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Adenoviral PR39 improves blood flow and myocardial function in a pig model of chronic myocardial ischemia by enhancing collateral formation

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Adenoviral PR39 improves blood flow and myocardial function in a pig model of chronic myocardial ischemia by enhancing collateral formation. Am J Physiol Regul Integr Comp Physiol 290: R494–R500, 2006. First published October 27, 2005; doi:10.1152/ajpregu.00460.2005.—Angiogenic therapy with individual growth factors or “master switch” genes is being evaluated for treatment of advanced coronary artery disease. In this study, we investigated the efficacy and mechanism of PR39, a gene capable of activating VEGF and fibroblast growth factor (FGF)-2-dependent pathways. PR39 enhances hypoxia-inducible factor-1α (HIF-1α)-dependent gene expression by selectively inhibiting proteasome degradation of this transcription factor. In addition, PR39 also stimulates expression of the FGF receptors (FGFR)-1 and syndecan-4. In a pig model of chronic myocardial ischemia, we used angiography, MRI, and microsphere regional blood flow to evaluate the efficacy of intramyocardial adenoviral protein arginine-rich peptide (Ad-PR39) injections. Ad-PR39 improved collateral scores, regional perfusion, and regional function in a dose-dependent manner. Local VEGF, VEGFR-1, VEGFR-2, syndecan, and FGFR-1 levels were 16–75% upregulated after Ad-PR39 injections as assessed by real-time PCR, suggesting upregulation of VEGF and FGF pathways. PR39 is an angiogenic peptide that improves perfusion and function of ischemic myocardium, at least in part, through collateral formation. The dual mechanism, i.e., stimulation of HIF-1α and FGF receptor expression, likely accounts for the functional benefits of PR39.

angiogenesis; growth factors; hypoxia-inducible factor-1α

GROWTH OF NEW VASCULATURE in ischemic tissue is a homeostatic response to maintain oxygen tension and is tightly regulated by angiogenic growth factors (3); “master switch” genes, such as HIF-1α (23); and extracellular matrix proteins (4, 17). Prevalence of chronic myocardial ischemia in patients with coronary artery disease suggests that the native angiogenic response is often insufficient to fully restore tissue perfusion. Since traditional revascularization approaches, such as percutaneous coronary intervention or coronary artery bypass surgery, frequently leave underperfused areas of myocardium (1), therapeutic interventions aimed at stimulating angiogenesis might be beneficial in a significant number of patients. Preclinical data have provided proof of the concept that administration of single angiogenic growth factors, such as FGF-2 or VEGF165, temporarily improve myocardial flow and function when administered in ways that ensure prolonged tissue exposure to these short-lived molecules (19). However, clinical experience with these agents has been less successful, possibly because of limited activity of a single growth factor in an adult diseased organism. Therapeutic use of master switch genes that regulate the activity of multiple angiogenic growth factors may offer benefit over single growth factor use. The best known gene is hypoxia-inducible factor-1α (HIF-1α), a transcription factor capable of inducing expression of a number of growth factors and receptors, including VEGF-A, VEGFR-1 (7), TGF-β3 (2), IGFl (5), and erythropoietin (24). Interestingly, the intracellular level of the HIF-1α level is primarily regulated by the rate of proteasomal degradation of the mature protein, which is inhibited by hypoxia (11) and is preceded by oxygen-dependent hydroxylation of critical proline moieties in HIF-1α.

We have recently described selective inhibition of proteasomal degradation of HIF-1α by a proline/arginine-rich peptide (PR39) (6, 14), which resulted in upregulation of HIF-1α-dependent genes including VEGF and VEGFR-1. In addition, PR39 upregulated expression of FGF receptors FGF-R1 and syndecan-4 expression, thus activating FGF signaling (9, 14, 25). When overexpressed in mice hearts under control of the MHC promoter or when mixed in a subcutaneously implanted Matrigel pellet, PR39 induces robust angiogenesis (14).

The present study was undertaken to test the potential therapeutic efficacy of adenoviral (Ad)-PR39 in a clinically relevant model of chronic myocardial ischemia and to relate its efficacy to the proposed mechanism of action that includes upregulation of HIF-1α-dependent genes and FGF receptors.

We find that sustained-release intramyocardial delivery of PR39 through adenoviral transfection improves myocardial perfusion and function of the ischemic territory in this porcine model of chronic myocardial ischemia.

MATERIALS AND METHODS

Adenovirus PR39, green fluorescent protein, and HIF-1α

The adenoviral (Ad) constructs for PR39 and green fluorescent protein (GFP) were based on adenovirus type 5 with E3 deletion and...
El replacement by a cytomegalovirus (CMV) promoter and a β-globin gene split by a polylinker region. Porcine PR39 or GFP was inserted in the polylinker region using EcoRI and ClaI restriction sites (Harvard Gene Therapy Initiative, Harvard Medical School, Boston, MA).

AdCA5 (AdTrack/HIF-1αCA5) was provided by Dr. Gregg Semenza, Johns Hopkins University (Baltimore, MD). The nucleotide sequence encoding HIF-1αCA5 [HIF-1α(1–391/521–826)] was excised from pCEP4/HIF-1α(1–391/521–826) and ligated into pAdTrack-CMV. Homologous recombination was performed in Escherichia coli. Recombinant plasmid pAdEasy-1. Recombinant plasmid was transfected into 293 cells for adenovirus production. Mismatch mutations (Pro567Thr and Pro658Gln) were introduced by PCR during construction of pCEP4/HIF-1α(1–391/521–826) and are required for constitutive activity of CA5 (12).

Cell Culture
HUVECs (Cambrex BioScience) cultured in endothelial basal medium (EBM)-2 medium were supplemented with endothelial growth medium (EGM)-2 SingleQuots (Cambrex) and FBS.

Transduction protocol. HUVECs, passage 5, plated overnight in six-well plates at density 3 × 10^5 cells/well were exposed to Ad-PR39 or AdCA5 at 100 plaque-forming units (PFUs) per cell in full medium for 24 h. Afterward, the medium was replaced with fresh medium without adenovirus construct, and cells were harvested 24–48 h later. Under these conditions, ~100% of cells were infected based on GFP expression as determined by fluorescence microscopy for AdCA5 (data not shown).

Immunoblotting. Cellular protein was extracted by radioimmunoprecipitation (RIPA) lysis buffer [50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% deoxycholate sodium supplement with protease inhibitor cocktail (Roche)]. The lysates were clarified by ultracentrifugation (14,000 g, for 15 min, at 4°C), and 35 µg cellular protein, determined by bicinchoninic acid assay ( Pierce), were subjected to SDS-PAGE (12% polyacrylamide gel). After transfer to Immobilon membranes (Millipore), the membranes were then blocked with 5% nonfat dried milk in TBS, pH 7.4, containing 0.1% Tween 20, and immoblotted with anti-VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Immuno reactive bands were visualized using the horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescent substrate (Pierce). Equal protein loading was assessed by reprobing the blot with anti-actin antibody (Santa Cruz Biotechnology).

Animal Model
Male Yorkshire pigs (n = 46, Parsons Farm, MA) weighing 20–25 kg were used for the in vivo study. The chronic ischemia model consisted of three phases (8). In brief, for amiodarone surgery and catheterization at 3 and 6 wk, the animals were anesthetized with 20 mg/kg ketamine im and 2 µg/kg fentanyl, intubated, mechanically ventilated with O2, and further anesthetized with 50–100 µg·kg^−1·h^−1 fentanyl and 0.2 mg·kg^−1·h^−1 versed. During MRI, 0.2 mg·kg^−1·h^−1 pancuronium was added to ensure breath hold. Postoperatively, all animals received antibiotics and analgesics for 48 h. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Institutional Animal Care Committee.

A plastic aortomer (ID: 1.75–2.25 mm, Research Instruments, Escondido, CA) was placed on the proximal left circumflex artery (LCX) through a left lateral fourth intercostal thoracotomy. Three weeks later (second phase, midstudy), pigs were reanesthetized and randomly assigned to either: 1) Ad-GFP 3 × 10^6, 2) Ad-PR39 3 × 10^8, or 3) Ad-PR39 3 × 10^10 PFUs. After randomization, right and left coronary catheterization was performed through standard femoral access after systemic coagulation with heparin 100 U/kg. Intravascular pressures and ECGs were recorded. Selective left and right angiography (General Electric; contrast: MD-R76, Mallinckrodt) confirmed complete occlusion of the LCX and allowed semiquantitative assessment of the presence of collaterals to the LCX territory, according to the Rentrop scoring system from 0 to 3, as previously described (15): 0 = none, 1 = filling of side branches, 2 = partial filling of the main artery via collateral channels, and 3 = complete filling of the main artery. For regional blood-flow measurements, nonradioactive isotope-labeled microspheres were injected at rest and during intravenous infusion of 16 µg·kg^−1·min^−1 dobutamine (see Regional Blood Flow). Directly thereafter, we performed function and perfusion MRI to quantify baseline regional cardiac function and perfusion. Through a second thoracotomy, the lateral wall (target ischemic area) of the ventricle was exposed, and 9–10 injections of 100 µl of adenovirus were given at a needle depth of 5 mm. Up to four injection sites were marked with a 6-0 Prolene suture (Ethicon, summerville, NJ).

Three weeks after therapy (final study), repeat angiograms were made and two additional sets of microspheres were injected to measure regional blood flow. Function and perfusion MRI was also repeated. Finally, animals were euthanized, and the hearts were excised. Eight animals were euthanized 4 days after therapy for molecular analyses.

Regional Blood Flow
Microspheres were injected into the left atrium as previously described (21). At midstudy and during the final study at rest and dobutamine (16 µg·kg^−1·min^−1) stress, 6 × 10^6 microspheres (BioPal, Wellesley, MA) were injected. Reference blood samples were drawn simultaneously at 4 ml/min using a withdrawal pump (Harvard Apparatus, Bedford, MA). At the end of the study (final study), a 1-cm thick cross section of left ventricle at midpapillary level was taken and divided into 12 radial segments as previously described (21, 22). Three segments in the LCX territory were further subdivided in an endocardial and epicardial piece. Tissue samples and reference blood samples were analyzed by a core lab (BioPal). Regional myocardial blood flow was calculated as blood flow (tissue sample X; ml·min^−1·g^−1) = [withdrawal rate (ml/min)/weight (tissue sample X; g)] × [y counts (tissue sample X)/γ counts (reference blood sample)]. Samples with significant infarct (>50% of tissue sample) were excluded from regional blood flow analysis.

Myocardial Magnetic Resonance Imaging Analysis
Arterial pulse-gated magnetic resonance imaging was performed in a 1.5-Tesla whole body Siemens Sonata. After scout images with turboFLASH technique, 24 sequential image frames were collected over 12 heartbeats during breath hold by using shared-center turboFLASH in each of the three standard views to assess myocardial function. For detection of optimal inversion time (typically 200–300 ms), a series of 32 diastolic images were acquired in the double-oblique four-chamber view during breath hold, while injecting 0.05 mmol/kg gadodiamide (T1-reducing contrast agent). The series of images was viewed as a movie to locate the zone with impaired contrast arrival. The short axis at the center of that zone (target zone) was prescribed graphically. Custom designed software was used to define myocardial borders and measure wall thickness. End-systolic and end-diastolic left ventricular volumes were computed from biplane measurement (apical 4-chamber and 2-chamber views), and used for calculating ejection fraction. Target wall motion was defined as the systolic endocardial movement toward a centroid in the ventricular lumen (radial shortening) at the level of maximal perfusion deficit and was expressed as percentage of the radial length at the end of diastole. Normal wall motion was measured at the septum. The area of delayed contrast arrival was defined as myocardium demonstrating distinctly slowed time (>1 cardiac cycle) to half-maximal signal intensity, using a two-dimensional map of contrast intensity vs. time (16).
RESULTS

A total of 46 male Yorkshire pigs weighing 25–30 kg underwent ameroid surgery. Eleven died prematurely, eight before and three after initiation of treatment (2 after low-dose Ad-PR39 and 1 after Ad-GFP). One animal (high-dose Ad-PR39 group) died during final angiography. Twenty-four animals had a complete follow-up (8 in each treatment group) of 21 days, and all eight animals marked for the gene expression study completed the 4-day follow-up. Two additional animals with a follow-up of 4 days were used for studies of transfection efficiency (Fig. 1). Small areas of myocardial necrosis (mean of 2.9, 2.6, and 2.4% of the total myocardial territory, P = not significant) were present in Ad-GFP, Ad-PR39 low-dose, and Ad-PR39 high-dose groups, respectively.

Angiography

Coronary angiograms were obtained before treatment and 3 wk later at the final study. Semiquantitative blinded analysis using a Rentrop scale, showed a significant increase in collateral index in the high-dose Ad-PR39 group, whereas low-dose Ad-PR39 and Ad-GFP had no significant effect (Fig. 2), suggesting induction of collateralization (arteriogenesis) by Ad-PR39.

Regional Blood Flow and Myocardial Perfusion

Myocardial blood flow was determined using neutron-activated microspheres at the peak of dobutamine infusion and at rest at both midstudy and final study. As previously observed, regional blood flow at stress increased in the ischemic territory of all groups. However, the high-dose Ad-PR39 resulted in a significantly better improvement than the control treatment (Table 1, P = 0.03), whereas the low dose had an intermediate effect. To exclude the possibility that these changes in regional blood flow were due to differences in perfusion pressures or to growth of the hearts, we also analyzed regional blood flow in the nonischemic territory (septum and part of anterior wall) and normalized the ischemic flow to nonischemic flow (Fig. 3). Regional blood flow in the nonischemic territory also improved over time, but this was similar in the three groups (F = 0.74, P = 0.49). Therefore, flow in the ischemic territory at stress and normalized for flow in the nonischemic territory, showed a similar trend as nonnormalized flow. The increase in the high-dose PR39 group was significantly higher than in the controls (P = 0.02 vs. GFP control). At rest, the differences

**VEGF-A, VEGF-1, VEGFR-2, FGFR-1, Syndecan, and PR39 Expression**

Total RNA from suture-marked samples was isolated and homogenized with TRI Reagent (Sigma, St. Louis, MO) and extracted according to the manufacturer’s protocol. RNA was quantified with a spectrophotometer and quality was checked by gel electrophoresis. cDNA was synthesized with random hexamers and ThermoScript RNase H − Reverse Transcriptase (Invitrogen, Carlsbad, CA) at 48°C for 30 min followed by Ribonuclease H (Invitrogen) treatment.

We used the following primers for porcine sequences of β-actin: 5′-ACGAGACCCATTCACACTGATC-3′ and 5′-AGGTCCCTCTCAGTGTCCACGT-3′; VEGF-A: 5′-CATGCAGATATTGCAG- GATCA-3′ and 5′-TTCCTCTCATGTGCTGGCCCTTG-3′; VEGFR-1: 5′-CCCCAAAGAGCCCAAGATT-3′ and 5′-GCAAGGTCAGCTA- GTTTTTCCA-3′; VEGFR-2: 5′-ACTGTTCTGGCCACACATC-3′ and 5′-TCTGTACCTCCACCTGTTTC-3′; syndecan: 5′-AGCTGCTTGAAGGTA- CAGGATGTGTCCAACAAGGT-GGTCCTTCG-3′; VEGFR-3: 5′-CTCTACCGCCTCCTGGAGCT-3′; Ad-PR39: 5′-GCCCTTCATAAT-TCCCCCA-3′, forward and reverse, respectively.

We performed quantitative real-time PCR on 10 ng reverse transcribed total RNA with 50–100 nM of both primers in 30 μl using SYBR Green PCR Master mix (Applied Biosystems) in the GeneAmp 5700 sequence detector (Applied Biosystems) under default conditions (AmpliTaq Gold activation: 95°C for 10 min and then PCR reaction: 40 cycles of 95°C, 15 s and 60°C, 1 min). The presence of a single amplicon was tested by dissociation curves at the predicted melting temperature.

To quantify absolute values of genes, RT-PCR products were subcloned in pCR 2.1-TOPO using the TOPO TA Cloning kit (Invitrogen), and sequence was verified. cDNAs were diluted in the subcloned microspheres at the peak of dobutamine infusion and at rest at both midstudy and final study. As previously observed, regional blood flow at stress increased in the ischemic territory of all groups. However, the high-dose Ad-PR39 resulted in a significantly better improvement than the control treatment (Table 1, P = 0.03), whereas the low dose had an intermediate effect. To exclude the possibility that these changes in regional blood flow were due to differences in perfusion pressures or to growth of the hearts, we also analyzed regional blood flow in the nonischemic territory (septum and part of anterior wall) and normalized the ischemic flow to nonischemic flow (Fig. 3). Regional blood flow in the nonischemic territory also improved over time, but this was similar in the three groups (F = 0.74, P = 0.49). Therefore, flow in the ischemic territory at stress and normalized for flow in the nonischemic territory, showed a similar trend as nonnormalized flow. The increase in the high-dose PR39 group was significantly higher than in the controls (P = 0.02 vs. GFP control). At rest, the differences

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**Fig. 1.** Two examples of β-galactosidase (blue cells) staining 4 days after injection of AdLaZ in porcine myocardium through the transepicardial route, suggesting high transfection efficiency and a reasonable spread of transfected cells away from the injection tract. Scale bar = 1 mm
RBFs were assessed before treatment (mid) and 3 weeks after treatment (fin). Analysis by paired sign test.

We have previously shown that delayed arrival of contrast on MRI studies designates the area of myocardium with impaired perfusion (16). Therefore, perfusion MRI was used to quantify the size of the area with abnormally perfused myocardium at rest and before and after Ad-PR39 therapy. The zone with delayed arrival before treatment was 16.7 ± 3.6% in the GFP group, 18.5 ± 4.7% in the low-dose PR39 group, and 16.2 ± 3.9% in the high-dose PR39 group (ANOVA, P = 0.50). Ad-PR39 resulted in a dose-dependent, albeit a modest, 20% decrease in the size of areas exhibiting delayed contrast arrival in both Ad-PR39 groups, whereas it slightly increased in the Ad-GFP group (Fig. 4; ANOVA, P = 0.04). Thus two different assessments of perfusion showed improved regional perfusion of the ischemic myocardium by Ad-PR39.

**Myocardial Function**

To determine whether PR39-induced improvement in myocardial perfusion resulted in functional improvement of myocardial contractility, MRI was used to assess regional radial wall motion in the ischemic territory before and after treatment initiation. Ad-PR39 treatment produced a dose-dependent increase in target wall motion (Table 2).

**Effect of Ad-PR39 on myocardial gene expression.** Myocardial gene expression by Ad-PR39 was ascertained in tissue samples taken from injection sites that were harvested 4 days after Ad-PR39 injection. Transcriptional analysis of gene expression by qRT-PCR showed a dose-dependent increase in mRNA expression of all genes studied with the greatest increase seen with PR39 hi. Ad-GFP did not affect gene expression. The increases in mRNA expression were highly significant (ANOVA, P < 0.01 compared with zero; †P < 0.01 compared with green fluorescent protein (GFP) and PR39 low).

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**Table 1. Regional blood flow at rest and stress in the normal territory and the target ischemic territory**

<table>
<thead>
<tr>
<th></th>
<th>GFP</th>
<th>PR39 low</th>
<th>PR39 high</th>
<th>F value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rest</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0.76±0.40</td>
<td>0.70±0.25</td>
<td>0.69±0.14</td>
<td>0.17</td>
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<tr>
<td>Mid</td>
<td>0.94±0.48</td>
<td>0.93±0.23</td>
<td>0.88±0.28</td>
<td>0.08</td>
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<tr>
<td>Fin</td>
<td>0.16±0.74</td>
<td>0.22±0.40</td>
<td>0.19±0.40</td>
<td>0.03</td>
</tr>
<tr>
<td>Ischemia</td>
<td>0.53±0.31</td>
<td>0.42±0.23</td>
<td>0.44±0.14</td>
<td>0.49</td>
</tr>
<tr>
<td>Mid</td>
<td>0.53±0.45</td>
<td>0.64±0.34</td>
<td>0.63±0.23</td>
<td>0.31</td>
</tr>
<tr>
<td>Fin</td>
<td>0.00±0.20</td>
<td>0.21±0.28</td>
<td>0.18±0.25</td>
<td>0.96</td>
</tr>
<tr>
<td>Stress</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>2.13±1.41</td>
<td>1.66±0.37</td>
<td>1.60±0.45</td>
<td>1.23</td>
</tr>
<tr>
<td>Mid</td>
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<td>2.00±0.45</td>
<td>2.34±0.85</td>
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<tr>
<td>Fin</td>
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<td>0.74±0.65</td>
<td>0.74</td>
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<tr>
<td>Ischemia</td>
<td>1.01±0.37</td>
<td>0.79±0.34</td>
<td>0.74±0.28</td>
<td>1.38</td>
</tr>
<tr>
<td>Mid</td>
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<td>1.42±1.16</td>
<td>1.72±0.68</td>
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<tr>
<td>Fin</td>
<td>0.36±0.20*</td>
<td>0.33±0.21*</td>
<td>0.97±0.62†</td>
<td>2.21</td>
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</table>

Data are regional blood flow (RBF; ml·min⁻¹·g⁻¹) in means ± SD, n = 8 for each group. RBFs were assessed before treatment (mid) and 3 weeks after treatment (fin). *P < 0.01 compared with zero; †P < 0.01 compared with GFP. RBFtarg/norm(mid) and RBFtarg/norm(fin), where targ is target (left circumflex artery territory) and norm is normal zone, septum, and left descending artery territory. The midstudy was performed just before treatment, and the final study was done 3 wk later. The increase in RBF was significantly higher in the high-dose Ad-PR39 group than in the Ad-GFP group (P = 0.02). Bars represent means ± SE, n = 8 for each group.
Table 2. MRI function

<table>
<thead>
<tr>
<th>Wall Motion, %</th>
<th>GFP</th>
<th>PR39 low</th>
<th>PR39 high</th>
<th>F value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Mid</td>
<td>31.0 ± 5.1</td>
<td>34.3 ± 4.0</td>
<td>37.6 ± 4.8†</td>
<td>3.88*</td>
</tr>
<tr>
<td>Fin</td>
<td>33.2 ± 5.4</td>
<td>37.5 ± 6.5</td>
<td>40.8 ± 5.7†</td>
<td>3.38*</td>
</tr>
<tr>
<td>Fin-mid</td>
<td>2.2 ± 2.5</td>
<td>3.1 ± 7.4</td>
<td>3.2 ± 6.2</td>
<td>0.07</td>
</tr>
<tr>
<td>Ischemia Mid</td>
<td>24.5 ± 6.2</td>
<td>22.2 ± 9.1</td>
<td>23.7 ± 5.9</td>
<td>0.19</td>
</tr>
<tr>
<td>Fin</td>
<td>20.5 ± 4.2</td>
<td>25.3 ± 9.6</td>
<td>29.2 ± 8.8</td>
<td>2.34</td>
</tr>
<tr>
<td>Fin-mid</td>
<td>−3.9 ± 6.5</td>
<td>3.1 ± 5.4</td>
<td>5.5 ± 9.9†</td>
<td>3.12*</td>
</tr>
</tbody>
</table>

Data are means ± SD, n = 8 for each group. Wall motion in the normal and target ischemia territory was measured just before treatment (mid) and 3 weeks after treatment (fin). ( *P < 0.05 for F test, †P < 0.05 for PR39 high vs. GFP, post hoc least significant difference test).

after Ad-PR39 or Ad-GFP administration in a separate set of experiments. Real-time PCR analysis of samples (n = 8) demonstrated that Ad-PR39 mildly but significantly increased the expression of HIF-1α-dependent genes including VEGF-A165 (41% vs. Ad-GFP P = 0.04), VEGFR-2 (75%, P = 0.03), and VEGFR-1 (25%, P = 0.0001). In addition, there was a significant increase in FGF-R1 (45%, P = 0.004), and a strong trend in syndecan-4 gene expression (16%, P = 0.07). These results are in agreement with the predicted mechanism of action, thus confirming a HIF-1α-dependent and a HIF-1α-independent gene expression profile of PR39 (Fig. 5, A and B). To compare Ad-PR39-induced changes in VEGF expression with Ad-HIF-1α induction, we then tested both constructs in cultured cells. VEGF expression was similar under both conditions (Fig. 5C). Ad-PR39 was still detectable after 3 wk at the sites of injection. No transgene was detected in the septum (Fig. 5D).

DISCUSSION

The present study provides proof of concept that PR39, administered as Ad-PR39 via direct intramyocardial injection, improves myocardial perfusion and function under conditions of chronic myocardial ischemia. We observed a dose-dependent increase in myocardial blood flow at the peak of dobutamine-induced stress, and a 20% decrease in the size of the abnormally perfused territory at rest. This improved perfusion resulted in improved regional ventricular function in the ischemic territory, as measured by target wall motion.

The improvement in blood flow with a maximum of \( \sim 1 \) ml·min\(^{-1}\)g\(^{-1}\) compares favorably with increases of 0.6 and 0.55 ml·min\(^{-1}\)g\(^{-1}\) observed after respective administration of intracoronary VEGF\(_{165}\) (22) or FGF-2 (21) proteins in the same model. The observed magnitude of improvement in the radial wall motion of the ischemic left ventricular segment is also higher than previously reported (21).

The increase in angiographically visible epicardial collaterals observed in the high-dose Ad-PR39 animals and in the high collateral index score and the absence of any effect in the low-dose and the GFP group, indicates that at higher-dosage PR39 induced the formation of collaterals and hence stimulated arteriogenesis. However, it should be noted that this study was not powered to directly compare collateral index, as ordinal parameter, between two treated and one control group.

In accord with previous in vitro and transgenic mouse observations, PR39 augmented the expression of HIF-1α-dependent
genes, such as VEGF and its receptor VEGF-R1, as well as the HIF-1α-independent genes VEGF-R2, FGFR-1, and syndecan-4 (14). Although VEGF-R1 is known to be a HIF-1α-responsive gene, VEGF-R2 is not. We attribute the increase in VEGF-R2 expression seen in this study, as well as in transgenic mice expressing PR39 in cardiac myocytes (14), to prolonged increase in tissue VEGF levels with a secondary increase in VEGF-R2 expression. The mechanism of PR39 dependent augmentation of FGF-R1 and syndecan-4 expression has not yet been determined, but is a consistent finding (14).

The combined effect of these changes in PR39-induced gene expression results in concerted activation of VEGF and FGF signaling cascades with FGF-R1 and syndecan-4 acting as mediators of FGF signaling (9). Synergism of FGF-2 and VEGF with respect to proangiogenic effects has been shown in an in vitro system (18). However, a direct inference with regard to synergism in the current study is difficult in the absence of data on the efficacy of a VEGF/FGF combination therapy in this model.

In this study, PR39 increased the levels of VEGF, VEGFR-2, and FGFR-1 mRNA by 40–50%, as measured by real-time PCR. Since ischemia by itself induces expression of VEGF and VEGF receptors, the activity of PR39 represents an additional increase over and above that value. This likely explains why the magnitude of the increase is less than ~200% increase seen after PR39 injection into normal tissues (14). The observed profile of gene expression is in accordance with the observed proangiogenic effect, such as increased regional blood flow.

We have elected to use adenoviral delivery of PR39 because of the short half-life of PR39 peptide in tissues. Single injection of adenoviruses in naive pigs produces prolonged construct expression as confirmed in our study. Whether this mode of therapy is eventually useful in patients with varying and sometimes high titers of anti-adenoviral antibodies is uncertain. Alternative viral therapies, such as adenovirus-associated virus and lentiviruses may be sought to overcome this potential shortcoming (10).

Because we used direct intramyocardial injection through the transepicardial route, which is clinically feasible as an adjunct to coronary artery bypass surgery, or through transcutaneous pericardial access in patients with a pristine pericardial space, the patient population that would benefit from this therapy would still be limited. In previous studies with adenoviral constructs we have shown that transendocardial injection with a catheter-based technique is equivalent to transepicardial injection in terms of protein expression (13, 20) and can be performed safely during a routine cardiac catheterization, thus greatly increasing the potential target population.

**Limitations**

This study was performed in a pig model of chronic myocardial ischemia, a commonly used model to study the efficacy of angiogenic interventions. The model is standardized in that it creates a LCX occlusion only and utilizes healthy, young pigs. Being an animal model, direct translation to potential clinical efficacy is highly questionable. Angiographic outcome was beneficial in the high-dose Ad-PR39 group, but the study was not powered for between-group comparison of ordinal data. Although regional blood flow by microsphere analysis and by perfusion MRI improved in a dose-dependent manner, it is clear that the GFP-treated group also improved, a phenomenon commonly seen in this model, which may be a preclinical correlate of the substantial placebo effect observed in most clinical trials.

In this model, global ventricular function is typically not compromised by the 10–20% hibernating part of the free ventricular wall and therefore cannot be used as a clinically relevant end point. Although it is beyond the scope of this study to fully discuss recent disappointing results of proangiogenic therapy in clinical trials, the negative outcome of the vascular endothelial growth factor in ischemia for vascular angiogenesis (VIVA) trial with respect to function was predicted from the results of a preclinical study in the pig ameroid model (22). It is, however, clear that studies in these animal models provide a proof of principle rather than an accurate prediction of clinical efficacy.

The transgene was still detectable 3 wk after injection, and it is assumed that the level of PR39 protein is augmented, as well. Expression of PR39 at the protein level has proven very hard to establish because the available antibodies are not suited for Western blot analysis and do not distinguish transgenic PR39 from native porcine PR39. In other species, this has not been a problem because PR39 analogs are sufficiently homologous to allow discrimination.

Despite these limitations, we have provided evidence that Ad-PR39 improves myocardial perfusion and function most likely through an increase in vessel size or number. PR39 might stimulate arteriogenesis and angiogenesis through several mechanisms. Important proangiogenic and arteriogenic growth factors and their receptors, such as VEGF, FGFR-1, and syndecan-4, are expressed at higher levels in the presence of PR39. Single administration of VEGF, as well as FGF-2, has led to similar phenotypes in the chronic myocardial ischemia model as observed for PR39 (15, 22). It is also possible that the improvement in perfusion is secondary to improved function with associated flow demand. Many growth factors, including FGF-2 and VEGF, are cardioprotective through antiapoptotic mechanisms (reviewed in Ref. 26), and indeed, PR39 has a direct antiapoptotic effect, as well (27). Detailed exploration of the temporal sequence of changes in perfusion and function would be required to resolve this question.

In conclusion, PR39 improves myocardial perfusion and function when used in an adenoviral vector injected directly into the ischemic myocardium. This effect is likely consequent to the well-established proangiogenic effect of PR39 and the observed effect on collateral formation. Further evidence is provided that PR39 exerts this effect partly via stimulation of HIF-1α and partly via induction of FGF receptor expression. This dual mechanism of activity likely accounts for the functional benefits of PR39 therapy. Final proof of efficacy of this protein awaits carefully designed randomized, double-blind, placebo-controlled, clinical efficacy trials.

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