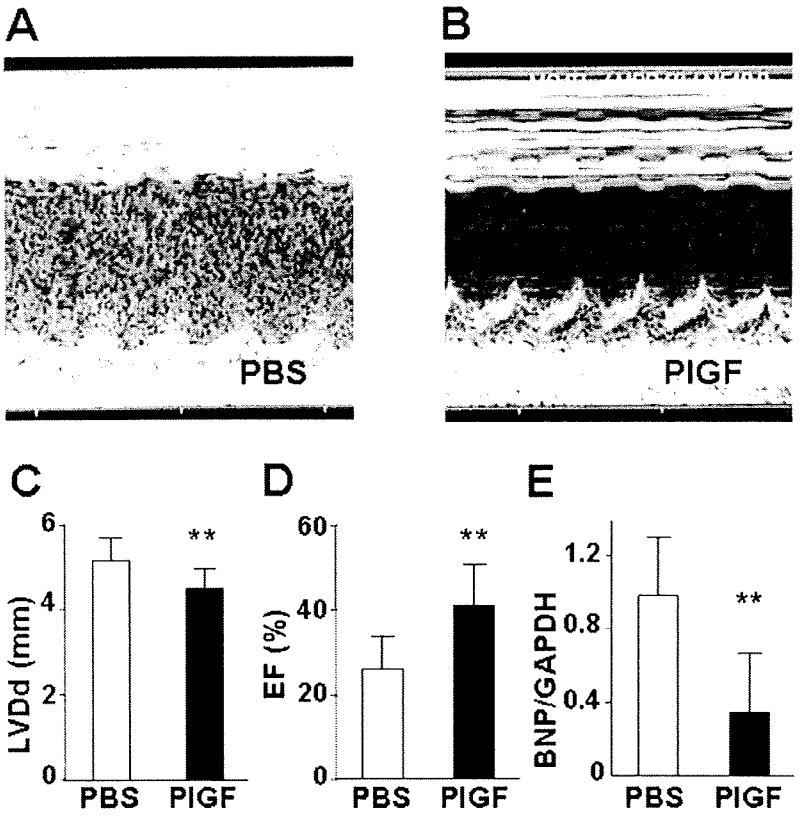


Figure 3. Echocardiography performed on the phosphate-buffered saline (PBS) (**A**) and recombinant human placental growth factor (rhPlGF) (**B**) groups 7 days after myocardial infarction. Left ventricular diastolic dimension and ejection fraction were analyzed (**C,D**). Reverse transcriptase-polymerase 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±SD; **P<0.01 (comparison between PBS and PlGF groups).

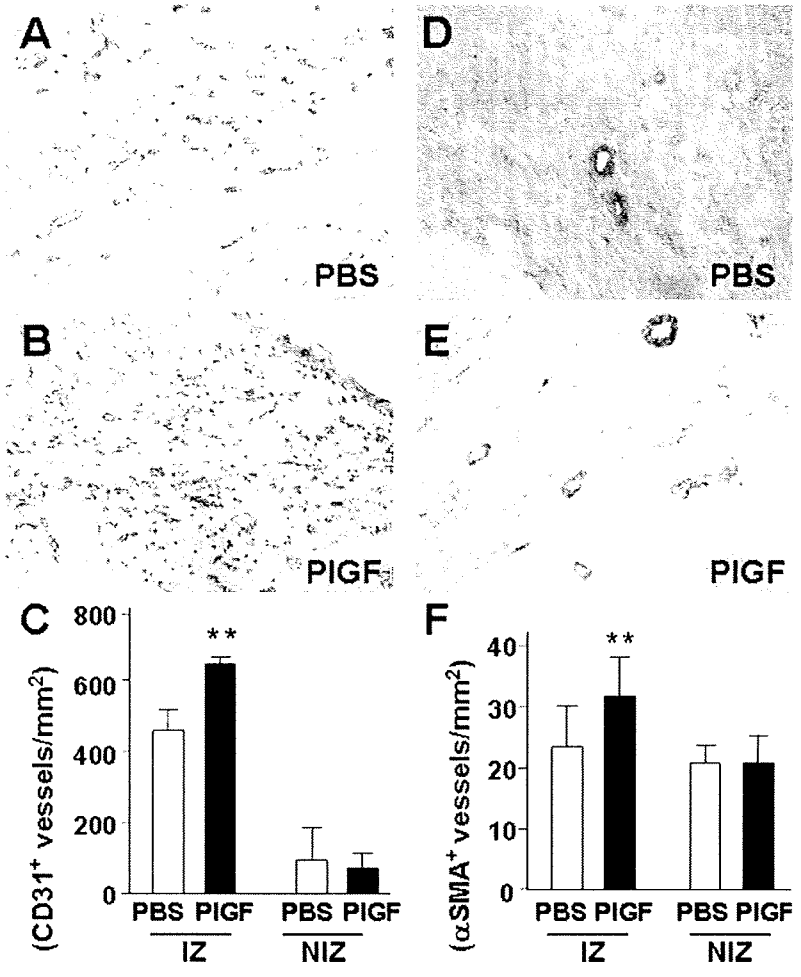


Figure 4. Regenerating vessels in infarcted myocardium analyzed by immunohistochemistry 7 days after myocardial infarction. The number of CD31-positive vessels was increased by recombinant human placental growth factor (rhPlGF) administration (×200) (**A–C**). The number of αSMA-positive mature blood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

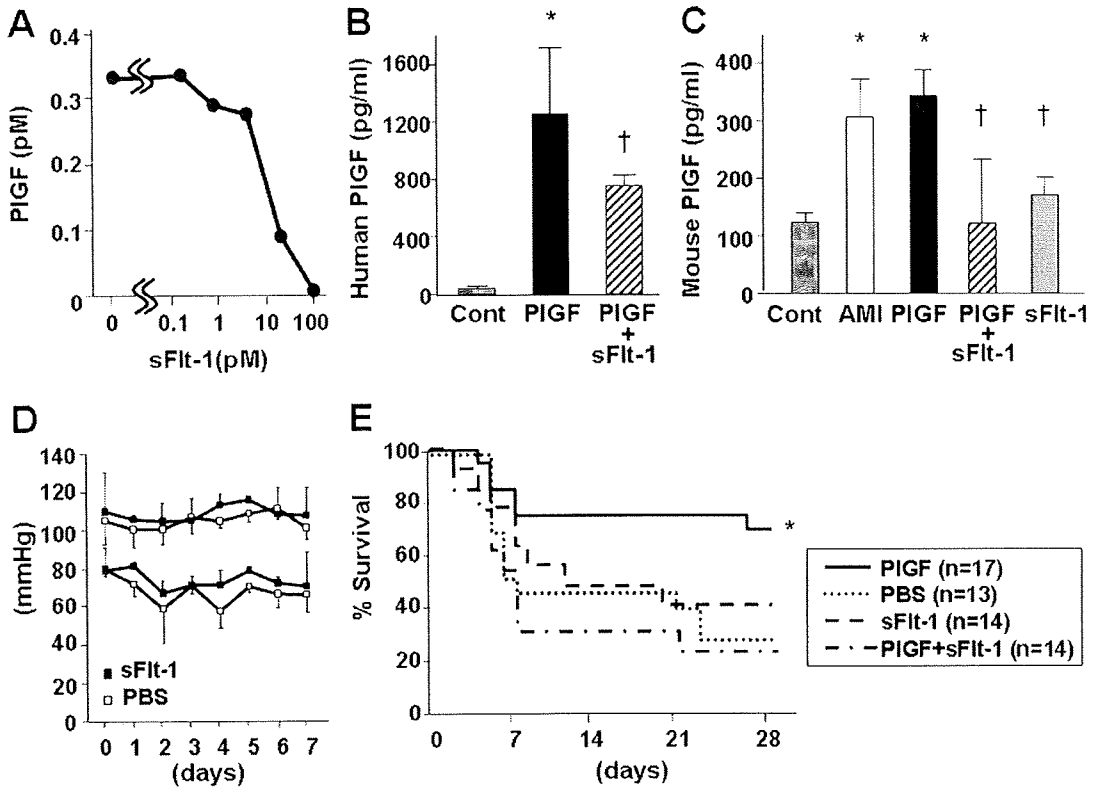


Figure 5. Co-administration of both rhPlGF and rhsFlt-1 in MI mice. Binding assay of rhPlGF with rhsFlt-1 (**A**). Recombinant human sFlt-1 can bind to rhPlGF in vitro. The efficacy of rhPlGF was inhibited by rhsFlt-1 in a dose-dependent manner. Human PlGF in peripheral blood was analyzed in normal mice using an ELISA kit (**B**). (Cont: normal mice; PlGF: rhPlGF-treated mice; PlGF+sFlt-1: co-administration of rhPlGF and sFlt-1.) rhPlGF was suppressed by rhsFlt-1 administration in vivo. Values are means±SD; *P<0.05 vs PlGF, †P<0.05 vs PlGF. Murine PlGF in peripheral blood in normal and MI mice was analyzed by ELISA kit (**C**). (C: normal mice; AMI: PBS-treated MI mice; PlGF: administration of rhPlGF with coronary ligation; PlGF+sFlt-1: co-administration of rhPlGF and rhsFlt-1 with coronary ligation; sFlt-1: administration of sFlt-1 with coronary ligation.) PlGF activity was suppressed by administration of sFlt-1. Values are means±SD; *P<0.05 vs Control, †P<0.05 vs PlGF. Blood pressure of rhsFlt-1- or PBS-treated mice was measured for 7 days (**D**). Survival of MI mice administered rhPlGF, PBS, sFlt-1, or rhPlGF and rhsFlt-1 simultaneously (**E**). Survival rate was improved in the rhPlGF 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±460 pg/ml, which is approximately 4-fold higher than the endogenous mouse PlGF plasma level 3 days after an AMI (307±68 pg/ml) (data not shown). During the administration of rhPlGF, blood pressure did not differ significantly between the PlGF and PBS groups (data not shown). At 28 days after development of AMI, 6 of 20 mice in the PlGF group and 12 of 17 mice in the PBS group had died. The mortality rate was thus improved by 52% by rhPlGF treatment. Kaplan-Meier analysis showed that administration of rhPlGF for 3 days improved survival rate significantly (P<0.05) compared with vehicle (**Figure 1**).

PlGF Improves Cardiac Function and Suppresses Remodeling

We next evaluated the effects of rhPlGF 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 rhPlGF group than in the PBS group (infarct area: 4.25±2.04 mm^2 vs 5.95±1.54 mm^2, P<0.01; infarct fraction: 26.46±1.59% vs 33.70±0.52%, P<0.05). In the chronic phase of AMI (28 days), both groups showed significant compensatory hypertrophy in non-infarcted myocardium. In contrast to the acute phase, the infarct area and infarct fraction were larger in the rhPlGF group than in the PBS group (infarct area: 5.58±2.76 mm^2 vs 3.37±2.00 mm^2, P<0.01; infarct fraction: 18.86±0.76% vs 11.25±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 rhPlGF 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 rhPlGF 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 rhPlGF 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±9.17% vs 25.7±7.23%, P<0.01) (**Figures 3A–D**).

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

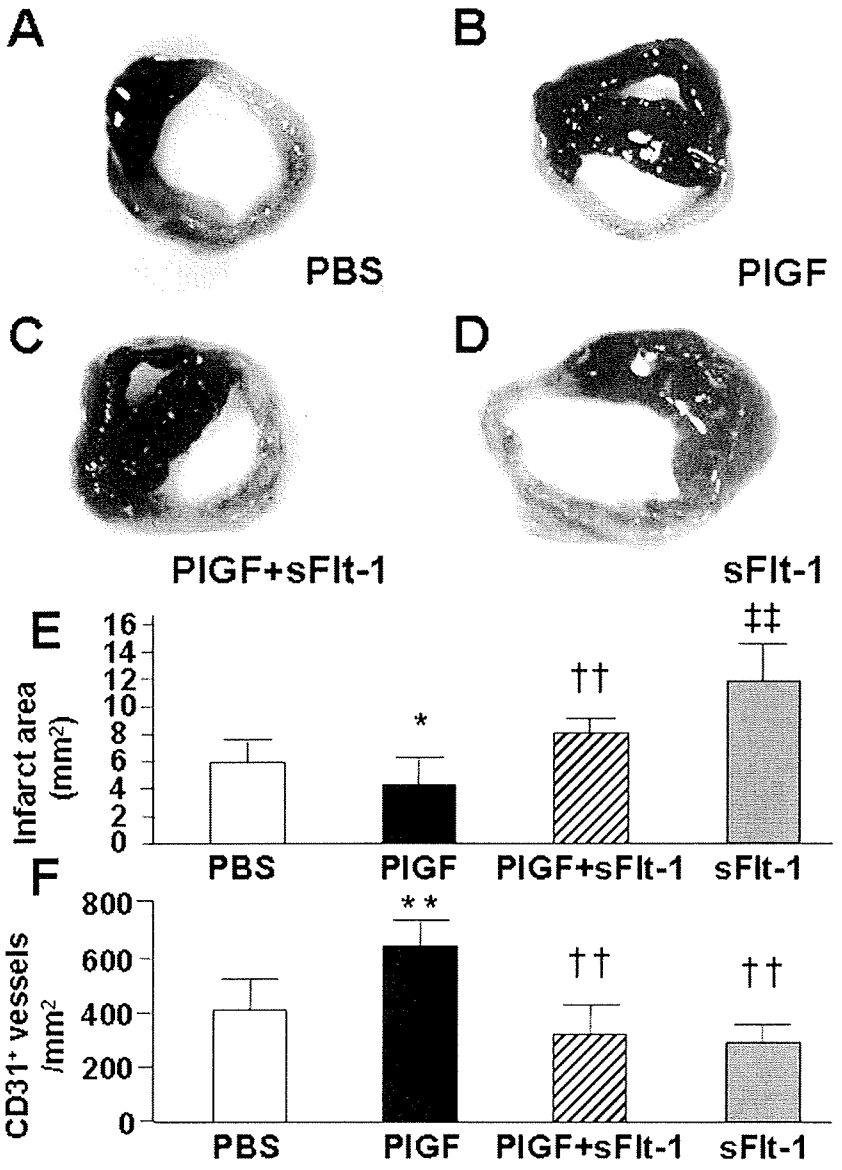


Figure 6. Left ventricular function of MI mice was analyzed by TTC staining 7 days after coronary ligation. Infarcted hearts treated with PBS (**A**), rhPlGF (**B**), co-administration of rhPlGF and rhsFlt-1 (**C**), and rhsFlt-1 (**D**) were stained with TTC. Infarct areas were smaller in the rhPlGF 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±SD; *P<0.05, **P<0.01 vs PBS, ††P<0.01 vs PlGF, ‡‡P<0.01 vs PlGF+sFlt-1. See text and Figures 1–4 for abbreviations.

PlGF Induces Angiogenesis and Arteriogenesis

To elucidate the mechanism underlying the beneficial effects of PlGF 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 rhPlGF group than in the PBS group (644.6±90.54/mm^2 vs 459.0±73.89/mm^2, P<0.01) (**Figures 4A–C**). Similarly, the number of α-SMA-positive vessels was increased in the same areas in the rhPlGF group relative to controls (rhPlGF: 31.6±7.20/mm^2, PBS: 23.5±7.41/mm^2, P<0.01) (**Figures 4 D–F**).

Beneficial Effect of Exogenous PlGF Blocked by rhsFlt-1

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

We confirmed before the experiment that rhsFlt-1 binds to PlGF 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 rhPlGF. When rhPlGF was administered alone, the plasma rhPlGF level was 1,252±460 pg/ml. Co-administration of rhsFlt-1 decreased the plasma rhPlGF level to 753±69 pg/ml, as shown in **Figure 5B**. The plasma level of endogenous PlGF was higher in AMI mice with rhPlGF 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 PlGF to 121±108 pg/ml (**Figure 5C**). Thus, rhsFlt-1 blocked both exogenous and endogenous PlGF by approximately 50%. We also proved that sFlt-1, like rhPlGF, did not affect hemodynamic function (**Figure 5D**).

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

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

As described earlier, TTC staining showed that the infarct

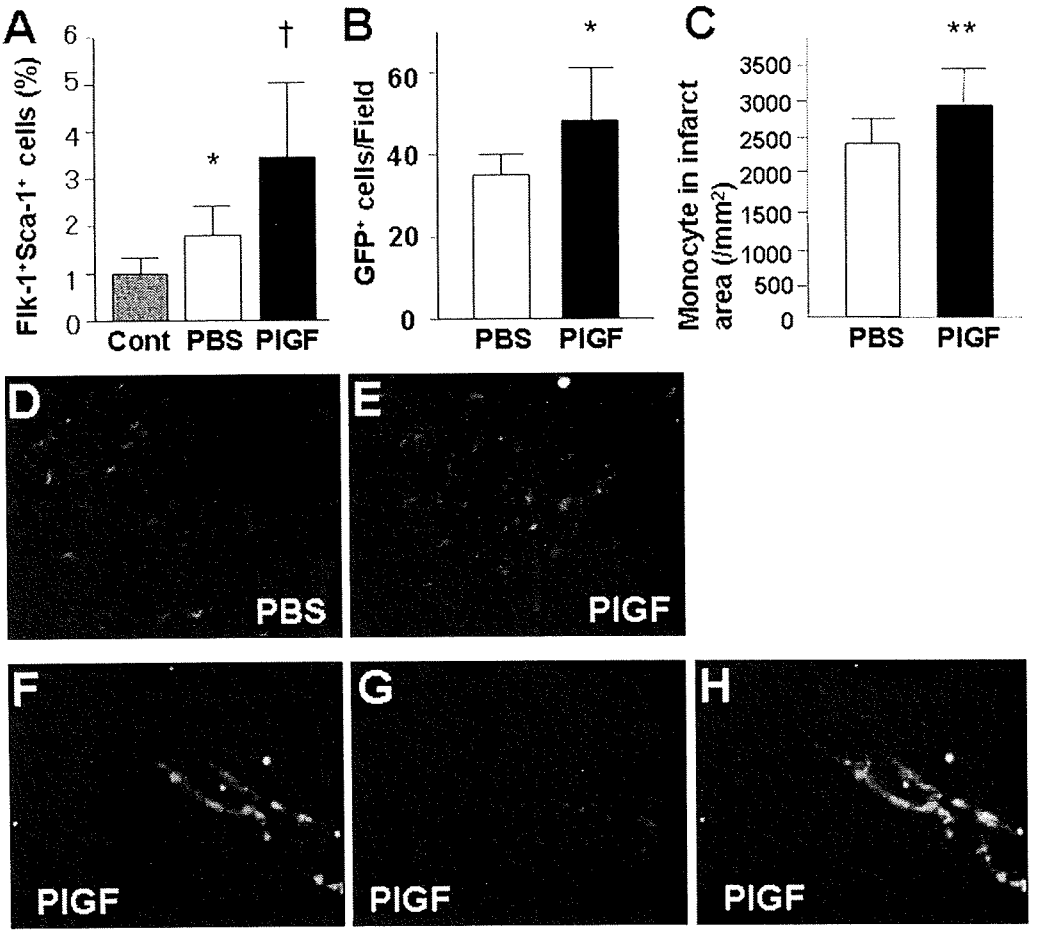


Figure 7. Mechanisms underlying the angiogenic effect of PlGF. EPCs (Flk-1+Sca-1+ cells) were mobilized into the peripheral circulation in MI mice (**A**). The percentage of EPCs was increased in MI mice, and was further increased in rhPlGF-treated MI mice. Values are means±SD; *P<0.05, †P<0.05 vs PBS. 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±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 (×400). GFP+CD31+ cells were observed in the vessels of the rhPlGF group (**F**: GFP, **G**: CD31, **H**: merge of **F** and **G**; ×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±1.33 mm^2 vs 4.25±2.04 mm^2, P<0.01) and was similar to that in the PBS group (5.96±1.54 mm^2). Interestingly, administration of rhsFlt-1 alone increased the infarct area to 11.90±2.90 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±90.5/mm^2; rhPlGF+sFlt-1: 335±103/mm^2; PBS: 407±121/mm^2; sFlt-1: 287±75.7 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±0.68%; Cont: 1.14±0.38%; rhPlGF: 2.38±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±17.58/field vs 37.4±5.26/field, P<0.05) (**Figure 7B**). CD68-

positive monocytes were also increased in the infarct areas in the rhPlGF group relative to the PBS group (2,878± 567/mm^2 vs 2,346±398/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 rhPlGF 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 (rhPlGF: 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 rhPlGF 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 rhPlGF in the circulation and thus inhibits its action. These findings suggest a possible use of PlGF as adjunctive therapy in AMI.

Exogenous PlGF 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-1[21] 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 PlGF in AMI for several reasons. Firstly, PlGF is a member of the VEGF family and induces not only angiogenesis but also arteriogenesis in vivo.[10] Secondly, PlGF is able to mobilize EPCs from bone marrow to the peripheral circulation,[17] and finally, we recently observed that PlGF mRNA expression is substantially upregulated in the endothelium of the coronary arteries and interstitial cells in the infarct regions, suggesting participation of PlGF in the pathology of AMI.[13] Some previous investigations revealed that transfer of the PlGF gene or direct injection of PlGF protein induced angiogenesis in ischemic hindlimb and myocardium.[9,10] Moreover, Autiero et al reported that intraperitoneal injection of a VEGF/PlGF heterodimer enhanced angiogenesis 2 weeks after AMI.[23] However, those studies focused primarily on the local angiogenic effect of PlGF 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 rhPlGF reduces infarct size, preserves EF and reduces mortality. Considering that PlGF mRNA expression was augmented in the infarct region and that the plasma PlGF level peaked on the 3rd day after the onset of AMI in our human study,[13] injection of exogenous PlGF would enhance the beneficial effect of endogenous PlGF.

sFlt-1 Cancels the Beneficial Effect of PlGF

Co-administration of rhFlt-1 with rhPlGF completely inhibited the PlGF-induced reduction of infarct size and improvement of survival rate, which confirms the beneficial effects of PlGF in AMI. However, sole administration of rhsFlt-1, which reduced the plasma level of endogenous PlGF by approximately 50%, did not affect the survival rate after AMI, though it increased infarct size. Considering that the plasma level of rhPlGF during administration was approximately 1,200 pg/ml and the peak plasma level of endogenous PlGF in AMI was approximately 300 pg/ml, augmentation of endogenous PlGF gene expression may not be sufficient to improve mortality. This supports the idea of using supplemental PlGF 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 PlGF 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 PlGF 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 PlGF.

Mechanisms of PlGF's Effect on Ischemic Myocardium

The current study could not elucidate the mechanism by which PlGF 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 PlGF, angiogenesis and arteriogenesis, are closely related to its reducing both infarct size and mortality. PlGF 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, PlGF 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 PlGF 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 PlGF-induced angiogenesis in the infarct regions. The present study showed that EPCs and monocytes were mobilized into the peripheral blood by rhPlGF; however, direct transdifferentiation of EPCs into the blood vessels was rarely observed. These findings suggest that angiogenesis induced by PlGF is not caused primarily by differentiation of the progenitor cells, but is probably related to angiogenic properties of rhPlGF itself and to other cytokines that are released by PlGF stimulation. Moreover, PlGF 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 PlGF knock-out mice.[32] In our results, the number of monocytes in the infarct area increased with rhPlGF 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 PlGF released by recruited monocytes indirectly stimulated angiogenesis and collateral artery growth in our study model. Furthermore, improvement of cardiac function by rhPlGF treatment might relate to mechanisms other than angiogenesis, such as inhibition of apoptosis.

In the present study, the infarct area of the rhPlGF 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 rhPlGF 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 PlGF-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 PlGF–Flt-1 pathway.

References

1. Pfeffer MA, Braunwald E. Ventricular remodeling after myocardial infarction. Experimental observations and clinical implications. *Circulation* 1990; **81:** 1161–1172.
2. Britten MB, Abolmaali ND, Assmus B, Lehmann R, Honold J, Schmitt J, et al. Infarct remodeling after intracoronary progenitor cell treatment in patients with acute myocardial infarction (TOPCARE-AMI): Mechanistic insights from serial contrast-enhanced magnetic resonance imaging. *Circulation* 2003; **108:** 2212–2218.
3. Schächinger V, Erbs S, Elsässer A, Haberbosch W, Hambrecht R, Hölschermann H, et al. Intracoronary bone marrow-derived progenitor cells in acute myocardial infarction. *N Engl J Med* 2006; **355:** 1210–1221.
4. Baumgartner I, Pieczek A, Manor O, Blair R, Kearney M, Walsh K, et al. Constitutive expression of phVEGF165 after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia. *Circulation* 1998; **97:** 1114–1123.
5. Losordo DW, Vale PR, Symes JF, Dunnington CH, Esakof DD, Maysky M, et al. Gene therapy for myocardial angiogenesis: Initial clinical results with direct myocardial injection of phVEGF165 as sole therapy for myocardial ischemia. *Circulation* 1998; **98:** 2800–2804.
6. Lunde K, Solheim S, Aakhus S, Arnesen H, Abdelnoor M, Egeland T, et al. Intracoronary injection of mononuclear bone marrow cells in acute myocardial infarction. *N Engl J Med* 2006; **355:** 1199–1209.
7. Ripa RS, Wang YZ, Jørgensen E, Johnsen HE, Hesse B, Kastrup J. Intramyocardial injection of vascular endothelial growth factor-A$_{165}$ plasmid followed by granulocyte-colony stimulating factor to induce angiogenesis in patients with severe chronic ischaemic heart disease. *Eur Heart J* 2006; **27:** 1785–1792.
8. Kastrup J, Jørgensen E, Rück A, Tägil K, Glogar D, Ruzyllo W, et al. Direct intramyocardial plasmid vascular endothelial growth factor-A$_{165}$ gene therapy in patients with stable severe angina pectoris: A randomized double-blind placebo-controlled study: The Euroinject One trial. *J Am Coll Cardiol* 2005; **45:** 982–988.
9. Carmeliet P, Moons L, Luttun A, Vincenti V, Compernolle V, De Mol M, et al. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. *Nat Med* 2001; **7:** 575–583.
10. Luttun A, Tjwa M, Moons L, Wu Y, Angelillo-Scherrer A, Liao F, et al. Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1. *Nat Med* 2002; **8:** 831–840.
11. Kolakowski S Jr, Berry MF, Atluri P, Grand T, Fisher O, Moise MA, et al. Placental growth factor provides a novel local angiogenic therapy for ischemic cardiomyopathy. *J Card Surg* 2006; **21:** 559–564.
12. Oura H, Bertoncini J, Velasco P, Brown LF, Carmeliet P, Detmar M. A critical role of placental growth factor in the induction of inflammation and edema formation. *Blood* 2003; **101:** 560–567.
13. Iwama H, Uemura S, Naya N, Imagawa K, Takemoto Y, Asai O, et al. Cardiac expression of placental growth factor predicts the improvement of chronic phase left ventricular function in patients with acute myocardial infarction. *J Am Coll Cardiol* 2006; **47:** 1559–1567.
14. Maglione D, Guerriero V, Viglietto G, Ferraro MG, Aprelikova O, Alitalo K, et al. Two alternative mRNAs coding for the angiogenic factor, placenta growth factor (PlGF), are transcribed from a single gene of chromosome 14. *Oncogene* 1993; **8:** 925–931.
15. Yasueda H, Nagase K, Hosoda A, Akiyama Y, Yamada K. High-level direct expression of semi-synthetic human interleukin-6 in Escherichia coli and production of N-terminus met-free product. *Biotechnology (NY)* 1990; **8:** 1036–1040.
16. Takaoka M, Uemura S, Kawata H, Imagawa K, Takeda Y, Nakatani K, et al. Inflammatory response to acute myocardial infarction augments neointimal hyperplasia after vascular injury in a remote artery. *Arterioscler Thromb Vasc Biol* 2006; **26:** 2083–2089.
17. Li B, Sharpe EE, Maupin AB, Teleron AA, Pyle AL, Carmeliet P, et al. VEGF and PlGF promote adult vasculogenesis by enhancing EPC recruitment and vessel formation at the site of tumor neovascularization. *FASEB J* 2006; **20:** 1495–1497.
18. Kawasuji M, Nagamine H, Ikeda M, Sakakibara N, Takemura H, Fujii S, et al. Therapeutic angiogenesis with intramyocardial administration of basic fibroblast growth factor. *Ann Thorac Surg* 2000; **69:** 1155–1161.
19. Misao Y, Arai M, Ohno T, Ushikoshi H, Onogi H, Kobayashi H, et al. Modification of post-myocardial infarction granulocyte-colony stimulating factor therapy with myelo-suppressives. *Circ J* 2007; **71:** 580–590.
20. Lin X, Fujita M, Kanemitsu N, Kimura Y, Tambara K, Premaratne GU, et al. Sustained-release erythropoietin ameliorates cardiac function in infracted rat-heart without inducing polycythemia. *Circ J* 2007; **71:** 132–137.
21. Takahashi K, Ito Y, Morikawa M, Kobune M, Huang J, Tsukamoto M, et al. Adenoviral-delivered angiopoietin-1 reduces the infarction and attenuates the progression of cardiac dysfunction in the rat model of acute myocardial infarction. *Mol Ther* 2003; **8:** 584–592.
22. Ferrarini M, Arsic N, Recchia FA, Zentilin L, Zacchigna S, Xu X, et al. Adeno-associated virus-mediated transduction of VEGF165 improves cardiac tissue viability and functional recovery after permanent coronary occlusion in conscious dogs. *Circ Res* 2006; **98:** 954–961.
23. Autiero M, Waltenberger J, Communi D, Kranz A, Moons L, Lambrechts D, et al. Role of PlGF in the intra-and intermolecular cross talk between the VEGF receptors Flt1 and Flk1. *Nat Med* 2003; **9:** 936–943.
24. Roncal C, Buysschaert I, Chorianopoulos E, Georgiadou M, Meilhac O, Demol M, et al. Beneficial effects of prolonged systemic administration of PlGF on late outcome of post-ischaemic myocardial performance. *J Pathol* 2008; **216:** 236–244.
25. Dull RO, Yuan J, Chang YS, Tarbell J, Jain RK, Munn LL. Kinetics of placenta growth factor/vascular endothelial growth factor synergy in endothelial hydraulic conductivity and proliferation. *Microvasc Res* 200; **61:** 203–210.
26. Taylor AP, Rodriguez M, Adams K, Goldenberg DM, Blumenthal RD. Altered tumor vessel maturation and proliferation in placenta growth factor-producing tumors: Potential relationship to post-therapy tumor angiogenesis and recurrence. *Int J Cancer* 2003; **105:** 158–164.
27. Bellik L, Vinci MC, Filippi S, Ledda F, Parenti A. Intracellular pathways triggered by the selective FLT-1-agonist placental growth factor in vascular smooth muscle cells exposed to hypoxia. *Br J Pharmacol* 2005; **146:** 568–575.
28. Sawano A, Takahashi T, Yamaguchi S, Aonuma M, Shibuya M. Flt-1 but not KDR/Flk-1 tyrosine kinase is a receptor for placenta growth factor, which is related to vascular endothelial growth factor. *Cell Growth Differ* 1996; **7:** 213–221.
29. Clauss M, Weich H, Breier G, Knies U, Röckl W, Waltenberger J, et al. The vascular endothelial growth factor receptor Flt-1 mediates biological activities: Implications for a functional role of placenta growth factor in monocyte activation and chemotaxis. *J Biol Chem* 1996; **271:** 17629–17634.
30. Hattori K, Heissig B, Wu Y, Dias S, Tejada R, Ferris B, et al. Placental growth factor reconstitutes hematopoiesis by recruiting VEGFR1(+) stem cells from bone-marrow microenvironment. *Nat Med* 2002; **8:** 841–849.
31. Li B, Sharpe EE, Maupin AB, Teleron AA, Pyle AL, Carmeliet P, et al. VEGF and PlGF promote adult vasculogenesis by enhancing EPC recruitment and vessel formation at the site of tumor neovascularization. *FASEB J* 2006; **20:** 1495–1497.
32. Scholz D, Elsaesser H, Sauer A, Friedrich C, Luttun A, Carmeliet P, et al. Bone marrow transplantation abolishes inhibition of arteriogenesis in placenta growth factor (PlGF) -/- mice. *J Mol Cell Cardiol* 2003; **35:** 177–184.
33. Heil M, Schaper W. Influence of mechanical, cellular, and molecular factors on collateral artery growth (arteriogenesis). *Circ Res* 2004; **95:** 449–458.
34. Arras M, Ito WD, Scholz D, Winkler B, Schaper J, Schaper W. Monocyte activation in angiogenesis and collateral growth in the rabbit hindlimb. *J Clin Invest* 1998; **101:** 40–50.
35. Mataveli FD, Han SW, Nader HB, Mendes A, Kanishiro R, Tucci P, et al. Long-term effects for acute phase myocardial infarct VEGF165 gene transfer cardiac extracellular matrix remodeling. *Growth Factors* 2009; **27:** 22–31.

Circulation
Cardiovascular Interventions
JOURNAL OF THE AMERICAN HEART ASSOCIATION

American Heart Association
Learn and Live

Coronary Spasm Preferentially Occurs at Branch Points: An Angiographic Comparison With Atherosclerotic Plaque

Hitoshi Nakagawa, Yoshinobu Morikawa, Yuji Mizuno, Eisaku Harada, Teruhiko Ito, Kunihiko Matsui, Yoshihiko Saito and Hirofumi Yasue

Circ Cardiovasc Interv 2009;2;97-104; originally published online Feb 20, 2009;
DOI: 10.1161/CIRCINTERVENTIONS.108.803767
Circulation: Cardiovascular Interventions is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
 Print ISSN: 1941-7640. Online ISSN: 1941-7632

Coronary Spasm Preferentially Occurs at Branch Points

An Angiographic Comparison With Atherosclerotic Plaque

Hitoshi Nakagawa, MD; Yoshinobu Morikawa, MD; Yuji Mizuno, MD; Eisaku Harada, MD; Teruhiko Ito, MD; Kunihiko Matsui, MD, MPH; Yoshihiko Saito, MD; Hirofumi Yasue, MD

Background—Coronary spasm plays an important role in the pathogenesis of ischemic heart disease. However, similarities and differences between coronary spasm and atherosclerosis are not known. We examined the angiographic characteristics of coronary spasm in comparison with those of atherosclerosis.

Methods and Results—Thirty-two left anterior descending arteries, 11 left circumflex arteries, and 23 right coronary arteries with spasm and atherosclerotic plaque were analyzed for the localization of spasm in comparison with that of plaque in 47 patients (38 men and 9 women, mean age 66.8±10.3 yrs). Spasm predominantly occurred at the branch point as compared with plaque in each of the 3 arteries (76.7% versus 23.3%, $P<0.0001$; 72.7% versus 9.1%, $P<0.039$; and 60.0% versus 10.0%, $P=0.002$, in the left anterior descending, left circumflex, and right coronary arteries, respectively). Spasm involved the proximal segment less frequently as compared with plaque in each of the 3 arteries (56.7% versus 93.3%, $P<0.0001$; 18.2% versus 81.8%, $P=0.016$; and 15.0% versus 75.0%, $P<0.0001$ in the left anterior descending, left circumflex, and right coronary arteries, respectively). Most spasms occurred at the nonplaque site in each of the 3 arteries (73.3%, $P=0.018$; 100%, $P<0.0001$; and 75.0%, $P=0.041$ in the left anterior descending, left circumflex, and right coronary arteries, respectively).

Conclusion—Coronary spasm preferentially occurred at branch points and nonplaque sites, whereas the atherosclerotic lesion was predominantly localized at the nonbranch points of the curved proximal segments. Coronary spasm may thus be a manifestation of a distinct type of arteriosclerosis different from the lipid-laden coronary atherosclerosis. (***Circ Cardiovasc Intervent.* 2009;2:97-104.**)

Key Words: atherosclerosis ■ coronary spasm ■ endothelium ■ nitric oxide ■ vasoconstriction

Coronary spasm is not only the cause of variant angina but also participates in the pathogenesis of unstable angina, acute myocardial infarction, and sudden death, particularly in Japan.[1–3] However, precise mechanisms by which coronary spasm occurs are not fully understood. We have shown that endothelial nitric oxide (NO) activity is deficient and endothelial function is impaired in the coronary arteries involved in spasm.[4] Endothelial NO enhances vascular functions, including vessel relaxation, survival of vascular endothelial cells, inhibition of platelet aggregation, and attenuation of leukocyte infiltration.[5,6] Impaired NO activity has been suggested as the earliest pathophysiological events contributing to atherosclerosis.[7,8]

Clinical Perspective see p 104

Flow-generated shear stress is an important physiological stimulus that enhances the production of NO and high shear stress augments the bioavailability of NO, whereas disturbed shear stress reduces it.[5,9,10] Although the entire vasculature is exposed to the atherogenic effect of systemic risk factors, atherosclerotic lesions form at specific arterial regions such as curvatures or branch sites where flow is disturbed.[9,10] Thus, local hemodynamic factors play a major role in the regional localization of atherosclerosis. It is, therefore, possible that coronary spasm also may preferentially occur at the sites of coronary arterial tree where flow is disturbed. However, no previous studies have examined this possibility and the relationship between coronary spasm and atherosclerosis is not clear. This study was designed to examine whether there are predilection sites for spasm in the coronary arteries and, if there are, whether these sites are similar to those of atherosclerosis.

Methods

Patients

Ninety-eight (67 men and 31 women, with a mean age of 65.5±10.1 years ranging from 35 to 86) Japanese patients in whom coronary

Received October 1, 2008; accepted February 11, 2009.

From the Division of Cardiovascular Medicine (H.N., Y. Morikawa, Y. Mizuno, E.H., T.I., H.Y.), Kumamoto Kinoh Hospital, Kumamoto Aging Research Institute; Clinical Education Center (K.M.), Kumamoto University Hospital, Kumamoto City; and First Department of Internal Medicine (Y.S.), Nara Medical University, Kashihara City, Japan.

The online-only Data Supplement is available at http://circinterventions.ahajournals.org/cgi/content/full/10.1161/CIRCINTERVENTIONS.108.803767/DC1.

Correspondence to Hirofumi Yasue, MD, Kumamoto Aging Research Institute, 6-8-1, Yamamuro, Kumamoto City 860-8518, Japan. E-mail yasue@juryo.or.jp

Circ Cardiovasc Intervent is available at http://circinterventions.ahajournals.org DOI: 10.1161/CIRCINTERVENTIONS.108.803767

Table 1. Clinical Characteristics of the Study Subjects

Variables	Normal Angiogram Group (n=51)	Atherosclerosis Group (n=47)
Age, yr	64.2±9.5	66.8±10.3
Gender (male/female)	29/22	38/9
Body mass index, kg/m^2	24.3±3.8	24.2±3.4
Hypertension	19/51 (37%)	25/47 (53%)
Diabetes mellitus	8/51 (16%)	17/47 (36%)
History of smoking	32/51 (63%)	28/47 (60%)
Leukocyte, per μL	6,160±1,668	6925±1944
Hemoglobin, g/dL	13.6±1.7	14.0±2.1
Platelet, $\times 10^4/\mu$	24.1±8.0	24.8±7.5
CRP, mg/L*	0.99 (0.32–2.81)	2.00 (0.48–3.52)
Total protein, g/dL	6.6±0.4	6.8±0.6
Albumin, g/dL	3.9±0.3	3.9±0.4
Fast plasma glucose level, mmol/L	5.86±1.51	5.69±1.01
AST, u/L	25.2±11.0	26.1±8.6
ALT, u/L	22.6±14.5	23.8±11.8
CK, u/L	105.4±71.8	106.7±71.3
Total cholesterol, mmol/L	5.11±0.81	5.35±1.00
LDL cholesterol, mmol/L	2.98±0.74	3.32±0.84
HDL cholesterol, mmol/L	1.54±0.40	1.32±0.38
Triglyceride, mmol/L	1.52±0.70	1.71±0.65

*Median (25th and 75th percentile). ALT indicates alanine aminotransferase; AST, aspartate aminotransferase; CK, creatine kinase; CRP, C-reactive protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

spasm was induced by intracoronary injection of acetylcholine (ACh; Daiichi-Sankyo Co, Tokyo, Japan) were the subjects of this study. They had been admitted to our hospital because of chest pain or ECG abnormalities suspected of ischemic heart disease between January 2003 and January 2009. The study consisted of the 2 parts: the first part of the 51 patients with normal or almost normal coronary angiogram (<25% stenosis of luminal diameter) in whom the confounding effect of organic stenosis on coronary flow could be excluded (normal angiogram group) and the second part of the 47 patients with organic stenosis (25% to 90% stenosis of luminal diameter) (atherosclerosis group) based on the consensus of 3 to 4 investigators. We defined coronary spasm as a total or subtotal occlusion or severe diffuse constriction of an angiographically demonstrable coronary artery associated with transient ischemic ST segment changes on ECG. In each spasm artery, we defined the site of spasm as that of total or subtotal occlusion or as that of the most severe and proximal constriction in the case of segmental diffuse or multifocal spasm and the site of atherosclerotic lesion (plaque) as the most narrowed based on the consensus of 3 to 4 investigators. Patients with recent myocardial infarction, acute coronary syndrome, left main trunk disease, severe organic stenosis of >90%, multivessel coronary disease with >75% organic stenosis, heart failure, liver disease, creatinine level >1.5 mg/dL, acute inflammation, malignant diseases, and cholesterol lowering medication within a month were excluded from the study. None of the study patients were on statins or other lipid-lowering drugs. The clinical characteristics of the study patients are presented in Table 1. Hypertension was defined as >140/90 mm Hg and diabetes mellitus as fasting plasma glucose level >7 mmol/L (126 mg/dL) or 2-hour postload glucose level >11.1 mmol/L (200 mg/dL).

The protocol of this study was approved by the institutional review board and each patient provided written informed consent.

Induction of Coronary Spasm

Ca-channel blockers and other vasodilators, if they had been administered, were stopped for at least 5 days. Coronary spasm was induced by intracoronary injection of ACh (Daiichi-Sankyo Co) after diagnostic catheterization in the morning. The details of the method were previously reported.[11] Briefly, ACh was injected in incremental doses of 20, 50, and 100 μg into the left coronary artery and then 20 and 50 μg into the right coronary artery (RCA) in 20 seconds under continuous monitoring of ECG and blood pressure. Coronary spasm induced by this method usually disappeared spontaneously within 1 to 2 minutes and both the left coronary artery and RCA could be examined separately unless severe spasm occurred in the left coronary artery and necessitated the prompt injection of isosorbide dinitrate (ISDN) into the arteries. After the end of the test, ISDN (0.1 mg) was injected into the coronary artery and angiography was again performed. The specificity of this test for variant or resting angina was 99%.[12] The test did not induce coronary spasm in any of the patients with normal coronary angiogram and without ischemic heart disease.[12,13] The specificity of this test for spasm arteries was also confirmed in the vitro study.[14]

Assessment of Coronary Artery Diameter and Length

We quantitatively measured the diameter of the coronary arteries and the distance from the branch point. An end-diastolic frame was digitized and the diameter of the index vessel was measured by CAAS II software (PIE Medical). We defined the branch point segment as that within 5-mm distal from the apex of the flow divider because the median distance between each adjacent branch was 14.3 mm (interquartile range was 9.1 to 21.1 mm). We divided the left anterior descending (LAD) artery, the left circumflex (LCx) artery, and the RCA into the proximal segments (segments 6, 7, and 9 in the LAD; segments 11 to 13 in the LCx; and segments 1 to 2 in the RCA) and the distal segments (segments 8 and 10 in the LAD; segments 14 and 15 in the LCx; and segments 3 and 4 in the RCA) according to the AHA coronary segment reporting system[15] and compared the incidence of spasm between the proximal and distal segments. The coronary diameter was expressed as percent narrowing in luminal diameter after ISDN injection. Total or subtotal obstruction or severe coronary spasm with a lumen diameter <0.4 mm could not be accurately quantified because of technical limitations of the computer-assisted quantitative coronary angiography.[16]

Laboratory Methods

Fasting blood samples were drawn by venipuncture 1 to 2 days before coronary angiography and the hematologic and biochemical analyses were done using standard laboratory procedures.

Statistical Analysis

Each of the 3 coronary arteries (LAD, LCx, and RCA) was separately analyzed. Discrete variables were expressed as counts and percentages and were compared using McNemar or binomial exact test between the paired data of the same artery. Probability value of <0.05 was considered to be statistically significant. Continuous data were expressed as mean±SD. However, when the variable was significantly skewed, the median (25th to 75th percentile) was reported. Statistical analysis was performed by using commercially available software (SPSS STATISTICS 17.0 BASE WIN, SPSS Japan Inc, Tokyo, Japan). The authors had full access to the data and take responsibility for its integrity. All authors have read and agreed to the manuscript as written.

Results

Table 2 shows the coronary angiographic findings of the 2 groups. In the normal angiogram group, spasm was induced in 106 (45, 28, and 33 in the LAD, LCx, and RCA, respectively) arteries. Of these, 9 (8.5%) were total occlusion, 18 (17.0%) subtotal occlusion, 50 (47.2%) segmental diffuse

Table 2. Coronary Angiographic Findings

	Normal Angiogram Group Spasm Site (n=106)	Atherosclerosis Group Spasm Site (n=66)	P	Plaque Site (n=66)
Entire artery spasm				
LAD, n	12	2		2
LCX, n	10	0		0
RCA, n	12	3		3
Total, n	34	5		5
LAD, n	33	30		30
Proximal segment, n (%)	23 (69.7)	17 (56.7)	<0.0001	28 (93.3)
LCX, n	18	11		11
Proximal segment, n (%)	12 (66.7)	2 (18.2)	0.016	9 (81.8)
RCA, N	21	20		20
Proximal segment, n (%)	4 (19.0)	3 (15.0)	<0.0001	15 (75.0)
Total, n	72	61		61

LAD indicates left anterior descending artery; LCA, left coronary artery; LCX, left circumflex artery; RCA, right coronary artery.

spasm involving the branch site and 29 (27.4%) diffuse and extensive spasm involving the entire arterial tree affecting the proximal and distal epicardial vessels and their branches. Five shifted from entire artery spasm into total occlusion. Accordingly, 34 (32.1%) of the 106 spasms involved the entire arterial tree in this group. Spasm of 1 vessel, 2 vessels, and 3 vessels was demonstrated in 16, 15, and 20 patients, respectively. Of the 44 patients in whom ACh was injected into both the left coronary artery and RCA, 15 had 1-vessel, 9 had 2-vessel, and 20 had 3-vessel spasm, and thus most (65.9%) patients had multivessel coronary spasm demonstrated. For the analysis of the localization of spasm at branch or nonbranch point, 34 (12 LAD, 10 LCx, and 12 RCA) entire artery diffuse spasms were excluded and the remaining 72 arteries (33 LAD, 18 LCx, and 21 RCA) were analyzed. Spasm occurred at the branch point in 27 (81.8%) of 33 LAD, 17 (94.4%) of 18 LCx, and 17 (81.0%) of 21 RCA. Coronary spasm thus preferentially occurred at the branch point in all of the 3 arteries (Figure 1). Spasm involved the proximal segment in 23 (69.7%) of the 33 LAD, 12 (66.7%) of the 18 LCx, and 4 (19.0%) of the 21 RCA. Thus, spasm occurred more frequently at the proximal than the distal segments in the LAD, whereas it occurred more frequently at the distal than the proximal segments in the RCA (Figure 2). This is probably related to the fact that branch site is more numerous at the proximal segment in the LAD, whereas it is more numerous at the distal segment in the RCA[15] and confirms the close relation of the branch point to spasm. In the atherosclerosis group, the organic stenosis was identified in the 66 (32 LAD, 11 LCx, and 23 RCA) arteries as shown in Table 2. Of these, 52 (22 LAD, 8 LCx, and 22 RCA) arteries had 25% to 75% and 14 (10 LAD, 3 LCx, and 1 RCA) arteries had 75% to 90% luminal diameter narrowing. Thus, most (78.8%) patients had mild to moderate organic stenosis in the atherosclerosis group. Spasm was induced in 66 (32 LAD, 11 LCx, and 23 RCA) arteries. Of these, 6 (9.1%) were total occlusion, 9 (13.6%) were subtotal occlusion, 48 (77.2%) were segmental diffuse spasm, and 3 (4.5%) were entire artery diffuse spasm. Two shifted from entire artery diffuse spasm into total occlusion. Accordingly, the entire artery diffuse spasm occurred in 5 (7.6%) of the 66 spasms in the atherosclerosis group (Table 2). For the analysis of the localization of spasm at the branch or nonbranch points, 5 entire artery diffuse spasms were excluded and the remaining 61 (30 LAD, 11

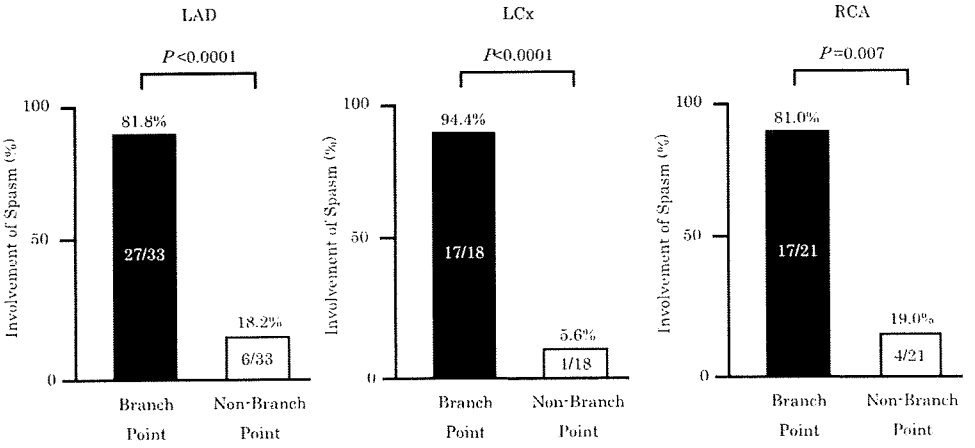


Figure 1. Comparison of the involvement of coronary spasm between the branch and nonbranch points in the normal angiogram group. LAD, left anterior descending coronary artery; LCx; left circumflex artery; RCA, right coronary artery.

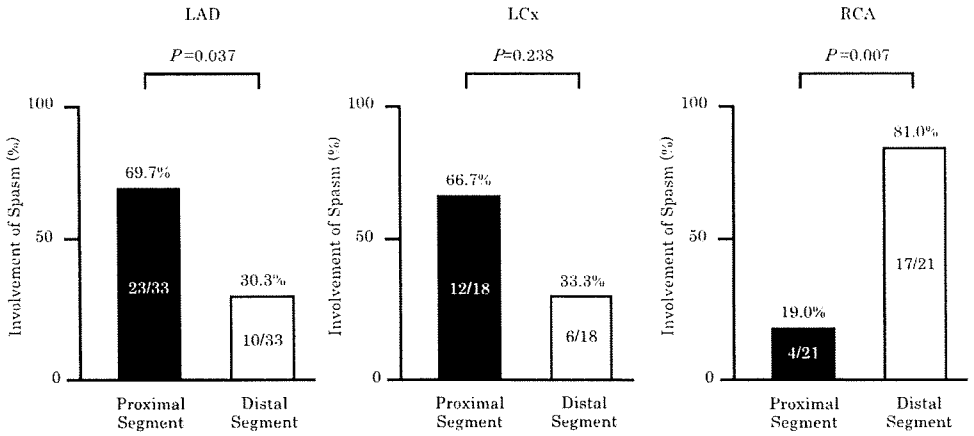


Figure 2. Comparison of the involvement of coronary spasm between the proximal and distal segments in the normal angiogram group. LAD indicates left anterior descending coronary artery; LCx, left circumflex artery; RCA, right coronary artery.

LCx, and 20 RCA) were analyzed for comparison of localization between spasm and plaque in the atherosclerosis group. Spasm occurred at the branch site in 23 (76.7%) of the 30 LAD, 8 (72.7%) of the 11 LCx, and 12 (60.0%) of the 20 RCA. These results are in agreement with those in the normal angiogram group. On the other hand, plaque was localized at the branch point in only 7 (23.3%) of the 30 LAD, 1 (9.1%) of the 11 LCx, and 2 (10.0%) of the 20 RCA. Thus, there was a significant difference in the involvement of the branch point between spasm and plaque in each of the 3 arteries ($P<0.0001$ in LAD, $P=0.039$ in LCx, and $P=0.002$ in RCA, respectively) (Figure 3). Spasm involved the proximal segment in 17 (56.7%) of the 30 LAD, 2 (18.2%) of the 11 LCx, and 3 (15.0%) of the 20 RCA, whereas plaque was localized at the proximal segment in 28 (93.3%) of the 30 LAD, 9 (81.8%) of the 11 LCx, and 15 (75.0%) of the 20 RCA. Thus, there was a significant difference in the involvement of the proximal segment between spasm and plaque in each of the 3 arteries ($P<0.0001$ in LAD, $P=0.016$ in LCx, and $P<0.0001$ in RCA, respectively) (Figure 4). In accordance with these results, most spasms occurred at the nonplaque site in each of the 3 arteries ($P=0.018$ in LAD, $P<0.0001$ in LCx, and $P=0.041$ in RCA, respectively) (Figure 5). Spasm thus preferentially occurred at branch points and nonplaque sites, whereas the plaque preferentially occurred at nonbranch point sites of the proximal segment in each of the 3 coronary arteries. Paired data using 2×2 tables for Figures 1 to 5 are shown in Online Data supplements.

Nineteen (17.9%) of the 106 spasms in the normal angiogram group and 11 (16.7%) of the 66 in the atherosclerosis group were associated with ST-segment elevation and the 87 (82.1%) and 55 (83.3%) with ST-segment depression on the ECG, respectively, indicating that coronary spasm with ST-segment depression is more numerous than that with ST-segment elevation in both groups ($P<0.0001$, respectively). Of the 11 spasms with ST-segment elevation, 8 (72.7%) involved the organic stenosis and 8 were total or subtotal occlusion in the atherosclerosis group.

Figures 6 and 7 show the representative angiograms of spasm in the normal angiogram group and those of atherosclerosis group, respectively.

Discussion

This study showed that most spasms were diffused and extensive, and the substantial number of these involved the entire arterial tree affecting the proximal and distal epicardial vessels and their intramural branches in the normal angiogram group. These findings are in agreement with the results of our previous angiographic study[17] and also with our intravascular ultrasound study, which revealed the existence

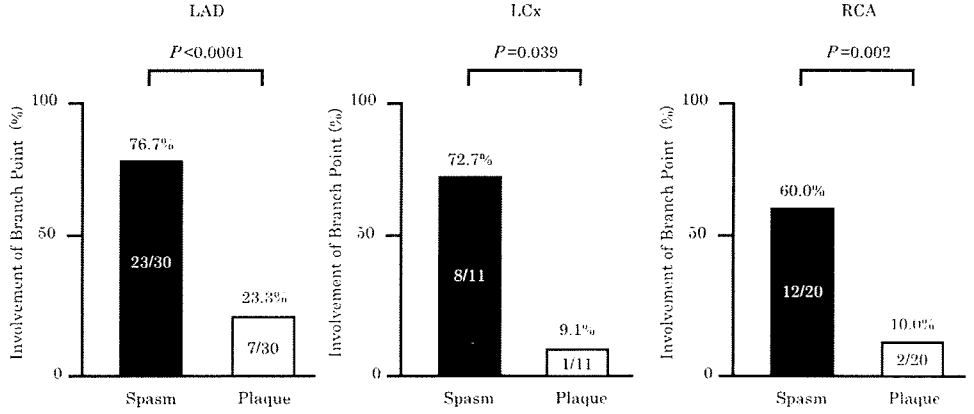


Figure 3. Comparison of the involvement of the branch point between spasm and plaque in the atherosclerosis group. LAD indicates left anterior descending coronary artery; LCx, left circumflex artery; RCA, right coronary artery.

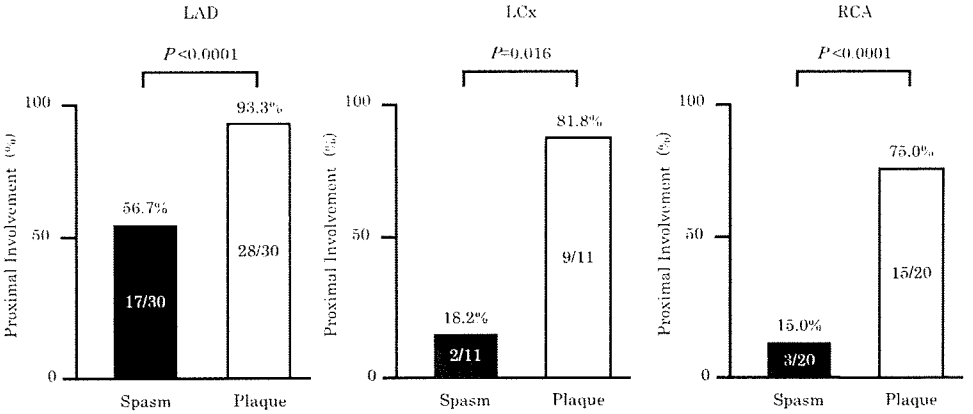


Figure 4. Comparison of the involvement of the proximal segment between spasm and plaque in the atherosclerosis group. LAD indicates left anterior descending coronary artery; LCx, left circumflex artery; RCA, right coronary artery.

of diffuse intimal thickening in an entire coronary artery in patients with coronary spasm and normal angiograms.[18] Accordingly, the results strongly suggest that systemic factors play an important role in the pathogenesis of coronary spasm.[18–21] On the other hand, the atherosclerotic plaque lesion was focal and largely localized to the proximal segments in agreement with previous studies.[7,8,22–24] This suggests that local factors are more important in the pathogenesis of atherosclerosis as compared with those of coronary spasm.

We have shown that endothelial NO activity is deficient and endothelial function impaired in the spasm arteries.[4] NO not only modulates vasomotor tone, but also inhibits inflammation, production of reactive oxygen species, vascular smooth muscle proliferation, and platelet aggregation,[5,6] and reduced endothelial NO activity represents the early steps in the development of atherosclerosis.[7–9] The endothelium is exposed to shear stress and unidirectional laminar shear stress in straight parts of the arterial tree potently stimulates NO production, whereas disturbed flows at curvatures or branches have the opposite effect.[5,9,10]

Studies of human coronary arteries provide evidence that regions prone to the development of atherosclerosis occur at sites of intimal thickening, which is mainly composed of smooth muscle cells (SMCs), suggesting that SMCs in intimal thickening play a pathogenic role in the initiation and development of atherosclerosis.[22–24] Low shear stress occurs at the curvature or upstream of stenosis, whereas oscillatory shear stress occurs downstream of stenosis or branch points.[9,10] Recent studies revealed that low–shear stress lesions contained fewer SMCs and more lipids and were larger and more progressive and vulnerable,[10,25,26] whereas oscillatory–shear stress lesions contained more SMCs and fewer lipids and are more stable.[25]

This study further showed that spasm preferentially occurred at the branch point or downstream of the flow divider where shear stress is presumed to be oscillatory[9,10] both in the normal angiogram and atherosclerosis groups. This is in agreement with the result of Selwyn's group, which showed that branch point constricted more intensely than nonbranch sites in response to ACh infusion.[27] On the other hand, the atherosclerotic stenosis was localized predominantly at the nonbranch point of the curved proximal segment where shear stress is presumed to be low.[9,10] Thus, there was a difference in the predilection site between the spasm and atherosclerotic plaque and most spasms occurred at different sites from those of the plaque. These results thus suggest that atherosclerosis does not contribute to the occurrence of spasm or rather tends

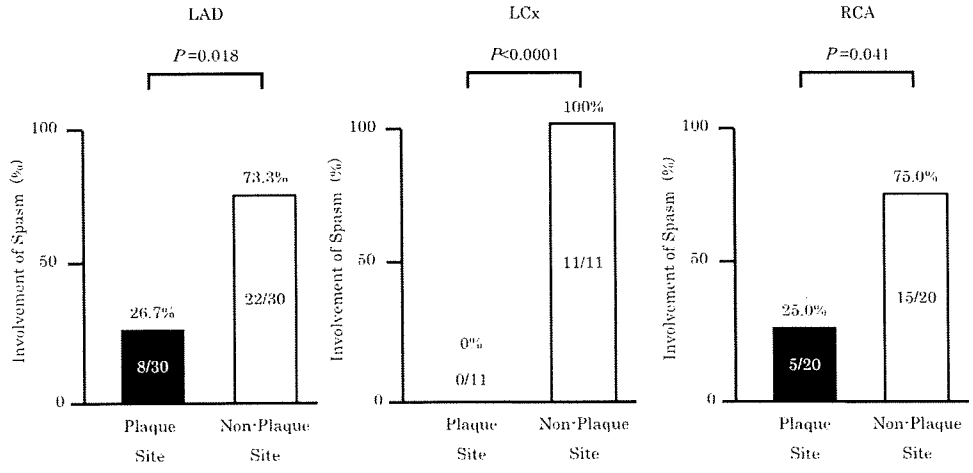


Figure 5. Comparison of the involvement of spasm between the plaque and nonplaque sites in the atherosclerosis group. LAD indicates left anterior descending coronary artery; LCx, left circumflex artery; RCA, right coronary artery.

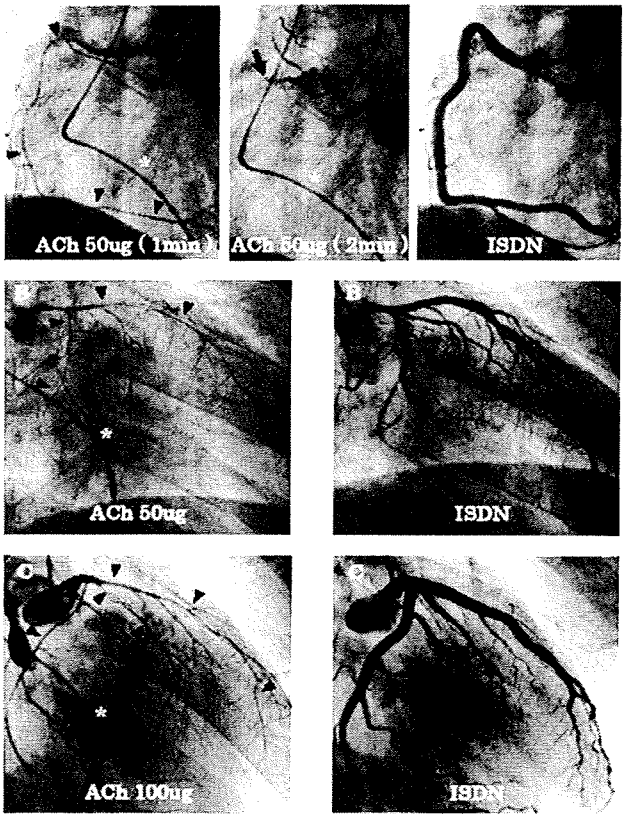


Figure 6. Coronary angiograms during spasm induced by ACh injection and after ISDN in the normal angiogram group. A, Severe diffuse spasm involving the entire RCA appeared after ACh (left) and converted into a total occlusion at the origin of the artery 2 minutes later (center, arrow). After ISDN, the artery was marked dilated and normal (right). B, Severe diffuse spasm involving the entire left coronary artery including intramural branches appeared after ACh (left, arrow heads) and disappeared after ISDN (right). C, Severe diffuse spasm involving the entire arterial tree of both the LAD and LCx appeared after ACh injection (left, arrow heads) and disappeared after ISDN (right). ACh indicates acetylcholine; ISDN, isosorbide dinitrate; LAD, left anterior descending artery; LCx, left circumflex artery; RCA, right coronary artery; *, a pacing catheter.

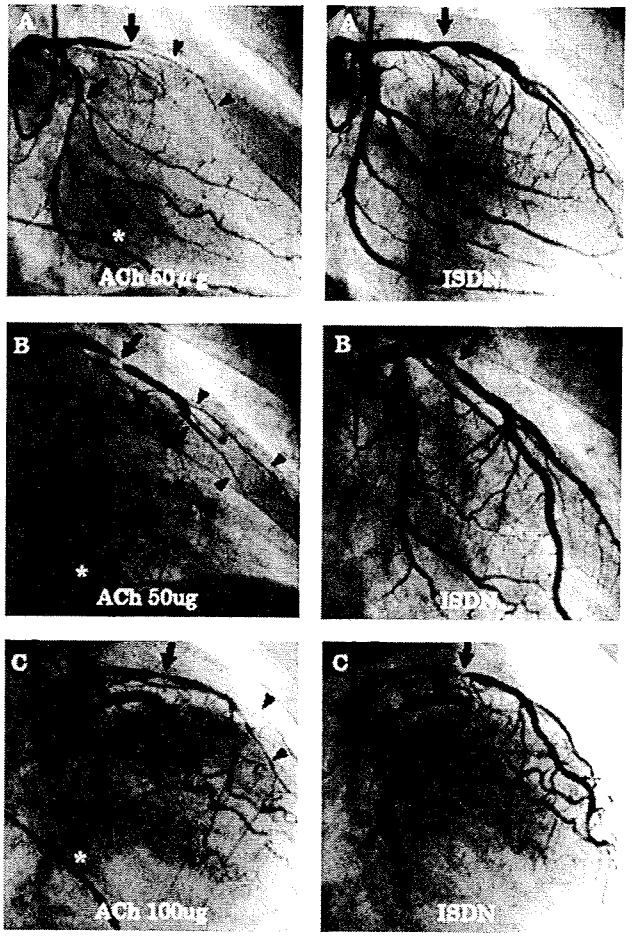


Figure 7. Coronary angiograms during spasm induced by ACh injection and after ISDN in the atherosclerosis group. A, A subtotal occlusion spasm with diffuse vasoconstriction involving the curved proximal segment of the LAD and a focal spasm at the branch site of the LCx appeared after ACh (left, arrows and arrow heads) and disappeared after ISDN (right). A significant organic stenotic lesion was revealed at the curved proximal segment (right, arrow). Spasm was superimposed on the lesion (left, arrow). B, Subtotal occlusion at the proximal segment and diffuse spasm at its distal branch site of the LAD appeared after ACh (left, arrow and arrow heads) and disappeared after ISDN, revealing a severe organic stenosis at the nonbranch site of the curved proximal segment (right, arrow). Despite severe stenosis, total occlusion did not occur at this site during spasm. C, Diffuse spasm occurred at the distal branches, not at the site of severe organic stenosis at the curved proximal segment of the LAD after ACh (left, arrow heads and arrow). A severe organic stenosis lesion was revealed at the nonbranch site of the curved proximal segment after ISDN (right). ACh indicates acetylcholine; ISDN, isosorbide dinitrate; LAD, left anterior descending artery; LCx, left circumflex artery; RCA, right coronary artery; *, a pacing catheter.

to suppress it and are in agreement with those of Maseri's group.[1,28] However, MacAlpin[29] reported on the basis of the literature that most spasms were localized at the site of an organic lesion. The discrepancy between his results and ours may probably be explained by the difference of the study subjects. He reported on the patients with "variant angina," ie, angina associated with ST elevation on ECG. On the other hand, most spasms were associated with ST depression and mild to moderate organic stenosis in the atherosclerosis group of this study.

Coronary spasm has risk factors, such as smoking and aging,[18–21] and is associated with endothelial dysfunction,[2,4] inflammation,[20,30] and intimal thickening.[18] It thus shares the common risk and pathogenetic factors with atherosclerosis.[7,8] However, atherosclerosis is characterized by subendothelial retention of atherogenic lipoproteins,[7,8,22–26] develops early from infants,[31] and is usually associated with hyperlipidemia,[7,8,32] whereas coronary spasm does not occur in the young but in the old patients (mean age of 65.5±10.1 in this study), and hyperlipidemia is not a risk factor for coronary spasm.[18–21] Indeed, Morikawa et al have recently reported by using intravascular optical coherence tomography that the spasm arteries with normal angiogram had a diffuse intimal thickening and contained almost no lipid deposits, whereas the no-spasm arteries with normal angiogram had either intimal thickening containing lipid deposits or had no intimal thickening.[33]

Coronary spasm is caused by abnormal contraction of vascular SMCs and therefore contractile and not synthetic phenotype SMCs are likely to play a crucial role in the

pathogenesis of coronary spasm. We, therefore, propose that coronary spasm may be a manifestation of coronary arteriosclerosis distinctly different from coronary atherosclerosis, which is characterized by lipid accumulation and SMCs of synthetic phenotype.[7,8,22–24,32] Recent studies showed that oxidized lipids suppress SMCs marker genes[34] and that lipid lowering promotes accumulation of mature SMCs.[35] To be noted in this connection is the fact that the patients with coronary spasm with angiographically normal or almost normal coronary arteries are less prone to develop acute myocardial infarction as compared with those with other types of unstable angina.[36,37] Intriguingly, the incidence of coronary spasm, particularly variant angina, has decreased recently,[38,39] whereas that of hyperlipidemia has risen in Japan.[40,41]

This study further demonstrates that most coronary spasms were associated with ST-segment depression rather than ST segment elevation on ECG and thereby confirms the concept that variant angina is only one aspect of the spectrum of coronary spastic myocardial ischemia.[42]

Study Limitations

In this study, we defined the site of spasm as that of total or subtotal obstruction or as that of the most severe and proximal constriction in the case of multifocal or segmental diffuse spasm and the site of atherosclerotic lesion as that of the most narrowed in each artery for the purpose of analysis. However, spasm is often, diffuse and or multifocal, or even migrates from site to site and thus the actual images of spasm may be more complex and dynamic than described in this study.[2] Atherosclerotic lesions also are often multifocal. In this study, however, most atherosclerotic lesions were mild and mostly monofocal, because we excluded the patients with multivessel or sever organic stenosis disease from the study. Thus, the results of this study may not necessarily be applicable to advanced atherosclerotic lesions with multiple plaques. Moreover, angiogram is not sensitive enough to detect atherosclerosis because it is highly likely that vascular remodeling may have occurred, and thus, the patients in the normal angiogram group in this study might not have been free from atherosclerosis.[10] In this study, we did not perform the intravascular ultrasound examination concurrently with angiography and thus could not present the data on shear stress and constituents of vessels walls. However, we have previously shown that the intimal thickening involved the entire arterial tree in the patients with coronary spasm and normal angiogram using intravascular ultrasound.[18]

Conclusions

Diffuse spasm involving the entire arterial tree occurred in a substantial number of angiographically normal or almost normal coronary arteries in the patients with chest pain. Spasm preferentially occurred involving branch points, whereas atherosclerosis was predominantly focal and localized at the nonbranch points of the curved proximal segments. Most spasms occurred at the sites different from those of the atherosclerotic plaque. These results strongly suggest that coronary spasm may be a manifestation of a distinct type of arteriosclerosis different from the lipid-laden coronary atherosclerosis.

Sources of Funding

This study was supported in part from Japan Heart Foundation, Tokyo; and Japan Vascular Disease Research Foundation, Kyoto, Japan.

Disclosures

None.

References

1. Maseri A, Davies G, Hackett D, Kaski JC. Coronary artery spasm and vasoconstriction. The case for a distinction. *Circulation*. 1990;81:1983–1991.
2. Yasue H, Kugiyama K. Coronary spasm: clinical features and pathogenesis. *Intern Med*. 1997;36:760–765.
3. Pristipino C, Beltrame JF, Finocchiaro ML, Hattori R, Fujita M, Mongiardo R, Cianflone D, Sanna T, Sasayama S, Maseri A. Major racial differences in coronary constrictor response between Japanese and Caucasians with recent myocardial infarction. *Circulation*. 2001;101:1102–1108.
4. Kugiyama K, Yasue H, Okumura K, Ogawa H, Fujimoto K, Nakao K, Yoshimura M, Motoyama T, Inobe Y, Kawano H. Nitric oxide activity is deficient in spasm arteries of patients with coronary spastic angina. *Circulation*. 1996;94:266–271.
5. Harrison DG, Widder J, Grumbach I, Chen W, Weber M, Searles C. Endothelial mechanotransduction, nitric oxide and vascular inflammation. *J Intern Med*. 2006;259:351–363.
6. Moncada S, Higgs EA. Nitric oxide and the vascular endothelium. *Handb Exp Pharmacol*. 2006;(176 Pt 1):213–254.
7. Libby P, Theroux P. Pathophysiology of coronary artery disease. *Circulation*. 2005;111:3481–3488.
8. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med*. 2005;352:1685–1695.
9. Gimbrone MA Jr, Topper JN, Nagel T, Anderson KR, Garcia-Cardeña G. Endothelial dysfunction, hemodynamic forces, and atherogenesis. *Ann N Y Acad Sci*. 2000;902:230–239.
10. Chatzizisis YS, Coskun AU, Jonas M, Edelman ER, Feldman CL, Stone PH. Role of endothelial shear stress in the natural history of coronary atherosclerosis and vascular remodeling: molecular, cellular, and vascular behavior. *J Am Coll Cardiol*. 2007;49:2379–2393.
11. Yasue H, Horio Y, Nakamura N, Fujii H, Imoto N, Sonoda R, Kugiyama K, Obata K, Morikami Y, Kimura T. Induction of coronary artery spasm by acetylcholine in patients with variant angina: possible role of the parasympathetic nervous system in the pathogenesis of coronary artery spasm. *Circulation*. 1986;74:955–963.
12. Okumura K, Yasue H, Matsuyama K, Goto K, Miyagi H, Ogawa H, Matsuyama K. Sensitivity and specificity of intracoronary injection of acetylcholine for the induction of coronary artery spasm. *J Am Coll Cardiol*. 1988;12:883–888.
13. Yasue H, Matsuyama K, Matsuyama K, Okumura K, Morikami Y, Ogawa H. Responses of angiographically normal human coronary arteries to intracoronary injection of acetylcholine by age and segment. Possible role of early coronary atherosclerosis. *Circulation*. 1990;81:482–490.
14. Kugiyama K, Murohara T, Yasue H, Kimura T, Sakaino N, Ohgushi M, Sugiyama S, Okumura K. Increased constrictor response to acetylcholine of the isolated coronary arteries from patients with variant angina. *Int J Cardiol*. 1995;52:223–233.
15. AHA Committee Report. A reporting system on patients evaluated for coronary artery disease. *Circulation*. 1975;51:7–37.
16. Waters D, Lespérance J, Craven TE, Hudon G, Gillam LD. Advantages and limitations of serial coronary arteriography for the assessment of progression and regression of coronary atherosclerosis. Implications for clinical trials. *Circulation*. 1993;87(3 Suppl):II38–II47.
17. Okumura K, Yasue H, Matsuyama K, Ogawa H, Kugiyama K, Ishizaka H, Sumida H, Fujii H, Matsunaga T, Tsunoda R. Diffuse disorder of coronary artery vasomotility in patients with coronary spastic angina. Hyperreactivity to the constrictor effects of acetylcholine and the dilator effects of nitroglycerin. *J Am Coll Cardiol*. 1996;27:45–52.
18. Miyao Y, Kugiyama K, Kawano H, Motoyama T, Ogawa H, Yoshimura M, Sakamoto T, Yasue H. Diffuse intimal thickening of coronary arteries

in patients with coronary spastic angina. *J Am Coll Cardiol.* 2000;36:432–437.
19. Takaoka K, Yoshimura M, Ogawa H, Kugiyama K, Nakayama M, Shimasaki Y, Mizuno Y, Sakamoto T, Yasue H. Comparison of the risk factors for coronary artery spasm with those for organic stenosis in a Japanese population: role of cigarette smoking. *Int J Cardiol.* 2000;72:121–126.
20. Itoh T, Mizuno Y, Harada E, Yoshimura M, Ogawa H, Yasue H. Coronary spasm is associated with chronic low-grade inflammation. *Circ J.* 2007;71:1074–1078.
21. Nakayama M, Yasue H, Yoshimura M, Shimasaki Y, Kugiyama K, Ogawa H, Motoyama T, Saito Y, Ogawa Y, Miyamoto Y, Nakao K. T-786–>C mutation in the 5′-flanking region of the endothelial nitric oxide synthase gene is associated with coronary spasm. *Circulation.* 1999;99:2864–2870.
22. Stary HC, Blankenhorn DH, Chandler AB, Glagov S, Insull W Jr, Richardson M, Rosenfeld ME, Schaffer SA, Schwartz CJ, Wagner WD. A definition of the intima of human arteries and of its atherosclerosis-prone regions. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Circulation.* 1992;85:391–405.
23. Nakashima Y, Fujii H, Sumiyoshi S, Wight TN, Sueishi K. Early human atherosclerosis: accumulation of lipid and proteoglycans in intimal thickenings followed by macrophage infiltration. *Arterioscler Thromb Vasc Biol.* 2007;27:1159–1165.
24. Doran AC, Meller N, McNamara CA. Role of smooth muscle cells in the initiation and early progression of atherosclerosis. *Arterioscler Thromb Vasc Biol.* 2008;28:812–819.
25. Cheng C, Tempel D, van Haperen R, van der Baan A, Grosveld F, Daemen MJ, Krams R, de Crom R. Atherosclerotic lesion size and vulnerability are determined by patterns of fluid shear stress. *Circulation.* 2006;113:2744–2753.
26. Chatzizisis YS, Jonas M, Coskun AU, Beigel R, Stone BV, Maynard C, Gerrity RG, Daley W, Rogers C, Edelman ER, Feldman CL, Stone PH. Prediction of the localization of high-risk coronary atherosclerotic plaques on the basis of low endothelial shear stress: an intravascular ultrasound and histopathology natural history study. *Circulation.* 2008;117:993–1002.
27. McLenachan JM, Vita J, Fish DR, Treasure CB, Cox DA, Ganz P, Selwyn AP. Early evidence of endothelial vasodilator dysfunction at coronary branch points. *Circulation.* 1990;82:1169–1173.
28. Newman CM, Maseri A, Hackett DR, el-Tamimi HM, Davies GJ. Response of angiographically normal and atherosclerotic left anterior descending coronary arteries to acetylcholine. *Am J Cardiol.* 1990;66:1070–1076.
29. MacAlpin RN. Relation of coronary arterial spasm to sites of organic stenosis. *Am J Cardiol.* 1980;46:143–153.
30. Soejima H, Irie A, Miyamoto S, Kajiwara I, Kojima S, Hokamaki J, Sakamoto T, Tanaka T, Yoshimura M, Nishimura Y, Ogawa H. Preference toward a T-helper type 1 response in patients with coronary spastic angina. *Circulation.* 2003;107:2196–2200.
31. Tuzcu EM, Kapadia SR, Tutar E, Ziada KM, Hobbs RE, McCarthy PM, Young JB, Nissen SE. High prevalence of coronary atherosclerosis in asymptomatic teenagers and young adults: evidence from intravascular ultrasound. *Circulation.* 2001;103:2705–2710.
32. Tabas I, Williams KJ, Borén J. Subendothelial lipoprotein retention as the initiating process in atherosclerosis: update and therapeutic implications. *Circulation.* 2007;116:1832–1844.
33. Morikawa Y, Uemura S, Ishigami K, Soeda T, Okayama S, Takemoto Y, Onoue K, Somekawa S, Nishida T, Takeda Y, Kawata H, Horii M, Nakajima T, Saito Y. Coronary spasm occurs in coronary arteries with diffuse intimal thickening without lipid deposition and/or calcification: evaluation with intravascular optical coherence tomography [abstract]. *Circulation* 2008;118(Suppl 2):S734.
34. Pidkovka NA, Cherepanova OA, Yoshida T, Alexander MR, Deaton RA, Thomas JA, Leitinger N, Owens GK. Oxidized phospholipids induce phenotypic switching of vascular smooth muscle cells in vivo and in vitro. *Circ Res.* 2007;101:792–801.
35. Aikawa M, Rabkin E, Voglic SJ, Shing H, Nagai R, Schoen FJ, Libby P. Lipid lowering promotes accumulation of mature smooth muscle cells expressing smooth muscle myosin heavy chain isoforms in rabbit atheroma. *Circ Res.* 1998;83:1015–1026.
36. Walling A, Waters DD, Miller DD, Roy D, Pelletier GB, Théroux P. Long-term prognosis of patients with variant angina. *Circulation.* 1987;76:990–997.
37. Yasue H, Takizawa A, Nagao M, Nishida S, Horie M, Kubota J, Omote S, Takaoka K, Okumura K. Long-term prognosis for patients with variant angina and influential factors. *Circulation.* 1988;78:1–9.
38. Sueda S, Kohno H, Fukuda H, Uraoka T. Did the widespread use of long-acting calcium antagonists decrease the occurrence of variant angina? *Chest.* 2003;124:2074–2078.
39. Japanese beta-Blockers and Calcium Antagonists Myocardial Infarction (JBCMI) Investigators. Comparison of the effects of beta blockers and calcium antagonists on cardiovascular events after acute myocardial infarction in Japanese subjects. *Am J Cardiol.* 2004;93:969–973.
40. Okamura T, Tanaka H, Miyamatsu N, Hayakawa T, Kadowaki T, Kita Y, Nakamura Y, Okayama A, Ueshima H; NIPPON DATA80 Research Group. The relationship between serum total cholesterol and all-cause or cause-specific mortality in a 17.3-year study of a Japanese cohort. *Atherosclerosis.* 2007;190:216–223.
41. Kitamura A, Sato S, Kiyama M, Imano H, Iso H, Okada T, Ohira T, Tanigawa T, Yamagishi K, Nakamura M, Konishi M, Shimamoto T, Iida M, Komachi Y. Trends in the incidence of coronary heart disease and stroke and their risk factors in Japan, 1964 to 2003: the Akita-Osaka study. *J Am Coll Cardiol.* 2008;52:71–79.
42. Maseri A, Severi S, Nes MD, L'Abbate A, Chierchia S, Marzilli M, Ballestra AM, Parodi O, Biagini A, Distante A. "Variant" angina: one aspect of a continuous spectrum of vasospastic myocardial ischemia. Pathogenetic mechanisms, estimated incidence and clinical and coronary arteriographic findings in 138 patients. *Am J Cardiol.* 1978;42:1019–1035.

CLINICAL PERSPECTIVE

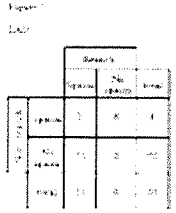
Coronary spasm plays an important role in the pathogenesis of ischemic heart disease. However, similarities and differences between coronary spasm and atherosclerosis are not known. This study examined the angiographic characteristics of coronary spasm in comparison with those of atherosclerotic plaque, first in the angiographically normal or almost normal coronary arteries and then in those with atherosclerotic plaque. The results showed that diffuse spasm involving the entire artery appeared in the substantial number of the angiographically normal arteries and that spasm preferentially occurred at branch points in both the angiographically normal arteries and those with plaque. On the other hand, plaque was predominantly localized at nonbranch point sites of the curved proximal segments. Most spasms did not occur at the sites of plaque. These results suggest that coronary spasm may be a manifestation of a distinct type of arteriosclerosis different from the lipid-laden coronary atherosclerosis. This study, thus, may provide a new insight into the pathogenesis not only of coronary spasm but also of atherosclerosis and may explain at least partially the decline of the number of coronary spasm with the increase of hyperlipidemia among Japanese in recent years.

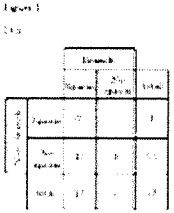
SUPPLEMENTAL MATERIAL

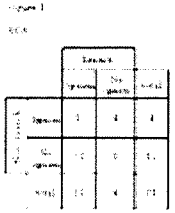
Coronary Spasm Preferentially Occurs at Branch Points

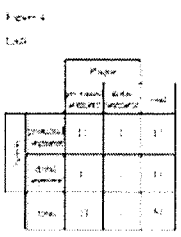
-an angiographic comparison with atherosclerotic plaque -

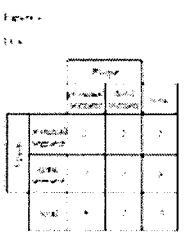
Hitoshi Nakagawa, MD, Yoshinobu Morikawa, Yuji Mizuno, MD,
Eisaku Harada, MD, MD, Teruhiko Ito, MD, Kunihiko Matsui, MD,
MPH*, Yoshihiko Saito, MD#,
Hirofumi Yasue, MD.

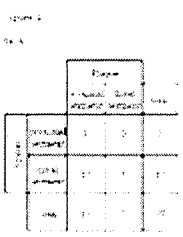


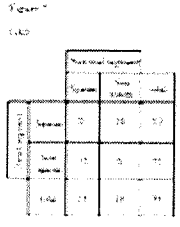


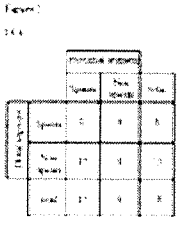


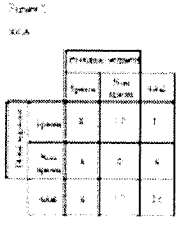


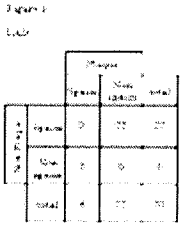


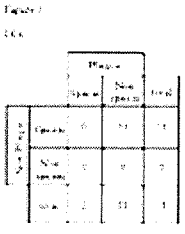


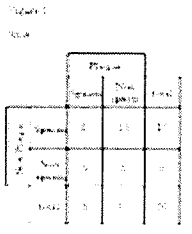


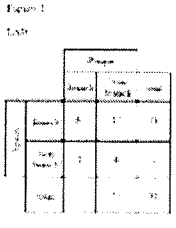


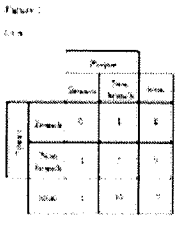












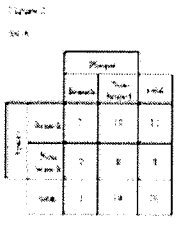


Figure legends

Figure 1. Comparison of the involvement of coronary spasm between the branch and non-branch points in the normal angiogram group. Branch indicates the branch point; Non-branch, the non-branch point; LAD, left anterior descending coronary artery; LCx; left circumflex artery; RCA, right coronary artery.

Figure 2. Comparison of the involvement of coronary spasm between the proximal and distal segments in the normal angiogram group. LAD indicates left anterior descending coronary artery; LCx, left circumflex artery; RCA, right coronary artery.

Figure 3. Comparison of the involvement of the branch point between spasm and plaque in the atherosclerosis group. LAD indicates left anterior descending coronary artery; LCx, left circumflex artery; RCA, right coronary artery.

Figure 4. Comparison of the involvement of the proximal segment between spasm and plaque in the atherosclerosis group. LAD indicates left anterior descending coronary artery; LCx, left circumflex artery; RCA, right coronary artery.

Figure 5. Comparison of the involvement of spasm between the plaque and non-plaque sites in the atherosclerosis group. LAD indicates left anterior descending coronary artery; LCx; left circumflex artery; RCA, right coronary artery.