Adeno-Associated Virus-Mediated Transduction of VEGF165 Improves Cardiac Tissue Viability and Functional Recovery After Permanent Coronary Occlusion in Conscious Dogs

Matteo Ferrarini,* Nikola Arsic,* Fabio A. Recchia, Lorena Zentilin, Serena Zacchigna, Xiaobin Xu, Axel Linke, Mauro Giacca, Thomas H. Hintze

Abstract—We have previously shown that VEGF165 gene delivery into ischemic skeletal muscle exerts not only proangiogenic, but also remarkable antiapoptotic and proregenerative activity. The aim of this study was to determine whether recombinant adeno-associated virus (rAAV)-mediated gene delivery of VEGF165 into cardiac muscle, during acute myocardial infarction, exerts a protective effect to promote long-term functional recovery. Acute infarction of the anterior LV wall was induced in 12 chronically instrumented dogs by permanent occlusion of the LAD coronary artery. Four hours after occlusion, rAAV-VEGF165 or rAAV-LacZ (n=6 each; 5×10^12 viral particles per animal) was directly injected with an echo-guided needle into the dysfunctional cardiac wall. LV and arterial pressure, dP/dt max, and ejection fraction were not significantly different between the two groups over time. In contrast, in the infarcted region, at four weeks after infarction, fractional shortening was 75±18% and −3±15% of baseline and length-pressure area was 54±15% and 0.8±15% of baseline in VEGF165 versus LacZ, respectively (P<0.05). Histological analysis of the border regions showed a marked increase in the number of α-SMA–positive arterioles (68±2.8 versus 100±3.8 vessels per microscopic field in LacZ and VEGF165 group, respectively; P<0.05). In both groups, the receptor VEGFR-2 was diffusely expressed on the surviving cardiomyocytes and, consistently, myocardial viability was significantly improved in the VEGF165-treated group, with several troponin T–expressing cardiomyocytes displaying nuclear positivity for the proliferation marker PCNA. Altogether, our results indicate that VEGF165 gene delivery exerts a marked beneficial action by enhancing both arteriologenesis and cardiomyocyte viability in infarcted myocardium. (Circ Res. 2006;98:954-961.)

Key Words: VEGF receptors ■ angiogenesis ■ myocardial infarction ■ cardiac regeneration ■ gene therapy ■ AAV vectors

Oxygen deprivation in myocardial tissue after an ischemic insult initiates a process of cellular damage, eventually leading to cell death, development of a fibrous scar, and loss of contractile function. Restoration of coronary artery patency in the early hours after myocardial infarction can rescue myocardial function and viability and can improve the long-term clinical outcome.1,2 On the other hand, no reliable strategies are available for the treatment of infarction many hours after myocardial ischemia, because it is generally held that reperfusion after 3 to 4 hours of ischemia has little effect on cardiac function or infarct size.3,4 Therefore, novel therapeutic strategies need to be established, which might provide better cardioprotection and limit postinfarction cardiac failure.

In the clinical setting, the possibility of delivering genes to the ischemic myocardium as a tool to promote neovascularization and, possibly, to induce tissue regeneration, has long been considered a very appealing opportunity.5,6 A large body of experimental data in small and large animal models, as well as the first clinical studies, has indeed supported the feasibility of a gene therapy approach.7 The recent results from more controlled clinical trials that tested the induction of therapeutic angiogenesis, however, suggest some caution. The outcome of the Euroinject One Trial, which used naked VEGF165 plasmid gene transfer via directed percutaneous intramyocardial injection, has been modest.8 More encouraging results have been obtained by the Kuopio Angiogenesis Trial (KAT) and the Angiogenic Gene Therapy (AGENT)
phase II trials,\textsuperscript{9,10} which exploited the intracoronary delivery of adenoviral vectors expressing vascular endothelial growth factor (VEGF) or fibroblast growth factor (FGF)-4. Taken together, these results clearly indicate that an optimal interventional strategy still needs to be devised in terms of therapeutic gene choice, delivery vector, and administration protocol. In particular, one of the pressing needs in this field appears to be the use of gene delivery vectors which might promote sustained, prolonged, and possibly regulated therapeutic gene expression, in the absence of immune reactivity or inflammation, all properties of the adeno-associated virus (AAV).\textsuperscript{11}

A cytokine that has been originally discovered for its powerful effect on endothelial cells and has subsequently turned out to exert other multiple pleiotropic effects, including myocyte protection from injury, is VEGF, a key regulator of blood vessel formation during both vasculogenesis and angiogenesis.\textsuperscript{12,13} In particular, the use of AAV vectors has recently offered us the possibility of exploring the effects of the prolonged expression of the 165–amino acid isoform of VEGF (VEGF hereafter for brevity) in the normoperfused skeletal muscles of adult rodents. We found that VEGF causes a powerful angiogenic response, with the formation of an impressive network of newly formed capillaries and small arteries.\textsuperscript{14} Besides this effect, we also serendipitously observed that VEGF receptors are overexpressed by mouse skeletal muscle satellite cells under hypoxic and cytoxic conditions and that VEGF promotes the growth of skeletal myogenic fibers and protects myogenic cells from apoptosis and degeneration both in vitro and in vivo.\textsuperscript{15}

In the present study we explored the efficacy of AAV-mediated human-VEGF165 gene delivery to promote the recovery of function of the infarcted myocardium after permanent coronary artery occlusion in dogs. This large animal model of acute myocardial infarction resembles the situation in the human heart, in which the diffusion distance for oxygen is great and limiting. Moreover, the timing of vector injections was chosen to mimic a clinical situation in which angioplasty and reperfusion may have little beneficial effect.

Materials and Methods

The protocols were approved by the Institutional Animal Care and Use and Biosafety Committees of the New York Medical College and conform to the Guiding Principles for the Care and Use of Animals published by the National Institutes of Health and the American Physiological Society.

Surgical Procedures, Instrumentation, and Hemodynamics

Twelve male mongrel dogs (25 to 28 kg; Chestnut Grove Kennel) were instrumented using sterile techniques.\textsuperscript{16} For details, see the supplementary data available online at http://circres.ahajournals.org.

Production, Purification, and Characterization of rAAV Vectors

These methods are as previously described\textsuperscript{16,15,17} and are further described in the online data supplement.

Experimental Protocol

Hemodynamics, regional shortening, and echocardiographic measurements were simultaneously performed in conscious dogs not more than 24 hours before acute myocardial infarction. Hemodynamics, regional contractility, and echocardiographic parameters were measured and arterial blood samples collected in conscious dogs at 48 hours and then at 1, 2, 3, and 4 weeks after myocardial infarction. Our previous study based on a similar experimental approach showed that the intramyocardial injection of vehicle at 4 hours after infarction had no effect on long-term functional recovery\textsuperscript{16} (see online data supplement).

Tissue Sample Collection and Analysis

All dogs were euthanized at 4 weeks after infarction with an overdose of sodium pentobarbital (60 mg/Kg). LV tissue samples were stored immediately in either liquid nitrogen or phosphate-buffered formalin solution (3%). Samples (\textasciitilde 1 cm\textsuperscript{3} each) were collected from the center of the infarct, from the border zone, and from a remote LV region as close as possible to the previously implanted piezoelectric crystals.

Immunohistochemical Staining

Immunohistochemical staining of 2-\mu m histological sections was performed using the following primary antibodies: monoclonal mouse \textalpha-FkI-1 (Santa Cruz Biotechnology; sc-6251), monoclonal mouse \textalpha-PCNA (Santa Cruz Biotechnology; sc-56), monoclonal mouse \textalpha-Tropinin T, cardiac isoform Ab-1 (Neo Markers; clone 13 to 11), mouse monoclonal \textalpha-smooth muscle actin (clone 1A4; Sigma), and rabbit polyclonal \textalpha-hCD117, c-kit (DakoCytomation). The staining procedures were according to the Vectorstain Universal ABC kit (Vector Laboratories), using 3,3'-diaminobenzidine as the substrate (Laboratory Vision Corporation) and Vector VIP (Vector Laboratories) for the peroxidase chromogenic reaction. In the case of double immunohistochemistry, expression of PCNA was first detected using the 3,3'-diaminobenzidine substrate, followed by re-blocking and staining of the same sections for \textalpha-Tropinin T, using the Vector VIP for signal development.

X-Gal Assay

Frozen transverse sections of dog hearts injected with the AAV-LacZ were prepared either from the border zone or from the center of the infarct, or from several remote areas, and stained to visualize \beta-galactosidase expression. After fixation in 0.2% glutaraldehyde (Sigma) for 15 minutes, sections were extensively washed in LacZ washing buffer (2 mmol/L MgCl\textsubscript{2}, 0.01% NaDOC, 0.02% Nonidet-P40, in PBS) and incubated overnight at 37\textdegree C in LacZ staining solution (1 mg/mL X-gal, 5 mmol/L K\textsubscript{3}Fe(CN)\textsubscript{6}, 5 mmol/L K\textsubscript{4}Fe(CN)\textsubscript{6}, in washing buffer). Samples were then rinsed in distilled water and counterstained with nuclear fast red.

Quantification of Vessel Number and Cardiomyocyte Area

The number of \textalpha-SMA–positive vessels in the infarct border was counted on digital images of sections containing the transition between normal and infarcted heart. The data are expressed as number of arterioles per 100\times microscopic field (40 fields were randomly chosen from 4 different regions of the heart of each animal). The cardiomyocyte area was quantified on the digital images of the transmural sections stained with an \alpha-Tropinin T antibody using the Image J (National Institute of Health, http://rsb.info.nih.gov/ij/) software. Data are expressed as a percentage of the cardiomyocyte area relative to the total section area. All counts were performed by three independent researchers blinded of the experimental procedures.

Real-Time PCR

Total RNA from frozen myocardial samples was extracted using Trizol reagent (Invitrogen) according to manufacturer instructions and reverse transcribed using hexameric random primers. The cDNA was then used as a template for real-time PCR amplification to detect the expression levels of human VEGF, as well as of the endogenous canine VEGF receptors (VEGFR-1, VEGFR-2, and NP-1). The housekeeping gene 18S was also amplified and used to normalize the
results. All the amplifications were performed on a 7000 ABI Prism Instrument (Applied Biosystems), using predeveloped assays for hVEGF and 18S (Applied Biosystems) while choosing appropriate probes and primers pairs in the context of canine genome sequences homologous to the human VEGF receptor cDNAs.

Statistical Analysis
All data are expressed as mean±SEM. Changes over time were compared by one-way ANOVA for repeated measurements, followed by post-hoc test when an overall significance was detected. Differences between the two groups were tested by two-way ANOVA, followed by a Scheffe post-hoc analysis. Segmental shortening was expressed as normalized and percent values, and regional stroke work was also normalized. Percent values of shortening were tested to determine their difference from zero, no change, after the 48 hours. For statistical analysis of histological and molecular evaluations, pair-wise comparison between groups was performed using the Student t test. *P<0.05 was considered statistically significant.

Results
Transgene Expression in Infarcted Myocardium
Figure 1A shows a schematic representation of the vector injection sites in relation to the localization of the piezoelectric crystals. Both VEGF and LacZ were expressed at 1 month after delivery. As shown in Figure 1B, human VEGF RNA transcript was found throughout the infarct, with the greatest expression in the border zone, which was injected with the highest vector dose. No human VEGF expression was detected in the heart of LacZ-treated dogs, indicating that no cross-reactivity exists between human and canine VEGF mRNA in this assay. In accordance with the pattern of transgene expression assessed by real time PCR, X-gal staining of frozen myocardial sections showed transgene expression throughout the infarct center (left and right upper panels) and border (right lower panel). The asterisks indicate the crystal wires left in place for the chronic heart instrumentation.

Figure 1. A, rAAV delivery. After acute LAD occlusion it was possible to identify three different myocardial zones (infarct center, infarct border, and remote zone), here represented by different gray shadings. Each heart was injected with a total of 600 μL of rAAV suspension (either AAV-VEGF or AAV-AAV-LacZ); two injections of 200 μL in the border zone and two additional injections of 100 μL in the infarcted area center, in close proximity to the piezoelectric crystals. The black dots indicate the injection sites. B, Quantification of human VEGF (hVEGF) expression in transduced hearts. Real-time PCR at 1 month showed that the transgene was highly expressed in the infarcted myocardium, particularly in the border zone that had been injected with the highest vector dose. No hVEGF expression was detected in LacZ-treated animals. Values are normalized by the levels of the housekeeping gene 18S (mean±SEM of 3 independent quantifications from 3 different dogs per group). C, β-galactosidase expression in hearts transduced with AAV-LacZ. The X-gal staining of frozen myocardial sections showed transgene expression throughout the infarct center (left and right upper panels) and border (right lower panel). The asterisks indicate the crystal wires left in place for the chronic heart instrumentation.

Effects of Acute Infarction on Hemodynamics and Global Cardiac Function
A detailed time course of short- and long-term changes in ejection fraction, mean arterial pressure, and LV systolic pressure is reported in supplemental Figure 1. Table 1 shows hemodynamic changes at selected time points. There was no significant difference between the two groups. Heart rate increased in both the VEGF- and LacZ-treated hearts at day 2 compared with preinfarction and then returned to control levels. At day 2, there was a slight reduction in LV systolic pressure in both groups, which was not statistically significant and remained constant up to 4 weeks. LV end-diastolic pressure increased slightly, but not significantly in both groups at 2 days.

At 1 and 6 hours after occlusion of the LAD, during anesthesia, there were no changes in septal function, either

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<td>MAP, mm Hg</td>
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<td>HR, b/min</td>
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<td>LVSP, mm Hg</td>
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<td>LVEDP, mm Hg</td>
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MAP indicates mean arterial pressure; HR, heart rate; LVSP, left ventricle systolic pressure; LVEDP, left ventricular end-diastolic pressure.
progressively increased up to 47.4% of baseline at 2 days in the AAV-LacZ group and displaying reduced function or paradoxical motion at 2 days (Figure 2). We included in the analysis cardiac segments was found by assessing regional contractile performance between the two groups, a striking effect of AAV-VEGF. Although global cardiac function was not statistically different between the two groups. Paradoxical motion of the ischemic heart could be assessed by echocardiography at 60 minutes after infarction and consisted of holosystolic bulging in the LV apex and in the adjoining septum. Because of the long half-life of pentobarbital anesthesia, the first time point considered for statistical comparison in the awake state was 2 days after infarction. At this point, segments were divided into: (1) reduced shortening or (2) paradoxical motion.

To estimate the infarct size, we divided the LAD coronary blood flow in the VEGF- and LacZ-treated hearts (34.3 ± 5.5 and 36.0 ± 4.7 mL/min, respectively) by the echo-estimated mass (118 ± 10 and 130 ± 6 g, respectively). Considering an estimated normal coronary flow of 0.8 mL/min/gram, which would result in total LV flows of 94 and 104 mL/min, the percentage of the LV that was potentially ischemic was 36% and 35% in the VEGF- and LacZ-treated hearts, respectively. This estimate includes the potential presence of preexisting collaterals in the dog heart, although the reduction in segment function in the two groups was not different for the first 24 hours after occlusion.

**Sustained Effect of AAV-VEGF on Regional Myocardial Contractility After Infarction**

Although global cardiac function was not statistically different between the two groups, a striking effect of AAV-VEGF was found by assessing regional contractile performance (Figure 2). We included in the analysis cardiac segments displaying reduced function or paradoxical motion at 2 days after infarction. In the first case, regional shortening fell to 8.7 ± 4.8% of baseline at 2 days in the AAV-LacZ group and progressively increased up to 47.4 ± 12.6% by 4 weeks. In contrast, in the AAV-VEGF group, segment shortening was much less compromised at day 2 (34.5 ± 4.8% of baseline) and almost completely recovered by 4 weeks (99.6 ± 13.9%). The difference between the two groups was statistically significant.

Accordingly, when examining the segments that displayed paradoxical motion at 2 days (−17 ± 4.9% and −6.7 ± 8.1% in AAV-LacZ and AAV-VEGF, respectively), a significant recovery was observed in the VEGF group (51 ± 17.5%) but not in the LacZ group (−11 ± 2.2%). The difference between the two groups was statistically significant. No significant changes occurred in untreated infarcted hearts at the same time points (data not shown; see also reference 16).

**VEGF-Induced Angiogenesis**

In accordance with our previous findings, the prolonged expression of human VEGF in myocardium resulted in massive formation of new blood vessels, particularly present in the border region of the infarct, as shown in Figure 3A. The

**TABLE 2. Short-Term Echocardiographic Changes After Myocardial Infarction**

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<th>Baseline</th>
<th>1 Hour</th>
<th>6 Hours</th>
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<tbody>
<tr>
<td>SV, mL</td>
<td>46±4.7</td>
<td>45±5.8</td>
<td>27±0.6</td>
</tr>
<tr>
<td>EF, %</td>
<td>63±2</td>
<td>66±1</td>
<td>52±4</td>
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SV indicates stroke volume; EF, ejection fraction.
number of α-SMA–positive arterioles (50 to 120 μm diameter) was significantly increased in AAV-VEGF–treated group (100.9 ± 3.83 vessels per 100× microscopic field) relative to the AAV-LacZ group (68.81 ± 2.51 vessels per 100× microscopic field), as represented by the histograms in Figure 3B.

Cardiomyocyte Viability
Cardiac tissue sections revealed the presence of myocytes positive for PCNA, a well-known marker of cellular proliferation, in both experimental groups (Figure 4A). These actively proliferating cardiac cells were mostly detected in the border zone of the infarct, possibly suggesting an ongoing process of active regeneration of the myocardium after infarction. Viable tissue quantitation indicated that AAV-VEGF caused a significant improvement in myocardial viability (Figure 4B).

Expression of VEGF Receptors
The prompt response of the infarcted hearts to VEGF delivery and the specific effect of VEGF expression on cardiac viability indicate a possible direct role of VEGF on cardiomyocytes. To evaluate this possibility, the levels of expression of the three primary VEGF receptors (VEGFR-2, VEGFR-1, and Neuropilin-1) in infarcted hearts were assessed by real-time PCR. As shown in Figure 5A through 5C, all three transcripts were found abundantly expressed, with prevalence in the border region of the infarct. Moreover, VEGFR-1 and VEGFR-2 were upregulated in all of the three regions of VEGF-treated hearts, although the major effect

Figure 4. A, Simultaneous detection of cardiac Troponin T (red) and of the nuclear proliferation marker PCNA (brown) by double immunohistochemistry at 30 days after treatment. B, Quantification of viable myocardium at 30 days after treatment. Forty transmural sections of the infarct central zone were analyzed for each animal, by measuring the area occupied by troponin-T-positive cardiomyocytes and expressing it as a percentage relative to the area of the total section. n=6 per group; *P<0.05 vs VEGF.

Figure 5. A through C, Levels of VEGF receptor mRNAs. The graphs show the results of the quantification of the levels of the three main endogenous VEGF receptors (VEGFR-2, VEGFR-1, Neuropilin-1) by real-time PCR at 30 days after treatment. n=6 per group; *P<0.05 vs VEGF. D, Expression of VEGFR-2 by cardiomyocytes. As detected by immunohistochemical staining, the survived cardiomyocytes (indicated by asterisks) at the border of the infarct or interspersed within the connective tissue of the scar abundantly express VEGFR-2.
was again observed in the border zone, which received the highest dose of rAAV-VEGF. In contrast, the expression of Neurontin-1 appeared to be independent of VEGF delivery. Even more interesting was the observation that VEGFR-2 protein was diffusely expressed by the surviving cardiomyocytes, as shown by immunohistochemical analysis in Figure 5D. VEGFR-2-positive cardiomyocytes were mostly found at the border of the infarct, interspersed within the connective tissue of the scar.

**Discussion**

The present study demonstrates that the sustained expression of human VEGF165, after rAAV vector-mediated delivery in infarcted canine heart, markedly improves tissue viability and restores cardiac function. This is the first evidence that a VEGF-based gene therapy approach might be of important therapeutic value in the early phases of acute myocardial infarction and that its beneficial effect extends beyond the well-known angiogenic properties of VEGF. Our results underscore the importance of exploiting large animals to assess the functional impact of new therapeutic strategies that might be ultimately translated to the clinics.

VEGF gene delivery resulted in marked improvement in contractile function of the infarcted region. At 4 weeks after infarction, regional fractional shortening and shortening work returned to 75% and 54% of controls, respectively, in the VEGF-treated animals, whereas the same indexes remained almost unchanged compared with the acute phase (0%) in the LacZ-treated animals. This remarkable result indicates that AAV-VEGF delivery determined the functional recovery of a vast portion of the infarcted myocardium. Besides the conspicuous angiogenic response observed in the AAV-VEGF–treated hearts at the end of one month, several of our observations suggest that this favorable effect of VEGF might result from direct or a paracrine indirect activity of the growth factor on cardiomyocytes. Indeed, the time at which the regional cardiac function in the two experimental groups (AAV-LacZ versus AAV-VEGF) starts to differ (48 hours) is too short to depend on the formation of a consistent number of new blood vessels. This strongly suggests that VEGF might have an effect on resident cells, either by protecting the differentiated cardiomyocytes from apoptosis or by promoting their proliferation. In addition, recent observations also indicate that VEGF exerts a direct effect on cardiomyocyte contractility after its interaction with VEGFR1 and the consequent activation of PLCy1.18 As far as gene expression is concerned, we have already observed that the transgene mRNA is detectable very early after AAV vector delivery in normal tissue.15 In ischemic conditions, the time lapse before the onset of AAV transgene expression is known to be even shorter.19

Alternatively, VEGF might have a role in the recruitment or activation of a putative population of cardiac stem cells, which could contribute to tissue repair after the onset of ischemia.16,20 Indirect support for the former possibility is provided by the observation that the three major VEGF receptors are highly expressed in the infarcted heart, and VEGF1 and VEGF2 receptors are specifically upregulated in the infarct border regions after injection of AAV-VEGF. The observation that VEGF can upregulate its own receptors is not new. The ability of VEGF to upregulate VEGFR1, VEGFR2, and VEGFR3 has been shown previously in endothelial cells21and in tumor cells.22,23 Accumulating evidence indicates that beyond angiogenesis, several cytokines, including G-CSF, erythropoietin, HGF, IGF-1, and FGF, have beneficial effects on cardiac function possibly by promoting survival and/or regeneration of cardiomyocytes.16,24–26 Recent data published by Anversa and colleagues and by us suggest that the injection of a combination of HGF and IGF-1 growth factors in the infarcted hearts of rats and dogs activates an endogenous cardiac stem cell population; under growth factor stimulation, these cells are able to proliferate and differentiate, eventually leading to a significant reconstitution of the dead myocardium.16,27,28 With reference to the number of arrhythmias, we did not record multiple simultaneous ECGs using Halter monitoring over an extended period of time and therefore did not quantify the number of arrhythmic events or whether they were unifocal or multifocal or the duration of these events. It should be noted that the primary time for arrhythmias after permanent coronary occlusion in the dog is 1 to 2 hours and again during reperfusion, when the fibrillation threshold is low. By injecting the AAVs after 4 hours and never reperfusing the heart we avoided serious, even lethal, arrhythmias.

Our previous results obtained by AAV-VEGF gene transfer in the skeletal muscle clearly indicate that this growth factor exerts a direct role on differentiating myoblasts, by promoting their resistance to apoptosis as well as their fusion to myotubes.15 The overall result of this effect is a marked improvement in muscle regeneration after various types of injury. In a similar manner, other recent studies have shown that VEGF is able to exert a protective effect against hypoxic and cytotoxic damage of other different cell types independently of angiogenesis. For instance, VEGF is critical in preventing neuronal degeneration29 and in reducing hepatotoxin-induced liver damage.30 Moreover, VEGF also promotes the proliferation and differentiation of embryonic and adult progenitor cells of various lineages.30,31 Taken together, these observations suggest that VEGF might exert protective or regenerative functions that extend far beyond its angiogenic activity. Does VEGF exert a similar effect also in the heart? The observation that both heart tissue and isolated cardiomyocytes (M.G. and L.Z., unpublished data, 2005) express abundant VEGF receptors and that AAV-VEGF delivery exerts such a potent effect on cardiac viability argues in favor of this possibility. In addition, in the infarct border zones we detected several cardiomyocytes positive for the proliferation marker PCNA, suggestive of a process of tissue regeneration. Further studies are clearly required to assess whether these PCNA-positive cells represent survived cardiomyocytes that re-entered the cell cycle. In this respect, recent experimental evidence has challenged the notion that adult cardiomyocytes are in a terminally differentiated state with little proliferative potential and have shown that at least a subset of these cells might be involved in myocardial regeneration as a physiological response to tissue damage.32–34 Alternatively, the detected PCNA-positive cardiomyocytes might derive from proliferating resident progenitor cells that
have just started their differentiation process. As far as the possibility that VEGF might activate a local stem cell population is concerned, it is worth noting that, when we looked at the presence of cells with a stem cell-like phenotype, we were only able to detect a few c-kit–positive cells (1 to 3 per 40× microscopic field) in the border regions of some of the VEGF-treated hearts (supplemental Figure II), whereas no such cells were ever detected in the remote regions. Whether these sporadic cells might significantly contribute to tissue repair still remains highly speculative.

A discussion of our model is particularly important. Firstly, experiments were designed to maximize survival of the dogs and to eliminate the need for defibrillation, a confounding influence. Furthermore, we created a small apical infarct to avoid the development of heart failure in the long term, another confounding factor, and to maximize the migration of stem cells from “niches” located in the apical region in the dog heart. We did not reperfuse the heart to avoid reperfusion arrhythmias which are often lethal and may necessitate defibrillation, adding another variable to the experimental design. Also a literature already exists suggesting that there is no recovery of segment function or wall thickening over 4 weeks after 4 hours of occlusion in the dog heart. Tissue samples were taken for histological examination only after 4 weeks at the time of sacrifice so that we might compare our physiological results with the already existing literature. We did not perform studies using microspheres to quantitate flow–function relationships because the tissue immediately around the implanted crystals was used for histological analyses and the flow–function relationship in the heart is already well established. In this context, it is tempting to speculate that a substantial portion of the recovery in the VEGF treated hearts was attributable to movement up the flow–function relationship, because segment function increased. However the oxygen-flow requirements of stem cell–derived cardiac cells is still unknown. Finally, there is no existing clinical treatment for permanent occlusion of a coronary artery.

In conclusion, these results indicate the feasibility and remarkable efficacy of a gene therapy approach based on the nonpathogenic noninflammatory AAV vectors to deliver VEGF after myocardial infarction. The effectiveness of this approach most likely stems from the dual effect of this factor in both promoting angiogenesis and inducing a protective/regenerative response on cardiac cells. Most notably, the translational value of these results is underscored by the fact that they were obtained in a large animal and in conditions, 4 hours after infarction, that resemble a clinical setting in which conventional revascularization provides no therapeutic benefit.

Acknowledgments

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References


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Adeno-associated virus-mediated transduction of VEGF165 improves cardiac tissue viability and functional recovery after permanent coronary occlusion in conscious dogs

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Department Physiology, New York Medical College, Valhalla, NY and *Molecular Medicine Laboratory, International Centre for Genetic Engineering and Biotechnology (ICGEB), Trieste, Italy. * contributed equally to the project.
Surgical procedures and instrumentation.

Twelve male mongrel dogs (25-28 kg) were sedated with 0.3 mg/kg, i.m. of acepromazine and anesthetized with 25 mg/kg i.v. of sodium pentobarbital. A thoracotomy was performed in the fifth intercostal space and a fluid-filled Tygon catheter was inserted into the descending thoracic aorta for blood pressure measurements and for arterial blood sampling. The pericardium was opened, then a Doppler ultrasonic flow transducer (Craig Hartley, Houston, TX) and, immediately distal, a hydraulic occluder were placed around the left anterior descending coronary artery (LAD) immediately distal to the origin of the first diagonal branch. A second Tygon catheter was inserted into the left atrial appendage. A miniature solid-state pressure gauge (Konigsberg Instruments) was inserted into the left ventricular cavity through the apex for LV pressure measurements. The area supplied by the LAD distal to the hydraulic occluder, ie. the potentially ischemic region of the LV wall, was identified and two pairs of piezoelectric crystals were implanted in the mid-myocardium, parallel and orthogonal to muscle fiber orientation, at a distance of 12-15 mm from each other, to assess regional shortening. An additional pair of crystals was inserted in the LV lateral wall, in the distribution territory of the left circumflex coronary artery (control region). The thorax was closed in layers. Wires and catheters were run subcutaneously and exited from the back of the dog’s neck. Dogs were treated with Buprenorphine (0.1 mg/kg im) for 5 days after surgery and with Amoxicillin (2 million units sq, BID) for 10 days after surgery. After 10 to 14 days of post-surgery recovery, the dogs were trained to lie quietly on the laboratory table.
**Hemodynamics recordings and calculated parameters.**

LV and aortic pressure, dP/dt\(_{\text{max}}\), LAD blood flow and segmental shortening were measured and stored digitally as previously described \(^1\). Percent segmental shortening and the area of the LV pressure-segment length loop, corresponding to regional stroke work, were calculated and used as indices of regional contractility \(^1\). Echocardiography was performed to measure ejection fraction, stroke volume, LV dimensions, volume and mass \(^1\).

**Production, purification and characterization of rAAV vectors**

rAAV vectors were prepared by the AAV Vector Unit at ICGEB Trieste (http://www.icgeb.org/RESEARCH/TS/COREFACILITIES/AVU.htm), as already described \(^2,3\). Briefly, infectious AAV2 vector particles were generated in HEK293 cells by co-transfecting each vector plasmid together with the packaging/helper plasmid, pDG, expressing AAV and adenovirus helper functions \(^4\). Viral stocks were obtained by CsCl\(_2\) gradient centrifugation; rAAV titers, determined by measuring the copy number of viral genomes in pooled, dialyzed gradient fractions, were in the range between ~1x10\(^{12}\) and ~1x10\(^{13}\) viral genome (vg) particles/ml.

**Hemodynamics.**

Hemodynamics, regional shortening and echocardiographic measurements were simultaneously performed in conscious dogs not more than 24 hours before acute myocardial infarction. The dogs were then anesthetized (sodium pentobarbital, 25 mg/Kg i.v.), intubated and ventilated. The coronary occluder was inflated permanently to
stop LAD blood flow. Changes in hemodynamics and regional contractility were recorded at 15, 30, 45 and 60 min after occlusion and then every hour for a total of 6 hours. At four hours after occlusion, if regional motion was still paradoxical (systolic elongation and clockwise rotation of the pressure-segment loop), indicating stable and marked contractile impairment, the chest right side was scrubbed with sterilizing solutions and a total of 600 µl of rAAV suspended in phosphate buffer, corresponding to ~5x10^{12} viral particles, was injected into the infarcted LV wall by echo-guided trans-thoracic injection: 200 µl of the suspension were injected in two separate sites of the border zone and 100 µl were injected in two separate sites of the central infarcted area, where paradoxical motion was more pronounced. We chose to inject rAAV at four hours after LAD occlusion since it is known that myocardial damage becomes irreversible at that time point \(^5\). The dogs were randomly divided in two groups: six received AAV human-VEGF165 (to distinguish it from canine VEGF) and six received AAV-LacZ. Measurements were taken for 6 hours after rAAV injection, while dogs were still anesthetized. Arterial blood samples were collected on the day before and, after coronary occlusion, every hour for a total of 6 hours. They were used to assess pH, pO\(_2\), pCO\(_2\), hematocrit and electrolytes. At the end of the 6-hour post-infarction monitoring, dogs were allowed to wake up and put back in their cages.

Hemodynamics, regional contractility and echocardiographic parameters were measured and arterial blood samples collected in conscious dogs at 48 hours and then at 1, 2, 3 and 4 weeks after myocardial infarction. Our previous study based on a similar experimental approach showed that the intramyocardial injection of vehicle at 4 hours after infarction had no effect on long-term functional recovery \(^1\).
References:


Online Figure legends

Supplementary Figure 1.

A. Changes in ejection fraction. Echocardiographic monitoring over time showed only a modest and self-limiting decrease in cardiac ejection fraction during the first two days after LAD occlusion. This parameter was completely normalized at the later time points in both of the experimental groups, indicating little impact of the infarction on global cardiac function.

B. The acute occlusion of the LAD coronary artery caused a slight increase in LV systolic pressure, followed by a prompt decrease between the 6 and the 24 hours after the infarction. No significant difference was detected between the two experimental groups.

C. Consistent with changes in LV systolic pressure, arterial pressure of animals treated with either AAV-LacZ or AAV-VEGF slightly increased immediately after infarction and returned to control levels during the following day. No additional variations of mean arterial pressure were observed at later time points.

Supplementary Figure 2.

In the border region of the infarct, a few c-kit-positive cells (1-3 per 40x field) were detected by immunohistochemistry, exclusively in the AAV-VEGF-treated group.
Suppl. Fig. 1