Gene therapy for chronic myocardial ischemia using platelet-derived endothelial cell growth factor in dogs

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Li, Wei, Kuniyoshi Tanaka, Akio Iiaya, Yasuhisa Fujibayashi, Shinji Takamatsu, Kouichi Morioka, Masato Sasaki, Takahiko Uesaka, Tetsuya Kimura, Narihisa Yamada, Takeshi Tsuda, and Yukio Chiba. Gene therapy for chronic myocardial ischemia using platelet-derived endothelial cell growth factor in dogs. Am J Physiol Heart Circ Physiol 288: H408–H415, 2005. First published September 16, 2004; doi:10.1152/ajpheart.00176.2004.—Platelet-derived endothelial cell growth factor (PD-ECGF), also known as thrombine phosphorylase (TP), has been reported to possess angiogenic activity and to inhibit apoptosis. This study was performed to determine whether PD-ECGF/TP can be used to ameliorate chronic myocardial ischemia. Myocardial ischemia was created in 40 mongrel dogs by placement of an ameroid constrictor on the proximal left anterior descending coronary artery (LAD). Plasmid vector encoding human PD-ECGF/TP cDNA (pCIhTP group; n = 12), empty vector pCI (pCI group; n = 12), or saline (Saline group; n = 12) was directly injected into the LAD territory 3 wk after ameroid constrictor implantation. Myocardial blood flow was detected using PET at baseline, 3 wk after ameroid constrictor implantation, and 2 wk after therapeutic treatment. At the end of the experiment, the hearts were isolated for biological and histological analysis. In the pCIhTP group, the transfected heart strongly expressed PD-ECGF/TP. The size of the infarct was smaller in the pCIhTP group than in the pCI or saline group. The number of apoptotic myocardial cells was decreased in the pCIhTP group compared with the control groups based on triple immunohistochemical staining for von Willebrand factor, -actin smooth muscle cells, and single-strand DNA. The level of proapoptotic protein Bax markedly decreased in the pCIhTP group compared with the other groups. Double immunohistochemical staining for von Willebrand factor and -actin smooth muscle cells demonstrated that angiogenesis and arteriogenesis occurred, and paralleled the changes in myocardial blood flow and myocardial function in the pCIhTP group. We conclude that genetic approaches using PD-ECGF/TP to target the myocardium are effective for alleviating chronic myocardial ischemia.

ANGIOGENESIS is a promising strategy for increasing blood flow in patients with severe ischemic heart or peripheral vascular disease, especially for individuals who are not candidates for standard revascularization techniques. Recent studies (3, 22, 28, 30) have established the feasibility of using recombinant protein formulations or gene transfer of angiogenic growth factors to expedite and augment collateral artery development in patients with ischemia. Preclinical studies (1, 10, 35, 36) using both plasmid and adenoviral-based gene transfer of VEGF, FGF, and hepatic growth factor (HGF) have shown functionally significant stimulation of angiogenesis in different models. A phase I clinical study using plasmid-VEGF 165 delivered via direct intramyocardial injection has shown that this therapy is safe and may reduce cardiac symptoms and improve myocardial perfusion in patients with severe coronary artery disease (22).

However, despite this proven efficacy, Epstein et al. (7, 8) have identified several potential complications of therapy with these angiogenic cytokines. For example, VEGF causes edema and induces the development of functionally abnormal blood vessels, and VEGF and FGF both trigger the growth of neoplasms and increase atherosclerotic plaque mass and instability, in addition to other problems. The side effects of these growth factors can limit their therapeutic usefulness, and stimulate the search for other angiogenic factors that are free of these complications.

Platelet-derived endothelial cell growth factor (PD-ECGF) is identical to thrombine phosphorylase (TP). This enzyme catalyzes the reversible phosphorolysis of thymidine to thymine and 2-deoxy-ribose-1-phosphate and plays a role in maintaining the nucleotide pool (2). Sengupta et al. (31) reported that equimolar concentrations of PD-ECGF/TP and VEGF induce a similar total monolayer recovery of wounded endothelium in vitro, suggesting that PD-ECGF/TP and VEGF may have similar angiogenic effects in vivo. Indeed, PD-ECGF/TP has been shown to possess angiogenic activity in vivo, stimulating chemotaxis for endothelial cells and conferring resistance to apoptosis induced by hypoxia (11, 13, 14, 31). Furthermore, Somjen et al. (32) reported that PD-ECGF/TP also inhibits DNA synthesis in vascular smooth muscle cells (VSMCs). All these characteristics of PD-ECGF/TP strongly suggest the possibility that it can be useful for gene therapy in the setting of myocardial ischemia by promoting angiogenesis, inhibiting apoptosis, and preventing proliferation of smooth muscle cells.

METHODS

Plasmid vector construct and delivery regimen. We cloned the full length of human PD-ECGF/TP cDNA restricted from pBluescript II SK (+)/TP (a gift from Roche Japan) as a 1.571-bp EcoRI-EcoRI fragment into the corresponding sites in the pCI backbone, an expression plasmid vector (Promega) that has been used to deliver transgenic.
forming growth factor-β to suppress ongoing inflammation in arthritis (33). We have termed this construct pCIhTP (Fig. 1A). The final expression construct of pCIhTP contained the human PD-ECGF/TP gene driven by a cytomegalovirus promoter and flanked by the late SV40 polyadenylation signal. To ensure that the pCIhTP plasmid expresses the human PD-ECGF/TP protein, we cultured rat VSMCs harvested from the thoracic aorta using the explant method (6). The pCIhTP or control pCI vector was transfected into VSMCs using the N-(1-[(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTAP) transfection reagent (Biontex), and the expression of PD-ECGF/TP by cells was detected by immunocytochemistry, Western blot analysis, and PD-ECGF/TP activity assay. The plasmid DNA for animal experiments was prepared from TOP 10F Escherichia coli (Invitrogen) by using an endotoxin-free plasmid extraction kit (QIAGEN).

Animal experiments. Forty mongrel dogs (13.6 – 18.4 kg) were purchased from Kitayama Labs. Thirty-six of the dogs were randomly divided into three groups: pCIhTP (n = 12), pCI (n = 12), and Saline (n = 12). The dogs were anesthetized with pentobarbital sodium (25 mg/kg intravenously), intubated, and ventilated with room air. Under sterile conditions, a left thoracotomy was performed through the fourth or fifth intercostal space and the heart was exposed. The proximal left anterior descending coronary artery (LAD) was encircled with a plastic ameroid constrictor (2.5 mm ID, Research Instruments; Escondido, CA). All visible epicardial collaterals connecting the LAD diagonals to the left circumflex coronary artery (LCx) were ligated to minimize collateral blood flow. Three weeks after the initial operation, four dogs were euthanized and the hearts were isolated. The fragment of LAD where the ameroid constrictor was placed was harvested for histological examination, and the hearts were stained with 2,3,5-triphenyltetrazolium chloride (TTC) and Evans blue dye as described below. The remaining 36 animals were anesthetized again, and a second thoracotomy was performed through the original incision. A total of 3.5 mg pCIhTP (pCIhTP group) or pCI plasmid DNA (pCI group) were diluted in 2 ml of saline, and 500 μl were directly injected into four sites supplied by the LAD with the use of a 1-ml syringe with a 27-gauge needle. The Saline group received similar injections of saline alone. Two weeks after gene delivery, a catheter was inserted into the left ventricle through the carotid artery, and the rate of developed pressure over time (dP/dt) and blood pressure were recorded. The hearts from eight dogs in each group were removed. Transmural myocardial specimens from the distribution of LAD and LCx and samples of the left lung and the liver were harvested, frozen immediately in liquid nitrogen, and stored at −80°C until use. For histological analysis, each of six transmural slices (~0.5 cm thickness) from the distribution of the LAD or LCx were cut from the apex to the base, fixed in 4% formalin, and embedded in paraffin (20, 21).

The hearts from the other four dogs in each group were used for assessment of infarct size. The region at risk and the extent of myocardial infarction were determined by Evans blue and TTC staining by a previously described technique with minimal modification (34). Briefly, two cannulas were inserted directly into the left and right coronary ostia, secured with a suture, and perfused with a solution of 0.25% Evans blue dye. Simultaneously, a 22-gauge catheter was inserted immediately distal to the point of LAD occlusion and fixed in place, and the LAD was perfused with 1% TTC in 0.1 mol/l sodium phosphate buffer (pH 7.4). The solutions were maintained at 37°C and infused for 15 min. After the staining procedure, the hearts were sliced transversely into 3-mm sections and fixed in 10% formalin. The area of the myocardium that was not stained with Evans blue dye was defined as the area at risk (AAR). In the AAR, the areas that were not stained by the TTC were defined as the area of infarction (AOI). Each slice was then photographed on both sides with a Canon digital camera. The AAR and AOI were quantified with the use of image analysis software (Image; Scion), and the mean values for both sides were used as the final value of this slice. The sum of 3 × AAR or 3 × AOI for each slice was defined as the total volume of AAR and AOI. Infarct size is expressed as a percentage of ARI/AAR.

The use of animals was in compliance with the Guidelines of the Institutional Animal Care and Use Committee of the Faculty of Medical Sciences, University of Fukui, and conforms to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1996).

The dogs received routine postoperative care after the surgical procedure. Cefazolin sodium (25 mg/kg) was administered twice daily for 5 days, and pain medication was given as needed.

Fig. 1. A: plasmid vector encoding human platelet-endothelial cell growth factor (PD-ECGF)/thymidine phosphorylase (TP) cDNA (pCIhTP), and immunocytochemical staining for human PD-ECGF/TP in pCIhTP-transfected cells. The brown-stained areas that were not stained with Evans blue dye were defined as the area at risk (AAR). In the AAR, the regions expressing PD-ECGF/TP in pCIhTP-transfected cells were defined as the total volume of AAR and AOI. Infarct size is expressed as a percentage of ARI/AAR.

The use of animals was in compliance with the Guidelines of the Institutional Animal Care and Use Committee of the Faculty of Medical Sciences, University of Fukui, and conforms to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1996).

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PET myocardial perfusion imaging. Myocardial blood flow (MBF) was measured with the use of a PET scanner (model SHR7700, Hamamatsu). After a 30-min transmission scan, 31-frame dynamic PET imaging (10 × 2 min frames) of the heart was performed using [13N]ammonia (15.4 ± 0.328 mCi). Myocardial ischemia was defined by absolute values for MBF that were reduced for the left ventricular wall supplied by the LAD. The analysis of PET images was conducted using an image analysis package (Dr. View) and a special, dedicated software package. The PET images were reoriented into short-axis images for all sets. Myocardial regions of interest were drawn in the territories of the LAD and LCx, and tracer uptake in each region was measured and expressed as the ratio of LAD/LCx. Eight dogs in the pCi/hTP and pCI groups were used in this experiment.

Molecular analyses. Total DNA and RNA were isolated from canine myocardium. Three micrograms of DNA were subjected to PCR using primers corresponding to sequences within the pCI plasmid (forward, 5'-ACTGACATCCACCTTTGCTTCTCT-3') and the human PD-ECGF/TP cDNA (reverse, 5'-CTTACCTGAGAATGGGAGGCTTCTGATG-3'). A specific PCR product represented transgenic PD-ECGF/TP DNA. One microgram of RNA was treated with DNase (Sigma) and used for first-strand cDNA synthesis by using the First Strand cDNA Synthesis Kit (Roche Diagnostics) as described by the manufacturer. The forward primer (5'-GAGCTAGGATGGAGGAGGCTTCTGATG-3') and reverse primer (5'-GAGATGAGGACTACCTTGGAGTGATGAG-3') bind to conserved regions of the human PD-ECGF/TP transcripts. The Advantage II PCR Kit (Clontech) was used for this protocol. To document that there were similar amounts of DNA or RNA in each lane, PCR for canine β-actin also was performed. Myocardial protein was extracted as described previously (20). Western blot analysis was performed with the use of antibodies against human thymidine phosphorylase (654-1, mouse monoclonal antibody, Roche), Bcl-2 (Pharmingen), and Bax (Pharmingen). In addition, blots were probed with an actin (Sigma) antibody as a loading control. PD-ECGF/TP activity was detected as described previously (20).

Histological analysis. Serial 4- to 5-μm-thick sections were cut and routine histological staining was performed with hematoxylin and eosin and Masson's trichrome stain. Standard immunohistochemical staining using the 654-1 antibody was performed to detect PD-ECGF/TP expression at the injection sites. Double immunohistochemical staining was performed with the use of von Willebrand factor (vWF; polyclonal rabbit anti human antibody, A0082, Dako, Japan) and β-actin smooth muscle cells (β-actin SMC, mouse anti-human monoclonal antibody, M0851, Dako) antibody to identify microvessel density and microvessel characterization. To determine the type of the cells undergoing apoptosis induced by ischemia, we stained tissue from the LAD area using a triple immunohistochemical staining technique for single-strand DNA (ssDNA; A4506, Dako), vWF, and β-actin SMC. ssDNA is a specific and sensitive cellular marker of apoptosis, and this antibody differentiates apoptosis from necrosis and identifies cell types in the early stages of apoptosis (9). Nuclei were stained with hematoxylin.

Microvessel density count and microvessel type analysis. Myocardial infarction is frequently heterogeneous in dogs and formed by complex interdigitations between necrotic and viable areas. Therefore, five subepicardial and five subendocardial cross-sectioned regions were randomly selected for analysis in each dog heart. Three researchers blinded to the group division performed the counting, and the average values were used for statistical analysis (20, 21). Microvessel densities are expressed as microvessel numbers per square millimeter. Capillaries were identified as a single layer of vWF-positive endothelial cells (×200 magnification, inside diameter ≤10 μm). Arterioles were identified as having an inside diameter ≥10 μm and an α-actin SMC-positive layer (×200 magnification). Venules were differentiated from arterioles by their large lumen diameter compared with vessel wall thickness, a thinner or absent smooth muscle layer, a less significant tunica adventitia, and an inner diameter of ≥10 μm (15).

Apoptotic cell type analysis. Five randomly selected areas of ssDNA-positive cells in each dog were photographed (×200 magnification), and the number of ssDNA-positive cells and total number of nuclei in the same area were counted using Mac Scope software (Mitani). The percentage of ssDNA-positive cells relative to the total number of nuclei was used for statistical analysis. Furthermore, apoptotic endothelial cells (ssDNA- and vWF-positive cells), SMCs (ssDNA- and α-actin SMC-positive cells), myocardium (not stained for vWF and α-actin SMC, but having abundant cytosome and ssDNA-positive nuclei), and other cells (e.g., inflammatory cells) were compared among groups based on the triple-staining results and presented as the percentage of total nuclei.

Statistical analysis. ANOVA or Mann-Whitney U-test was used for intergroup comparisons. The Friedman test was used for the analysis of
among all samples from the three groups. Values are reported as means ± SE. \( P < 0.05 \) was considered significant.

**RESULTS**

**Plasmid vector-mediated transfection with human PD-ECGF/TP gene stimulates expression of transgene and production of functional protein in rat VSMCs.** Immunocytostaining with antibodies against human PD-ECGF/TP demonstrated that the transgenic VSMCs expressed this protein (Fig. 1B). Western blot analysis of cell lysates performed 24 h after transfection confirmed that the transgenic rat VSMCs produced human PD-ECGF/TP (Fig. 1C). There was no detectable PD-ECGF/TP activity in VSMCs transfected with pCI, but in the pCIhTP-transfected cells, the PD-ECGF/TP activity still was markedly increased 72 h after transfection (Fig. 1D, \( n = 3 \)).

**Plasmid vector-mediated intramyocardial delivery of human PD-ECGF/TP gene leads to expression of transgene and production of functional protein in dogs.** No postoperative deaths occurred in any group. Two weeks after gene transfection, transgene DNA and PD-ECGF/TP mRNA were expressed in the distribution of the LAD in the pCIhTP group, but not in the distribution of the LCx, liver, or lung in the pCIhTP group or anywhere in the pCI or Saline groups (Fig. 2, A and B; \( n = 8 \), data for liver and lung not shown). Correspondingly, Western blot analysis demonstrated that human PD-ECGF/TP protein was expressed only in extracts prepared from the LAD area injected with pCIhTP (Fig. 2C, \( n = 8 \)). Immunohistochemical staining confirmed that PD-ECGF/TP was expressed in sites of the plasmid injection (Fig. 2D). The assessment of PD-ECGF/TP activity in myocardial extracts demonstrated that the activity was higher in the LAD area than in the LCx area in the

![Image](http://ajpheart.physiology.org/)

Fig. 3. Photomicrographs of tissue after hematoxylin and eosin staining (A–C) or Masson’s trichrome staining (D–F), showing that histology of the myocardium in the pCIhTP group is almost normal, but the myocardium in the pCI and Saline groups is fibrotic. Double immunohistochemical staining for von Willebrand factor (vWF, red) and \( \alpha \)-actin smooth muscle cells (SMC; \( \alpha \)-actin SMC, brown), with nuclei stained with hematoxylin (G–I), showing that the density of microvessels is greater in the pCIhTP group than in the pCI or Saline groups. Triple immunohistochemical staining for vWF (red), \( \alpha \)-actin SMC (bluish purple), and single-stranded DNA (ssDNA, brown), with nuclei stained with hematoxylin (J–L), showing that the density of apoptotic cells is lower in the pCIhTP group than in the pCI or Saline groups. All representative tissue samples are from the endomyocardium. Scale bars equal 50 \( \mu \)m.
Gene therapy for myocardial ischemia

Plasmid vector mediated intramyocardial delivery of human PD-ECGF/TP gene protects the heart from ischemia and reduces infarct size. No epicardial infarction was identified in any canine heart. Three weeks after ameroid constrictor implantation, 36.7% (36.7 ± 6.4%, n = 4) of the left ventricle was in the AAR, but no grossly visible infarction was found in the AAR by TTC staining. Hematoxylin and eosin staining confirmed ameroid occlusion in these four dogs, and demonstrated that scattered infarction existed at this time point (data not shown). Two weeks after gene delivery, the hearts were removed and cut open along the ventricular septum; there was no gross evidence of endocardial infarction in the pCIhTP-transduced hearts. In contrast, endocardial infarction was obvious in the pCI- and saline-injected hearts. Hematoxylin and eosin staining demonstrated near-normal morphology in the pCIhTP-transduced hearts, with only small scattered areas of subendocardial infarction, and abundant thread-like microvessels present in the myocardium (Fig. 3A). In the pCI and Saline groups, granulation tissue was present in the myocardium, consistent with infarction (Fig. 3, B and C). Masson’s trichrome staining demonstrated that the endocardium in the pCI- and saline-injected hearts was fibrotic (Fig. 3, D–F). TTC and Evans blue staining demonstrated that the AAR was equal in the three groups (32.4 ± 7.4% of the left ventricle in pCIhTP, 34.6 ± 5.7% of the left ventricle in pCI, and 33.2 ± 8.6% of the left ventricle in the Saline group; n = 4 for each group). The ratio of AOI/AAR in the pCIhTP group was markedly lower than in the pCI or Saline groups, and no significant difference was present between the pCI and Saline groups (Fig. 4).

Plasmid vector mediated intramyocardial delivery of human PD-ECGF/TP gene promotes angiogenesis and inhibits apoptosis. Double immunohistochemical staining for vWF and α-actin SMC showed higher microvessel densities in the pCIhTP group and less neovascularization in the pCI and Saline groups (Fig. 3, G–J). Furthermore, based on this staining, we confirmed that the thread-like microvessels detected by hematoxylin and eosin staining were capillaries or arterioles. The densities of total microvessels, capillaries, and arterioles were higher in the pCIhTP group than in the pCI or Saline groups (Table 1), and no difference was found between the pCI and Saline groups. The number of microvessels was similar among the groups. Further evaluation of the relative percentages of arterioles, capillaries, and venules in randomly selected areas showed that pCIhTP treatment resulted in a significantly (P < 0.01) higher percentage of arterioles than in the control groups. There were no differences among the groups with respect to the number of myocyte nuclei in the analyzed areas.

Apoptotic cells were present mainly in the granulation tissue and the endocardial region, which is known to be sensitive to ischemia. There were fewer apoptotic cells in the pCIhTP group than in the pCI or Saline group (Fig. 3, J–L, and Table 2). Furthermore, based on the triple immunohistochemical staining, there were fewer apoptotic cardiac myocytes in the pCIhTP group than in control groups. The number of apoptotic endothelial cells in the pCIhTP group was lower than in the Saline group. No difference was found among groups for SMC (Table 2). The total number of nuclei in the counted area was

![Infarct size assessment. Two weeks after vector treatment, the area at risk (AAR) and area of infarction (AOI) were determined by Evans blue and 2,3,5-triphenyltetrazolium chloride staining, respectively, and infarct size is presented as the percentage of AOI relative to AAR (n = 4, in each group).](image)

### Table 1. Microvessel densities and microvessel characterization

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Arterioles</th>
<th>Capillaries</th>
<th>Venules</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCIhTP</td>
<td>Mean</td>
<td>3,580.8*</td>
<td>199.6†</td>
<td>3,338.6‡</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>229.8</td>
<td>42.1</td>
<td>233.2</td>
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<tr>
<td></td>
<td>%Total</td>
<td>5.6%§</td>
<td>93.2%</td>
<td>1.2%</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>1.2%</td>
<td>1.4%</td>
<td>0.4%</td>
</tr>
<tr>
<td>pCI</td>
<td>Mean</td>
<td>2,816.6</td>
<td>93.8</td>
<td>2,674.6</td>
</tr>
<tr>
<td></td>
<td>SD</td>
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<td>32.2</td>
<td>361.1</td>
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<tr>
<td></td>
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<tr>
<td></td>
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<td>1.4%</td>
<td>1.1%</td>
<td>0.5%</td>
</tr>
<tr>
<td>Saline</td>
<td>Mean</td>
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<td>88.2</td>
<td>2,689</td>
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<tr>
<td></td>
<td>SD</td>
<td>165.6</td>
<td>18.5</td>
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</tr>
<tr>
<td></td>
<td>%Total</td>
<td>3.1%</td>
<td>95.3%</td>
<td>1.6%</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>0.5%</td>
<td>0.8%</td>
<td>0.4%</td>
</tr>
</tbody>
</table>

*SD, standard deviation; pCIhTP, plasmid vector encoding human thymidine phosphorylase. †P < 0.0007, total microvessel density per square millimeter, pCIhTP vs. pCI and Saline. ‡P < 0.0002, arteriole densities in the same areas, pCIhTP vs. pCI and Saline. §P < 0.001, capillary densities in the same areas, pCIhTP vs. pCI and Saline. ¶P < 0.01, percentage of arterioles relative to total microvessels, pCIhTP vs. pCI and Saline.

### Table 2. Apoptotic cell densities and apoptotic cell characterization

<table>
<thead>
<tr>
<th></th>
<th>Total Apoptotic Cells</th>
<th>Myocytes</th>
<th>Endothelium</th>
<th>SMC</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCIhTP</td>
<td>Mean 10.4%*</td>
<td>7.3%†</td>
<td>1.7%‡</td>
<td>1.1%</td>
<td>0.8%</td>
</tr>
<tr>
<td></td>
<td>SD 7.8%</td>
<td>4.9%</td>
<td>1.6%</td>
<td>1.4%</td>
<td>1.6%</td>
</tr>
<tr>
<td>pCI</td>
<td>Mean 28.6%</td>
<td>23.1%</td>
<td>3.6%</td>
<td>1.9%</td>
<td>0.3%</td>
</tr>
<tr>
<td></td>
<td>SD 3.7%</td>
<td>0.9%</td>
<td>2.2%</td>
<td>2.0%</td>
<td>0.4%</td>
</tr>
<tr>
<td>Saline</td>
<td>Mean 27.9%</td>
<td>17.4%</td>
<td>5.2%</td>
<td>3.5%</td>
<td>1.3%</td>
</tr>
<tr>
<td></td>
<td>SD 8.6%</td>
<td>5.5%</td>
<td>2.2%</td>
<td>2.1%</td>
<td>3.1%</td>
</tr>
</tbody>
</table>

Values are reported as a percentage of total nuclei. *P < 0.002, pClhTP vs. pCI and Saline. †P < 0.005, pClhTP vs. pCI and Saline; ‡P = 0.02, pClhTP vs. Saline.
Plasmid vector-mediated intramyocardial delivery of human PD-ECGF/TP gene increased regional myocardial blood flow and improved myocardial function. We measured the MBF in the pChlTP and pCI groups at three time points using PET: start of the experiment (Baseline MBF), 3 wk after ameroid constrictor placement (Ischemic MBF), and 2 wk after gene delivery (Therapeutic MBF). There was no difference in Baseline MBF between the two groups. Three weeks after ameroid constrictor implantation, MBF in the LAD distribution had decreased to 91.4 ± 4.3% in the pCI group and 90.4 ± 1.4% in the pChlTP group of the baseline flow (P < 0.05, Ischemic MBF vs. Baseline MBF; n = 8), and MBF in the two groups was similar (P = 0.15). Two weeks after gene injection, MFB decreased further in the pCI group to 68.5 ± 8.5% of the baseline value. In contrast, in the pChlTP group, MBF recovered to 99.5 ± 2.1% of the baseline value. The difference in the MBF between these two groups was statistically significant 2 wk after vector treatment (P < 0.05; Fig. 6). These data indicate that PD-ECGF/TP gene directly injected into the ischemic myocardium restores regional MBF, and this restoration in regional MBF results in a decrease in the size of infarct.

Myocardial function was evaluated by measuring dP/dt with an intraventricular catheter. The maximum dP/dt was higher in the pChlTP group (4,320 ± 346 mmHg/s) than in the pCI group (3,310 ± 129 mmHg/s vs. pChlTP; \( P = 0.009 \)) or the Saline group (3,180 ± 421 mmHg/s vs. pChlTP group; \( P = 0.007 \)).

Several groups have demonstrated cardiomyocyte apoptosis in specimens from patients with heart failure, suggesting that gene transfer techniques that promote myocyte survival may be beneficial (12). In vivo delivery of the potent caspase inhibitor p35 gene significantly improved contractility in the failing myocardium (18). Similarly, experiments using transgenic mice that overexpress the anti-apoptotic human Bcl-2 in the heart have demonstrated that Bcl-2 overexpression reduces myocardial reperfusion injury and improves cardiac function (4). The present study used antibodies against ssDNA to demonstrate that the number of apoptotic cells is markedly decreased in pChlTP-treated hearts, with less of the proapoptotic protein, Bax, detected in the pChlTP group than in the pCI or Saline group (Fig. 5).

Western blot analysis of myocardial extracts demonstrated that expression of the anti-apoptotic protein, Bcl-2, was similar in all three groups (data not shown), but there was less of the proapoptotic protein, Bax, detected in the pChlTP group than in the pCI or Saline group (Fig. 5).

**DISCUSSION**

The present study demonstrates that plasmid-mediated gene transfer of PD-ECGF/TP stimulates angiogenesis and arteriogenesis in chronically ischemic myocardium, inhibits apoptosis of hibernating cardiac cells, decreases myocardial infarct size, restores myocardial blood flow, and improves myocardial function. To the best of our knowledge, this is the first reported use of the PD-ECGF/TP gene for the treatment of myocardial ischemia.

PD-ECGF was first isolated in 1987 (23). Five years later, it was shown to be a previously characterized intracellular enzyme, TP (24). A large volume of experimental evidence has established a relationship between PD-ECGF/TP and tumor angiogenesis (17, 27). In our previous study (20), we also found that angiogenesis induced by transmyocardial laser revascularization correlates with the expression of PD-ECGF/TP, suggesting that PD-ECGF/TP also has angiogenic effects in the myocardium. The present study extended these findings by showing that myocardial injection of plasmid encoding the human PD-ECGF/TP gene promoted angiogenesis and arteriogenesis in the ischemic canine myocardium. Of potential clinical significance, the histological evidence of neovascularization corresponded to an increase in MBF, which, in turn, translated into decreased myocardial infarct size and improved myocardial function. On the basis of the results of the microvessel characterization, and the percentage of arterioles markedly increased in pChlTP-treated hearts, we then hypothesize that one possible mechanism of the rapid increase of the myocardial blood (2 wk long) might be development of collateral arteries.

Several groups have demonstrated cardiomyocyte apoptosis in specimens from patients with heart failure, suggesting that gene transfer techniques that promote myocyte survival may be beneficial (12). In vivo delivery of the potent caspase inhibitor p35 gene significantly improved contractility in the failing myocardium (18). Similarly, experiments using transgenic mice that overexpress the anti-apoptotic human Bcl-2 in the heart have demonstrated that Bcl-2 overexpression reduces myocardial reperfusion injury and improves cardiac function (4). The present study used antibodies against ssDNA to demonstrate that the number of apoptotic cells is markedly decreased in pChlTP-treated hearts, with less of the proapoptotic protein, Bax, detected in the pChlTP group than in the pCI or Saline group (Fig. 5).

**Fig. 5.** Western blot analysis of myocardial extracts for Bax and actin, showing that the level of Bax relative to actin was lower in the pChlTP group than in the pCI and Saline groups.

**Fig. 6.** Myocardial blood flow (MBF) detected by positron emission tomography. MBF was higher in the pChlTP group than in the pCI group 2 wk after gene delivery. Baseline MBF, MBF before the first operation; ischemic MBF, MBF after 3 wk of LAD constriction; therapeutic MBF, MBF 2 wk after gene transfection.
decreased by PD-ECGF/TP gene treatment. Although this decrease in the number of apoptotic cells may be secondary to improved myocardial blood flow, we cannot exclude a direct effect of PD-ECGF/TP on the inhibition of apoptosis. Mori et al. (25) reported that PD-ECGF/TP suppresses Fas-induced apoptosis. The present study did not find any change in Bcl-2, an antiapoptotic protein, expression but levels of Bax, which is a proapoptotic protein, were lower in the PD-ECGF/TP-treated group than in pCI or Saline groups. It has been shown that the Bax protein, when present above a threshold level, triggers the apoptosis cascade (16). Our data also suggest that direct myocardial injection of the PD-ECGF/TP gene inhibited myocardial apoptosis, but the mechanism is not clear, whether it is via inhibiting Bax expression should be discussed further.

PD-ECGF/TP has been reported to inhibit DNA synthesis in cultured smooth muscle cells in vitro. In fact, we have also demonstrated that, after serum starvation for 48 h, the serum-stimulated proliferation of PD-ECGF/TP gene transsected rat VSMC was significantly decreased and the cell cycle was arrested at the G1 phase (W. Li, unpublished observation). This effect of PD-ECGF/TP has important therapeutic implications because it could decrease neointimal smooth muscle cell proliferation, and thereby reduce the neointimal mass in atherosclerotic vessels and inhibit further growth. However, although VSMC is necessary in vivo for vascular maturation and angiogenesis (5), which is the process whereby capillaries acquire a coat of VSMC and thereby gain the ability to regulate blood flow through rapid alternating in internal diameter, this does not negate the effect of TP on VSMC. The origin of VSMC is complex, and may vary depending on the tissues involved (5). It is generally believed that VSMC differentiate from mesenchymal cells in situ, with potential precursors, including cell types such as pericytes (26, 29), stromal cells, myoepithelial cells, and myofibroblasts (19). VSMC can also transdifferentiate from endothelial cells, or from bone marrow precursors or macrophages (5). Under pathological conditions (such as restenosis or atherosclerosis), smooth muscle cells often “de-differentiate” to an embryonic phenotype, reverting from their “contractile” to synthetic phenotype (5). Data from Somjen et al. (32) and our group were obtained from the cultured VSMCs. Therefore, we speculate that PD-ECGF/TP only influences the synthetic phenotype of VSMC, but not the VSMC differentiation from other origins. We are now characterizing the detailed mechanism responsible for the inhibited effect of PD-ECGF/TP on VSMC.

The goal of therapeutic angiogenesis in patients with severe ischemic heart disease is to improve myocardial function and quality of life. The improvement in myocardial function and myocardial blood flow, along with the decrease in infarct size, produced by PD-ECGF/TP therapy suggests that this gene delivery method has a potent beneficial effect on the ischemic myocardium. Other authors have reported angiogenic effects of VEGF, FGF, and HGF on the myocardium. In addition to the angiogenic or arteriogenic effect of these reported growth factors, PD-ECGF/TP has beneficial effects, such as inhibition of VSMC proliferation and inhibition of apoptosis. Our data suggest that PD-ECGF/TP may be a prime candidate for gene therapy for myocardial ischemia.

Some limitations exist in this study. First, in this animal model, we performed an additional occlusion of the end of the LCx branches to decrease the collateral circulation. Therefore, it is not true chronic ischemic model and myocardial infarction appeared 3 wk after ameroid constrictor implantation. However, by using this model, we confirmed the therapeutic effects of PD-ECGF/TP. Second, the 2-wk period between the plasmid injection and the time of evaluation is relatively short. Although we did not detect the expression of the delivered gene in the left lung or liver, further long-term studies should be done in the future to evaluate the safety of this therapy.

In summary, plasmid vector-mediated gene transfer of PD-ECGF/TP-stimulated angiogenesis and arteriogenesis in the ischemic myocardium, inhibited myocardial apoptosis, decreased the size of myocardial infarction, improved myocardial blood flow, and improved myocardial function. These data suggest that genetic approaches using PD-ECGF/TP to target ischemic myocardium are worthy of further study.

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