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Adeno-Associated Viral Vector Delivered Cardiac-Specific and Hypoxia-Inducible VEGF Expression in the Ischemic Mouse Hearts

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Abbreviations:

AAV: adeon-associated virus; VEGF: vascular endothelial growth factor; HRE: hypoxia-responsive element; Epo: erythropoietin gene; MLC: myosin light-chain;

Abstract:

We have shown previously that the adeno-associated viral vector (AAV) can deliver VEGF gene efficiently into ischemic mouse myocardium. However, the AAV genomes can be found in extra-cardiac organs after intra-myocardial injection. To limit unwanted VEGF expression in organs other than heart, we tested the use of the cardiac myosin light-chain-2v (MLC-2v) promoter and the hypoxia-response element (HRE), to mediate cardiac-specific and hypoxia-inducible VEGF expression. An AAV vector, MLCVEGF, with 250-bp of the MLC-2v promoter and 9 copies of HRE driving VEGF expression was constructed. Gene expression was studied *in vitro* by infection of rat cardiomyocytes, rat skeletal myocytes and mouse fibroblasts with the vector and *in vivo* by direct injection of the vector into normal and ischemic mouse hearts. With MLCVEGF infection, VEGF expression was higher in cardiomyocytes than the other two cell lines and was hypoxia inducible. VEGF expression was also higher in ischemic hearts than normal hearts. No VEGF expression was detectable in with organs detectable MLCVEGF vectors other than the heart. MLCVEGF-injected ischemic hearts had more capillaries and small vessels around the injection site, smaller infarct size, and better cardiac function than the negative controls. Hence, MLCVEGF can mediate cardiac-specific and hypoxia-inducible VEGF expression, neo-angiogenesis, infarct-size reduction, and cardiac functional improvement.

Introduction:

Angiogenic factors have been investigated for the treatment of ischemic heart disease for more than a decade. Animal and clinical data indicate that angiogenic factors such as FGF(1-3), VEGF(4-6) and angiopoietins(7) can induce angiogenesis in ischemic myocardium and improve cardiac function. However, a potential problem associated with prolonged and high-level expression of angiogenic factors, such as VEGF, is hemangioma formation in the heart(8, 9) and the limb(10). Hence, it is important to modulate the expression of the angiogenic proteins. For the treatment of ischemic heart disease, an ideal control is for the expression of angiogenic factors to respond to hypoxia. By using 9 copies of HRE isolated from the erythropoietin (Epo) gene enhancer, we showed previously that VEGF gene expression could be regulated to respond to hypoxia-induction *in vitro* and *in vivo*(11). However, with intra-myocardial delivery in the rat, the injected vectors have been found to infect extra-cardiac tissues(12). Unwanted angiogenesis may also occur in these tissues even with HRE controlled angiogenic gene expression, as hypoxia may exist in these tissues as well due to vascular diseases, cardiac failure, or occult tumors.

In this study, we first investigated AAV vector distribution after intra-myocardial injection in the mouse and found the vectors in several extra-cardiac organs. We then tested the use of a cardiac-specific promoter combined with the HRE, to

mediate both cardiac-specific and hypoxia-inducible gene expression. We chose the MLC-2v promoter because this contractile protein is abundant in slow twitch skeletal and cardiac muscles(13). Although the MLC-2v promoter is 3kb long, critical elements that mediate cardiac-specific gene expression are located within first 250-bp(14, 15). We constructed an AAV vector with the 250-bp MLC-2v promoter and 9 copies of Epo HRE driving VEGF gene expression. VEGF expression mediated by this vector was studied *in vitro* and *in vivo*. Angiogenesis and functional improvement mediated by this vector in ischemic mouse hearts were also analyzed.

Material and Methods:

AAV vector construction and production

A primer set, 5'-GGCCAGATCTTAGACAATGGCAGGACCCAG (sense) and 5'-GGCCAAGCTTGAATTCAAGGAGCCTGCTGG (anti-sense), was used to PCR amplify the 250 bp of rat MLC-2v promoter. This promoter was cloned between 9 copies of Epo HRE and human $VEGF_{165}$ cDNA in an AAV vector to generate MLCVEGF (Fig. 1). CMVVEGF (AAV-VEGF), and CMVLacZ (AAV-LacZ) constructed for previous experiments(11, 16) were used as control (Fig. 1). AAV serotype 2 vectors were prepared by using the three plasmids co-transfection system(17).

Measurement of VEGF production by cultured cells infected with VEGF expressing vectors

Rat cardiomyocytes (H9C2)(18), rat skeletal myocytes (L6) and mouse fibroblasts (NIH3T3) (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum. H9C2 and L6 cells were cultured in 10% CO_2 and NIH3T3 in 5% CO_2. Anoxia induction and measurement of VEGF expression were conducted using the previous described methods(11).

Inoculation of vectors into ischemic heart

Mice were housed in the animal care facility of University of California, San Francisco (UCSF), and experiments were conducted according to guidelines for rodent surgery and a protocol approved by the Institutional Animal Care and Use Committee of UCSF. CD1 mice (male and female) (Charles River, Wilmington, MA) were used. Left anterior descending coronary artery (LAD) ligation and viral vector inoculation were performed as previous described(11, 16).

PCR and real-time RT-PCR analyses

The TRIzol RNA isolation system (Invitrogen Life Technologies, Philadelphia, PA) was used to isolate RNA and genomic DNA. 2μg of genomic DNA were used

for PCR amplification of AAV genomes. Total RNA was DNase treated and purified using RNeasy Mini Kit (Qiagen Inc., Valencia, CA). First-strand DNA was synthesized with SuperScript II reverse transcriptase (Invitrogen Life Technologies). 2μg aliquot of the treated total RNA was used for each sample. 5μl of cDNA from each sample was used for PCR amplification. RNA without RT were also amplified and used as negative control to rule out possible genomic DNA contamination. Mouse hypoxanthine phosphoribosyl transferase (mHPRT) was used as internal control. For real-time PCR, the cDNA was diluted 1:10, and 5 μl was added to each reaction. PCR were performed using Taqman Master Mix and Taqman Real-Time PCR detection system (ABI PRISM 9700HT, Applied Biosystem). The primers sequences and the method used for real-time PCR data analysis are described in the supplement.

Histological and immunohistochemical analyses

Serial frozen sections were made from the apex of the heart to the site of the ligation. Infarct size was reconstructed with serial Trichrome stained sections. CMVLacZ infected cells were detected by LacZ staining. Anti PECAM-1 (Santa Cruz Biotechnology, Inc., Santa Cruz) and anti smooth muscle α-actin (Sigma, St. Louis, MO) antibodies were used to stain endothelial cells and vascular smooth muscles. Immunohistochemical staining was conducted using the standard protocol of Elite Vectastain ABC Kit (Vector Laboratories, Inc., Burlingame, CA).

Echocardiogram

Transthoracic echocardiogram on conscious mice was performed with an Acuson Sequoia c256 using a 15-MHz linear array transducer (15L8). After the anterior chest was shaved, the mouse was inserted into a plastic cone (Mouse Decapicone, Braintree Scientific Inc., Braintree, MA) in a prone position, to restrain its activity. The cone was fixed with adhesive tapes and warm ultrasound transmission gel (Aquasonic 100) was used to fill the space between the chest and the cone. Care was taken to avoid excessive pressure on the thorax, which can induce bradycardia, and two-dimensional imaging was obtained.

Statistical Analysis

Student's t-tests and ANOVA testing were used to comparing the differences among groups, with statistical significance considered if $P \leq 0.05$. The data are presented as mean±SD.

Results:

Detection of AAV genome in multiple extra-cardiac organs after intra-myocardial injection

AAV vector distribution in the mouse after intra-myocardial injection of CMVVEGF was analyzed by PCR genomic DNA isolated from the brain, heart, lung, liver, kidney, spleen, gonad and diaphragm 2 weeks (3 mice) and 6 months (2 mice) after the injections. Genomic DNAs isolated from a normal uninjected heart and a HEPES buffer injected heart were used as negative controls. Vectors were detected from all three mouse hearts collected 2 weeks after vector inoculation and 1 of the 2 mouse hearts collected 6 months after vector injection (Fig. 2, top panel). No vector sequence was detected from the uninjected and HEPES buffer injected hearts. The vector genome was also detected in the livers of all the vector injected mice and in the brain, the kidney, the spleen, the gonad or the diaphragm of some of the mice (Fig. 2, bottom panel). In the mouse in which we detected no vector sequence in the heart at the 6 month time point, vector sequences were detected in other organs. Most likely, the vector was injected into the chamber of left ventricle instead of the myocardium in this animal. No vector sequence was detected in the lung of any of the animals. These results suggest that the AAV vector had leaked out of the heart at or after intra-myocardial injection.

Enhancement of anoxic response in cultured cardiomyocytes by MLCVEGF

To limit the VEGF expression in heart and to achieve hypoxia-inducible gene expression, an AAV vector (MLCVEGF, Fig. 1) with 9 copies of Epo HRE and

250 bp MLC-2v promoter driving VEGF was constructed. The gene expression mediated by MLCVEGF vector was tested *in vitro* using H9C2 cells (rat cardiac myocyte), L6 (rat skeletal myocyte) and NIH 3T3 (mouse fibroblast). NIH3T3 have been used in our previous study(11) and expressed LacZ 10 times higher in anoxic condition than normoxic condition after infection with an AAV vector carrying 9 copies Epo HRE and minimum SV40 promoter driving LacZ gene. CMVVEGF was also used to infect these cell lines as control. The cells were cultured in normoxic and anoxic conditions after the infection. The ratios of VEGF expression between anoxic and normoxic conditions of all the three cell lines were near one after CMVVEGF infection. In contrast, these ratios with MLCVEGF infection were 8.7 for H9C2, 2.9 for L6 and 3.7 for NIH3T3. The ratios of VEGF expression between H9C2 cell and NIH3T3 cell were 1.8 (normoxic condition) and 4.2 (anoxic condition), between H9C2 and L6 were 6.2 (normoxia condition) and 8.7 (anoxic condition) (Table 1). Thus, MLCVEGF vector mediated hypoxia-inducible VEGF expression predominantly in the cardiomyocyte (H9C2).

Induction of VEGF gene expression in ischemic mouse hearts

To investigate if the MLCVEGF could mediate hypoxia-inducible VEGF expression *in vivo*, $5X10^{10}$ copies of MLCVEGF mixed with 10% ($5X10^{9}$ copies) of CMVLacZ were injected into normal and ischemic hearts. The CMVLacZ vector, in which the LacZ gene was driven by CMV promoter, was used as an indicator of the location and the efficiency of vector inoculation. We also injected

$5X10^{10}$ copies of CMVVEGF mixed with 10% CMVLacZ to normal and ischemic hearts as controls. 6 mice were injected in each group. Gene expression was quantitated by real-time RT-PCR 2 weeks after the vector inoculation. We observed, in our previous experiment, that the hematocrits of mice received intramyocardial injection of AAV-Epo (serotype 2) began to rise after two weeks and reached a plateau at about 5 weeks. Previous study with brain showed that Hif-1α accumulated in tissue at the onset of hypoxia and decreased gradually to normoxic level by 3 weeks despite the continuous low arterial oxygen tension(19). Thus, we chose to analyze the hypoxia responsive gene expression 2 weeks after the LAD ligation and vector injection. Compared to normal hearts, MLCVEGF-mediated VEGF expression increased by 74% in ischemic hearts and CMVVEGF remained unchanged (Fig. 3). The differences in gene expression between ischemic and normal hearts were significant ($p=0.01$) with MLCVEGF inoculation.

Heart specific VEGF gene expression mediated by MLCVEGF

Genomic DNA analysis showed that liver is the most frequent organ that has detectable viral sequences followed by spleen and diaphragm after intramyocardial injection. We compared VEGF expression in the liver (by real-time RT-PCR) and the diaphragms (by RT-PCR) in which vector genomes were detected. Whereas VEGF expression was detected in the livers and diaphragms of CMVVEGF/CMVLacZ injected mice, no expression was detected in the livers

and diaphragms of mice infected with MLCVEGF/CMVLacZ, although the level of LacZ expression mediated by the co-injected CMVLacZ vector were about the same in CMVVEGF and MLCVEGF injected mice (Fig. 4 and Fig. 5). Thus, about the same amount of the AAV vector genomes were present in the livers and diaphragms of these animals. The lack of VEGF gene expression in the livers and diaphragms of MLCVEGF injected mice was due to the inactivity of MLC-2v promoter in these organs.

Improved cardiac function in ischemic hearts injected with MLCVEGF

To study the effect of MLCVEGF mediated gene expression on angiogenesis, infarct size and cardiac function, five groups of mice were injected into the myocardium with: 1) 10^{10} copies of MLCVEGF mixed with 10% of CMVLacZ; 2) CMVVEGF mixed with 10% of CMVLacZ; 3) 10^{10} copies of CMVLacZ alone; 4) HEPES buffer; and 5) uninjected, immediately after the LAD occlusion. The number of mice used for final analyses is shown in Table 2. Cardiac function was assessed 4 weeks after the surgery. Left ventricular end diastolic dimension (LVDd) and end systolic dimension (LVDs) were measured. The percentage of fractional shortening (FS%) was calculated as (LVDd-LVDs)/LVDdX100. The FS% was 31.4±6.4 for CMVVEGF group and 31.2±9.9 for MLCVEGF group. The FS% for control groups were 26.6±8.9 for CMVLacZ group, 21.8±4.6 for HEPES buffer group and 24.8±5.6 for uninjected group. The FS% for normal mouse was 53.8±6.2 (Table 2). The differences in FS% between VEGF vectors injected

groups and HEPES buffer group are significant, at P≤0.05. The LVDd and LVDs of VEGF vectors injected mice were significantly smaller than the HEPES buffer injected mice (P≤0.05) (Table 2, Fig. 6). Thus, MLCVEGF mediated VEGF expression improved cardiac function. The functional improvement was equivalent to that mediated by CMVVEGF.

Induction of neovasculature and reduction of infarct size in ischemic hearts injected with MLCVEGF

Hearts collected after echocardiography were sectioned and stained with anti-PECAM-1 and smooth muscle α-actin antibodies. Vessels were counted on 6 areas, 3 on anterior wall and 3 on posterior wall in cross-sections of the left ventricle (Fig. 7a). Area 1 is made up entirely of muscle tissue, area 2 has both muscle and scar and area 3 has scar only. Vectors were injected into area 2 at the anterior wall. Hence, comparison between the injected areas in the anterior and the corresponding uninjected posterior areas would indicate the effect of VEGF expression. Capillary density was expressed as the ratio of capillary to cardiac myocyte for area 1, and as the number of capillary per mm^2 for area 2 and 3. The density of α-actin positive vessels was expressed as the number of vessels per mm^2 for all areas. In the two VEGF vector injected groups, the capillaries and α-actin positive vessels in all 3 areas of the anterior walls were increased compared to the posterior walls in the same hearts. The anterior walls in the hearts of these groups also had more capillaries and α-actin positive

vessels than the anterior walls of the three control groups (CMVLacZ, HEPES and uninjected groups) (Fig. 7b). In anterior area 1, the differences in capillary to muscle ratios and density of α-actin positive vessels were significant between MLCVEGF group and all three control groups ($P<0.01$). In anterior area 2, significant differences were seen between MLCVEGF and HEPES for the α-actin positive vessels, and uninjected group for capillary density ($P\leq0.03$). For anterior area 3, significant differences were shown between MLCVEGF group and LacZ or HEPES groups ($P=0.03$) on the density of α-actin positive vessels. CMVVEGF group has similar densities of capillaries and α-actin positive vessels as MLCVEGF group.

Infarct size was reconstructed on Trichrome stained serial sections and expressed as percentage of the left ventricular wall. The CMVVEGF and MLCVEGF injected groups had smaller infarct size compared to the three control groups (Table 2). The differences between the VEGF-injected groups and all three control groups were significant ($P\leq0.05$ for all). Thus, MLCVEGF mediated new blood vessel formation around injection sites and resulted in smaller infarcts in treated mice compared to control mice.

Discussion:

Ischemic heart disease remains the leading cause of morbidity and mortality in the Western world. Current therapeutic approaches aim to relieve symptoms and

cardiac events by reducing myocardial oxygen demand with medical therapy, or by restoring blood flow by coronary angioplasty or bypass surgery. However, the ability to accomplish revascularization in some patients with diffuse disease is limited. Therapeutic angiogenesis, using angiogenic growth factors or cytokines to stimulate collateral blood vessel formation is being tested as an alternative treatment(20, 21).

Gene therapy has the potential advantage of maintaining expression of the angiogenic factors at a sufficient concentration over a long period from a single administration. We have used AAV vector to deliver the VEGF gene into the ischemic myocardium of mice, and were able to induce neo-vasculature formation in ischemic hearts(16). However, uncontrolled exogenous VEGF expression has been shown to cause tumor-like vessels and hemangiomas(8-10, 22-24). To limit unregulated VEGF expression, we used the Epo HRE to regulate VEGF expression to respond to hypoxia induction(11). It is possible that injected vectors could enter the blood stream and become distributed to extra-cardiac organs, where they might cause unwanted angiogenesis. In this study, we demonstrated by PCR vector sequences in genomic DNA isolated from brains, hearts, livers, kidneys, spleens, gonads and diaphragms of mice that had received intra-myocardial injection of AAV vectors. The liver was the most frequent extra-cardiac organ that showed positive vector genome. It has been shown that AAV delivered 4 copies of Epo HRE and a minimum SV40 promoter driving transgene expression were detected in ischemic liver and skeletal

muscle(25). Thus, even with HRE controlled VEGF expression, angiogenesis at unwanted site cannot be completely prevented, as hypoxia might occur in organs other than the heart due to vascular disease affecting other organs or to congestive heart failure. The finding of AAV vector in extra-cardiac organs may be due to the leakage of the vector into the left ventricular cavity during the injection, as the wall of the mouse ventricle is thin. This may explain the absence of vector DNA in the lung. Perhaps in ventricles that have thicker walls, leakage would be less likely. Nevertheless, for safe angiogenic therapy for ischemic hearts, it will be desirable to have the gene expression both cardiac specific and hypoxia responsive.

In this study, we used the 250-bp MLC-2v promoter and 9 copies of Epo HRE to drive VEGF gene expression specifically in the heart in response to hypoxia induction. MLC-2v promoter is the best characterized promoter for ventricular-specific gene expression in embryonic and adult hearts(26). Although the MLC-2v promoter is 3 kb long, transgenic mouse studies have identified the first 250-bp sequence of the promoter to be adequate for ventricular-specific gene expression in embryonic and adult hearts(13, 27). The key element for ventricular-specific gene expression is the 28-basepair HF-1(14, 15, 28). The endogenous cardiac MLC-2v gene is expressed at high levels in both mouse cardiac and slow twitch skeletal muscles. However, high level of luciferase activity was only detected in cardiac muscle of transgenic mice harboring the 250- base pair rat cardiac MLC-2v promoter driving luciferase. Luciferase was

not expressed at detectable levels in slow twitch skeletal muscle of these mice(13). When inserted into an adenoviral or an AAV vector, this promoter mediated cardiac specific reporter gene expression both in neonatal rat and adult mouse after intravenous and intra-ventricular cavity injection(29-31). In our study, this promoter was used to mediate VEGF expression in the acute ischemic adult mouse heart. We demonstrated that the 250-bp MLC-2v promoter plus 9 copies of Epo HRE increased VEGF expression by 74% in ischemic hearts compared to normal hearts. Since RNA was isolated from whole heart in our study, the hypoxia induction of gene expression may be under estimated. Although MLCVEGF DNA sequence was detected in livers and diaphragms of MLCVEGF injected mice, no VEGF expression was detected. In contract, LacZ expression mediated by co-injected CMVLacZ vector was detected in these organs and VEGF expression was found in the organs of CMVVEGF injected mice that have detectable vector sequences. With cardiac-specific gene expression, the densities of capillaries and small vessels were increased in the myocardium and in the scar around MLCVEGF injection site. Also, the MLCVEGF-injected mice, compare to non-VEGF injected controls, had smaller infarct sizes and better cardiac functions, that were comparable to the CMVVEGF injected group.

In conclusion, both cardiac-specific and hypoxia-inducible VEGF expression were achieved with the 250-bp MLC-2v promoter and 9 copies of Epo HRE. Intra-myocardial injection of this AAV vector resulted in neo-vasculature formation, reduction of infarct size and improvement of cardiac function in

ischemic hearts. This approach may lead to a safe and effective angiogenic gene therapy for ischemic heart disease in potential clinical application.

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Figure Legends:

Figure 1. AAV constructs. The name in parentheses was used in previous publications(16). ITR: inverted terminal repeat. 9HRE: 9 copies of Epo HRE. MLCp: 250-bp of MLC-2v promoter.

Figure 2. PCR detection of AAV vector sequences in hearts and several extra-cardiac organs after intra-myocardial injection of the vectors. The top panel shows the PCR results with heart DNA. The bottom panel shows an example of organs in one of the mice collected 2 weeks after intra-myocardial injection. N: normal heart; 2 weeks: hearts collected 2 weeks after vector injection; HEPES: HEPES-injected heart; 6 months: hearts collected 6 months after vector injection; B: brain; H: heart; L: lung; Li: liver; K: kidney; S: spleen; O: ovary and D: diaphragm.

Figure 3. Induction of VEGF expression in ischemic hearts by MLCVEGF vector. Expression of human VEGF mRNA was shown as the relative threshold of VEGF and LacZ mRNA and was calculated based on the threshold cycle (C_T). ΔC_T: relative threshold cycle; $\Delta\{\Delta C_{T(VEGF\text{-}LacZ)}\}=\Delta C_{TVEGF}-\Delta C_{TLacZ}$ where $\Delta C_{TVEGF}=C_T$VEGF-C_TmHPRT; $\Delta C_{TLacZ}=C_T$LacZ-C_TmHPRT. Y axis indicated the relative threshold cycles. The larger number, the more cycles were required to reach the threshold, indicating lower gene expression. The relative thresholds for CMVVEGF were similar for normal heart (1.50±0.80) and ischemic hearts (1.51±0.79). While the relative threshold for MLCVEGF injected hearts decreased from 3.11±0.38 (normal hearts) to 2.31±0.34 (ischemic heart), indicating a 74% increase of VEGF expression in ischemic hearts compare to normal hearts.

Figure 4. VEGF and LacZ expression in livers of mice that have AAV sequences after receiving intra-myocardial injection of CMVVEGF/CMVLacZ (A) and MLCVEGF/CMVLacZ (B & C). A & C: normal heart mice; B: ischemic heart mice. Y axis: gene Expression (relative value) was calculated based on the threshold cycle (C_T). Using the expression of mouse HPRT as the basis for comparison, the relative value of VEGF expression were calculated as $2^{-\Delta C_T VEGF}$ where $\Delta C_T VEGF = C_T VEGF - C_T mHPRT$ and of LacZ were calculated as $2^{-\Delta C_T LacZ}$ where $\Delta C_T LacZ = C_T LacZ - C_T mHPRT$. The relative value of VEGF expression in livers of CMVVEGF injected normal heart mice was 26615.9±1.37. VEGF expression was not detectable in livers of MLCVEGF injected mice with either normal or ischemic hearts. The relative values of LacZ expression in livers were 10085.5±2.03 (CMVVEGF/CMVLacZ group), 11585.2±1.53 (MLCVEGF/CMVLacZ group with ischemic hearts) and 9410.1±1.21 (MLCVEGF/CMVLacZ group with normal hearts).

Figure 5. PCR detection of AAV genomes, and VEGF and LacZ gene expressions in hearts and diaphragms 2 weeks after LAD ligation and intromyocardial vector injection. The templates used for the PCR were genomic DNA (DNA), cDNA (RT+) and RNA (RT-). H: heart; D: diaphragm. VEGF and

Figure 6. Echocardiogram of the left ventricle. A: anterior wall; P: posterior wall. Note: the greater movement of the anterior wall of the MLCVEGF injected heart compared to the CMVLacZ and HEPES injected hearts.

Figure 7. a. A diagram of cross section of left ventricle. The areas used for analyses of vessels density are marked. b. Blood vessel densities in different areas. MLC: MLCVEGF injected group; CMV: CMVVEGF injected group; LacZ: CMVLacZ injected group; HEPES: HEPES injected group and Uninj: uninjected group. CAP: capillary. M: muscle cell. *: the differences between MLCVEGF group and control groups (CMVLacZ, HEPES and uninjected groups) are significant. **: the differences between CMVVEGF group and control groups are significant.

MLCVEGF

CMVVEGF (AAV-VEGF)

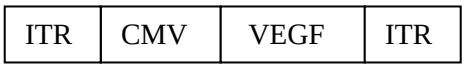


Fig. 1

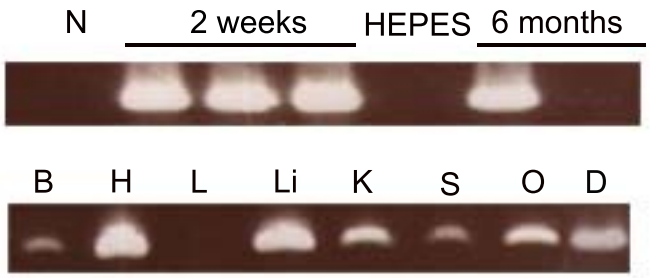


Fig. 2

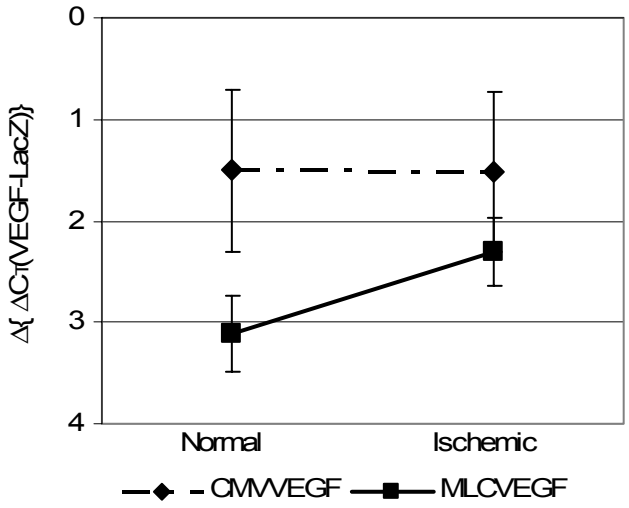


Fig. 3

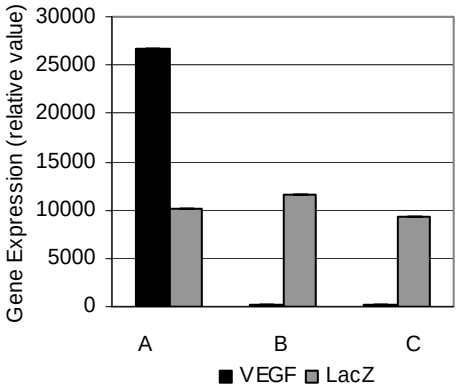

Gene Expression (relative value)
30000
25000
20000
15000
10000
5000
0
A
B
C
VEGF
LacZ

Fig. 4

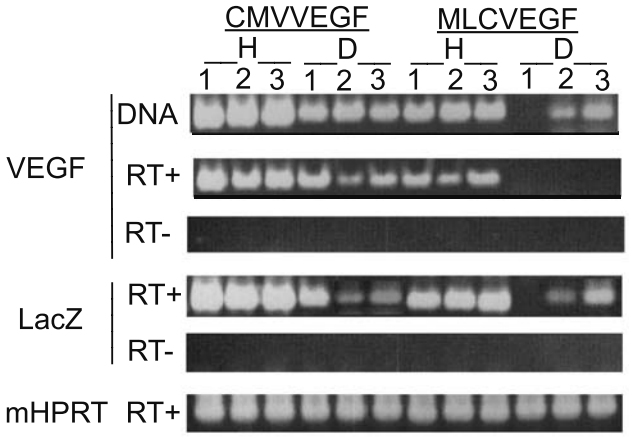

CMVVEGF
MLCVEGF
H
D
H
D
1 2 3 1 2 3 1 2 3 1 2 3
VEGF
DNA
RT+
RT-
LacZ
RT+
RT-
mHPRT
RT+

Fig. 5

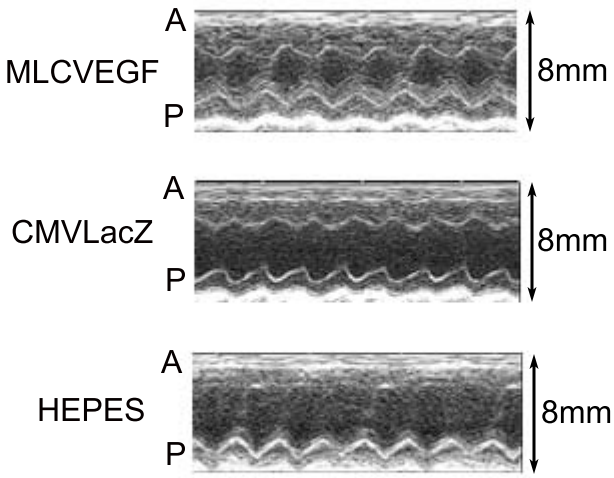
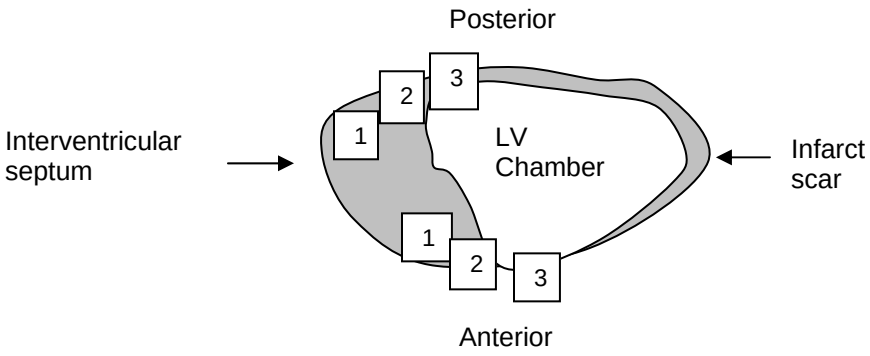


Fig. 6

a.


Posterior
Interventricular septum
LV Chamber
Infarct scar
Anterior
1
2
3

b.

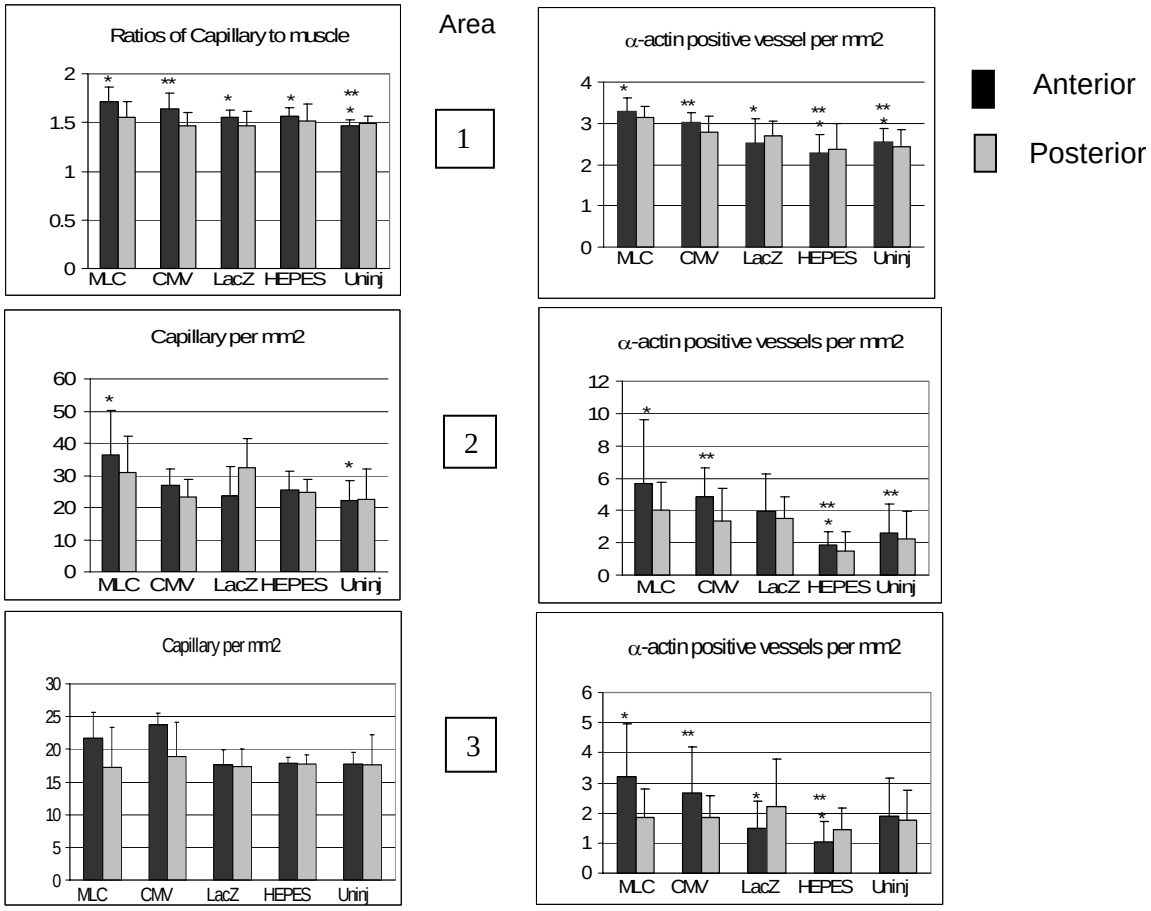

Area
Anterior
Posterior
Ratios of Capillary to muscle
α-actin positive vessel per mm2
Capillary per mm2
α-actin positive vessels per mm2
Capillary per mm2
α-actin positive vessels per mm2
MLC
CMV
LacZ
HEPES
Uninj
1
2
3

Fig. 7

Table 1. VEGF Expression by AAV Vectors under Normoxic and Anoxic Conditions (pg/ml/16 hour culture)

	MLCVEGF			CMVVEGF		
	Anoxia	Normoxia	A/N* ratios	Anoxia	Normoxia	A/N* ratios
H9C2	156±73	18±2.1	8.7	119±42	103±24	1.2
L6	18±4.6	6.2±0	2.9	110±38	92±6	1.2
NIH3T3	37±5.5	10±3	3.7	88±15	105±22	0.84

* A: anoxia, N: normoxia.

Table 2, Infarct Size and Echocardiogram

Vectors (n)	Infarct Size (%)	LVDd (mm)	LVDs (mm)	FS (%)
CMVVEGF (5)	37±11	4.45±0.19	3.06±0.41	31.4±6.4
MLCVEGF (8)	38±7	4.42±0.60	3.09±0.87	31.2±9.9
CMVLacZ (6)	46±6	4.54±0.20	3.34±0.51	26.6±8.9
HEPES (5)	46±5	4.72±0.17	3.69±0.25	21.8±4.6
Uninjected (7)	49±8	4.57±0.18	3.43±0.28	24.8±5.6
Normal (4)	0	3.60±0.18	1.67±0.29	53.8±6.2

LVDd: left ventricular end diastolic dimension; LVDs: left ventricular end systolic dimension; FS(%): percentage of fractional shortening, calculated as (LVDd-LVDs)/LVDdX100. (n): number of animals used in each group. Infarct size was measured as percentage of left ventricular wall.

Supplements:

PCR primers

1. Human VEGF specific primers: 5'-GGCAGAAGGAGGAGGGACAGAATC (sense) and 5'-CATTTACACGTCTGCGGATCTTGT (anti-sense).
2. LacZ primers: 5'-GGCGTAAGTGAAGCGACCCG (sense) and 5'-GCGTGCAGCAGTGGCGATGG (antisense).
3. Mouse HPRT primers: 5'-AAGGACCTCTCGAAGTGTTGGATA (sense) and 5'-CATTTAAAAGGAACTGTTGACAACG (antisense)
4. Primers and probes for real-time PCR analyses: human VEGF and mHPRT were purchased from Applied Biosystems (Foster City, CA). LacZ primer sequences: 5'-GCGTTAACTCGGCGTTTCAT (sense) and 5'-GCGCTCAGGTCAAATTCAGAC (anti-sense); LacZ probe sequence: 5'-CGGTTACGGCCAGGACAGTCGTTTG.

Real-time PCR data analysis:

Gene expression was calculated based on the threshold cycle (C_T). The relative threshold cycles of VEGF and LacZ to mouse HPRT were calculated as $\Delta C_{TVEGF}=C_T$VEGF-C_TmHPRT and $\Delta C_{TLacZ}= C_T$LacZ-C_TmHPRT. The relative values of VEGF and LacZ expression were calculated as 2's negative power of relative thresholds; $2^{-\Delta C_T VEGF=C_T VEGF-C_T mHPRT}$ for VEGF and $2^{-\Delta C_T LacZ= C_T LacZ-C_T mHPRT}$ for LacZ. The relative threshold of VEGF to LacZ was calculated as $\Delta(\Delta C_T)= \Delta C_{TVEGF} - \Delta C_{TLacZ}$ and presented as $\Delta\{\Delta C_T$(VEGF-LacZ)$\}$. The ratio of VEGF

expression in ischemic and normal hearts was calculated as percentage of 2's negative power of VEGF relative threshold in ischemic heart [$\Delta(\Delta C_T)$ ischemic] subtracted by relative threshold in normal heart [$\Delta(\Delta C_T)$ normal] ($2^{-\{\Delta(\Delta C_T)\text{Ischemic}-\Delta(\Delta C_T)\text{Normal}\}}$X100 percent).