

# Cardiac Expression of Placental Growth Factor Predicts the Improvement of Chronic Phase Left Ventricular Function in Patients With Acute Myocardial Infarction

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<b>OBJECTIVES</b>	Our aim was to investigate cardiac expression of placental growth factor (PIGF) and its clinical significance in patients with acute myocardial infarction (AMI).
<b>BACKGROUND</b>	Placental growth factor is known to stimulate wound healing by activating mononuclear cells and inducing angiogenesis. The clinical significance of PIGF in AMI is not yet known.
<b>METHODS</b>	Fifty-five AMI patients and 43 control subjects participated in the study. Peripheral blood sampling was performed on days 1, 3, and 7 after AMI. Blood was also sampled from the coronary artery (CAOs) and the coronary sinus (CS), before and after acute coronary recanalization. Cardiac expression of PIGF was analyzed in a mouse AMI model.
<b>RESULTS</b>	In AMI patients, peripheral plasma PIGF levels on day 3 were significantly higher than in control subjects. Plasma PIGF levels just after recanalization were significantly higher in the CS than the CAOs, which indicates cardiac production and release of PIGF. Peripheral plasma levels of PIGF on day 3 were negatively correlated with the acute phase left ventricular ejection fraction (LVEF), positively correlated with both acute phase peak peripheral monocyte counts and chronic phase changes in LVEF. Placental growth factor messenger ribonucleic acid expression was 26.6-fold greater in a mouse AMI model than in sham-operated mice, and PIGF was expressed mainly in endothelial cells within the infarct region.
<b>CONCLUSIONS</b>	Placental growth factor is rapidly produced in infarct myocardium, especially by endothelial cells during the acute phase of myocardial infarction. Placental growth factor might be over-expressed to compensate the acute ischemic damage, and appears to then act to improve LVEF during the chronic phase. (J Am Coll Cardiol 2006;47:1559–67) © 2006 by the American College of Cardiology Foundation

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After acute myocardial infarction (AMI), reducing the total amount of necrotic myocardium and minimizing ventricular remodeling are the most effective ways of preserving long-term left ventricular function and improving prognosis (1). In that regard, a variety of cytokines and circulating cells are known to participate in the wound healing processes that are ongoing in the injured myocardium of the post-myocardial infarction heart, and it has been suggested that appropriate manipulation of those molecules and/or cells could be used to exert a beneficial effect that promotes long-term improvement of cardiac function after AMI (2–4).

Recent progress in stem cell research has opened the possibility of regenerating lost cardiomyocytes and/or vascular tissue by recruiting autologous bone-marrow-derived stem cells to the heart after AMI. Among the candidate cytokines considered for this purpose, vascular endothelial growth factor (VEGF) has been extensively studied because of its established angiogenic capacity and its ability to mobilize endothelial progenitor cells (5). Vascular endothelial growth factor is

known to be strongly expressed in the ischemic myocardium, and treatment with exogenous VEGF has been clearly demonstrated to enhance vessel formation in ischemic myocardium (6,7). Still, *in vivo* treatment with VEGF gene or recombinant VEGF protein does not always lead to improved ventricular function because of the development of non-functioning or unstable capillary vessels (8,9).

Placental growth factor (PIGF) is a member of the VEGF family that acts via VEGF receptor-1 (flt-1) (10,11). Like VEGF, PIGF has been shown to play a key role in vascular development under pathological conditions; for instance, by stimulating both angiogenesis and arteriogenesis, PIGF enhances not only capillary but also collateral formation in ischemic tissue (12). In addition, PIGF appears to promote mobilization of flt-1-positive hematopoietic stem cells that might be involved in regeneration of vessels and myocardium from bone marrow to the peripheral circulation (13,14).

With that as background, our aim in the present study was to assess cardiac expression of PIGF in both human AMI patients and a mouse model of AMI, and to determine the impact of PIGF expression on the clinical features of AMI in humans.

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#### Abbreviations and Acronyms

AMI	= acute myocardial infarction
CAos	= coronary artery ostium
CK	= creatine kinase
CS	= coronary sinus
flt-1	= vascular endothelial growth factor receptor-1
IR	= ischemia-reperfusion
LVEF	= left ventricular ejection fraction
MI	= myocardial infarction
PCI	= percutaneous coronary intervention
PIGF	= placental growth factor
VEGF	= vascular endothelial growth factor

## METHODS

**Clinical study. PATIENT POPULATION.** Fifty-five patients admitted to Nara Medical University Hospital with AMI were enrolled in this study. In addition, 43 age-matched healthy subjects (mean age  $62.7 \pm 1.5$  years, 33 men, 10 women) served as control subjects. Acute MI was diagnosed when patients experienced chest pain within 24 h before admission and lasted more than 30 min and was not relieved by sublingual nitroglycerin, and exhibited ST-segment elevation and/or abnormal Q waves on an electrocardiogram and elevated serum creatine kinase (CK) levels. Exclusion criteria were AMI more than 24 h from the onset, a history of renal dysfunction requiring dialysis, evidence of malignant disease, or an unwillingness to participate. All patients received coronary angiography on admission. When patients had an occluded coronary artery suitable for percutaneous coronary intervention (PCI) using coronary stents, the patients underwent emergency PCI. After PCI, patients underwent left ventriculography to assess the left ventricular ejection fraction (LVEF) in acute phase. Then they were routinely treated with heparin, isosorbide dinitrate, nicorandil, ticlopidine, aspirin, and an angiotensin-converting enzyme inhibitor or angiotensin II receptor blocker.

The protocol was approved by our institutional ethics committee (#2002–009, Nara Medical University Ethics Committee), and written informed consent was obtained in all of cases from either the patient or his/her family members.

**CLINICAL PARAMETERS OF AMI PATIENTS.** The clinical parameters assessed included age, gender, and coronary risk factors (smoking, hypertension, diabetes mellitus, hyperlipidemia, and obesity). The diagnostic criteria for the coronary risk factors were as follows: hypertension, blood pressure more than 140/90 mm Hg, and/or a history of taking antihypertensive medication; diabetes mellitus, fasting plasma glucose more than 126 mg/dl, or casual plasma glucose more than 200 mg/dl, or a diabetic pattern in 75 g OGTT; hyperlipidemia, serum total cholesterol levels of  $>220$  mg/dl or serum triglyceride levels of  $>150$  mg/dl; obesity, body mass index of more than  $25 \text{ kg/m}^2$ . Infarct site, peak CK, peak CK-MB, LVEF, and coronary angiographical findings were selected for analysis as indicators of MI severity.

**BLOOD SAMPLING AND ANALYSIS.** Peripheral blood samples were collected from all subjects upon admission and then on the third and seventh hospital days. In 30 patients, moreover, blood was sampled during emergency cardiac catheterization from the coronary artery ostium (CAos) and coronary sinus (CS) using a 4-F coronary catheter (Goodman, Tokyo, Japan) and a 6-F CS catheter (Goodman, Tokyo, Japan) before and 30 to 45 min after emergency PCI. Plasma samples were collected in EDTA anticoagulant tubes and stored at  $-80^\circ\text{C}$  until assayed.

White blood cell counts, profiles, and peripheral blood monocyte counts were obtained using Sysmex SE9000 (Sysmex, Kobe, Japan). Blood biochemistry data were obtained using commercially available assays.

Levels of PIGF and VEGF were measured using commercially available enzyme-linked immunoadsorbent assay kits (DPG00, DVE00, respectively; R&D Systems, Minneapolis, Minnesota). This enzyme-linked immunoadsorbent assay kit also cross-reacts with human recombinant PIGF-2 isoform (50%) and also reacts with human recombinant VEGF/hrPIGF heterodimer (5%). For peripheral blood samples, maximum values on the day of admission and on the third and seventh hospital days were considered to be the peak levels for each cytokine at those times.

**ANALYSIS OF CORONARY ANGIOGRAPHY AND LEFT VENTRICULOGRAPHY.** Coronary angiograms and left ventriculograms were analyzed by two independent angiographers without knowledge of the patients' background. Antegrade flow in the infarct-related coronary artery at the initial examination, after recanalization and during follow-up, was graded according to the Thrombolysis in Myocardial Infarction classification (15). Global LVEF was obtained from the right anterior oblique projection of contrast left ventriculography (QLV-CMS; MEDIS, Leiden, the Netherlands).

**Experimental study. MOUSE MODEL OF AMI.** After anesthetizing 10- to 12-week-old male mice (C57BL/6, SLC, Hamamatsu, Japan) using isoflurane with artificial ventilation, thoracotomy was performed in the left intercostal space, and a proximal site of the left coronary artery was ligated as described previously (16). The duration of coronary artery ligation was 40 or 60 min in the ischemia-reperfusion (IR) model, and permanent ligation in the MI model. Mice were sacrificed under general anesthesia with pentobarbital, and the heart was excised on days 3 and 7 after surgery in the IR model and on days 1, 3, 7, and 28 after surgery in the MI model.

**QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION.** Frozen infarct heart tissue, excised on day 3 after surgery, was homogenized in Trizol reagent (Invitrogen Corp., Carlsbad, California) using the standard protocol. After DNase processing, PIGF cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen Corp.). Relative PIGF mRNA levels were then determined using real-time polymerase chain reaction carried out using cDNA samples with Assay-on-Demand Product, Taqman

Probe and an ABI Prism 7700 Sequence Detection System (ABI, PE Biosystems, Foster City, California). Levels of PIGF mRNA were normalized to those of glyceraldehyde-3-phosphate dehydrogenase mRNA.

**IMMUNOHISTOCHEMICAL AND IMMUNOFLUORESCENT STAINING OF PIGF IN INFARCT MYOCARDIUM.** The left ventricular apex, including the infarct lesion, was perfused with 4% paraformaldehyde and harvested, then cut into 3- $\mu$ m sections followed by immersion fixated in 4% paraformaldehyde for overnight. The sections were labeled with primary mouse anti-PIGF (AF465 1:100 dilution, R&D Systems) and then with secondary biotin-labeled anti-goat IgG (BA-9500 1:800 dilution; Vector Laboratories, Burlingame, California) antibodies, and processed using the ABC-DAB (3,3'-diaminobenzidine tetrahydrochloride) method (Vectastain Elite ABC Kit, Vector Laboratories). Hematoxylin was used for nuclear staining.

For immunofluorescent staining, acetone-fixed sections were incubated for 1 h at room temperature with a primary anti-PIGF (sc-1882, 1:100 dilution, Santa Cruz Biotechnology Inc., Santa Cruz, California) and/or fluorescein isothiocyanate-conjugated anti-alpha-smooth muscle actin (F3777, 1:400 dilution, Sigma-Aldrich Inc., St. Louis, Missouri) and/or anti-von Willebrand factor (A0082, 1:200 dilution, DakoCytomation Corp., Carpinteria, California) and/or biotin-labeled anti-mouse CD11b (13-0112, eBioscience, San Diego, California). After washing, the sections were incubated for 30 min at room temperature with Cy3-conjugated donkey anti-goat IgG (705-165-003, Jackson Immunoresearch Laboratories, Inc., West Grove, Pennsylvania) for PIGF and/or anti-rabbit IgG (A21441, Molecular Probes Inc., Eugene, Oregon) for von Willebrand factor and/or Cy2-conjugated anti-biotin (016-220-084, Jackson Immunoresearch Laboratories Inc.) for CD11b. 4',6-Diamidino-2-phenylindole was used for nuclear staining.

**MEASURING THE BLOOD VESSEL DENSITY IN THE INFARCT MYOCARDIUM.** We quantified the blood vessel density in the MI and IR models in mice sacrificed on day 7 after surgery. Sliced sections with 3  $\mu$ m thick of the left ventricle, including the infarct lesion, were labeled with mouse anti-alpha-smooth muscle actin (monoclonal anti-actin, alpha-smooth muscle-alkaline phosphatase antibody produced in mouse clone 1A4) (A5691, 1:200 dilution, Sigma-Aldrich Inc.) and processed using 5-bromo-4-chloro-3-indoxyl phosphate/nitro blue tetrazolium chloride substrate (K0598, DakoCytomation Corp.) for measuring the blood vessel density in the marginal area of infarct myocardium. Blood vessels were counted in a stained slide from at least 10 randomly selected fields under  $\times 400$  confocal microscopy (AX70, OLYMPUS, Tokyo, Japan) and expressed as count per high power field.

**Statistical analysis.** Continuous data are expressed as means  $\pm$  SE. Comparisons between control and AMI groups were made using paired or unpaired *t* tests, as appropriate. To assess correlations between two parameters, simple linear regressions

were calculated using the least squares method. Values of  $p < 0.05$  were considered significant. The relationship between the improvement of LVEF and clinical baseline parameters including PIGF were analyzed using simple and multiple linear regression analysis. All statistics were calculated using Stat View for Windows, version 5.0 (SAS Institute Inc., Cary, North Carolina).

## RESULTS

**Patient characteristics.** Among the 55 enrolled patients, 39 received follow-up cardiac catheterization six months after the onset of AMI. Of the 16 patients who did not receive follow-up cardiac catheterization, 3 died during hospitalization because of pump failure, 5 were transferred to other hospitals, and 8 refused a follow-up examination. Table 1 shows the clinical characteristics of the AMI patients participating in this study. There were 43 men and 12 women with a mean age of  $66.7 \pm 1.8$  years (range, 31 to 88 years). The mean interval from the onset of AMI to reperfusion was  $10.8 \pm 1.8$  h. The mean value of the maximum CK level was  $4,117 \pm 502$  IU/l, and mean LVEF during the acute phase was  $57.1 \pm 1.7\%$ . The mean peak monocyte count was  $702 \pm 40$  cells/ $\mu$ l, which was apparently unrelated to the severity of the AMI estimated from the LVEF and maximum CK levels. The mean interval from admission to the peak monocyte count was  $3.2 \pm 0.1$  days.

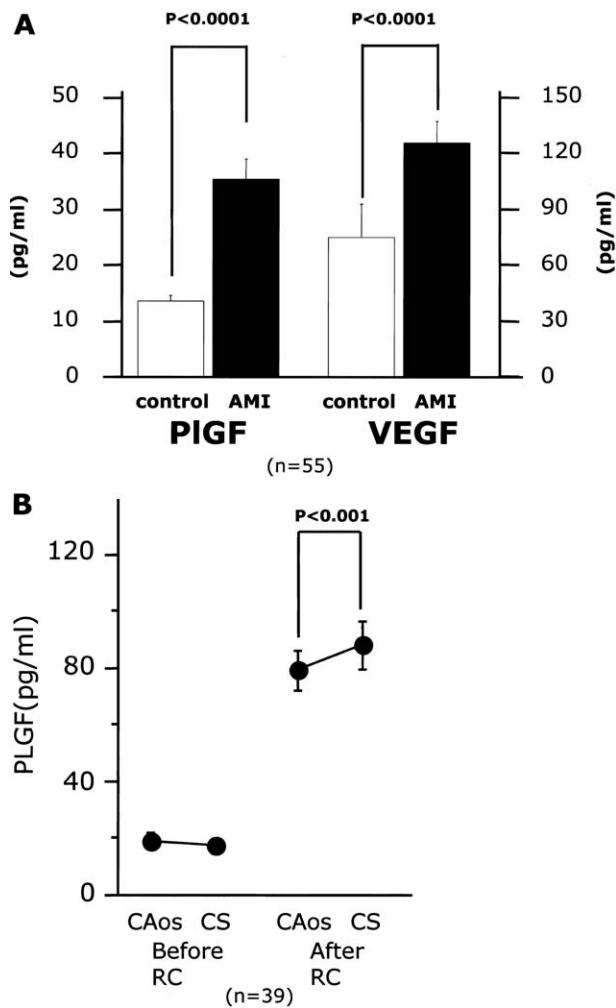
**Changes in peripheral plasma PIGF levels after AMI.** When measured in peripheral plasma obtained on the 1st, 3rd, and 7th days after the onset of AMI, levels of both PIGF and VEGF were found to peak on day 3 after AMI in most cases, at which time they were significantly higher than in control subjects (Fig. 1A). They then tended to decline about one week after AMI (data not shown).

**Table 1.** Clinical Characteristics of AMI Patients

Age (yrs)	66.7 $\pm$ 1.8
Gender (male/female)	43/12
Reperfusion time (h)	10.8 $\pm$ 1.8
Max CK (IU/l)	4,117 $\pm$ 502
Max CK-MB (IU/l)	274 $\pm$ 34
LVEF at the onset of MI (%)	57.1 $\pm$ 1.7
Peak WBC counts (cells/ $\mu$ l)	11,158 $\pm$ 494
Peak PBMC (cells/ $\mu$ l)	702 $\pm$ 40
Culprit lesion	
LAD	27
LCx	4
RCA	24
Coronary risk factor	
Diabetes	23
Hypertension	29
Hyperlipidemia	35
Smoking	25
Obesity	7

Data are expressed as mean value  $\pm$  SE.

AMI = acute myocardial infarction; CK = creatine kinase; LAD = left anterior descending artery; LCx = left circumflex artery; LVEF = left ventricular ejection fraction; Max = maximum; MI = myocardial infarction; PBMC = peripheral blood; RCA = right coronary artery; WBC = white blood cell.



**Figure 1.** (A) Peripheral blood plasma cytokine levels in patients with acute myocardial infarction (AMI). Placental growth factor (PIGF) and vascular endothelial growth factor (VEGF) were significantly higher than control in blood samples from AMI patients. **Bars** represent means  $\pm$  SE. (B) Placental growth factor levels in plasma samples taken from the coronary artery ostium (CAOs) and coronary sinus (CS) before and just after percutaneous coronary intervention. Before recanalization (RC) of an infarct-related artery, plasma PIGF levels were similarly low in the CAOs and CS. After RC, PIGF levels were significantly increased in both the CAOs and CS, as compared to before RC, and levels in the CS were significantly higher than in the CAOs. **Closed circles and bars** represent means  $\pm$  SE.

**Plasma PIGF levels in the coronary circulation during AMI.** To determine whether the elevated PIGF levels in the peripheral blood plasma reflected release of the cytokine from the infarct heart, we measured PIGF levels in plasma samples collected from the CAOs and CS at the time of emergency coronary intervention (Fig. 1B). Before reperfusion of the infarct-related artery, PIGF levels were similarly low in the CAOs and CS ( $20.2 \pm 3.1$  pg/ml and  $18.4 \pm 2.7$  pg/ml, respectively), and were comparable to the levels in healthy subjects. After reperfusion, however, PIGF levels in both the CAOs and CS were significantly elevated, and the levels in the CS were significantly higher than those in the CAOs ( $90.1 \pm 8.8$  pg/ml vs.  $79.7 \pm 7.6$  pg/ml;  $p < 0.01$ ), which is indicative of cardiac production and release of PIGF.

**Clinical determinants of plasma PIGF levels.** To identify the clinical determinants governing the plasma PIGF levels, we evaluated the relationship between plasma PIGF levels and the clinical characteristics of our patients. We found that plasma PIGF levels were unaffected by gender or by the presence or absence of any of the coronary risk factors (Table 2). Likewise, age, the extent of coronary atherosclerosis, site of the culprit lesion, reperfusion time, and maximum CK did not correlate with plasma PIGF levels (data not shown).

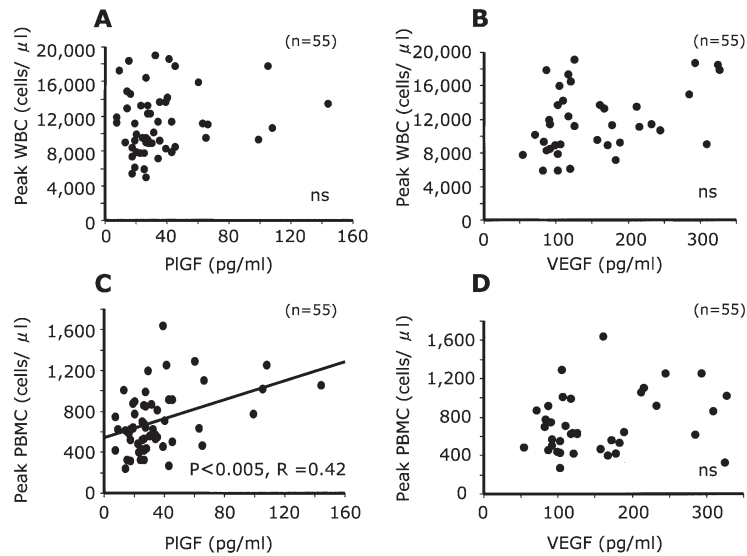
**Clinical significance of PIGF during AMI.** Plasma PIGF levels on day 3 post-MI positively correlated with peak monocyte counts ( $r = 0.415$ ,  $p = 0.0016$ ), but did not correlate with total peripheral white blood cell counts, suggesting PIGF may be involved in the peripheral mobilization of mononuclear cells during AMI. By contrast, plasma VEGF levels on day 3 post-MI did not correlate with either the total white blood cell counts or the monocyte counts (Fig. 2). At the same time, plasma PIGF levels negatively correlated with acute phase LVEF ( $r = 0.434$ ,  $p = 0.021$ ), though plasma VEGF levels did not (Figs. 3A and 3B). In addition, plasma PIGF levels on day 3 post-MI were positively correlated with the subsequent changes in LVEF observed during the chronic phase 6 months after the onset of AMI ( $\Delta$ EF,  $R = 0.38$ ,  $p = 0.0196$ ; Fig. 3C), which reflects improvement in left ventricular function. The corresponding VEGF levels did not correlate with  $\Delta$ EF (Fig. 3D). Multiple regression analysis revealed that the plasma PIGF level was the strongest independent predictor of improvement of LVEF during the chronic phase ( $p = 0.0098$ ) (Table 3). Furthermore, when we studied the subgroup of patients with substantially impaired left ventricular function (LVEF  $< 60\%$ ), we found that patients who showed improvement in LVEF ( $\Delta$ LVEF  $\geq 0$ ) ( $n = 13$ ), as  $\Delta$ LVEF  $> 0$ , in the chronic phase had significantly higher plasma PIGF levels than patients without improvement in LVEF ( $\Delta$ LVEF  $< 0$ ) ( $n = 8$ ) ( $47.3 \pm 22.6$  pg/ml vs.  $27.9 \pm 12.6$  pg/ml, respectively), supporting that peak PIGF levels in the acute phase, independent from baseline LVEF, is the determinant factor for the improvement of LVEF in the chronic phase. Placental growth factor might be over-expressed to compensate for ischemic damage during AMI.

**Table 2.** Plasma PIGF Levels on Third Day After AMI and the Coronary Risk Factors

Risk Factors	PIGF (pg/ml)		p Value
	(+)	(-)	
Diabetes	$31.4 \pm 21.2$	$37.8 \pm 29.8$	NS
Hypertension	$35.9 \pm 28.2$	$33.7 \pm 23.7$	NS
Hyperlipidemia	$33.4 \pm 23.2$	$37.0 \pm 30.1$	NS
Smoking	$31.8 \pm 20.3$	$37.8 \pm 30.8$	NS
Obesity	$26.1 \pm 8.7$	$36.4 \pm 28.0$	NS
	Male	Female	p Value
	$31.1 \pm 3.0$	$40.7 \pm 11.4$	NS

Data are expressed as mean value  $\pm$  SE.

AMI = acute myocardial infarction; PIGF = placental growth factor.

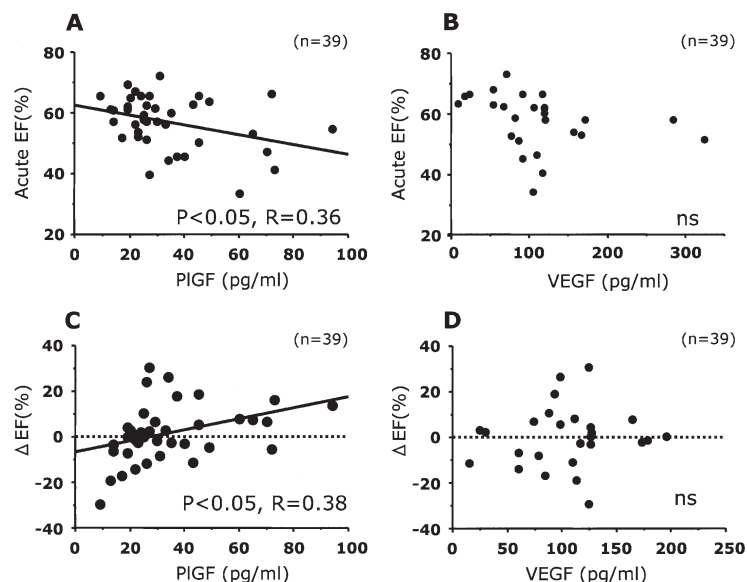


**Figure 2.** Relationship between plasma placental growth factor (PIGF) levels on day 3 post-myocardial infarction and peripheral white blood cell (WBC) counts. Neither PIGF (A) nor vascular endothelial growth factor (VEGF) (B) correlated with total peripheral WBC counts. Placental growth factor levels positively correlated with peak monocyte counts (C), though VEGF levels did not (D). PBMC = peripheral blood.

**Expression of PIGF in a mouse AMI model.** To confirm the augmented production of PIGF in infarct hearts, we assessed the expression of PIGF mRNA in a mouse model of MI. We found that levels of PIGF transcript were increased 26.6-fold ( $p < 0.0001$ ) in infarct hearts of MI models, as compared to sham-operated hearts, on days 1 and 3 post-MI, and that transcript levels had returned to control levels on days 7 and 28 post-MI (Fig. 4).

Immunohistochemical analysis with confocal microscopy (AX70, OLYMPUS) showed that PIGF protein was strongly expressed in vascular tissues, especially vascular endothelial cells of both capillaries and small vessels in

the infarct myocardium (Fig. 5), within the infarct myocardium, but was scarcely detected in non-infarct regions, and was not detected at all in sham-operated hearts (data not shown). Although we cannot totally deny the PIGF expression in vascular smooth muscle cell in the same vessel, results from double immunofluorescent staining indicate that endothelial cells of vascular tissue in infarct myocardium is the major site of PIGF production (Fig. 6). We also found a small number of PIGF-positive cells in interstitial space in infarct myocardium. However, PIGF-positive cells did not cross-react with known macrophage antibody (anti-CD11b) (Fig. 6).



**Figure 3.** Relationship between plasma placental growth factor (PIGF) levels on day 3 post-myocardial infarction and left ventricular ejection fraction (EF) during the acute phase. Whereas plasma PIGF levels negatively correlated with left ventricular EF at the onset of acute myocardial infarction (A), plasma vascular endothelial growth factor (VEGF) levels did not (B). Plasma PIGF levels on day 3 post-myocardial infarction were positively correlated with the changes of left ventricular EF ( $\Delta$ EF) that occurred between the acute and chronic phases (C); VEGF levels were not (D).

**Table 3.** Multiple Regression Analysis for Chronic Phase Left Ventricular Function

Clinical Parameters	p Value
PIGF	<0.05
Age	NS
Max CK	NS
Max CK-MB	NS
Reperfusion time	NS

Data are expressed as mean value ± SD.

CK = creatine kinase; Max = maximum; PIGF = placental growth factor.

Next, we evaluated the effect of severity of ischemia on the PIGF mRNA expression and the angiogenesis in ischemic myocardium. Placental growth factor mRNA expression on day 3 after surgery did not increase in mice with 40-min ischemia, but significantly increased in the 60-min ischemia model (1.9-fold increase), as well as in the MI model (26.6-fold increase) compared with sham-operated mice. Furthermore, PIGF mRNA expression levels were significantly higher in MI than in IR. Blood vessel densities in the marginal area of infarct myocardium were also significantly increased in both IR and MI models compared with sham, and blood vessel density of the MI model was significantly higher (2.5-fold increase) compared with the IR model (Fig. 7B).

## DISCUSSION

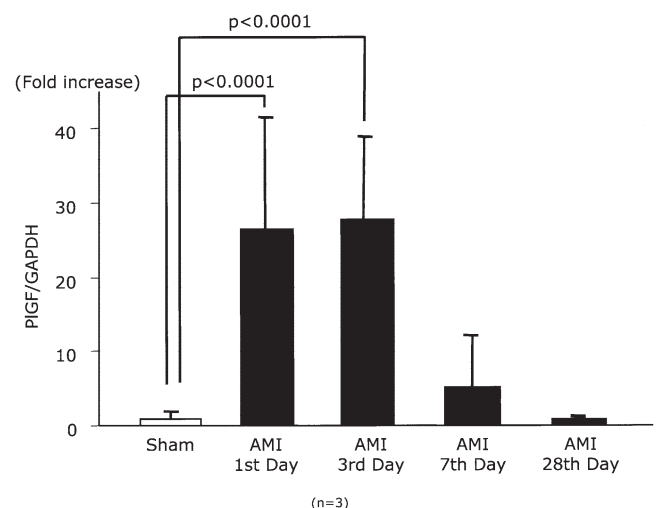
The present study demonstrates for the first time that: 1) cardiac production of PIGF is increased after AMI; 2) the major site of augmented expression of PIGF is the endothelium of vessels within the infarcted region; 3) PIGF production after AMI is determined, in part, by the amount of injured myocardium; and 4) plasma PIGF levels on day 3 post-MI are positively correlated with both the circulating monocyte counts during the acute phase and the degree of improvement of LVEF during the chronic phase of MI. Taken together, these findings may suggest that cardiac expression of PIGF has a beneficial effect on wound healing processes after AMI, possibly by inducing peripheral mobilization of mononuclear cells and enhancing angiogenesis.

**Cardiac expression of PIGF during AMI.** Earlier studies showed that PIGF is expressed in placental tissue, trophoblasts, leukocytes, and endothelial cells under normal physiological conditions (17–19). The present findings indicate that, during AMI, vascular tissue, especially the endothelium within the infarct myocardium, is also a major site of PIGF production. Clinically, we found that LVEF during the acute phase is negatively correlated with plasma PIGF levels on day 3 post-MI, making it likely that the degree of injury to the vascular endothelium within the infarct myocardium is a key determinant of cardiac PIGF production after AMI. Furthermore, our observation that induction of PIGF begins very early (within 24 h) after the onset of cardiac ischemia and is sustained for about a week thereafter suggests that tissue PIGF is importantly involved in the early phase of tissue healing after ischemic injury (e.g.,

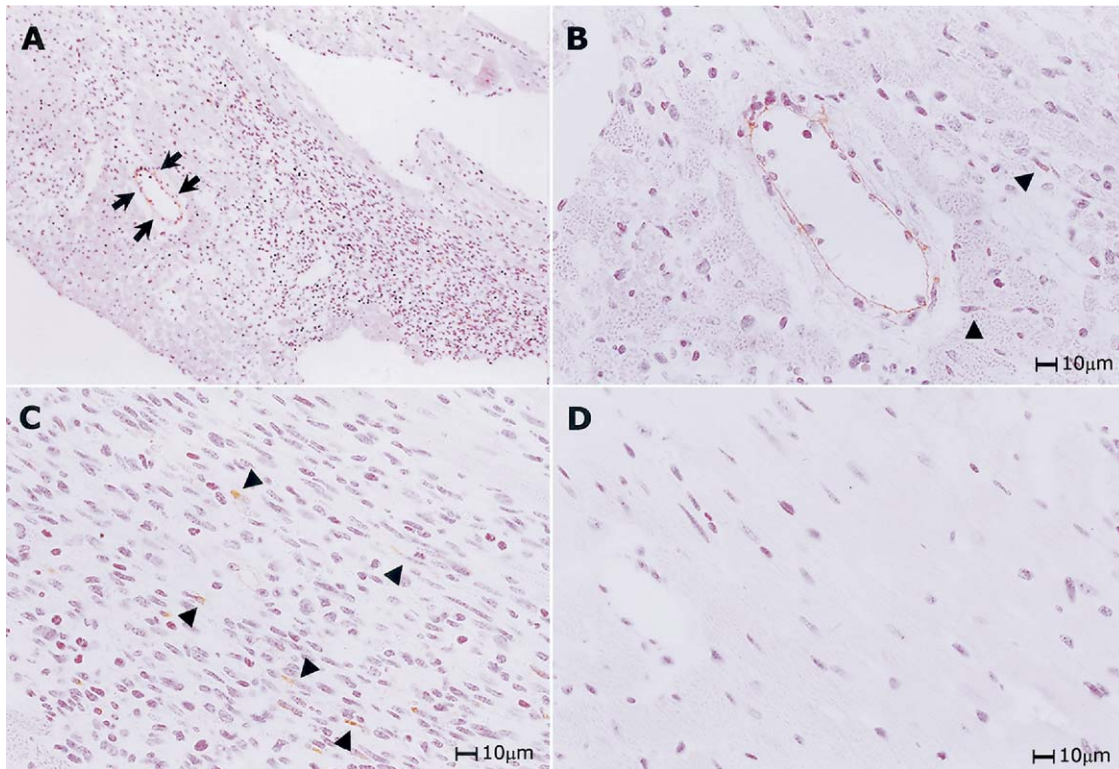
angiogenesis). Although the signaling pathway leading to up-regulation of PIGF expression in the ischemic myocardium is not yet clear, expression level of PIGF mRNA levels was regulated by the degree and duration of myocardial ischemia in this study. Supporting our observations, one in vitro study reported that PIGF is induced by hypoxia (20). In that regard, the promoter region of the PIGF gene does not contain a hypoxia-inducible, factor-responsive element, but it does contain other hypoxia-responsive elements, such as those for metal transcription factor-1 and Sp-1 (20,21). It is, thus, plausible that hypoxia is an important stimulus of PIGF expression within the infarct myocardium.

Although their study population and the study design are not the same with the present study, Heesch et al. (22) have reported that plasma levels of PIGF in patients with non-ST-segment elevated MI and refractory unstable angina pectoris were significantly elevated compared with healthy control subjects, supporting that prolonged and/or severe ischemia induces the cardiac expression of PIGF.

**Clinical significance of PIGF during healing after AMI.** The function of PIGF after AMI has not been systematically studied. We noticed, however, that patients with higher plasma PIGF levels on day 3 post-MI showed greater improvement in LVEF during the chronic phase (6 months post-MI) than patients with lower plasma PIGF levels, and also that patients with improvement in LVEF in the chronic phase had significantly higher plasma levels of PIGF in the acute phase compared with patients without improvement, again suggesting the involvement of PIGF in the healing of the injured myocardium. Thus, PIGF might be over-expressed to compensate the ischemic damage during AMI. Like VEGF, PIGF is known to have powerful angiogenic properties, especially under pathological circumstances, such



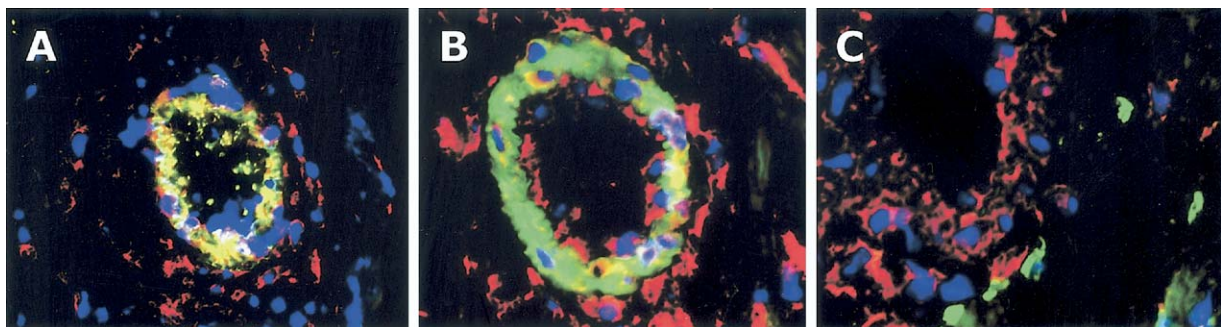
**Figure 4.** Expression of placental growth factor (PIGF) mRNA in the hearts of mice subjected to myocardial infarction induced by coronary artery ligation. Quantitative real-time polymerase chain reaction revealed that expression of PIGF mRNA was significantly up-regulated in infarct hearts, as compared to sham-operated hearts on days 1 and 3 post-myocardial infarction, but had returned to levels that did not differ from control by days 7 and 28. AMI = acute myocardial infarction.



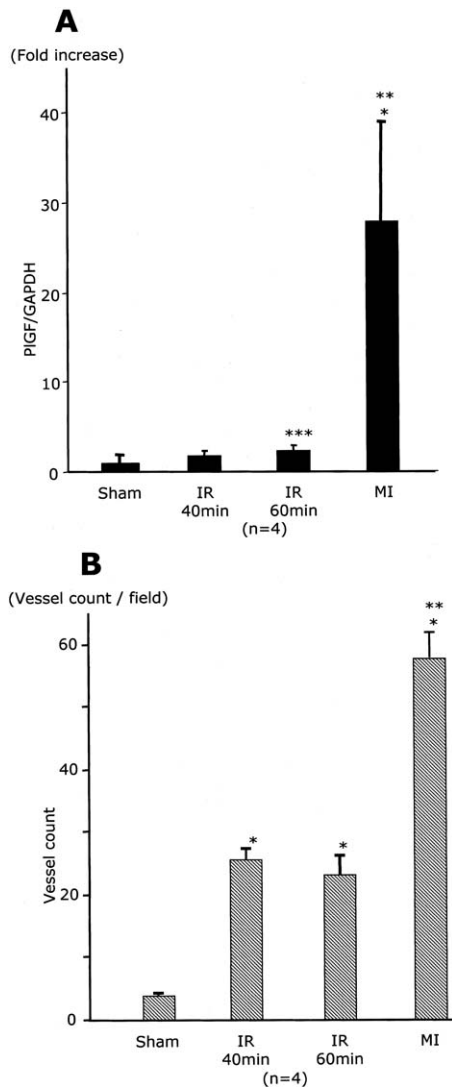
**Figure 5.** Photomicrographs showing immunohistochemical staining of placental growth factor in mouse heart on day 3 post-myocardial infarction. (A) Infarct myocardium (original magnification, 60 $\times$ ). (B and C) Higher magnification (240 $\times$ ) of the corresponding box (A). The staining shows strong expression of placental growth factor protein in the endothelium within the infarct region (B), as well as in interstitial cells (C). The staining shows an absence of placental growth factor protein in the non-infarct area (D). Arrows indicate vascular tissue, arrowheads interstitial cells.

as in cancer and limb ischemia (23,24). This suggests that, in cases of AMI, locally expressed PIGF might directly stimulate angiogenesis in both the infarct and infarct-border regions. In mouse experiments of our study, we observed that both temporal and permanent occlusion of coronary artery promoted PIGF gene expression, as well as angiogenesis in ischemic myocardium. As shown in Figure 7, the increment of vessel density and PIGF expression in the permanent occlusion model was substantially higher than that in temporal ischemia. Accordingly, cardiac PIGF expression seems to be involved in the angiogenesis under the

situation of severe myocardial ischemia. Moreover, we observed that plasma PIGF levels on day 3 post-MI were significantly correlated with peak monocyte counts  $3.2 \pm 0.1$  days post-MI, suggesting that PIGF released from the infarct heart exerts a stimulatory effect on monocyte mobilization. Consistent with that idea, Hattori et al. (13) observed an increase in circulating stem cells after adenovirus-mediated gene transfer of PIGF to BALB/c mice (14). They suggested that PIGF induces stem cell mobilization via flt-1, which is expressed on monocyte, CD34 $^{+}$  cells, and hematopoietic stem cells (25). Furthermore, recent observations by Pipp et



**Figure 6.** Photomicrographs showing immunofluorescent staining of a small artery and infiltrating cell within the infarct myocardium. (A) Combination staining of the target vessel with anti-placental growth factor (red) and anti-von Willebrand factor (green) antibodies. Yellow coloration indicates placental growth factor expression within vascular endothelial cells. (B) Combination staining with anti-placental growth factor (red) and anti-alpha-smooth muscle actin (green) antibodies. Note that placental growth factor expression is restricted to the intima of the target vessel. (C) Combination staining with anti-placental growth factor (red) and anti-CD11b (green) antibodies. Placental-growth-factor-expressing cell in the interstitium did not cross-react with CD11b-positive cell (macrophage). Original magnification, 800 $\times$ . DAPI was used for nuclear staining (blue).



**Figure 7.** Placental growth factor (PIGF) gene expression and angiogenesis in ischemic myocardium. **(A)** Relation between the duration of ischemia and the PIGF gene expression three days after surgery in mouse models of myocardial infarction (MI, as permanent occlusion) and ischemia-reperfusion (IR). **Bars** represent PIGF mRNA levels of infarct heart tissue. Placental growth factor mRNA expression levels were significantly increased in both in 60 min. Ischemia-reperfusion and MI models compared with sham operated mice. **(B)** Relation between the duration of ischemia and the blood vessel density in the marginal area of infarct myocardium in mouse models of MI and IR. Heart tissue was sampled seven days after surgery. **Bars** represent alpha-smooth muscle actin-positive blood vessel counts in infarct lesion per field under  $\times 400$  microscopy. Blood vessel densities in both MI and IR are significantly increased compared with sham, and blood vessel density in MI is significantly higher ( $2.5\times$ ) compared with IR. \* $p < 0.001$  for MI and IR vs. sham; \*\* $p < 0.001$  for MI vs. IR; \*\*\* $p < 0.01$  for IR 60 min vs. sham.

al. (26) indicate that, in animal ischemic limb models, PIGF administration significantly enhances collateral vessel formation by activating and recruiting mononuclear cells to the ischemic region in an *flt-1*-dependent fashion. We speculate that same mechanisms might work in MI. It has also been reported that monocytes infiltrating into the injured tissue directly stimulate collateral vessel growth by releasing various cytokines, including basic fibroblast growth factor and mono-

cyte chemoattractant protein-1 (27), which is consistent with the idea that, in addition to its direct angiogenic activity, PIGF facilitates wound healing by helping home circulating mononuclear and bone marrow-derived stem cells to the injured myocardium by inducing the expression of adhesion molecules on the surfaces of both endothelial and mononuclear cells (28). Furthermore, macrophage is also known to express *flt-1*, so elevated PIGF might enhance wound healing by activating and recruiting macrophages that have important roles in removing necrotic tissue from an injured area (27,29).

A crucial consideration when interpreting the results of the present study is to determine whether plasma PIGF levels in the range of 30 to 120 pg/ml, which were the highest concentrations observed in our AMI patients, are sufficient to exert a biological effect, such as angiogenesis or mobilization of mononuclear cells from bone marrow. It is noteworthy in that regard that an earlier report showed that PIGF at a concentration of 100 pg/ml stimulates DNA synthesis in *flt-1*-expressing endothelial cells (30), which suggests that the plasma PIGF levels we observed in our patients were indeed sufficient to be biologically active.

**Differences between PIGF and VEGF.** We found that, like PIGF, plasma VEGF levels were elevated in patients' blood, but they did not correlate with either monocyte counts or improvement in LVEF. One possible explanation for the difference between these two angiogenic cytokines is that PIGF only binds to *flt-1*, whereas VEGF binds to both *flt-1* and VEGF receptor 2 (31). Another possible explanation is that *flt-1*-mediated intracellular signaling is ligand-specific. For example, PIGF-mediated stimulation of *flt-1* induces MAP kinase activation in porcine aortic endothelial cells, which, in turn, leads to enhanced DNA synthesis (30). By contrast, VEGF-mediated stimulation of *flt-1* does not activate the MAP kinase pathway (30).

**Clinical implications.** Blood vessel growth is a principle component of the wound healing process. Recently, administration of bone marrow-derived or peripheral blood-derived mononuclear cells into infarct-related coronary arteries was shown to improve left ventricular function by enhancing angiogenesis and regeneration of the myocardium (32–34). Similarly, Hojo *et al.* (35) reported that patients showing improved left ventricular systolic function after MI exhibited significantly higher monocyte counts than patients without improvement. We, therefore, suggest that an efficient and safe method for mobilizing bone marrow mononuclear cells may be a useful approach to treating AMI. Our finding that local expression of PIGF is associated with increased monocyte counts and improved LVEF after AMI suggests administration of human PIGF might be a possible means of improving left ventricular function in the chronic phase. On the other hand, several studies have reported a negative relationship between monocyte counts and long-term prognosis after AMI (22,36,37), which suggests peripheral monocytosis may also have adverse effects. Additional studies analyzing the relation be-

tween PIGF levels and monocyte counts during the acute phase of MI and subsequent left ventricular function and long-term prognosis in MI patients are needed to address this issue.

**Conclusions.** Placental growth factor is rapidly produced in infarct myocardial tissue, especially in the endothelium of vessels within the infarct myocardium during the acute phase of MI. The resultant elevation in plasma PIGF levels appears to contribute to the improved LVEF seen during the chronic phase, probably by activating monocytes and enhancing angiogenesis.

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