. We first tested the responsiveness of the ANF 5'-flanking region -638/62 linked to GFP in a plasmid to transfect cultured rat neonatal cardiomyocytes stimulated with isoproterenol, a known ANF inducer (27) (Online Figure 3). In response to isoproterenol, the ANF element was able to drive approximately one-third of the GFP expression found in cells transfected with CMV-GFP. To compensate for the weaker promoter in vivo, we delivered 5×10^{13} AAV carrying ANF-VEGF-B in the left coronary artery of 15 dogs, 2 days before starting the pacing protocol (**Figure 1**). As a control group, 5 of these dogs did not undergo cardiac pacing and were killed 28 days later. **Figure 4A** shows that LVEDP and dP/dt_{max} were significantly more altered compared with the AAV-CMV-VEGF-B group; however, they remained within levels consistent with moderate/compensated HF. Alternatively, LV systolic and mean arterial pressures were not significantly different between the 2 groups. Non-paced dogs did not show any significant functional or morphological changes over time (data not shown).

A peculiarity of tachypacing-induced HF is its gradual functional recovery over a few weeks after restoration of spontaneous heart rate (18,28). We exploited this characteristic to test whether ANF-VEGF-B gene expression was silenced after post-failure recovery. In 5 of the 15 dogs transduced with ANF-VEGF-B, the pacemaker was disconnected after

FIGURE 3 VEGF-B Gene Transfer Halts the Progression to Decompensated HF

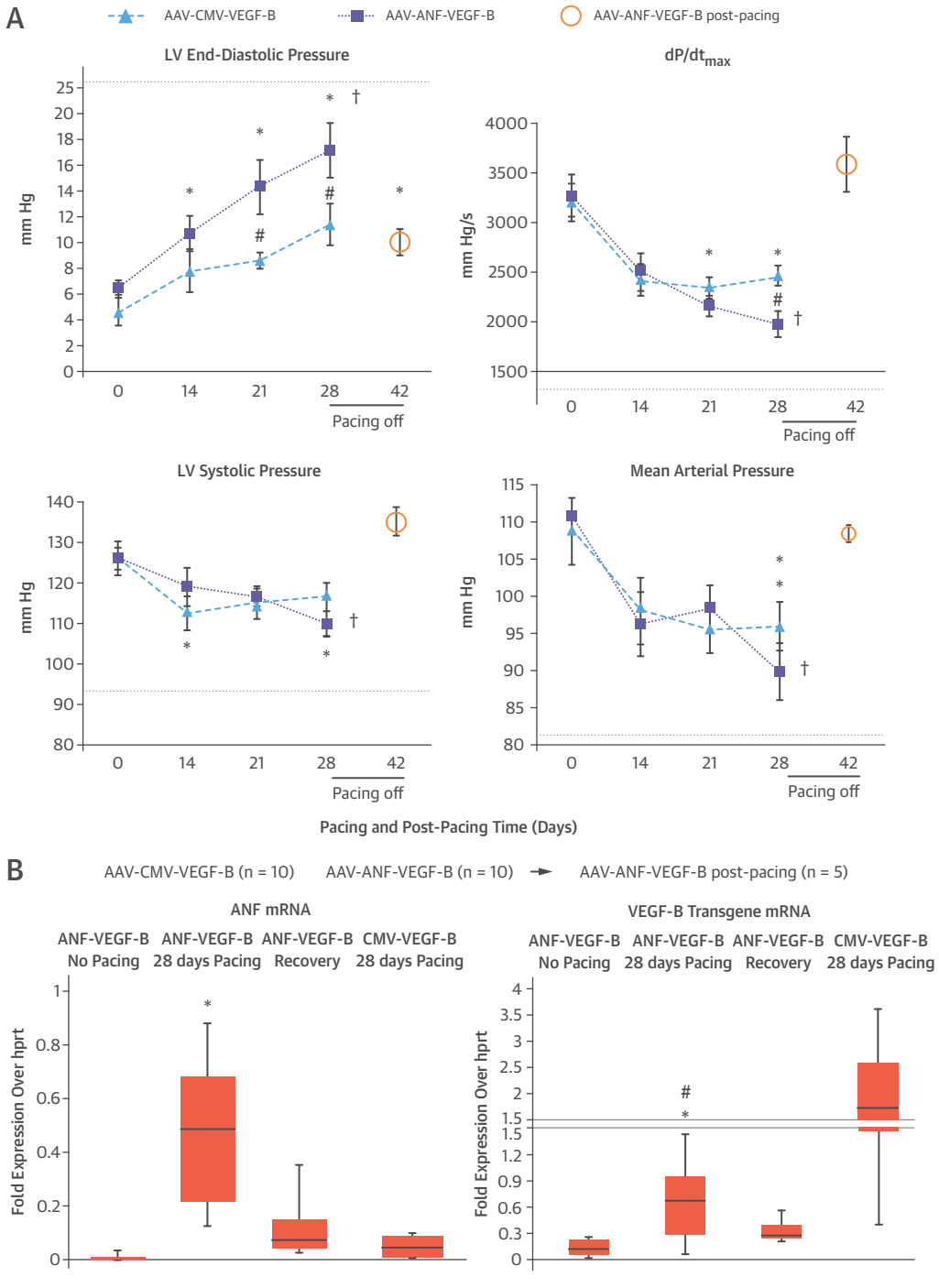


Gene transfer therapy appears effective in halting heart failure (HF) progression as seen in comparisons between main hemodynamic changes, over 4 weeks of chronic cardiac pacing, in dogs with intracoronary infusion of AAV-CMV-GFP (control, n = 10) or AAV-CMV-VEGF-B. This latter was administered at the beginning of the pacing protocol (n = 10) or after 14 days of pacing (delayed AAV-CMV-VEGF-B, n = 8). *p < 0.05 versus day 0 (baseline) within group; †p < 0.05 versus day 14 within group; #p < 0.05 versus AAV-CMV-GFP at the same time point. LV = left ventricular; other abbreviations as in Figures 1 and 2.

28 days of pacing, the functional parameters were monitored, and the animals were killed 2 weeks later. As expected, these dogs' hemodynamic values returned to normal or quasi-normal values (**Figure 4A**). We chose this time point, on the basis of a previous study in a similar dog model, in which circulating ANF was already normalized 1 week after turning off the pacemaker (28). We found a marked and significant increase in median ANF gene expression (normalized by the housekeeping gene hypoxanthine phosphoribosyl-transferase) in HF versus normal LV tissue (0.86 [range 0.2 to 1.85] vs. 0.04 [range 0.03 to 0.06]). Similarly, ANF expression was very low in hearts not subjected to pacing and transduced with ANF-VEGF-B, although ANF-VEGF-B was mildly expressed in the left ventricle, likely due to some degree of basal transcription, also noticed in vitro (Online Figure 3).

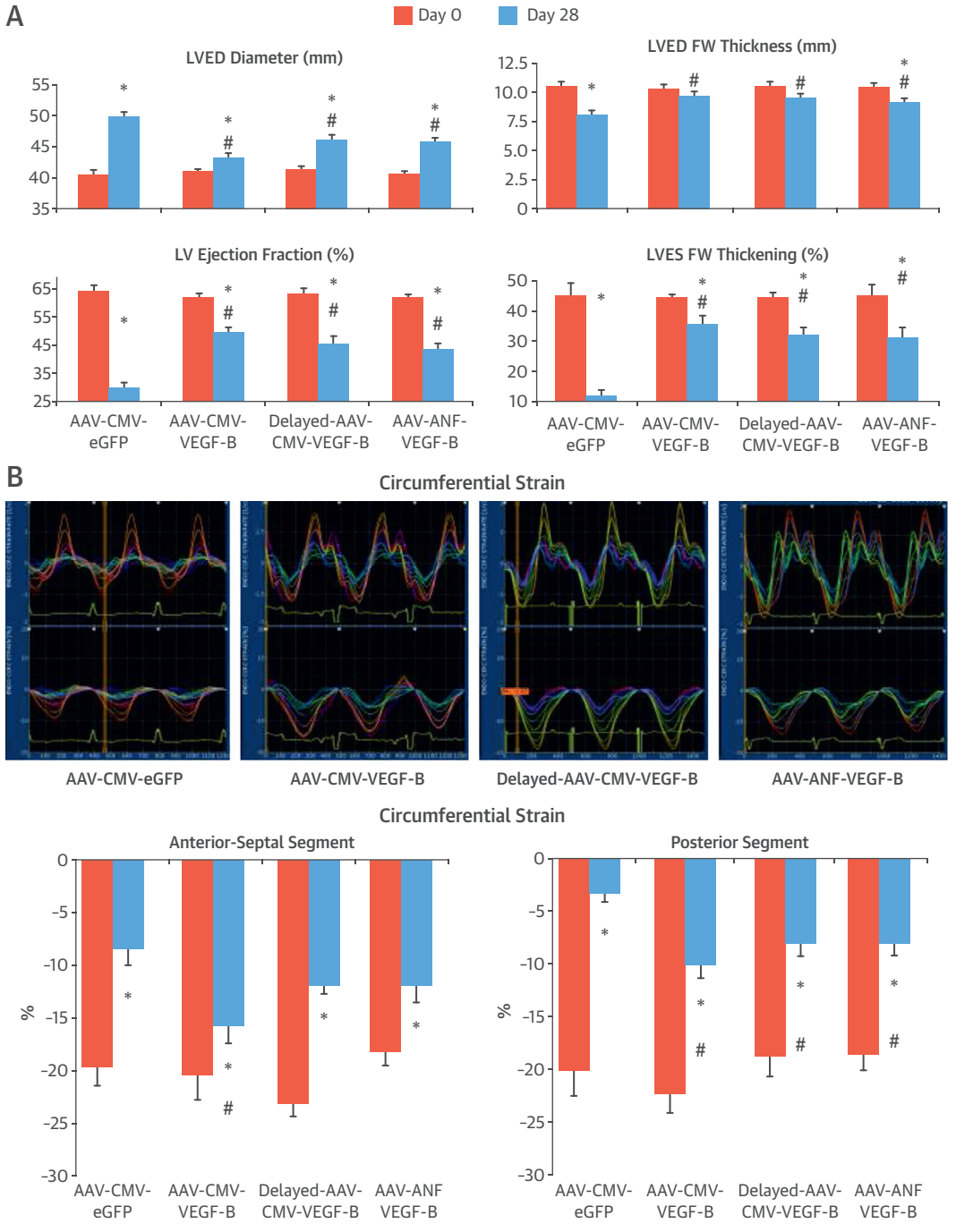
However, ANF-VEGF-B transgene expression markedly increased in LV tissue after 28 days of pacing, consistent with pathological ANF up-regulation, returning to almost control levels after post-pacing functional recovery when ANF levels were normalized (**Figure 4B**). Of note, ANF-VEGF-B gene expression in LV tissue was significantly lower than CMV-VEGF-B expression, which could partially explain the difference between the effects of the 2 therapeutic approaches on hemodynamics. Furthermore, hearts transduced with CMV-VEGF displayed ANF levels not significantly different from nonpaced hearts.

FIGURE 4 ANF-VEGF-B: Inducible Therapeutic Transgene



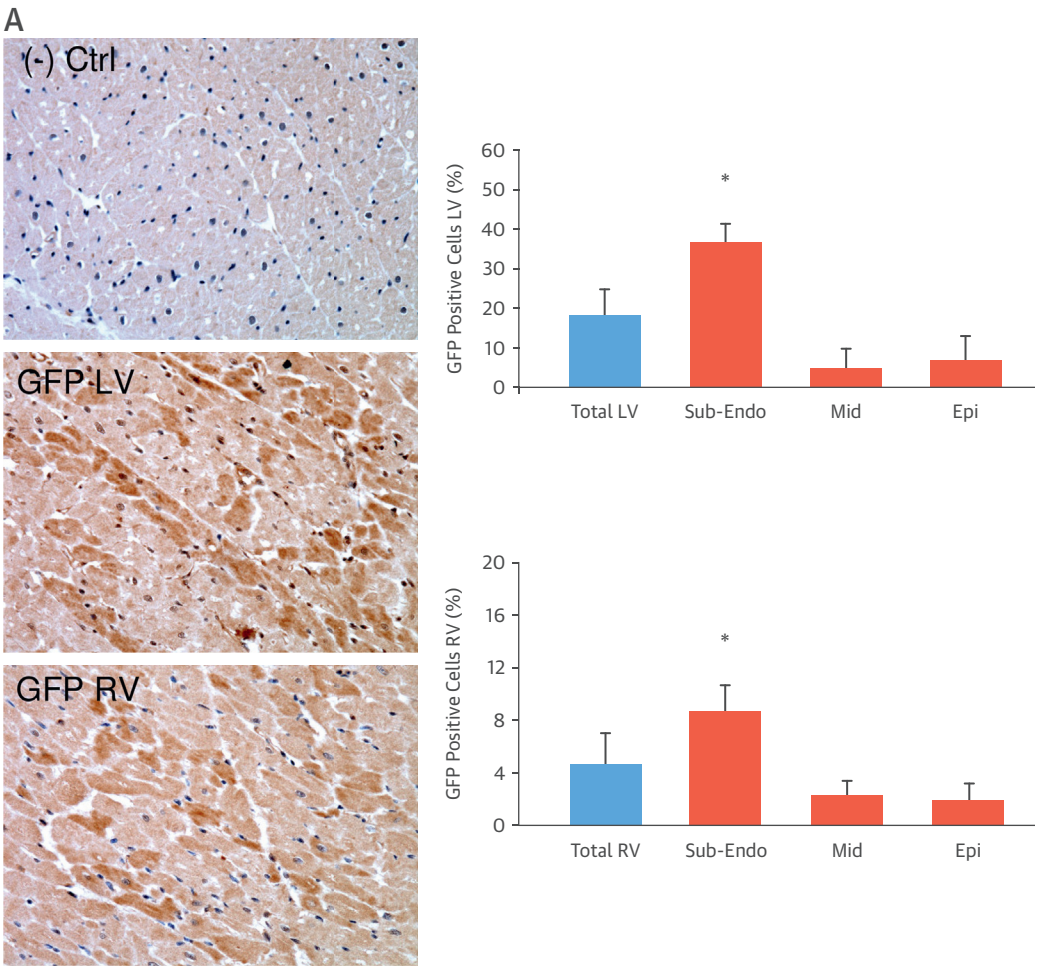
(A) Comparison between the main hemodynamic changes, over 4 weeks of chronic cardiac pacing, in dogs with intracoronary infusion of AAV-CMV-VEGF-B or AAV-ANF-VEGF-B (n = 10 per group). Five dogs receiving AAV-ANF-VEGF-B were observed for an additional 14-day period after stopping cardiac pacing (post-pacing). The dotted line in each panel indicates the average value of the respective hemodynamic parameter found in the control group after 28 days of pacing (as in Figure 2). *p < 0.05 versus day 0 (baseline) within group; †p < 0.05 versus day 14 within group; #p < 0.05 versus AAV-CMV-GFP at the same time point. **(B)** Gene expression of ANF, CMV-VEGF-B, and ANF-VEGF-B in the left ventricle. Messenger ribonucleic acid (mRNA) was quantified as fold expression of the housekeeping gene hypoxanthine phosphoribosyl-transferase (hprt); n = 5 for all groups. Data are medians with percentiles. *p < 0.05 versus control group (hearts transduced with ANF-VEGF-B but not undergoing pacing); # p < 0.05 versus CMV-VEGF-B. Abbreviations as in Figures 1 and 2.

FIGURE 5 **Cardiac Functional and Morphological Changes**



VEGF-B attenuates cardiac function and morphological derangement as seen in **(A)** echocardiographic parameters and **(B)** strain analysis with representative tracings recorded at 28 days of pacing. The latter include strain rate **(upper)** and circumferential strain **(lower)**. The **colored lines** indicate different segments of the left ventricular circumference, from the posterior-alter wall **(blue lines)** to the antero-septal wall **(orange lines)**. Measurements were taken with the pacemaker off. Group sizes as for Figures 1 and 2. *$p < 0.05$ versus day 0; #$p < 0.05$ versus AAV-CMV-GFP at 28 days. FW = free wall; LVED = left ventricular end-diastolic; LVES = left ventricular end-systolic; other abbreviations as in Figures 1 and 2.

FIGURE 6 Histological Analysis



(A) Immunohistochemical detection of GFP in cardiac tissue demonstrated heterogeneous transduction efficiency after intracoronary AAV delivery. Representative photomicrographs from negative control group, left ventricle (LV) and right ventricle (RV); n = 5 per group. **(B)** VEGF-B gene transfer attenuated myocardial apoptosis and prevented microvascular rarefaction. Nuclei are stained with 4′,6-diamidino-2-phenylindole (DAPI), apoptotic nuclei identified by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), and cardiomyocytes stained with α-sarcomeric actin (SA); n = 5 per group. Microvascular density normalized by the number of cardiomyocyte fibers; total microvessel numbers were quantified by using immunofluorescence staining for endothelial cells (lectin positive), whereas microvessels with muscular wall were identified by using positive immunofluorescence staining of smooth muscle (SM) actin. *$p < 0.05$ versus normal; #$p < 0.05$ versus AAV-CMV-GFP. Ctrl = control; Epi = subepicardial; Mid = mid-myocardial; Sub-endo = subepicardial; other abbreviations as in Figures 1 and 2.

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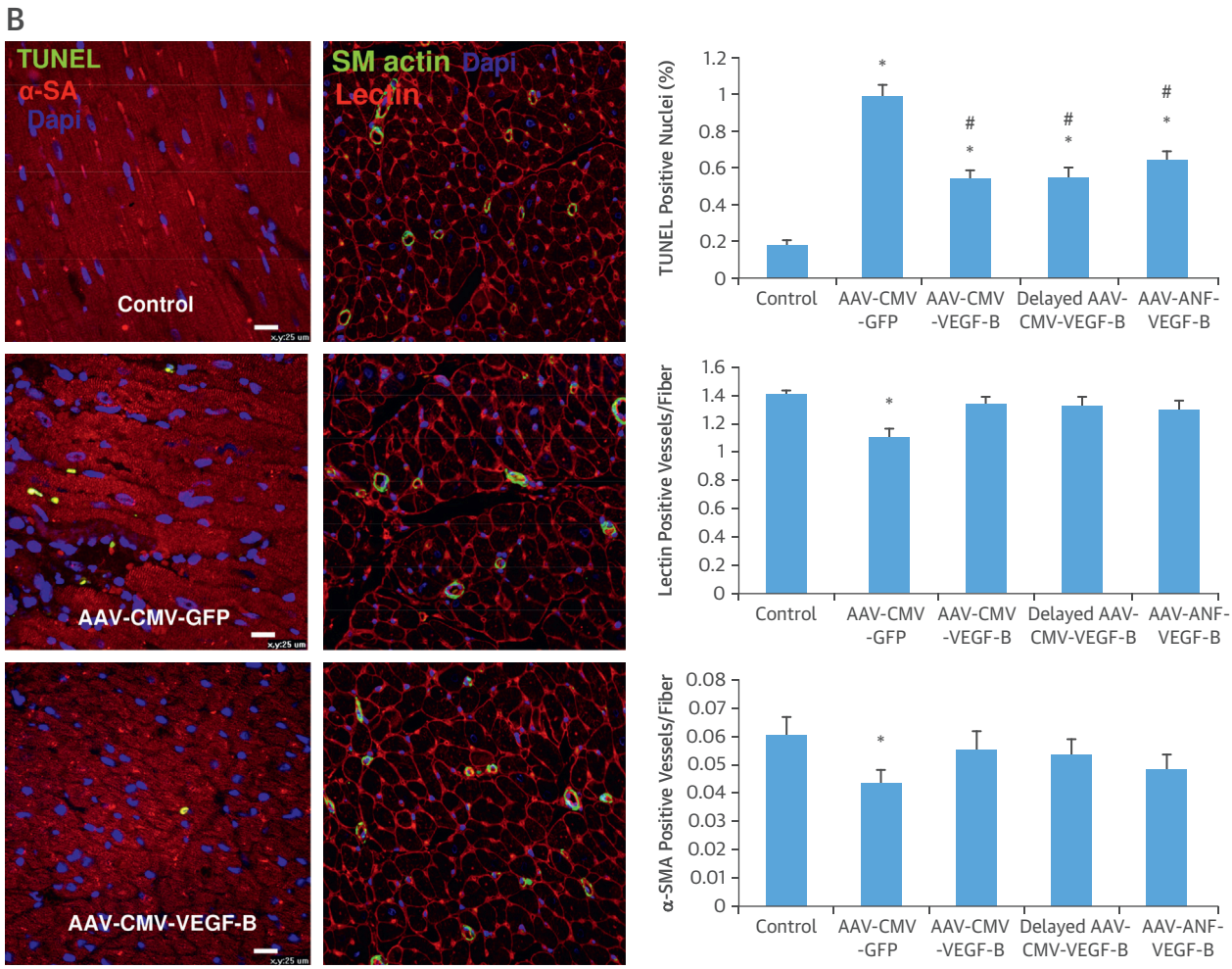
LV end-diastolic diameter increased by ~25% and LV end-diastolic thickness decreased by ~30% after 28 days of pacing in hearts transduced with GFP, indicating development of DCM (**Figure 5A**) (17,18,28,29). The increase in diameter was significantly attenuated by AAV-CMV-VEGF-B as well as by AAV-ANF-VEGF-B administration. Such a beneficial effect was even more pronounced on LV end-diastolic thickness. Cardiac remodeling in control HF was associated with >50% reduction in LV ejection fraction and >70% reduction in LV systolic wall thickening, 2 commonly used indexes of contractility. Cardiac transduction with both CMV-VEGF-B and ANF-VEGF-B attenuated these changes, although they remained significant versus baseline.

LV tachypacing causes dyssynchronous contraction, leading to an asymmetric contractile impairment, more pronounced in the LV free wall compared with the septum (30). In our experiments, AAV was delivered intracoronarily, without targeting specific regions of the heart. Therefore, we assessed whether gene therapy was similarly beneficial in the LV free

FIGURE 6 Continued



wall and septum. Echocardiography-based strain analysis was used to assess maximal circumferential shortening of the 2 opposite walls of the LV chamber. We confirmed the asymmetric functional impairment in both LV sites, but circumferential shortening was more preserved in hearts receiving the VEGF-B transgene (Figure 5B). However, the best protective effect in the septum occurred in hearts that received AAV-CMV-VEGF-B at the beginning of the pacing protocol.

The significant functional effects of VEGF-B gene delivery indicated achievement of an adequate, therapeutic level of myocardial transduction. By localizing the reporter gene GFP expression with immunohistochemistry in the control HF group, we could precisely quantify the percent and topographic distribution of transduced cells after intracoronary gene delivery. In both the left and right ventricles, transduction efficiency ranged widely from a maximal value in the subendocardial layers of the myocardium to a minimal value in the subepicardial layers (Figure 6A). Normal control, nontransduced cardiac tissue was obtained from chronically instrumented dogs killed for unrelated studies. Overall, expression efficiency was higher in the left ventricle versus the right ventricle.

Increased myocardial apoptosis is a known hallmark of human and experimental DCM (10–12,15). Histochemical analysis indicated a reduced percentage of apoptotic cells after 28 days of pacing in hearts transduced with CMV-VEGF-B or ANF-VEGF-B compared with control HF (Figure 6B). Another characteristic of DCM, the absence of major lesions of large coronary arteries associated with myocardial microvascular rarefaction (16), was confirmed in our canine HF model, and CMV-VEGF-B or ANF-VEGF-B gene transfer preserved the density of capillaries and smooth muscle actin-positive microvessels. Finally, the number of T lymphocytes in the myocardium did not

FIGURE 7 VEGFR-1 Agonists Activate Antioxidant Defenses in Cardiomyocytes

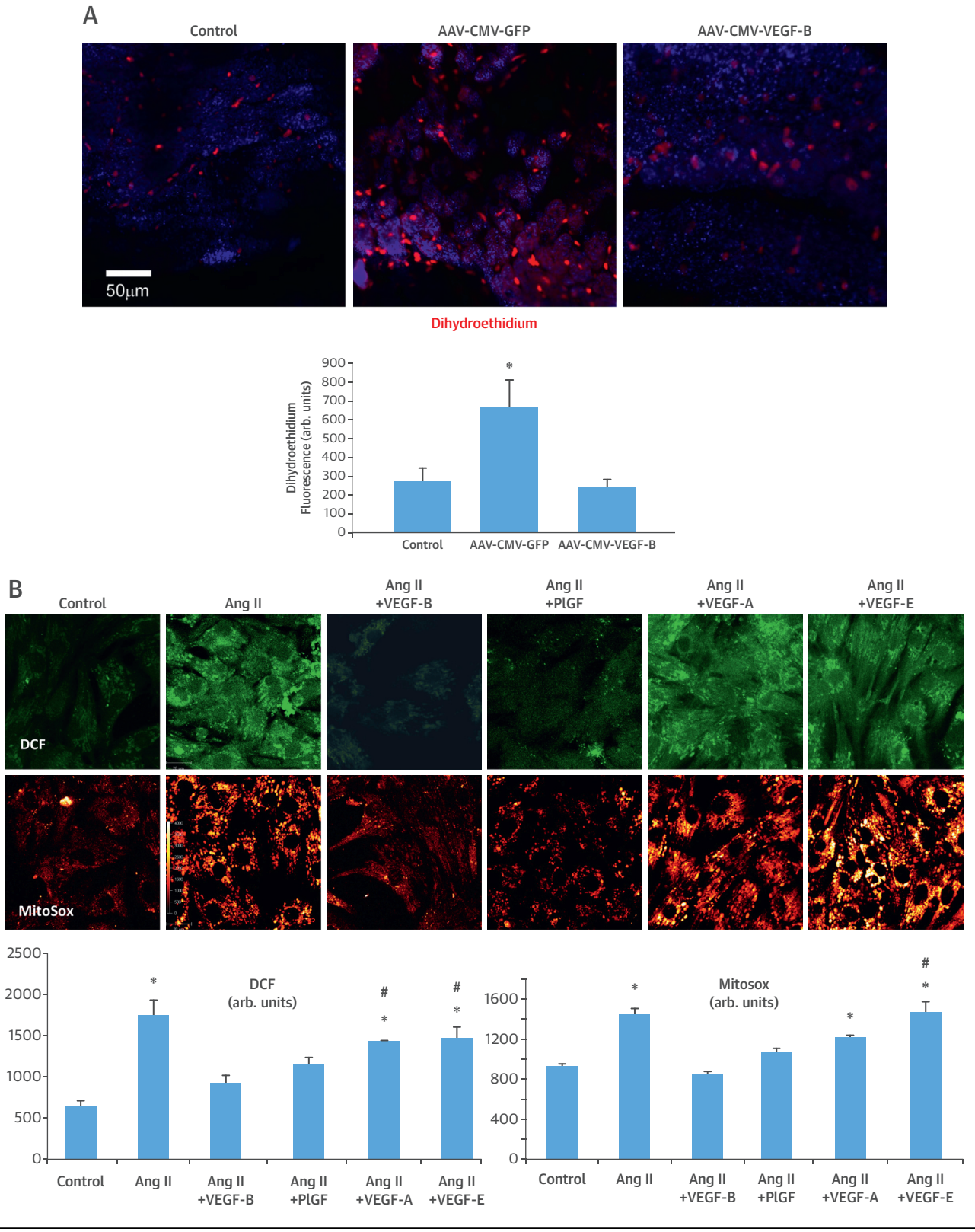


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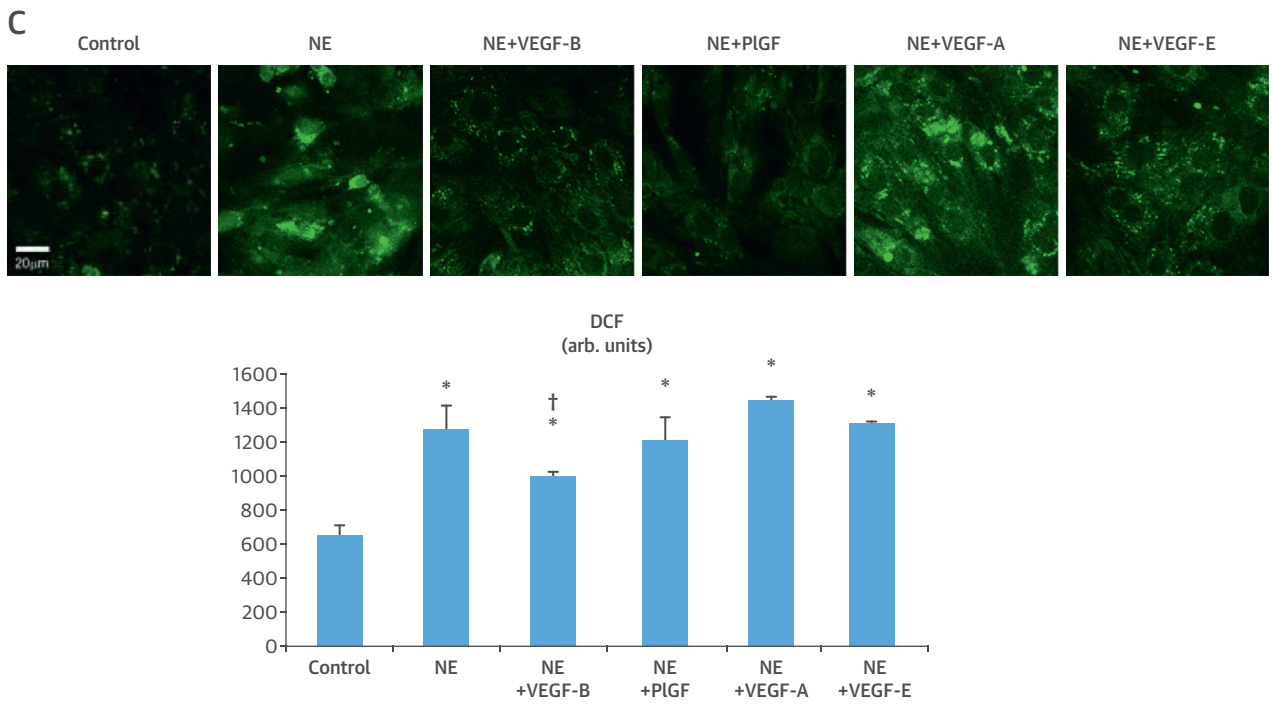
FIGURE 7 Continued



Use of vascular endothelial growth factor receptor (VEGFR)-1 agonists to activate antioxidant defenses in cardiomyocytes is seen in **(A)** representative photomicrographs and quantification of superoxide detection by dihydroethidium (DHE) in cardiac tissue slices (n = 3 per group); **(B)** representative images in angiotensin II (Ang II)–treated cultured neonatal cardiomyocytes showing fluorescence of MitoSOX (detecting mitochondrial superoxide) and 2′,7′-dichlorofluorescein diacetate (DCF; detecting cytosolic H_2O_2) and relative quantifications in the bar graph (n = 5 to 12 per group); and **(C)** representative images showing DCF fluorescence in norepinephrine (NE)–treated cultured neonatal cardiomyocyte and relative quantifications in the bar graph (n = 5 to 12 per group). *p < 0.05 versus control group. †p < 0.05 vs NE. #p < 0.05 vs Ang || +VEGF-B. arb. = arbitrary; PlGF = placental growth factor; other abbreviations as in **Figures 1 and 2**.

change significantly in any of the transduced hearts compared with nontransduced hearts after 28 days of pacing (Online Figure 4).

VEGF-B ACTIVATES ANTIOXIDANT DEFENSES. Dihydroethidium staining of cross sections from freshly harvested LV tissue and subsequent quantification of fluorescence intensity (19) indicated increased reactive oxygen species production in failing versus normal hearts (**Figure 7A**). This production was significantly lower in tissue slices harvested from paced hearts that had been transduced with VEGF-B. We therefore performed experiments in cultured rat neonatal cardiomyocytes to test whether: 1) VEGF-B attenuates reactive oxygen species generation in response to both angiotensin II and norepinephrine, 2 major promoters of oxidative stress (31,32); and 2) these effects are specific of VEGFR-1 ligands or shared by VEGFR-2 ligands. We compared VEGF-B with placental growth factor (another selective VEGFR-1 ligand), VEGF-A (a ligand of both VEGFR-1 and VEGFR-2) (8), and VEGF-E (a selective VEGFR-2 ligand) (33). **Figure 7B** shows that increased mitochondrial superoxide and cytosolic H_2O_2 production, detected, respectively, by MitoSOX red and 2′,7′-dichlorofluorescein diacetate, were significantly attenuated only by VEGF-B and placental growth factor but not by equivalent concentrations of VEGF-A and VEGF-E. However, norepinephrine-induced cytosolic reactive oxygen species elevation was attenuated in neonatal cardiomyocytes pretreated with VEGF-B but not placental growth factor, VEGF-A, or VEGF-E (**Figure 7C**).

We next explored the most obvious mechanisms potentially involved in the protection against angiotensin II, namely potentiation of mitochondrial superoxide dismutase and inhibition of nicotinamide adenine dinucleotide phosphate oxidase (NOX2). Pre-treatment of cultured cardiomyocytes with short-interfering ribonucleic acid against mitochondrial superoxide dismutase enhanced the MitoSOX signal in response to angiotensin II (Online Figure 5), confirming the importance of this enzyme as a

mitochondrial antioxidant defense. Importantly, the anti-mitochondrial superoxide dismutase short-interfering ribonucleic acid abrogated the beneficial effects of VEGF-B.

The activation of the isoform 2 of the superoxide-generating enzyme NOX2 in response to angiotensin II was tested by quantifying the translocation of the enzyme subunit p47 to plasma membrane rafts, a mandatory step for the assembling of this enzyme complex. Angiotensin II caused a marked translocation of p47 to plasma membrane, as expected, which was largely prevented by VEGF-B but not by VEGF-A (Online Figure 5). However, VEGF-B did not affect the NOX2 catalytic subunit gp91phox protein expression (data not shown).

DISCUSSION

We previously provided proof of concept of the beneficial effects of the VEGF-B_{167} gene delivery by direct intramyocardial injections in canine pacing-induced HF, whereas VEFG-A was ineffective (7). The present study successfully addresses remaining important questions; that is, whether intracoronary AAV-VEGF-B infusion (which is more realistic in clinical practice) is similarly effective and whether VEGFR-1 is the sole mediator of cardiomyocyte protection against oxidative stress.

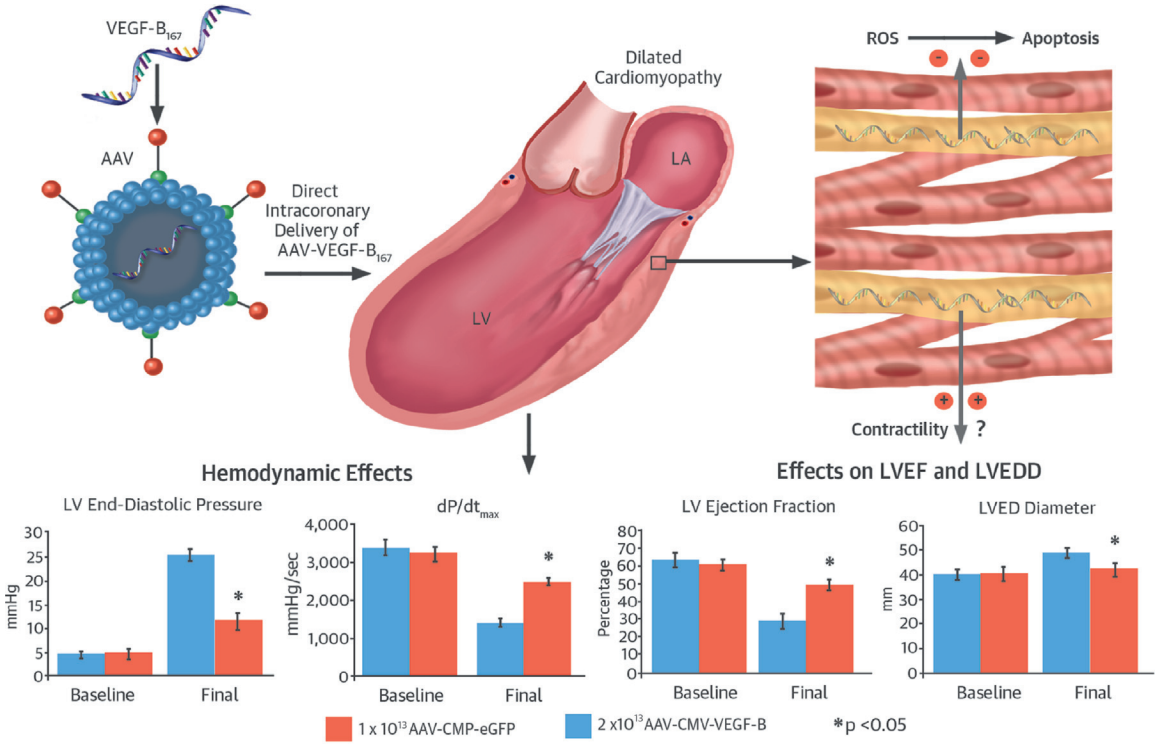
Different from intramyocardial injections, intravascular infusions are challenging because viral vectors can be rapidly flushed away from the target cells. We found that intracoronary AAV-VEGF-B delivery, conducted with procedures feasible at any coronary catheterization unit, was well tolerated, and displayed marked therapeutic efficacy halting progression toward decompensated HF (**Central Illustration**). Hemodynamic alterations and cardiac remodeling were blunted, if not completely prevented; consistently, at tissue level, the rate of apoptosis (a major DCM pathogenic determinant) was markedly reduced. Of note, LVEDP, an index of central congestion and diastolic dysfunction, remained within the almost physiological range of 6 to 10 mm Hg even after 28 days of tachypacing. Considering the severity and the elevated cardiac stress characterizing this model of HF, such results are promising.

Another important aspect was the equally high therapeutic efficacy of AAV-VEGF-B delivered to dogs with compensated HF, corresponding to the stage when most patients seek medical care for initial symptoms. In those dogs, gene therapy prevented further worsening of any functional alteration already developed after 2 weeks of pacing. Such rapidly occurring beneficial effect suggests that VEGF-B-mediated cytoprotection may not be the only mechanism involved. Other authors have found that VEGFR-1 agonists stimulate contractility by enhancing cytosolic calcium ion transients in neonatal ventricular myocytes (34); therefore, part of the therapeutic action we found in dogs could be due to direct support of contractile function. It is known that myocardial VEGFR-1 is down-regulated in DCM, whereas VEGF-B does not change significantly (7,16).

Although intracoronary AAV infusion has been previously used in several large animal and human studies, to our knowledge, no detailed description of myocardial transduction efficiency and regional heterogeneities was provided. We chose the serotype 9 AAV for its known cardiotropism (3,35). However, by using the GFP reporter, we found that the transduction efficiency was relatively high only in the subendocardial layers of ventricular walls and minimal in others. This finding was surprising; nonetheless, it supported the high efficacy/transduction ratio attained with AAV-VEGF-B. Conceivably, the action of VEGF-B synthesized in transduced cells extended to remote cells in a paracrine fashion.

Dogs with sustained VEGF-B expression were observed for a maximal period of 6 weeks. During that time, we detected no clinical or functional change indicative of harmful adverse effects; cardiac tissue analysis did not reveal specific alterations, including a possible increase in T lymphocyte infiltration. We did not expect any, as other authors found only moderate morphological changes and no functional alterations in transgenic mice with cardiac-specific VEGF-B overexpression (36). However, definitive conclusions about adverse effects will require long-term monitoring of dogs transduced with VEGF-B because this factor has also been implicated in pathological processes (37). Thus, an important finding of the present study is the curative efficacy achieved with very mild, hence theoretically safe, myocardial transduction. Moreover, we tested the inducible transgene strategy, which renders unnecessary the CMV promoter, further reducing possible risks related to long-term expression. Ideally, therapeutic transgenes should be induced by pathological molecular changes and silenced when the curative effect has been achieved. Although this strategy is not novel (38,39), it has not been previously applied to cardiac gene therapy in large animal models. The present results are encouraging because, similar to ANF, ANF-VEGF-B expression increased in response to chronic pacing and proved, at least in part, to be therapeutically efficacious. The reversibility of transgene expression was indicated by the return of ANF-VEGF-B messenger ribonucleic acid to low control levels

CENTRAL ILLUSTRATION **VEGF-B_{167} Gene Therapy in Dilated Cardiomyopathy**



Woitek, F. et al. J Am Coll Cardiol. 2015; 66(2):139–53.

Intracoronary infusion of adeno-associated virus serotype 9 (AAV)–vascular endothelial growth factor (VEGF)-B delays development of pacing-induced heart failure. A putative cardioprotective mechanism is inhibition of reactive oxygen species (ROS) production, which, in turn would prevent apoptotic cell death **(upper panel)**. This and other potential mechanisms preserve cardiac function in the VEGF-B–treated group compared with the green fluorescent protein (GFP) control group as indicated, for instance, by the left ventricular end-diastolic pressure (LVEDP) and left ventricular end-diastolic diameter (LVEDD), as well as by the higher dP/dt_{max} and left ventricular ejection fraction (LVEF) **(bottom panel)**. CMV = cytomegalovirus promoter.

after post-pacing recovery, mirroring LV ANF normalization. Additional testing will help refine this strategy and maximize its efficacy.

CYTOPROTECTIVE MECHANISMS. Oxidative stress is increased in HF and has been proposed as a primary pathogenic factor responsible for progressive cardiac tissue damage (31,32,40–43). Angiotensin II and norepinephrine, 2 mediators whose production/release is abnormally up-regulated in failing hearts, promote oxidative stress by activating NOX2 (31) and feeding the H_2O_2-generating enzyme monoamine oxidase (32). The present novel finding is that only the selective VEGFR-1 ligands VEGF-B and placental growth factor prevented mitochondrial superoxide and cytosolic H_2O_2 overproduction in cultured neonatal cardiomyocytes exposed to angiotensin II. VEGF-A, a dual VEGFR-1 and VEGFR-2 ligand, exerted a smaller, nonsignificant effect, whereas VEGF-E, a selective VEGFR-2 ligand, was ineffective. We further showed, for the first time, that VEGF-B (but not the other members of the VEGF family) could mitigate H_2O_2 overproduction in cultured cardiomyocytes exposed to norepinephrine. These results strongly suggest that an important mechanism underlying the therapeutic action of VEGF-B in vivo might consist of antagonizing the pro-oxidant effects of angiotensin II and norepinephrine.

Our data also suggest that antioxidant effects are exerted only by the VEGFR-1 ligands of the VEGF family, which perhaps can explain why, in our previous study, VEGF-A gene transfer did not prove beneficial in tachypacing-induced HF (7). Redox

equilibrium is finely regulated by a conspicuous number of pro-oxidant and antioxidant enzymes. However, in view of future investigations, we focused on 2 major enzymes: mitochondrial superoxide dismutase, a mitochondrial defense against superoxide generation, and NOX2, the superoxide-generating enzyme activated by angiotensin II. The excess mitochondrial superoxide production in cells exposed to angiotensin II could not be prevented by VEGF-B when mitochondrial superoxide dismutase overexpression was prevented by a specific short-interfering ribonucleic acid, indicating this enzyme's important involvement. Moreover, VEGF-B blocked the activation of NOX2. We tested these mechanisms in cardiomyocytes, but we cannot exclude the possibility that the protective action of VEGF-B in the intact heart benefits other important cell types such as endothelium and fibroblasts.

STUDY LIMITATIONS. First, due to the characteristics of our dog model, we could only test the therapeutic effects of VEGF-B gene transfer over a relatively short period, whereas human chronic HF develops over many years. Second, as conducted by other authors, our in vitro studies were performed in neonatal cardiomyocytes because, compared with adult cardiomyocytes, they are easier to obtain in large quantities for numerous experiments and to be stably maintained in culture for days without undergoing degenerative processes. However, some additional tests were performed in isolated adult cardiomyocytes, and we found similar responses to angiotensin II and VEGF-B, supporting the reliability of our results in neonatal cardiomyocytes (Online Figure 6). Finally, our experiments aimed at identifying molecular mechanisms responsible for the protective effects of VEGFR-1 ligands against oxidative stress are preliminary and warrant more in-depth studies at the cellular level.

CONCLUSIONS

In this pre-clinical model, VEGF-B gene transfer emerged as an efficacious and safe therapy for DCM. The perspective of blocking with a single intracoronary infusion, at early stages of HF, the malignant evolution of cellular/molecular processes otherwise hardly delayed by chronic polypharmacological treatments is appealing.

ACKNOWLEDGMENTS The authors are grateful to Sandhya Vallem for her valuable assistance with the in vitro reactive oxygen species detection analysis, and to Dr. John Edwards, New York Medical College, who provided the ANF promoter and enhancer.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Pharmacological options for the treatment of patients with DCM are currently limited, but gene delivery of VEGF-B can activate cytoprotective mechanisms in myocardium and was efficacious in a pre-clinical model of this disease state.

TRANSLATIONAL OUTLOOK: Further research involving large animals are needed to more fully evaluate the potential of therapy with transgenes and using various viral vectors and modalities of delivery for the treatment of DCM and other myocardial disorders.

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KEY WORDS gene therapy, heart failure, translational approach

APPENDIX For an expanded Methods section and additional figures, please see the online version of this article.