Treatment With Recombinant Placental Growth Factor (PlGF) Enhances Both Angiogenesis and Arteriogenesis and Improves Survival After Myocardial Infarction

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Background: Placental growth factor (PlGF), a homolog of vascular endothelial growth factor, is reported to stimulate angiogenesis and arteriogenesis in pathological conditions. It was recently demonstrated that PlGF is rapidly produced in myocardial tissue during acute myocardial infarction (MI). However, the effects of exogenous PlGF administration on the healing process after MI are not fully understood. The purpose of the present study was to examine whether PlGF treatment has therapeutic potential in MI.

Methods and Results: Recombinant human PlGF (rhPlGF; 10 µg) was administered continuously for 3 days in a mouse model of acute MI. rhPlGF treatment significantly improved survival rate after MI and preserved cardiac function relative to control mice. The numbers of CD31-positive cells and α -smooth muscle actin-positive vessels in the infarct area were significantly increased in the rhPlGF group. Endothelial progenitor cells (Flk-1⁺Sca-1⁺ cells) were mobilized by rhPlGF into the peripheral circulation. Furthermore, rhPlGF promoted the recruitment of GFP-labeled bone marrow cells to the infarct area, but only a few of those migrating cells differentiated into endothelial cells.

Conclusions: Exogenous PlGF plays an important role in healing processes by improving cardiac function and stimulating angiogenesis following MI. It can be considered as a new therapeutic molecule. (Circ J 2009; 73: 1674–1682)

Key Words: Myocardial infarction; Placental growth factor; Soluble Flt-1

In patients with acute myocardial infarction (AMI), reperfusion of the occluded coronary artery is of great importance in salvaging ischemic myocardium and minimizing ventricular infarct size. Although reperfusion therapy has contributed to approximately 30% decline in the mortality rate of AMI over the past decade, the rate of rehospitalization because of congestive heart failure that occurs as a result of left ventricular dysfunction with myocardial infarction (MI) is still high.¹ To improve both the cardiac function and the prognosis of patients with AMI, several adjunctive therapies using cell transplantation^{2,3} and some growth factors^{4,5} have been tried clinically in the past decade. Lunde et al reported that intracoronary injection of autologous mononuclear bone marrow cells (BMCs) had no effect on AMI over a 6-month follow-up.⁶ Although gene therapy using vascular endothelial growth factor (VEGF) has successfully induced angiogenesis in rodent models by mobilizing endothelial progenitor cells (EPCs) to the peripheral circulation, there are few reports that VEGF has improved cardiac function in patients with ischemic heart diseases.^{7,8} Adjunctive therapies, therefore, have not

yet demonstrated an improvement in the clinical prognosis of AMI.

Placental growth factor (PlGF), a homolog of VEGF, is reported to stimulate angiogenesis and arteriogenesis^{9–11} with an efficacy at least comparable to that of VEGF, but without any of the side-effects that normally accompany VEGF therapy, such as edema, hypotension,¹² and hemangioma-genesis. We have recently demonstrated that PlGF is produced rapidly in infarcted myocardial tissue, and that the plasma level of PlGF after MI is positively correlated with the improvement in left ventricular function in patients with AMI.¹³ These findings suggest that PlGF is involved in post-AMI pathology, infiltration of inflammatory cells, neoangiogenesis, and the development of fibrosis and thus the healing process after AMI. However, therapeutic effects of PlGF administration have not been studied, so in the present study we investigated the result of exogenous administration of PlGF protein in regard to cardiac function and prognosis after MI.

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Methods

Preparation of Recombinant Human PIGF (rhPIGF)

A DNA sequence encoding the mature human PIGF protein¹⁴ (amino acid residues 21–149 of the 149-amino acid form of PIGF) was amplified by polymerase chain reaction (PCR) with human placental cDNA (TaKaRa, Ohtsu, Japan) as a template. *Escherichia coli* BL21 was transformed with the expression vector, and the thioredoxin-PIGF fusion protein was expressed and purified essentially as previously described.¹⁵ Proteins were concentrated and suspended in a solution containing 20 mmol/L PB (pH 7.4), 0.5 mol/L NaCl, 1 mmol/L dithiothreitol, and 8 mol/L urea, and purified by HisTrap FF (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) column chromatography. Following dialysis against graded concentrations of urea containing 50 mmol/L Tris-HCl (pH 8.0), the fusion protein was resuspended in 50 mmol/L Tris-HCl (pH 8.0). After digestion by EKMax Enterokinase (Invitrogen, Carlsbad, CA, USA), proteins were suspended again in a solution containing 1 mmol/L dithiothreitol and 8 mol/L urea and applied onto a HisTrap FF column in order to remove the tag protein. The purified PIGF was dialyzed against graded concentrations of urea containing 20 mmol/L Tris-HCl (pH 8.0). Finally, the protein solution was fractionated by gel filtration on a Superdex 75 10/300GL column (GE Healthcare Bio-Sciences).

Preparation of Recombinant Human Soluble Flt-1 (rhsFlt-1)

A DNA fragment encoding amino acids 1–338 of human Flt1 (sFlt1 (D1-3) containing 3 Ig-like domains at the N-termini) was amplified by PCR reaction with specific primers (forward primer: 5'-CATCCATGGATCCTGAAC-TGAGTTTAAAG-3', reverse primer: 5'-CATGGATCC-TCAATGTTTCACAGTGATGAATGC-3') and human placental cDNA (Clontech) as a template. The expression vector was used for transformation of BL21 star (CE3) competent cells (Invitrogen). For bacterial expression, the transformant expressing sFlt1 (D1-3) was cultured in LB medium containing 50 µg/ml of Kanamycin at 37°C. Four hours after IPTG was added to the final concentration of 1 mmol/L, the cells were collected by centrifuge. The cells were lysed by incubation with 2 mg/ml Lysozyme and sonication. Inclusion bodies were collected by centrifuging for 20 min at 10,000 rpm, and were suspended and solubilized in 20 mmol/L PB containing 0.5 mol/L NaCl, 6 mol/L urea, 1 mmol/L DTT, and 20 mmol/L imidazole followed by His-Trap FF (GE Healthcare Bio-Sciences) column purification. Elute was dialyzed against a graded concentration of urea in 20 mmol/L Tris (pH 8.0) containing 0.5 mol/L NaCl. Following the final dialysis against 20 mmol/L Tris (pH 8.0) containing 0.5 mol/L NaCl, purified sFlt-1 was harvested.

Animals

C57BL/6 mice were purchased from SLC (Shizuoka, Japan). Transgenic mice (C57BL/6 background) that ubiquitously express enhanced GFP (GFP mice) were a generous gift from Dr Masaru Okabe (Osaka University, Suita, Japan). All experimental procedures were performed in accordance with protocols approved by the Ethics Review Committee for Animal Experimentation of Nara Medical University.

Preparation of Mouse Model of MI

C57BL/6 mice (12 weeks old) were anesthetized with diethyl ether inhalation and an osmotic minipump (Durect

Corp, Cupertino, CA, USA) filled with 10 µg of rhPIGF or 40 µg of rhsFlt-1 was implanted subcutaneously. A polyethylene tube connected to the osmotic minipump was extended to the peritoneal cavity and stitched to the oblique abdominal muscle using a 7-0 silk suture. The next day, coronary ligation was performed in the same mouse, as previously reported.^{13,16} rhPIGF was infused for 3 days and rhsFlt-1 was infused for 7 days. In total, 95 mice were used for assessing survival rate and 74 mice for histological, physiological and biochemical analyses.

Bone Marrow Transplantation (BMT)

BMT was performed as described previously.¹⁶ Six weeks after BMT, the mice were treated with rhPIGF (10 µg) and coronary ligation was performed. Five days after AMI, peripheral blood was collected for fluorescence-activated cell sorting analysis, and hearts were harvested for immunohistochemical examination.

Enzyme-Linked Immunosorbent Assay (ELISA)

Levels of human and murine PIGF were measured by ELISA (R&D System, Germany). The assay for human PIGF recognizes recombinant and natural human PIGF, but not murine PIGF. Murine PIGF also reacts only with natural murine PIGF. Both human and murine PIGF assays recognize free PIGF, but not PIGF bound to sFlt-1.

Binding Assay of rhPIGF With rhsFlt-1 In Vitro

To confirm whether rhsFlt-1 binds to PIGF, we performed an in vitro binding assay. rhsFlt-1, which contains a histidine-tag, was diluted with a solution consisting of 20 mmol/L imidazole, 0.5 mol/L NaCl, 20 mmol/L Tris, and 0.1 mg/ml bovine serum albumin (BSA), at 5 concentrations: 1 ng/20 µL, 10 ng/20 µL, 100 ng/20 µL, 1,000 ng/20 µL, and 10,000 ng/20 µL, respectively. The rhsFlt-1 solution was mixed with Ni-agarose gel (Qiagen, Valencia, CA, USA), which can bind sFlt-1 that is histidine-tagged. After centrifugation, the supernatant was removed and rhPIGF solution (10 ng/100 µL: 100 mmol/L NaCl, 50 mmol/L Tris, 20 mmol/L imidazole, 0.1 mg/ml BSA) was added to the precipitation including the Ni-agarose-rhsFlt-1 complex. The solution was incubated overnight at 4°C. After centrifugation in order to remove the Ni-agarose-rhsFlt-1 complex bound to rhPIGF, uncombined rhPIGF in the supernatant was measured by ELISA.

Reverse Transcriptase-PCR

The apex of the infarcted heart was homogenized and RNA was extracted using Trizol reagent (Life Technologies, Grand Island, NY, USA). Reverse transcription was performed and PCR was then conducted using ABI Prism7700 (Applied Biosystems, Carlsbad, CA, USA). The level of brain natriuretic peptide (BNP) in infarcted hearts was detected using Taqman Gene Expression Assays (Applied Biosystems).

Evaluation of Infarct Size

In order to evaluate infarct size, TTC (2,3,5-triphenyltetrazolium chloride; Sigma Chemical Co, St Louis, MO, USA) staining was performed. At 7 or 28 days after coronary ligation, the hearts were excised, washed with phosphate-buffered saline (PBS), and the ventricles were cut into transverse slices. The slices were placed on a culture plate, stained for 15 min at room temperature with 1.0 ml of 1.5% TTC solution to determine the infarct area, and photo-

Figure 1. Recombinant human placental growth factor (rhPIGF) protein was produced and administered to C57BL/6 mice. Survival of mice with a myocardial infarct in the rhPIGF group was compared with the phosphate-buffered saline (PBS) group for 28 days after coronary ligation. Values are means $\pm$ SD; * P <0.05 vs Control.

graphed under a microscope (Olympus Co, Tokyo, Japan). Infarct area and infarct fraction were evaluated. Infarct fraction was calculated as: infarct area/(infarct area+non-infarct area). Fibrin deposition within the infarct area was evaluated by Masson-trichrome staining: areas of fibrin deposition and area of viable tissue were directly measured, and the ratio of fibrin deposition area to viable tissue area (fibrin deposit area/viable tissue area) was calculated. Sequential sections were used to analyze the TTC and

Masson-trichrome staining.

Functional Analysis

The left ventricular end-diastolic dimension (LVEDd) and ejection fraction (LVEF) were measured by 2-D guided M-mode echocardiography (Toshiba, Tokyo, Japan).

Immunohistochemistry

The hearts of adult mice were fixed and embedded in frozen sections. The following antibodies were used: anti CD68 antibody (cloneFA-11, UK-Serotec, Oxford, UK), biotin-conjugated monoclonal mouse anti-CD31 antibody (clone390, eBioscience, San Diego, CA, USA), alkaline phosphatase-conjugated anti- α smooth muscle actin (SMA) antibody (clone1A4, Sigma Chemical Co), and Cy3-conjugated anti- α SMA antibody (clone1A4, Sigma Chemical Co).

Statistical Analysis

All results are expressed as the means $\pm$ SD. Differences between groups were evaluated for statistical significance using Kaplan-Meier analysis, Student's t -test and 1-factor ANOVA. Values of P <0.05 were considered significant.

Results

Recombinant hPIGF Administration and Survival of AMI Mice

The plasma rhPIGF level on the 3rd day of administration by

Figure 2. Infarct size and left ventricular function analyzed by TTC staining and echocardiography, respectively. Infarcted hearts of mice treated with both phosphate-buffered saline (PBS) and recombinant human placental growth factor (rhPIGF) were stained with TTC 7 and 28 days after coronary ligation (A–D). After staining, viable myocardium appeared red and non-viable myocardium appeared white. Infarct area (E), and infarct fraction (F) were calculated. Representative images of Masson-trichrome staining of a PBS- (G) and a rhPIGF- (H) treated mouse. The ratio of fibrin deposition area to viable tissue area (fibrin deposit area/viable tissue area) was calculated (I). Values are means $\pm$ SD; * P <0.05, ** P <0.01 (comparison between PBS and rhPIGF groups). †† P <0.01 (comparison between the same color bars).

Figure 3. Echocardiography performed on the phosphate-buffered saline (PBS) (A) and recombinant human placental growth factor (rhPIGF) (B) groups 7 days after myocardial infarction. Left ventricular diastolic dimension and ejection fraction were analyzed (C,D). Reverse transcriptase-polymerase chain reaction was performed to compare the brain natriuretic peptide level in infarcted tissue of the PBS and rhPIGF groups (E). GAPDH was used as a control. Values are means $\pm$ SD; ** $P < 0.01$ (comparison between PBS and PIGF groups).

Figure 4. Regenerating vessels in infarcted myocardium analyzed by immunohistochemistry 7 days after myocardial infarction. The number of CD31-positive vessels was increased by recombinant human placental growth factor (rhPIGF) administration ($\times 200$) (A–C). The number of α SMA-positive mature blood vessels was also increased after rhPIGF treatment ($\times 100$) (D–F). Values are means $\pm$ SD; ** $P < 0.01$ vs phosphate-buffered saline (PBS). IZ, infarct zone; NIZ, non-infarct zone.

Figure 5. Co-administration of both rhPIGF and rhsFlt-1 in MI mice. Binding assay of rhPIGF with rhsFlt-1 (A). Recombinant human sFlt-1 can bind to rhPIGF in vitro. The efficacy of rhPIGF was inhibited by rhsFlt-1 in a dose-dependent manner. Human PIGF in peripheral blood was analyzed in normal mice using an ELISA kit (B). (Cont: normal mice; PIGF: rhPIGF-treated mice; PIGF+sFlt-1: co-administration of rhPIGF and sFlt-1.) rhPIGF was suppressed by rhsFlt-1 administration in vivo. Values are means $\pm$ SD; * P <0.05 vs PIGF, † P <0.05 vs PIGF. Murine PIGF in peripheral blood in normal and MI mice was analyzed by ELISA kit (C). (C: normal mice; AMI: PBS-treated MI mice; PIGF: administration of rhPIGF with coronary ligation; PIGF+sFlt-1: co-administration of rhPIGF and rhsFlt-1 with coronary ligation; sFlt-1: administration of sFlt-1 with coronary ligation.) PIGF activity was suppressed by administration of sFlt-1. Values are means $\pm$ SD; * P <0.05 vs Control, † P <0.05 vs PIGF. Blood pressure of rhsFlt-1- or PBS-treated mice was measured for 7 days (D). Survival of MI mice administered rhPIGF, PBS, sFlt-1, or rhPIGF and rhsFlt-1 simultaneously (E). Survival rate was improved in the rhPIGF group, but not in the other groups. Survival rate in the rhsFlt-1 group was the same as that in the PBS group. * P <0.05 vs the other groups. rhsFlt-1, recombinant human soluble Flt-1. See text and Figures 1–4 for abbreviations.

osmotic minipump was $1,252\pm 460$ pg/ml, which is approximately 4-fold higher than the endogenous mouse PIGF plasma level 3 days after an AMI (307 ± 68 pg/ml) (data not shown). During the administration of rhPIGF, blood pressure did not differ significantly between the PIGF and PBS groups (data not shown). At 28 days after development of AMI, 6 of 20 mice in the PIGF group and 12 of 17 mice in the PBS group had died. The mortality rate was thus improved by 52% by rhPIGF treatment. Kaplan-Meier analysis showed that administration of rhPIGF for 3 days improved survival rate significantly (P <0.05) compared with vehicle (Figure 1).

PIGF Improves Cardiac Function and Suppresses Remodeling

We next evaluated the effects of rhPIGF on infarct size and cardiac function after induction of MI. TTC staining of the heart revealed that 7 days after MI, both infarct area and infarct fraction were significantly smaller in the rhPIGF group than in the PBS group (infarct area: 4.25 ± 2.04 mm² vs 5.95 ± 1.54 mm², P <0.01; infarct fraction: $26.46\pm 1.59\%$ vs $33.70\pm 0.52\%$, P <0.05). In the chronic phase of AMI (28 days), both groups showed significant compensatory hyper-

trophy in non-infarcted myocardium. In contrast to the acute phase, the infarct area and infarct fraction were larger in the rhPIGF group than in the PBS group (infarct area: 5.58 ± 2.76 mm² vs 3.37 ± 2.00 mm², P <0.01; infarct fraction: $18.86\pm 0.76\%$ vs $11.25\pm 0.22\%$, P <0.01) (Figures 2A–F). In order to quantify the fibrin deposition within the infarct area at 28 days after surgery, we performed Masson-trichrome staining. A larger area of viable myocardial tissue was observed in the infarct area of the rhPIGF group (Figure 2H) compared with the PBS group (Figure 2G). The ratio of fibrin deposit area to viable tissue area (fibrin deposit area/viable tissue area) in the infarct area was significantly lower in the rhPIGF group than in PBS group (1.5 ± 1.04 , 4.63 ± 1.85 , P <0.05) (Figure 2I).

Echocardiographic findings at 7 days after coronary ligation showed that LVEDd was smaller in the rhPIGF group than in the PBS group (4.50 ± 0.46 mm vs 5.14 ± 0.38 mm, P <0.01) and LVEF was significantly higher ($40.6\pm 9.17\%$ vs $25.7\pm 7.23\%$, P <0.01) (Figures 3A–D).

The level of BNP mRNA in the infarcted myocardium was significantly lower in the rhPIGF-treated group than in the PBS group (BNP mRNA/GAPDH: 0.46 ± 0.42 vs 1.14 ± 0.44 , P <0.01) (Figure 3E).

Figure 6. Left ventricular function of MI mice was analyzed by TTC staining 7 days after coronary ligation. Infarcted hearts treated with PBS (A), rhPIGF (B), co-administration of rhPIGF and rhsFlt-1 (C), and rhsFlt-1 (D) were stained with TTC. Infarct areas were smaller in the rhPIGF group than in the PBS group, but larger in the rhsFlt-1 group (E). Regenerating vessels in infarcted myocardium were analyzed by immunohistochemistry 7 days after MI (F). The number of CD31-positive vessels decreased with rhsFlt-1 administration. Values are means $\pm$ SD; *P<0.05, **P<0.01 vs PBS, ††P<0.01 vs PIGF, ‡‡P<0.01 vs PIGF+sFlt-1. See text and Figures 1–4 for abbreviations.

PIGF Induces Angiogenesis and Arteriogenesis

To elucidate the mechanism underlying the beneficial effects of PIGF on post-AMI pathology, we analyzed angiogenesis and arteriogenesis in the infarcted myocardium. The number of CD31-positive vascular endothelial cells in the infarct areas (including the border zone) was significantly higher in the rhPIGF group than in the PBS group ($644.6\pm90.54/\text{mm}^2$ vs $459.0\pm73.89/\text{mm}^2$, $P<0.01$) (Figures 4A–C). Similarly, the number of α -SMA-positive vessels was increased in the same areas in the rhPIGF group relative to controls (rhPIGF: $31.6\pm7.20/\text{mm}^2$, PBS: $23.5\pm7.41/\text{mm}^2$, $P<0.01$) (Figures 4D–F).

Beneficial Effect of Exogenous PIGF Blocked by rhsFlt-1

In order to further evaluate the actions of exogenously administered rhPIGF, we examined whether or not its beneficial effects were blocked by the simultaneous administration of rhsFlt-1, a PIGF antagonist.

We confirmed before the experiment that rhsFlt-1 binds to PIGF in vitro and in vivo. As shown in Figure 5A, an in-vitro binding assay showed that 10^{-11} mol/L rhsFlt-1 binds

to 0.17×10^{-12} mol/L rhPIGF. When rhPIGF was administered alone, the plasma rhPIGF level was $1,252\pm460$ pg/ml. Co-administration of rhsFlt-1 decreased the plasma rhPIGF level to 753 ± 69 pg/ml, as shown in Figure 5B. The plasma level of endogenous PIGF was higher in AMI mice with rhPIGF treatment than in control mice (307 ± 68 pg/ml vs 128 ± 8.8 pg/ml). The co-administration of rhsFlt-1 also reduced the plasma level of endogenous PIGF to 121 ± 108 pg/ml (Figure 5C). Thus, rhsFlt-1 blocked both exogenous and endogenous PIGF by approximately 50%. We also proved that sFlt-1, like rhPIGF, did not affect hemodynamic function (Figure 5D).

As shown in Figure 5E, co-administration of rhsFlt-1 with rhPIGF lowered the survival rate of mice given rhPIGF alone, validating the improvement in the survival rate seen in mice treated with rhPIGF alone. The survival rate of control mice and those given only rhsFlt-1 was similar.

Soluble Flt-1 Inhibits rhPIGF Effects on Infarct Size and Angiogenesis

As described earlier, TTC staining showed that the infarct

Figure 7. Mechanisms underlying the angiogenic effect of PlGF. EPCs (Flk-1+Sca-1+ cells) were mobilized into the peripheral circulation in MI mice (A). The percentage of EPCs was increased in MI mice, and was further increased in rhPlGF-treated MI mice. Values are means $\pm$ SD; * P <0.05, † P <0.05 vs PBS. The number of GFP-positive cells in the infarct area in bone marrow transplant mice (B). The number of infiltrated monocytes (CD68-positive cells) in the infarct area was also increased by rhPlGF treatment compared with PBS treatment (C). Values are means $\pm$ SD; * P <0.05 vs PBS. Bone-marrow-derived cells were examined by immunohistochemistry. GFP-positive (green cells) were observed in both PBS- (D) and rhPlGF- (E) treated mice hearts ($\times 400$). GFP+CD31+ cells were observed in the vessels of the rhPlGF group (F: GFP, G: CD31, H: merge of F and G; $\times 1,200$). Green represents GFP and red represents CD31. See text and Figures 1–4 for abbreviations.

area in rhPlGF-treated mice was significantly smaller than that of the PBS-treated group. However, the infarct area in the co-administered group was significantly larger than that in mice given rhPlGF alone ($7.99\pm 1.33\text{ mm}^2$ vs $4.25\pm 2.04\text{ mm}^2$, P <0.01) and was similar to that in the PBS group ($5.96\pm 1.54\text{ mm}^2$). Interestingly, administration of rhsFlt-1 alone increased the infarct area to $11.90\pm 2.90\text{ mm}^2$, which was significantly larger than the infarct area of the PBS group (P <0.01) (Figures 6A–E).

CD31-positive cells were counted to confirm the inhibitory effect of rhsFlt-1 on angiogenesis. In the MI mice, the number of newly formed vessels was increased in the rhPlGF group, but was decreased by co-administration of rhsFlt-1 to a quantity similar to that in the PBS group (rhPlGF: $644\pm 90.5/\text{mm}^2$; rhPlGF+sFlt-1: $335\pm 103/\text{mm}^2$; PBS: $407\pm 121/\text{mm}^2$; sFlt-1: $287\pm 75.7/\text{mm}^2$) (Figure 6F).

Mechanisms of PlGF-Induced Protective Effect on Ischemic Heart

Given that PlGF has been reported to mobilize EPCs into the peripheral circulation,¹⁷ we hypothesized that EPCs

mobilized from bone marrow as a result of PlGF administration may mediate the angiogenesis and healing that occur after AMI, as well as the improvements seen in survival rates. In this study, the number of CD34-positive cells increased in the peripheral circulation and the peak PlGF level significantly correlated with the CD34-positive cell count in patients with AMI (data not shown).

Based on the human result, we performed a mouse analysis. Flk-1+Sca-1+ cells (murine EPCs) in the peripheral circulation were significantly increased in AMI mice (PBS group) relative to control sham-operated mice without AMI, and they were further augmented by administration of rhPlGF (PBS: $1.84\pm 0.68\%$; Cont: $1.14\pm 0.38\%$; rhPlGF: $2.38\pm 1.02\%$) (Figure 7A).

Next, to evaluate the differentiation of EPCs into vascular endothelial cells in newly developed vasculature in the infarct regions, we generated mice in which bone marrow was substituted with GFP-positive BMCs using a BMT technique. GFP-positive BMCs in the heart were increased in rhPlGF-treated mice compared with the PBS group ($51.68\pm 17.58/\text{field}$ vs $37.4\pm 5.26/\text{field}$, P <0.05) (Figure 7B). CD68-

positive monocytes were also increased in the infarct areas in the rhPIGF group relative to the PBS group ($2,878 \pm 567/\text{mm}^2$ vs $2,346 \pm 398/\text{mm}^2$ $P < 0.01$; **Figure 7C**). Immunofluorescent histological analysis revealed a few GFP and CD31 double-positive cells or BMCs directly differentiated into endothelial cells existing in the infarct area in both rhPIGF and PBS groups (**Figures 7D,E**). However, the number of these cells was very small and there was no significant difference between the 2 groups (rhPIGF: 6–8 per 10 high-power fields; PBS: 1–2 per 10 high-power fields) (**Figures 7F–H**).

Discussion

The present study demonstrates that exogenous administration of rhPIGF enhances angiogenesis and arteriogenesis, reduces infarct size, and improves cardiac function and survival rate following AMI. These beneficial effects were blocked by co-administration of rhsFlt-1, which traps rhPIGF in the circulation and thus inhibits its action. These findings suggest a possible use of PIGF as adjunctive therapy in AMI.

Exogenous PIGF Improves the Prognosis of MI Via Angiogenesis and Arteriogenesis

Earlier experimental studies have shown that several growth factors and cytokines, including basic fibroblast growth factor,¹⁸ G-CSF,¹⁹ erythropoietin,²⁰ angiopoietin-1²¹ and VEGF,²² improve cardiac function after AMI by promoting angiogenesis. However, no molecule has been clinically proved to be useful as an adjunctive therapy in AMI. In the present study we assessed the therapeutic use of PIGF in AMI for several reasons. Firstly, PIGF is a member of the VEGF family and induces not only angiogenesis but also arteriogenesis *in vivo*.¹⁰ Secondly, PIGF is able to mobilize EPCs from bone marrow to the peripheral circulation,¹⁷ and finally, we recently observed that PIGF mRNA expression is substantially upregulated in the endothelium of the coronary arteries and interstitial cells in the infarct regions, suggesting participation of PIGF in the pathology of AMI.¹³ Some previous investigations revealed that transfer of the PIGF gene or direct injection of PIGF protein induced angiogenesis in ischemic hindlimb and myocardium.^{9,10} Moreover, Autiero et al reported that intraperitoneal injection of a VEGF/PIGF heterodimer enhanced angiogenesis 2 weeks after AMI.²³ However, those studies focused primarily on the local angiogenic effect of PIGF and did not examine its broader role; for instance, its influence on cardiac function and survival rate, which are important in terms of the molecule's clinical application. The present study demonstrates that 3-day intraperitoneal administration of rhPIGF reduces infarct size, preserves EF and reduces mortality. Considering that PIGF mRNA expression was augmented in the infarct region and that the plasma PIGF level peaked on the 3rd day after the onset of AMI in our human study,¹³ injection of exogenous PIGF would enhance the beneficial effect of endogenous PIGF.

sFlt-1 Cancels the Beneficial Effect of PIGF

Co-administration of rhFlt-1 with rhPIGF completely inhibited the PIGF-induced reduction of infarct size and improvement of survival rate, which confirms the beneficial effects of PIGF in AMI. However, sole administration of rhsFlt-1, which reduced the plasma level of endogenous PIGF by approximately 50%, did not affect the survival rate after

AMI, though it increased infarct size. Considering that the plasma level of rhPIGF during administration was approximately 1,200 pg/ml and the peak plasma level of endogenous PIGF in AMI was approximately 300 pg/ml, augmentation of endogenous PIGF gene expression may not be sufficient to improve mortality. This supports the idea of using supplemental PIGF as an adjunctive therapy in AMI. We also found there was a discrepancy in that rhsFlt-1 administration increased infarct area without suppressing angiogenesis compared with PBS treatment. This result suggests that endogenous PIGF production in the injured myocardium does not have potent stimulatory effects on angiogenesis by itself, but rather it may have direct protective effects on the ischemic myocardium by a mediating pathway except for angiogenesis. It is well recognized that the loss of ischemic cardiomyocytes after MI induces compensatory hypertrophy of the non-ischemic remote myocardium. Roncal et al reported that PIGF induces not only enlargement of vessel size, but also compensatory hypertrophy of cardiomyocyte in remote non-infarcted myocardium.²⁴ We assume this may be one of the mechanisms by which sFlt-1 exacerbates cardiac remodeling via suppressing endogenous PIGF.

Mechanisms of PIGF's Effect on Ischemic Myocardium

The current study could not elucidate the mechanism by which PIGF reduces infarct size and ultimately improves survival rate. Given previous evidence that VEGF did not improve cardiac function and mortality in either animal or human studies, despite its strong angiogenic properties, it is likely that the dual properties of PIGF, angiogenesis and arteriogenesis, are closely related to its reducing both infarct size and mortality. PIGF is known to induce new vascular formation by 2 mechanisms: stimulation of local proliferation of endothelial cells,^{10,25–27} as well as recruitment of BMCs to target tissue.¹⁷ In particular, PIGF exerts its biological activities by binding specifically to Flt-1 and causing its autophosphorylation.²⁸ Flt-1 is expressed not only on endothelial cells, but also on macrophages, hematopoietic progenitor cells, and EPCs.^{29,30} Li et al revealed that PIGF enhances EPC recruitment from the bone marrow into peripheral tissues.³¹ In this context, we have also investigated whether EPCs and Flt-1-positive monocytes contribute to PIGF-induced angiogenesis in the infarct regions. The present study showed that EPCs and monocytes were mobilized into the peripheral blood by rhPIGF; however, direct transdifferentiation of EPCs into the blood vessels was rarely observed. These findings suggest that angiogenesis induced by PIGF is not caused primarily by differentiation of the progenitor cells, but is probably related to angiogenic properties of rhPIGF itself and to other cytokines that are released by PIGF stimulation. Moreover, PIGF enhances both vessel maturation and collateral growth by inducing monocyte infiltration. Sholz et al reported that collateral growth in a hindlimb ischemia model was delayed in PIGF knock-out mice.³² In our results, the number of monocytes in the infarct area increased with rhPIGF administration, which may result in augmentation of arteriogenesis.³³ Earlier studies reported that mobilized monocytes produce MCP-1, bFGF, and TNF- α ,³⁴ so it is possible that cytokines other than PIGF released by recruited monocytes indirectly stimulated angiogenesis and collateral artery growth in our study model. Furthermore, improvement of cardiac function by rhPIGF treatment might relate to mechanisms other than angiogenesis, such as inhibition of apoptosis.

In the present study, the infarct area of the rhPIGF group was significantly larger than that of the PBS group because of preservation of viable myocardium and suppression of fibrin deposition in the infarct area. The reason why rhPIGF treatment suppressed fibrin deposition in the infarct area in the chronic phase remains unknown, but preservation of functional blood supply to in the infarct area by the PIGF-mediated arteriogenesis plays an important role in ameliorating tissue hypoxia, which stimulates extracellular matrix production by interstitial cells in the infarct area.³⁵

Further studies are necessary to elucidate the mechanisms underlying the angiogenesis and arteriogenesis induced by the PIGF-Flt-1 pathway.

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