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AAV Serotype-1 Mediates Early Onset of Gene Expression in Mouse Hearts and
Results in better Therapeutic Effect

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Summary:

Adeno-associated viral vectors (AAV) are attractive tool for gene therapy for coronary artery disease. However, gene expression in myocardium mediated by AAV serotype 2 (AAV2) does not peak until 4-6 weeks after gene transfer. This delayed gene expression may reduce its therapeutic potential for acute cardiac infarcion. To determine whether earlier gene expression and better therapeutic effect could be achieved using a different serotype, CMV promoter driving the EPO gene (AAV-EPO) was packaged into AAV serotypes 1 to 5 capsids and injected into mouse myocardium. EPO expression was studied by measuring the hematocrits and EPO mRNA. After we found that AAV1 mediates the highest gene expression after 4 days of gene transduction, AAV-LacZ (CMV promoter driving LacZ gene), and MLCVEGF (hypoxia-inducible and cardiac-specific VEGF expression) were packaged into AAV1 and 2 capsids. LacZ expression was detected in AAV1-LacZ but not in AAV2-LacZ injected hearts one day after vector injection. Compared to AAV2-MLCVEGF that mediated no significant VEGF expression, AAV1-MLCVEGF mediated 13.7-fold induction of VEGF expression in ischemic hearts 4 days after gene transduction and resulted in more neovasculatures, better cardiac function and less myocardial fibrosis. Thus, AAV1 mediates earlier and higher transgene expression in myocardium and better therapeutic effects.

Introduction:

AAV vectors possess several advantages over other vectors such as adenoviral vectors for gene delivery to heart as (1) they do not exhibit pathogenicity in human;[1] (2) they have low immune reactivity and the ability to enable transgene expression for considerable length of time; [2] and (3) they can deliver genes into myocardium effectively.[2-5] In a previous study, we used AAV serotype 2 (AAV2) vector mediated VEGF gene transfer to induce new blood vessel formation in ischemic murine hearts. [6] To avoid the harmful effect of excessive angiogenesis, we used 9 copies of hypoxia-response element (HRE) isolated from the EPO gene enhancer and the promoter of myosin light chain 2v (MLC-2v) to regulate AAV vector mediated VEGF gene expression to be cardiac-specific and hypoxia-inducible.[7,8] Cardioprotective heme oxygenase gene has also been delivered to murine hearts by AAV2 vector and resulted in reduction of infarct size after coronary artery occlusion.[9,10] AAV2 mediated transgene expression in myocardium has a "time lag". Significant transgene expression was first detected 2-4 weeks after gene transfer.[2,3] In an acute heart attack, metabolic changes begin with 8-10 minutes [11] and irreversible myocardial injury may occur within 6 hours. In our ischemic mouse heart model, we found minimal viable myocardium in the ischemic area 2 weeks after occlusion of the left anterior descending coronary (LAD). With delayed gene expression, the therapeutic effect for acute myocardial infarction is severely compromised. To achieve a better therapeutic result, AAV2 vectors carrying HRE controlling heme oxygenase gene were injected into mouse heart 6 weeks before

LAD ligation.[9] However, this strategy if applied to humans can only be used on patients with previous history of heart attack or blockage of the coronary artery.

In addition to AAV2, other AAV serotypes have been isolated and their capsid genes have been cloned and used for package of AAV vectors.[12-17] A number of *in vivo* studies showed that some of these serotypes displayed tissue preference and, therefore, improved gene transduction efficiency and therapeutic effect in their respective tissues.[12,18-23] Differential gene delivery by different AAV serotypes was also observed in hearts. Du et al have shown that AAV2 has greater transduction efficiency in neonatal cardiomyocytes *in vitro*, AAV1 mediates higher transduction efficiency in adult murine cardiomyocytes both *in vitro* and *in vivo* and adult human cardiomyocytes *in vitro*.[24] The early gene expression which is crucial for the treatment of acute myocardial infarction has not been analyzed in this study. Although AAV6 has been shown to mediate widespread and early myocardial maker gene expression in rat model,[25] no therapeutic effect has been analyzed.

In this study, we compared gene expression and therapeutic effect of AAV vectors packaged in different serotypes. Gene expression mediated by AAV vectors packaged with serotypes 1 to 5 was studied in the normal mouse myocardium first. Our result shows that the onset of gene expression delivered by AAV serotype 1 and 4 has minimal delay. Since AAV4 has been shown to be inefficient in transducing human cells [15] and could not sustain long-term gene expression in murine hearts,[24] we focused our studies on comparing AAV1 and 2 mediated transgene expression. We packaged AAV-

LacZ and MLCVEGF into the capsids of these two serotypes and injected them into normal and ischemic mouse hearts. Gene expression and therapeutic effect of these two vectors mediated VEGF gene transfer were compared.

Results

AAV1 mediates earlier and higher gene expression in hearts than other serotypes

Most studies of intramyocardial gene transfer used AAV2 as vector. Previous studies have shown that LacZ gene expression could only be detected by β-gal staining in the myocardium 2-4 weeks after AAV2 mediated gene transfer. [2,3] This delayed onset of gene expression could severely reduce the therapeutic effect for acute myocardial infarction mediated by AAV2 vector, as viable myocardium may not persist in the ischemic area for such a long period. We did triphenyltetrazolium chloride (TTC) staining on 3 hearts collected 2 weeks after the occlusion of left anterior descending coronary artery (LAD) and found minimal viable myocardium in the ischemic area. Thus, early onset of gene expression is essential for the treatment of acute myocardial infarction.

Many new AAV serotypes have been cloned and have showed differential gene expression in different tissues.[24] To test if an AAV serotype other than 2 can mediate earlier onset of gene expression in myocardium, we packaged AAV-EPO into capsids of 1 to 5 AAV serotypes. 5×10^{10} genome copies of each vector were injected into normal

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mouse hearts intramyocardially with10 mice for each serotype. The EPO gene was used as a surrogate marker for the study, because hematocrit increase in response to EPO over-expression can be easily measured in the same animals at different time points after the gene transduction. Hematocrits measured 3, 7, 10 and 14 days after the vector injections showed that AAV1, 4 and 5 mediated earlier and higher hematocrit increase than AAV2. Although the hematocrits of AAV4-EPO injected mice were higher than those of other groups in day 3 after gene transfer, hematocrits of AAV1 injected mice overtook those mediated by AAV4, and became the highest 7 days after the gene transfer. The differences in hematocrits between AAV1 and AAV2 groups were significant at day 7, 10 and 14 (P<0.01) (Fig. 1).

To verify the hematocrit results, the mRNA expression of EPO gene in myocardium mediated by the 5 AAV serotypes was also analyzed by real-time RT-PCR. 5×10^{10} genome copies of AAV-EPO packaged in each capsid were injected to normal heart intramyocardially. 6 mice were used for each serotype. The results were consistent with the hematocrit measurements. AAV4 mediated earliest gene expression. Gene expression mediated by AAV1 overtook AAV4 and became the highest since day 4. Gene expression mediated by AAV3 was the lowest (Fig. 2). Gene expression mediated by AAV1 and 4 were 2.5 and 5 folds higher than that mediated by AAV2 at day 1, and 3.1 and 2.1 folds higher at day 7. Thus, the onset of gene expression delivered by AAV 1 and 4 has minimal delay.

LacZ gene expression is detected in the hearts one day after AAV1 mediated LacZ gene transfer

To compare the onset and the extent of gene expression mediated by AAV1 and 2, we packaged AAV-LacZ vector with these capsids. 5×10^{10} genome copies of AAV-LacZ packaged in each serotypes were injected directly into myocardium of normal hearts and ischemic hearts at the border of ischemic area immediately after LAD ligation. Normal hearts were collected one and 14 days after vector injections, ischemic hearts were collected 14 days after LAD ligation and vector injections. 3 normal and 3 ischemic hearts were used for each serotype for each time point. Gene expression was detected by β-gal staining. One day after AAV1-LacZ injection, LacZ expression was detected in the normal myocardium, while no LacZ expression could be detected in the hearts injected with AAV2-LacZ vector (Fig. 3a). At day 14, robust LacZ gene expression was detected in the AAV1-LacZ injected myocardium (Fig. 3b). AAV1 vectors also transduced a larger area (3 to 5 mm^3 surrounding the injection site) than AAV2 (1-2 mm^3). LacZ gene expression was mainly observed in cardiomyocytes in both serotype infected hearts by intramyocardial injection (Fig. 3b, bottom 2 enlarged squares). In the ischemic hearts, AAV1-LacZ infected almost all the cardiomyocytes around the infarct scars, while only a few cells were infected in the AAV2-LacZ injected hearts (Fig. 3c). There were less LacZ positive myocytes in ischemic hearts than normal hearts because a large part of the myocardium that were infected by CMVLacZ vectors were died and were replaced by fibrous tissues that were not infected by the virus. However, almost all of the remaining myocytes were expressing LacZ in AAV1-

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CMVLacZ infected heart, while in AAV2-CMVLacZ infected heart, only a few cardiomyocytes were LacZ positive. These data show that AAV1 can mediate early and robust gene expression in normal and ischemic myocardium.

AAV1 mediates hypoxia-inducible VEGF expression 4 days after gene transfer

Hypoxia inducible factor-1 (Hif-1) is a heterodimeric protein,[26,27] and has a labile α subunit (Hif-1α) and a constitutively expressed β subunit. The α subunit is stabilized in hypoxic tissues and binds to β subunit to form functional Hif-1 protein. Hif-1 activates transcription of several genes, including EPO and VEGF genes in hypoxic cells by binding to HRE in their enhancers.[28] Studies on rat brain have shown the Hif-1α increases immediately after the onset of hypoxia.[29] We found that Hif-1α was increased within 2 days after the occlusion of mouse LAD.[7] To test if AAV1 can mediate hypoxia-induction of VEGF expression at an earlier time than AAV2, MLCVEGF was packaged in AAV serotype 1 and 2. 5×10^{10} copies of each vector were injected into normal and ischemic mouse myocardium, 6 mice for each group. Hearts were collected 4 days after vector injection and VEGF gene expression were analyzed by real-time RT-PCR. VEGF expression mediated by AAV1-MLCVEGF was 13.7-fold higher in ischemic hearts than in normal hearts. AAV2-MLCVEGF did not mediate significant VEGF expression in both normal and ischemic hearts at this time point (Fig. 4). Hence, AAV1 mediated earlier hypoxia-inducible VEGF expression in ischemic hearts than AAV2.

AAV1 mediated intramyocardial VEGF gene transfer results in less myocardial fibrosis and better cardiac function due to earlier and more neovascular formation

To determine the benefit of early onset of VEGF expression after LAD occlusion, 5×10^{10} genome copies of MLCVEGF and CMVVEGF (packaged in AAV1 and 2) were injected into myocardium at the border of ischemic area immediately after LAD ligation. AAV2-LacZ (5×10^{10} genome copies per heart) and HEPES buffer were injected to separate groups of mice in the same way as VEGF vectors and serve as controls. 8 mice were used in each group. The cardiac function of mice was analyzed 4 weeks after LAD ligation and vector injections. The left ventricular end diastolic dimension (LVDd) and left ventricular end systolic dimension (LVDs) were measured with echocardiogram on conscious mice. The percentage of left ventricular fractional shortening (FS%) was calculated as (LVDd-LVDs)/LVDd X 100. All the VEGF injected mice had better cardiac function (greater FS%) than the LacZ and HEPES buffer injected mice (P<0.05). The fractional shortening of AAV1-CMVVEGF (38.5 ± 4.7) and AAV1-MLCVEGF (38.3 ± 5.3) injected hearts were significantly greater than AAV2-CMVVEGF (30.4 ± 5.4) and AAV2-MLCVEGF (31.2 ± 5.9) injected hearts (P<0.05) (Fig. 5 and 6). Percentage circumferential fibrous areas of these hearts were measured on trichrome stained serial sections. Although AAV2-CMVVEGF (37 ± 5) and AAV2-MLCVEGF (38 ± 7) injected hearts appeared to have smaller fibrous areas than AAV-LacZ (39 ± 5) and HEPES buffer (39 ± 5) injected hearts, the differences were not statistically significant. In contrast, AAV1-CMVVEGF (30 ± 6) and AAV1-MLCVEGF (31 ± 6) injected hearts had significantly smaller fibrous areas than AAV2-CMVVEGF, AAV2-MLCVEGF, AAV-LacZ

or HEPES buffer injected hearts (P<0.05) (Fig. 6). Thus, the early onset of VEGF expression in the LAD ligated hearts is beneficial; as it results in less myocardial fibrosis and better left ventricular function.

To study the mechanism of the better therapeutic effect mediated by AAV1 delivered VEGF gene transfer, we stained sections of hearts collected 4 days and 4 weeks after the vector injection with PCNA (proliferating cell nuclear antigen), PECAM-1 (platelet endothelial cell adhesion molecule) and smooth muscle α-actin (α-SMA) antibodies to visualize proliferating cells, capillaries and α-SMA positive vessels. Blood vasculatures at the border of infarct were counted blindly. Capillary (CAP) density was expressed as capillary numbers per cardiomyocyte. The density of α-SMA positive vessels was expressed as vessel numbers per mm^2. We detected significantly more CAP and proliferating cells in AAV1-MLCVEGF injected ischemic hearts 4 days after the vector injection than AAV2-MLCVEGF and HPEPS buffer injected hearts (Fig. 7a & b). The CAP density was about the same in AAV2-MLCVEGF and HEPES injected hearts at this early time point, which is consistent with our real-time RT-PCR data. Hypoxia-induction of VEGF expression were shown on AAV1-MLCVEGF injected hearts whereas no significant VEGF gene expression were detected in AAV2-MLCVEGF injected normal and ischemic hearts 4 days after vector injection (Fig. 4). Four weeks after the vector injection, we observed significantly more CAP (P<0.05) and α-SMA positive vessels (P<0.01) in AAV1-MLCVEGF (1.89±0.10 and 7.73±1.02) and AAV1-CMVVEGF (1.85±0.14 and 6.58±0.85) injected hearts than AAV2-MLCVEGF (1.71±0.15 and 5.27±0.52) and AAV2-CMVVEGF (1.64±0.16 and 4.84±0.36) injected

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hearts (Fig. 7 c, d, e &f). We have also detected more PCNA positive cells in AAV1-VEGF injected hearts than AAV2-VEGF and control hearts (data not show). Thus the better cardiac function and less myocardial fibrosis we observed in hearts received AAV1 mediated VEGF gene transfer were due to earlier and more neovascular formation in these hearts than the hearts received AAV2 mediated VEGF gene transfer.

Discussion:

AAV vectors have gained favor in gene therapy for ischemic heart disease due to their low toxicity and long-term expression of introduced genes. However, the therapeutic efficacy of commonly used AAV2 vectors for acute myocardial infarction has been limited by the delayed onset of transgene expression.[2,3] The cause of delayed gene expression is not clear. It has been suggested that second-strand synthesis of viral DNA is the rate-limiting step for gene expression from AAV vector.[30] However, second-strand synthesis was shown to occur within 1 day after AAV vector injection into skeletal muscle, whereas onset of expression was associated with the occurrence of high-molecular weight AAV concatamers 2 weeks after gene transfer into skeletal muscle.[31] Modification of virus by serotype selection, genetic manipulation of virion coat protein tropism may lead to better AAV transduction. The availability of alternative serotypes with improved tissue tropism has improved the application of AAV vectors. For example, AAV8 vector has been reported to increase transduction of cells in liver by 100-fold compared to an analogous AAV2 vector.[17] Differential infection of different tissues and cells with different AAV serotypes has also been reported.[18,32-35] Studies by Zabner at

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el. [19] have shown that AAV5 was more efficient in transducing both human and murine airway epithelia compared to AAV2 or AAV4. In rat retina, serotypes 5 and 4 were shown to be the most efficient. [36] Interestedly, AAV5 transduced both retinal pigmented epithelium (RPE) and photoreceptor cells, with higher level of transduction in photoreceptors, whereas AAV4 transduction was unambiguously restricted to RPE cells. [18] Recently, Blankinship et al[37] and Wang et al[38] have achieved robust gene transduction of whole body skeletal muscle and cardiac muscle by AAV6 and AAV8 mediated gene transduction. Differential gene delivery was also observed in hearts. [24,25] Thus, using an AAV serotype other than AAV2 is one of the methods to improve the therapeutic effect of AAV mediated gene therapy for ischemic hearts.

As early gene expression is necessary to prevent cardiac myocyte death caused by acute coronary artery occlusion and post-infarct remodeling, we studied the early gene expression profile *in vivo* and analyzed gene expression as early as one day after the intramyocardial gene transfer. LacZ gene expression mediated by AAV serotype 1 was detected in myocardium one day after intramyocardial injection of the vectors. We observed robust LacZ gene expression 14 days after AAV1 mediated LacZ gene delivery; a level never observed with same dose of AAV2 mediated gene transfer. More importantly, hypoxia-induction of gene expression was observed 4 days after intramyocardial injection of AAV1-MLCVEGF vector and resulted in more capillaries in the injected hearts than control hearts. At that time, no significant gene expression could be observed in normal and ischemic hearts inoculated with same dose of AAV2-MLCVEGF, and no increase of vascular density was observed in these hearts either.

The induction level of VEGF expression with AAV1 mediated gene transfer at day 4 was higher than that of equivalent dose of AAV2 at 14 days (13.7-fold vs 1.7-fold).[8] The higher hypoxia-induction of gene expression we observed in AAV1-MLCVEGF injected hearts at day 4 than AAV2-MLCVEGF injected hearts at day 14 may due to the higher Hif-1 protein in ischemic hearts at day 4 than day 14, as study in the brain showed that Hif-1α increase immediately after the onset of ischemic and reduce to half at day 14. [29] Since we have packaged the same vectors into AAV1 and AAV2 capsids and the same dose of the vectors were injected into normal and ischemic hearts using exactly the same method, we reason that the differences in gene expression were due to the differences of capsid proteins. Thus, our data indicated that AAV1 is superior to AAV2 to 5 in transduction of myocardium by intramyocardial injection. Wang et al have also indicated that although AAV8 could mediate robust intramuscular and intramyocardial gene transfer by intravenous and intraperitoneal injection, AAV1 was superior to AAV8 in direct infection of muscle tissues *in vitro*.[24] Although, it has been known for several years that AAV1 can transduce skeletal muscle more efficiently that AAV2, [21] the mechanism of AAV1 viral entry is still not clear. Since the primary receptor for AAV2 is heparin sulfate proteoglycan, [39] abundant non-cell-associated heparin sulfate proteoglycan in the extracellular matrix in myocardium [25] may absorb AAV2 particles after intramyocardial injection and limit its travel distance and infection efficiency. Unlike AAV2, the infection of AAV1 was not blocked by heparin. [40,41] Heparin binding properties or the heparin sulfate proteoglycan receptor are not required for AAV1-mediated gene transduction in muscle.[40] Thus, AAV1 may use a mechanism of uptake distinct from AAV2. Lack of influence of heparin on AAV1 mediated gene transfer may

be one reason why AAV1 is distributed to a larger area than AAV2 after intramyocardial injection.

Previous studies using marker genes, such as LacZ and alkali phosphotase receptor have shown that AAV serotype 1 and 6 can mediate higher and in AAV6, also earlier transgene expression than AAV2 after intramyocardial injection. In the present study, we used AAV1 and AAV2 to package identical VEGF vectors and injected same dose of each vectors into ischemic mouse hearts. The therapeutic effects mediated by these 2 serotypes were compared by measuring the fibrous area and cardiac function. Our results indicate that earlier onset and higher VEGF expression mediated by AAV1 result in a better therapeutic effect (less myocardial fibrosis and better cardiac function) than AAV2. AAV1 mediated VEGF gene transfer also resulted in earlier and more neovascular formation than AAV2. Except angiogenesis, VEGF has been reported to induce vasodilation[42,43] and attract stem cell homing. [44] The better myocardial protection against ischemic injury provided by AAV1 mediated VEGF gene expression may also be due to vasodilation and stem cell homing at early stage and neovasculature formation thereafter.

Since scar formed after myocardial infarction is surrounded by hibernating but viable myocardium, the extent of tissue damage, restoration of heart function and survival of the patient are therefore dependent on the rapid restoration of blood flow. [45] Early onset of VEGF expression will speed up the naturally occurred arteriogenesis process after ischemic stimuli and salvage hibernating myocardium. Thus we conclude that AAV

serotype 1 by mediates early and robust gene expression in the ischemic murine myocardium is likely to be a preferable vector of gene therapy for acute myocardial infarction.

Material and Methods:

AAV constructs and viral production

AAV-EPO and AAV-LacZ vectors and serotype 2 capsid plasmid were provided by Avigen (Alameda, CA). AAV serotypes 1, 3, 4 and 5 capsid plasmids were kindly provided by Xiao Xiao (University of Pittsburgh).[35] AAV vectors were prepared by using a three-plasmid co-transfection system.[46]

Ischemic heart model and vector inoculation

Mice were housed in the animal care facility of the University of California, San Francisco (UCSF), and experiments were conducted according to guidelines for rodent surgery with a protocol approved by the Institutional Animal Care and Use Committee of UCSF. CD1 mice (male and female) (Charles River, Wilmington, MA) were used. LAD ligation was performed as described previously. [6,7] After the LAD ligation, the corresponding area become pale. Viral vectors ($5X10^{10}$ genome copies in 50 μl HEPES buffer) were injected into myocardium of normal hearts and to ischemic hearts at the anterior border of the areas that look pale immediately after LAD ligation.

Hematocrit Measurement

Blood samples (100 μl/sample) were collected from retro-orbital veins using heparinized 75mm hematocrit tubes (Drummond Scientific Company, Broomall, PA). The tubes were loaded into a micro-centrifuge (International Equipment Co., Needham Heights, MA) and spun at 11,500 rpm for 5 minutes. Hematocrit was measured by reading the volume percentage of the red blood cells in the tubes by using a micro-capillary reader (International Equipment Co.).

Real-time RT-PCR analyses

The TRIzol RNA isolation system (Invitrogen Life Technologies, Philadelphia, PA) was used to isolate RNA. 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). 2 μg aliquot of the treated total RNA was used for each sample. 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, Foster City, CA). Primers and probes for human EPO, human VEGF and mouse HPRT were purchased from Applied Biosystems.

Histological staining:

Hearts were embedded in OCT (optimum cutting temperature compound, Sakura
Finetek U. S. A., Inc., Torrance, CA), and frozen in liquid nitrogen. Serial sections were
made from the apex of the heart to the site of the ligation and stained with Hematoxylin
and Eosin (H&E) or trichrome to study morphology and myocardial fibrosis. Percentage
circumferential fibrous area of each heart was measured on trichrome stained serial
sections and compared between different groups. To reduce the variation of the
measurement, 1ml of 0.1M of $CdCl_2$ was injected into the right ventricle to stop the heart
beat at diastolic stage. For β-gal staining, sections were fixed in 2% formaldehyde and
0.2% glutaraldehyde for 5 minutes and incubated in X-gal staining solution (0.2% 4-CL-
5-Br-3-indolyo-β-galactosidase) at 37^0C for 16 hours and counterstained with Eosin.
Anti PECAM-1 and anti PCNA (Santa Cruz Biotechnology, Inc., Santa Cruz), and anti
smooth muscle α-actin (Sigma, St. Louis, MO) antibodies were used to stain endothelial
cells, proliferating nuclears and vascular smooth muscles. Immunohistochemical
staining was conducted using the standard protocol of Elite Vectastain ABC Kit (Vector
Laboratories, Inc., Burlingame, CA).

Echocardiography

Transthoracic echocardiography was performed on conscious mice with a commercially
available system (Acuson Sequoia c256) using a 15-MHz linear array transducer
(15L8). Animals were habituated to the experimental environment prior to the
echocardiogram by inserting them into a soft plastic cone for 10 minutes every day for

three days. After the anterior chest was shaved, the mice were inserted into the plastic cone and laid naturally prone. Two-D long-axis images of the LV were obtained at the plane of the mitral and aortic valves at the largest LV cavity and adequate visualization of the LV apex, and a short-axis image was recorded at the level of the papillary muscles as described previously. [47] A 2D guided M-mode echocardiogram was recorded through the anterior and posterior walls at a sweep speed of 200 mm/sec. Images were digitally acquired and stored on magnetooptical disk. All measurements were made from digital images captured on cine loops at the time of study with the use of a specialized software package (Acuson Sequoia). The LVDd and LVDs, were measured. LV percent fractional shortening (%FS) was calculated as 100X(LVDd-LVDs)/LVDd.

Statistical Analysis

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

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Titles and Legends to Figures:

Figure 1, Kinetics of hematocrit increase after intramyocardial injection of AAV-EPO vector packaged in AAV serotypes 1-5 capsids. Standard deviations for EPO-1 and EPO-2 groups were showed. The differences of hematocrit between AAV1-EPO and AAV2-EPO injected groups were statistically significant ($p<0.01$) at day 7, 10 and 14 (*).

Figure 2, Comparison of EPO mRNA expression mediated by 1-5 AAV after intramyocardial injection. EPO mRNA in different days was measured by real-time RT-PCR. * indicate that gene expression mediated by other serotypes are significantly different from that mediated by serotype 2 ($p<0.05$). Note: AAV4 mediated the highest gene expression at day one and gene expression mediated by AAV 1 overtook AAV4 and became the highest at day 4 and day 7.

Figure 3, LacZ gene expression (blue color) in normal and ischemic hearts injected with AAV1-LacZ (left column) and AAV2-LacZ (right column). (a) Normal hearts collected one day after the vector injections. LacZ positive cells were detected in the heart injected with AAV1-LacZ, but not in the heart injected with AAV2-LacZ. (b) Normal hearts collected 14 days after the vector injections. Cross sections and enlarged views showed that LacZ expression was mainly located in cardiomyocytes. (c) Ischemic hearts collected 14 days after the vector injections. In the AAV1-LacZ injected heart, almost all myocytes around the fibrous scar tissues were expressing LacZ, while in

AAV2-LacZ injected heart, only a few LacZ positive cells were detected. Sections in (b) and (c) were counterstained with Eosin. Black bars indicate 100 μm.

Figure 4, Induction of VEGF expression 4 days after intramyocardial injection of AAV1-MLCVEGF and AAV2-MLCVEGF vectors in normal (N) and ischemic (I) hearts. Gene expression mediated by AAV1 was 13.7-fold higher in ischemic hearts than normal hearts. AAV2-MLCVEGF did not mediate significant VEGF expression either in normal or ischemic hearts.

Figure 5, Echocardiogram of the left ventricles. A: anterior wall; P: posterior wall. Note: the greater movement of the anterior wall of the AAV1-MLCVEGF and AAV2-MLCVEGF injected heart compared to the AAV2-LacZ and HEPES injected hearts. White arrows indicate the anterior walls of left ventricles at diastolic stage.

Figure 6, Left ventricular percentage fractional shortening (black) and fibrous areas (grey). Fibrous areas are presented as percentage of the LV wall. 1: AAV1; 2: AAV2; CMV: CMVVEGF; MLC: MLCVEGF. *The differences between this group and control groups (AAV2-LacZ and HEPES) are significant. **The difference between this group and AAV2-CMVVEGF is significant. Data are presented as mean ± SD

Figure 7, Vessel densities and proliferating cells in VEGF treated hearts. (a) Sections stained with anti-PECAM-1 antibody, showing CAP in hearts collected 4 days after vector injections. AAV1-MLCVEGF injected heart has more CAP than AAV2-

MLCVEGF and HEPES injected heart. (b) PCNA stained sections of hearts collected 4 days after vector injections. AAV1-MLCVEGF injected heart has more proliferating cells that AAV2-MLCVEGF and HEPES buffer injected hearts. (c) & (d) CAP numbers per cardiomyocyte (CMC) and densities of α-SMA positive vessels per mm^2 in hearts collected 4 weeks after vector injection. *The differences between AAV1 injected hearts and AAV2 injected hearts are statistically significant. MLC: MLCVEGF; CMV: CMVVEGF. (e) & (f) Sections stained with anti-PECAM-1 (e) and anti-α-SMA antibodies (f) showing CAP and α-SMA positive vessels in hearts collected 4 weeks after vector injections. Black bar indicates 20 μm.
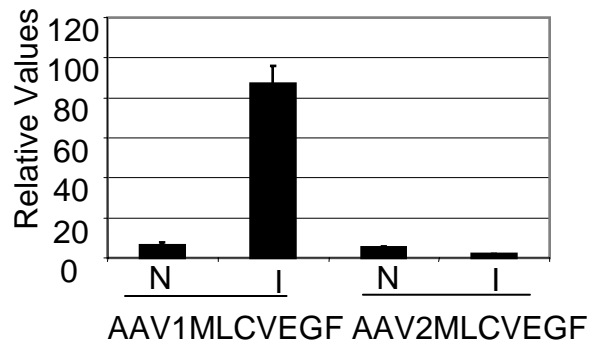
Fig. 1

Fig. 2

a   AAV-1   AAV-2

b

c

Fig. 3

Fig. 4

AAV1-MLCVEGF

AAV2-MLCVEGF

AAV2-LacZ

HEPES

Fig. 5

Fig. 6

Fig. 7