Efficacy of Therapeutic Angiogenesis by Intramyocardial Injection of pCK-VEGF165 in Pigs

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Background. Intramyocardial injection of vascular endothelial growth factor (VEGF) plasmid DNA was studied to demonstrate improvement of regional myocardial function.

Methods. Twenty-one pigs that had undergone ligation of the left anterior descending coronary artery were randomly allocated to one of two treatments: intramyocardial injection of pCK-VEGF165 (VEGF group) or pCK-Null (control group) into the ischemic border zone. Electrocardiogram-gated single-photon emission computed tomography was performed 30 and 60 days after the coronary ligation. Segmental variables of perfusion and function were automatically quantified using a 20-segment model. In the segmental analysis, 119 segments were selected for analysis (71 segments in the VEGF group; 48 segments in the control group). Histologic analysis was also performed in the myocardial tissue of the ischemic border zone.

Results. At day 30, there were no significant differences in segmental perfusion, wall thickening, and wall motion between the two groups. In the VEGF group, all variables of perfusion, wall thickening, and wall motion were significantly improved at day 60 compared with those at day 30 ($p < 0.05$), while there were no differences in the control group. At day 60, perfusion ($p = 0.018$), wall motion ($p = 0.004$), and wall thickening ($p = 0.068$) of the VEGF group were improved compared with those of the control group. Histologic analysis showed that microcapillary density was significantly higher in the VEGF group than the control group ($p < 0.001$).

Conclusions. Intramyocardial injection of pCK-VEGF165 significantly augmented neoangiogenesis in the ischemic area and improved regional myocardial function as well as myocardial perfusion.

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Vascular endothelial growth factor (VEGF), existing as four isoforms of 206, 189, 165, and 121 amino acid residues [1], is a direct-acting endothelial cell mitogen and angiogenic factor with high endothelial selectivity [2]. Vascular endothelial growth factor has been used in the study of growth factor therapy for ischemic heart disease since the concept of therapeutic angiogenesis [3] was first introduced.

Although previous studies [4–6] showed that gene transfer of naked plasmid DNA encoding VEGF165 promoted collateral vessel development or perfusion increase without any evidence of arteriovenous anomaly or inflammatory reaction, they did not demonstrate improvement in regional myocardial function. The aim of this study was to elucidate the efficacy of therapeutic angiogenesis induced by intramyocardial injection of pCK expression vector containing the human VEGF165 gene (pCK–VEGF165) in a porcine model.

Material and Methods

pCK–Vascular Endothelial Growth Factor 165

Complementary DNA (cDNA) encoding VEGF165 was cloned from total RNA prepared from human vascular smooth muscle cells by reverse transcription polymerase chain reaction amplification. The polymerase chain reaction primers were 5′-AAGATGAACCTTCTGCTGTCT-3′ and 5′-TCTAGATCACCCGCTCGTGTACATCT-3′. The amplified cDNA was initially cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA) and its nucleotide sequence was confirmed by sequencing. The construct pCK–VEGF was created by the insertion of the cDNA encoding VEGF165 into the pCK vector. The major characteristics of pCK that differentiate it from many other human cytomegalovirus promoter-based expression vectors are that it contains not only the full-length immediate–early promoter of human cytomegalovirus but also its entire 5′ untranslated region, consisting of the entire exon 1 and intron and a part of exon 2 to increase the rate of gene expression. It replaces the β-lactamase gene with the kanamycin resistance gene to avoid allergic reactions caused by residual ampicillin in the final DNA solution used for transfection [7].
Porcine Myocardial Infarction Model
Twenty-one male pigs (mean body weight, 31.2 ± 1.9 kg) underwent surgical manipulation to create left ventricular myocardial infarction. Xylazine (2 mg/kg), ketamine (20 mg/kg), and atropine (0.05 mg/kg) were injected intramuscularly. Thiopental sodium (10 mg/kg) was injected intravenously, and endotracheal intubation was performed by means of the orotracheal route. Anesthesia was maintained by inhalation of enflurane. Electrocardiograms, oxygen saturation, and arterial blood pressure were monitored continuously. After performing the left thoracotomy, the pericardium was opened and the left anterior descending coronary artery (LAD) was exposed. Before further experimental manipulation, lidocaine (1 mg/kg) was administered intravenously and ischemic preconditioning was performed by snaring the LAD just distal to the first diagonal branch with 4-0 polypropylene sutures buttressed with a small piece of Nélaton catheter (4F) (Sewoon, Qingdao, China). Preconditioning consisted of two 3-minute occlusions, each followed by a 5-minute period of reperfusion. After the ischemic preconditioning, the LAD just distal to the second diagonal branch was ligated, and ST-segment depression or elevation on the monitored electrocardiogram was confirmed. Additional lidocaine (1 mg/kg) was injected intravenously 15 minutes after the ligation. The pericardium and thoracotomy wounds were closed. A single 28F chest tube was inserted. It was connected to wall suction and was removed just after sufficient spontaneous respiration returned. The endotracheal tube was removed after full recovery of self-respiration. All animals received humane care in compliance with the “Principles of Laboratory Animal Care” formulated by the Clinical Research Institute at Seoul National University and the 1996 “Guide for the Care and Use of Laboratory Animals” recommended by the U.S. National Institutes of Health.

Intramyocardial Injection of pCK–Vascular Endothelial Growth Factor
Thirty days after the coronary ligation (day 30), a redo-thoracotomy was performed. The animals were randomly allocated to one of two treatments in an operator-blinded fashion: intramyocardial injection of pCK–VEGF165 (VEGF group) or intramyocardial injection of pCK-Null (control group). Seven of the 21 pigs died during the trial, and 8 pigs from the VEGF group and 6 pigs from the control group were included in the analysis. A total of 1000 µg of the plasmid dissolved in 1 mL of phosphate-buffered saline solution was divided into five 200-µg doses. Each 200-µg dose of plasmid was injected, using a 27-gauge needle, in 1.5-cm intervals at five points along the anterolateral ischemic border zone. The anterolateral ischemic border zone was defined as the area located between the fibrotic infarction area and grossly normal myocardium along the course of the second diagonal branch. Suture tags of 5-0 polypropylene marked the injection points.

Transthoracic Echocardiography
All anesthetized pigs were examined by echocardiographic imaging and Doppler ultrasonography (SONOACE 8800; Medison, Seoul, Korea) with a 2.5/2.0-MHz transducer. Pigs showing pericardial disease, valvular disease, abnormal regional ventricular wall motion, or arrhythmia were excluded from this study. Thirty days after the surgical induction of myocardial infarction by coronary ligation (day 30), transthoracic echocardiography was performed to evaluate the establishment of a porcine myocardial infarction model. The variables measured on M-mode echocardiography were interventricular septal wall thickness, left ventricular posterior wall thickness, left ventricular end-systolic dimension (LVESD), and left ventricular end-diastolic dimension (LVEDD). The end-systolic volume (ESV), end-diastolic volume (EDV), and left ventricular ejection fraction (EF) were calculated using the following formulas:

\[
\text{ESV} = \frac{7.0}{(2.4 + \text{LVESD})} \times \text{LVESD}^3
\]
\[
\text{EDV} = \frac{7.0}{(2.4 + \text{LVEDD})} \times \text{LVEDD}^3
\]
\[
\text{EF} = (\text{EDV} - \text{ESV})/\text{EDV}
\]
Electrocardiography-Gated Single-Photon Emission Computed Tomography

Thirty days after the surgical induction of myocardial infarction (day 30), 99mTc-sestamibi myocardial single-photon emission computed tomography (SPECT) was performed as a baseline using a dual-head gamma camera (Vertex EPIC; ADAC Labs, Milpitas, CA). The SPECT was repeated 30 days after the injection of pCK–VEGF or null vector plasmids (day 60). The acquired and reconstructed images were analyzed by an automatic quantifying program (AutoQUANT; ADAC Labs) for regional perfusion and function. The program has been well validated as a regional analysis tool [8, 9]. Regional perfusion was quantified and expressed as a percentage of the maximal perfusion pixel. For regional function measurements, two variables of systolic wall thickening and wall motion were analyzed. Wall thickening was expressed as a percentage of end-diastolic wall thickness, and wall motion was expressed as a distance between the endocardial surfaces at end-diastole and end-systole.

A 20-segment model was adopted for the regional analysis (Fig 1). Segments of cardiac base (segments 15 through 20) and right coronary artery territory (segments 6, 12, 17, 18) were excluded from the analysis because they were easily influenced by imaging artifacts and are remote from the distal LAD territory where the coronary ligation and gene injection were performed. Nonviable infarcted segments in the apex (segments 1, 2) were also excluded. The segments not affected by the coronary ligation, defined as a segment with a perfusion measurement greater than 80% (mean ± 1 standard deviation of the remaining segments), were also excluded because they would not receive additional benefit from therapeutic angiogenesis. A total of 119 segments (48 segments in the control group; 71 segments in the VEGF group) were included in the analysis. Ejection fraction, ESV, and EDV were also automatically calculated by AutoQUANT.

Histologic Analysis of Microcapillary Densities

After the final SPECT were performed, the pigs were euthanized and the hearts were extracted. The indoxyltetrazolium method using alkaline phosphatase was performed to stain microcapillaries selectively [10]. The five injection sites marked by 5-0 polypropylene suture tags were sampled and frozen at −80°C. The tissue samples were embedded in blocks of OCT compound (Miles Inc, Elkhart, IN) and frozen. A cryotome (HM550, MICROM International GmbH, Waldorf, Germany) was used to cut frozen sections from the tissue blocks. Sections were fixed for 5 minutes in cold acetone and then washed with

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Table 1. Echocardiographic Evaluation of Porcine Myocardial Infarction Model

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control Group (n = 6)</th>
<th>VEGF Group (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 30</td>
</tr>
<tr>
<td>IVST (mm)</td>
<td>7.9 ± 1.6</td>
<td>4.8 ± 2.2</td>
</tr>
<tr>
<td>LVPWT (mm)</td>
<td>7.2 ± 1.4</td>
<td>7.1 ± 1.7</td>
</tr>
<tr>
<td>ESV (mL)</td>
<td>26.3 ± 12.0</td>
<td>36.8 ± 9.7</td>
</tr>
<tr>
<td>EDV (mL)</td>
<td>57.9 ± 20.1</td>
<td>62.3 ± 14.5</td>
</tr>
<tr>
<td>EF</td>
<td>0.482 ± 0.092</td>
<td>0.416 ± 0.100</td>
</tr>
</tbody>
</table>

EDV = left ventricular end-diastolic volume; EF = ejection fraction; ESV = left ventricular end-systolic volume; IVST = interventricular septal thickness; LVPWT = left ventricular posterior wall thickness; VEGF = vascular endothelial growth factor.

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Table 2. Regional Functional Measurements by Gated Myocardial Single-Photon Emission Computed Tomography

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control Group (n = 48 segments)</th>
<th>VEGF Group (n = 71 segments)</th>
<th>p Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Segmental perfusion (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 30</td>
<td>56.9 ± 18.8</td>
<td>58.9 ± 16.3</td>
<td>1.000</td>
</tr>
<tr>
<td>Day 60</td>
<td>55.5 ± 19.4</td>
<td>64.7 ± 17.7</td>
<td>0.018</td>
</tr>
<tr>
<td>p Value</td>
<td>0.480</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Wall thickening (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 30</td>
<td>26.4 ± 12.3</td>
<td>29.1 ± 13.8</td>
<td>0.544</td>
</tr>
<tr>
<td>Day 60</td>
<td>28.6 ± 16.1</td>
<td>34.8 ± 14.9</td>
<td>0.068</td>
</tr>
<tr>
<td>p Value</td>
<td>0.470</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Wall motion (mm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 30</td>
<td>4.1 ± 1.8</td>
<td>4.7 ± 2.1</td>
<td>0.270</td>
</tr>
<tr>
<td>Day 60</td>
<td>4.6 ± 2.2</td>
<td>5.8 ± 1.9</td>
<td>0.004</td>
</tr>
<tr>
<td>p Value</td>
<td>0.218</td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

*p Value was adjusted by Bonferroni correction.
distilled water. Alkaline phosphatase substrate (NBT/BCLP, fast-red; Sigma-Aldrich, St. Louis, MO) dissolved in 50 mmol/L sodium acetate buffer (pH 5.0) was applied to the slides, and they were counterstained 3 minutes later with eosin. Stained capillaries from each slide at 200× magnification were counted directly from five randomly selected 0.6-mm² sites. Microcapillary density was calculated as the number of capillaries per square millimeter.

**Statistical Analysis**

In the analysis of regional perfusion, wall motion, wall thickening, and vascular density, within-group or between-group comparisons were performed using two-tailed and paired or independent Student’s t tests, respectively. For multiple comparisons, the Bonferroni correction for potentially increasing type 1 error was performed and the p value was adjusted. Statistical
analyses for other variables (EF, ESV, and EDV) were performed using nonparametric tests (Mann–Whitney U test or Wilcoxon signed-rank test). Discrete data were analyzed using Fisher’s exact test. All data were analyzed using SPSS software (version 11.0, SPSS Inc, Chicago, IL). All values were expressed as mean and standard deviation, and *p* less than 0.05 was assumed to be statistically significant.

**Results**

**Echocardiographic Evaluation of Porcine Myocardial Infarction Model**

Echocardiography performed 30 days after coronary ligation showed significantly increased ESV and decreased EF in both groups, when compared with those measurements performed before coronary ligation (Table 1).

**Myocardial Single-Photon Emission Computed Tomography**

**SEGMENTAL PERFUSION.** There was no difference in the perfusion uptake between the two groups at day 30 (Table 2; Figs 2, 3A). In the VEGF group, myocardial SPECT at day 60 showed significantly improved perfusion in the LAD territory, compared with the SPECT on day 30 (58.9% ± 16.3% versus 64.7% ± 17.7%; *p* < 0.001). When the perfusion measurements at day 60 were compared between the two groups, the VEGF group showed a significantly higher perfusion than the control group (64.7% ± 17.7% versus 55.5% ± 19.4%; *p* = 0.018).

**WALL THICKENING.** There was no difference in the wall thickening between the two groups at day 30 (Table 2; Fig 3B). The wall thickening significantly increased in the VEGF group at day 60 compared with that of day 30 (29.1% ± 13.8% versus 34.8% ± 14.9%; *p* < 0.001), while there was no significant improvement in the control group. When the measurements of wall thickening at day 60 were compared between the two groups, the wall thickening of the VEGF group was higher than the control group (34.8% ± 14.9% versus 28.6% ± 16.1%; *p* = 0.068).

**WALL MOTION.** There was no difference in wall motion between the two groups at day 30 (Table 2; Fig 3C). The wall motion in the VEGF group significantly increased at day 60 (4.7 ± 2.1 mm versus 5.8 ± 1.9 mm; *p* < 0.001), compared with the control group (*p* = 0.218). When the wall motion at day 60 was compared between the two groups, the VEGF group showed a significantly increased wall motion than the control group (5.8 ± 1.9 mm versus 4.6 ± 2.2 mm; *p* = 0.004).

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Fig 3. Comparison of segmental perfusion (A), wall thickening (B), and wall motion (C) measured by gated myocardial single-photon emission computed tomography on day 30 (white bars) and day 60 (hatched bars). (VEGF = vascular endothelial growth factor.)
EJECTION FRACTION, END-SYSTOLIC VOLUME, AND END-DIASTOLIC VOLUME. There were no significant differences in the interval changes of EF, ESV, and EDV between the two groups (Table 3).

Histologic Analysis
Histologic review of thin-sectioned myocardium showed no evidence of arteriovenous anomaly or active inflammatory reaction (Fig 4). The VEGF group was found to have significantly higher microcapillary density (386 ± 110/mm² versus 291 ± 127/mm²; p < 0.001) in histologic quantitative analysis, suggesting that the perfusion improvement that was demonstrated in the segmental perfusion analysis resulted from the augmented angiogenesis.

Comment
This blinded, randomized controlled study demonstrated two major findings. First, direct intramyocardial injection of naked plasmid DNA encoding VEGF165 improved regional myocardial function as well as regional perfusion as measured by myocardial SPECT. Second, direct intramyocardial injection of naked plasmid DNA encoding VEGF165 augmented neoangiogenesis in the ischemic area without any histologically abnormal reaction.

Therapeutic angiogenesis can be achieved by administration of either the gene coding for VEGF (gene therapy) or the VEGF protein (protein therapy). Either plasmid or virus can be used as a vector for delivery in gene therapy, and can be administered by direct intramyocardial injection or through coronary arteries. Gene therapy

Table 3. Global Myocardial Function Measurements by Gated Myocardial Single-Photon Emission Computed Tomography

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control Group (n = 6)</th>
<th>VEGF Group (n = 8)</th>
<th>Comparison of % Changes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 30</td>
<td>Day 60</td>
<td>p Value</td>
</tr>
<tr>
<td>ESV (mL)</td>
<td>33.3 ± 13.8</td>
<td>34.0 ± 15.1</td>
<td>0.833</td>
</tr>
<tr>
<td>EDV (mL)</td>
<td>62.3 ± 13.6</td>
<td>68.2 ± 15.6</td>
<td>0.172</td>
</tr>
<tr>
<td>EF</td>
<td>0.490 ± 0.117</td>
<td>0.517 ± 0.102</td>
<td>0.528</td>
</tr>
</tbody>
</table>

*% changes = [(day 60 − day 30)/day 30] × 100.

ESV = left ventricular end-systolic volume; EDV = left ventricular end-diastolic volume; EF = ejection fraction.

Fig 4. (A) More microvessels stained by alkaline phosphatase were observed in the vascular endothelial growth factor (VEGF) group than in the control group (original magnification, ×200). (B) Comparison of the numbers of microvessels between the two groups.
has potential advantages, such as relatively prolonged production of VEGF protein by the transfected myocyte [11–13], avoidance of potential systemic hypotension caused by protein administration, and a lower cost than protein therapy, which requires a large quantity of human-quality recombinant protein to achieve the same effect as gene therapy. Viral vectors may have the risk of immune and inflammatory responses [10, 14]. Direct intramyocardial administration, compared with intra-coronary injection, provides higher levels of VEGF at the target area and limits the VEGF production to the area of new collateral blood vessels [15].

Previous phase I clinical trials using direct intramyocardial injection of the plasmid [5, 6] demonstrated myocardial perfusion improvement, although these studies did not include a placebo control group. Another clinical study [16] showed an improved regional wall motion by ventriculography without demonstration of significant myocardial perfusion improvement. The other study [17] showed better left ventricular contractility (maximum rate of increase of left ventricular pressure) as measured using a pressure transducer–tipped catheter in the VEGF-injected group, but did not demonstrate histologic or myocardial perfusion improvement.

In the present study, we evaluated the efficacy of therapeutic angiogenesis induced by intramyocardial injection of pCK expression vector containing the human VEGF165 gene (pCK–VEGF165) in a porcine model. Our quantitative analysis using SPECT demonstrated a significantly increased segmental perfusion 4 weeks after the pCK–VEGF injection, compared with the decreased segmental perfusion of the control group. This study also showed significantly increased regional wall thickening and wall motion, which may be the result of improved local perfusion of hibernating myocardium adjacent to the infarcted area. However, we failed to demonstrate improvement in EF and ESV values 30 days after plasmid DNA injection. These results suggest that pCK–VEGF plasmid injection did not interrupt or reverse the postinfarction ventricular remodeling process, although regional myocardial perfusion and function were significantly improved. Longer follow-up may be needed because ventricular remodeling continues for several months until the distending forces are counterbalanced by the tensile strength of the collagen scar [18]. This negative result might be underrepresented because of the small sample size and small estimated standardized difference. The low statistical power may produce a high probability of type 2 error, making this result less convincing.

One of the limiting factors when using naked DNA for intramyocardial gene transfer is its lower transfection efficiency, compared with viral vectors [19]. However, sufficient therapeutic angiogenesis is possible even without viral transfection because VEGF receptors are highly selective and almost exclusively restricted to vascular endothelial cells [20, 21] and because VEGF contains at its amino terminus the signal sequence that permits it to be actively secreted by intact cells and to achieve meaningful effect despite the low transfection rate [22]. The plasmid vector pCK used in the present study demonstrated a higher gene expression rate both in vivo and in vitro than the naked DNA vector [7]. The use of a total of 1,000 μg of pCK–VEGF165 (five doses of 200 μg per each injection) in this study was based on a previous study [23]. Studies of the pharmacokinetics of pCK–VEGF165 in the skeletal muscle of Balb/C mice showed that the level of VEGF165 expression from pCK was maintained for the entire duration of analysis (16 days) [7]. These pharmacokinetics may be augmented in ischemic myocardium because ischemic myocardium takes up DNA more efficiently and reaches a higher gene expression level than other tissues [24], and the uptake and expression of plasmid DNA is increased in a hypoxic state [25]. Previous studies demonstrated increased angiogenesis by immunohistologic evidence [26] or ex vivo coronary angiography [15]. The present study, using quantified histologic analysis of microcapillary densities, demonstrated that microcapillary density was significantly increased in the VEGF group, which suggests that the regional perfusion increase might result from the augmented neoangiogenesis. This study also did not show any histologic evidence of abnormal inflammatory reaction, which supported the safety of intramyocardially injected naked DNA found in previous studies [4, 16].

There are limitations to the present study that must be recognized. First, the proper timing of plasmid administration is not known. Serum VEGF levels were maintained at a high level between days 7 and 21 after acute myocardial infarction in a human study by Hojo and associates [27]. Li and colleagues [28] demonstrated that the VEGF receptors (flk-1 and flt-1) in the myocardium increased until 6 weeks after myocardial infarction in rats. However, there has been no long-term observation of VEGF levels or the remodeling process in pigs. The time course of VEGF gene expression after coronary ligation and after plasmid injection needs to be studied. Second, there is a possibility that a microcapillary-enriched area was included for myocardial tissue sampling. Third, accurate evaluation of the ischemic extent may have been limited because only resting myocardial SPECT was performed without a stress test. However, it is unrealistic and impractical to perform a stress SPECT in pigs because the heart rate and blood pressure changes are irreproducible according to Simons and coworkers [29].

In summary, intramyocardial injection of pCK–VEGF165 significantly augmented neoangiogenesis in the ischemic area and improved regional wall motion as well as myocardial perfusion.

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References


