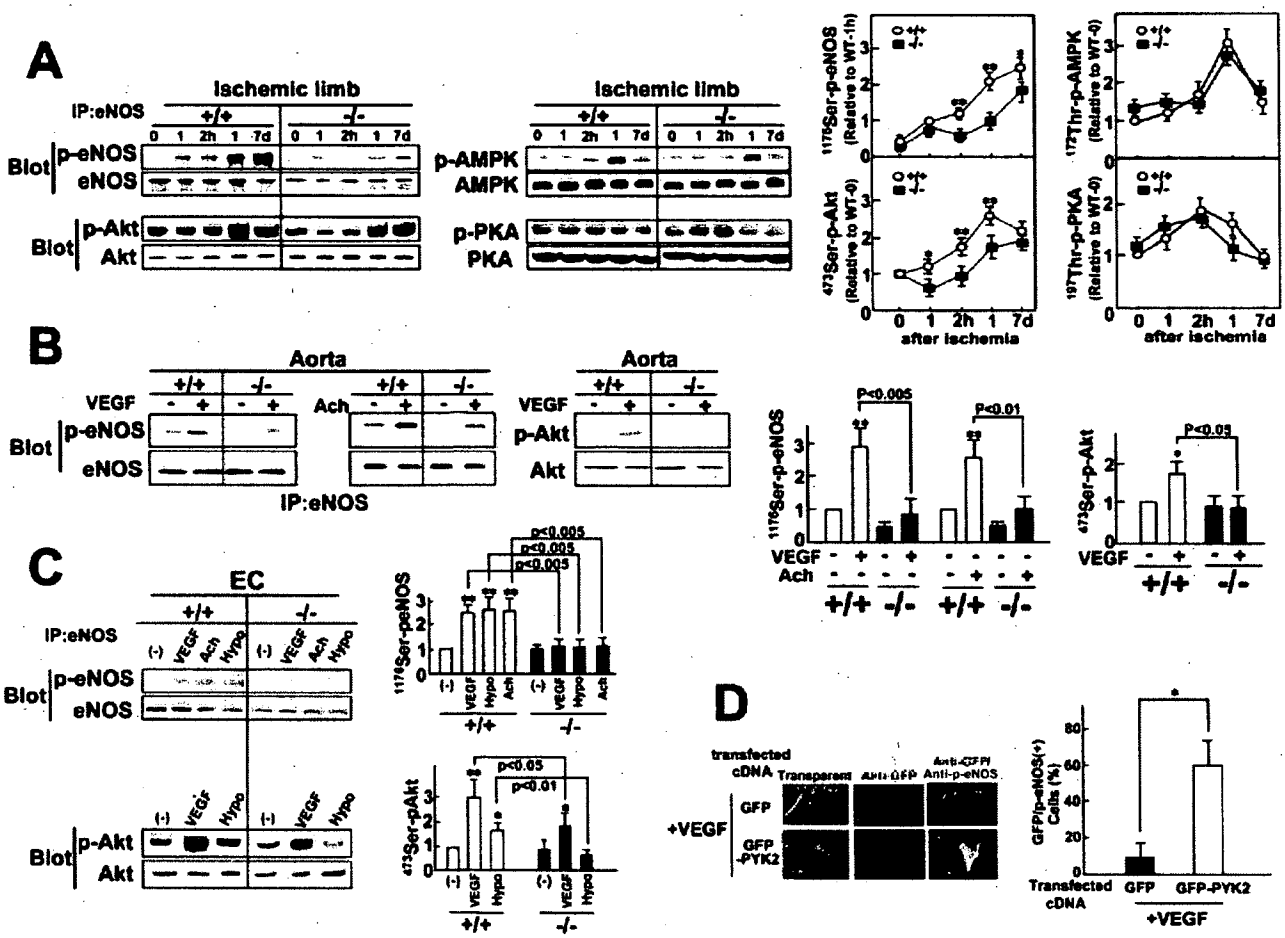

Figure 2. Impaired eNOS/Akt activation in PYK2-deficient mice. A, Skeletal muscle (n=10) was excised at the indicated time after ischemia. B, Aortic tissue (n=6 for each stimuli) was stimulated with VEGF (100 ng/mL) or Ach (1 μmol/L) for 5 minutes. C, Aortic ECs (n=6 for each stimuli) were stimulated with VEGF (100 ng/mL) or Ach (1 μmol/L) for 5 minutes or 1% hypoxia for 18 hours (n=6 each). Cell lysates were subjected to immunoprecipitation with anti-eNOS antibody, followed by immunoblot with antibodies against ^{1176}Ser-phosphorylated eNOS or eNOS. In addition, lysates were immunoblotted with antibodies against ^{473}Ser-phosphorylated Akt or Akt. Relative phosphorylation levels of eNOS and Akt are shown. Open circles and closed squares indicate the wild-type and PYK2$^{-/-}$ mice, respectively. A, *P<0.05, **P<0.01 vs the same time points of the PYK2$^{-/-}$ mice. B, C, *P<0.05, **P<0.005 vs the nonstimulated control. D, Aortic ECs were transfected with GFP- or GFP-PYK2-cDNA-plasmid. Forty-eight hours after transfection, cells were stimulated with VEGF (100 ng/mL), fixed with 4% paraformaldehyde, permeabilized with 0.02% Triton/PBS, and immunostained with antibodies against GFP- (red) or ^{1176}Ser-phosphorylated eNOS (green). Ratio of the GFP/p-eNOS double-positive cells (%) (yellow) to the total GFP-positive cells (red) was evaluated. *P<0.005; n=4.

microscopy (Figure 4A). In the wild-type ECs, VEGF increased NO levels by 3.2-fold (P<0.005 versus untreated cells), whereas this increase was severely impaired in the PYK2-deficient ECs. NO metabolites in the 24-hour urine sample were significantly lower in the PYK2$^{-/-}$ mice than the wild-type mice (4084±820 versus 9326±1163 nmol; P<0.005; Figure 4B). Oral administration of N^G-nitro-L-arginine methyl ester (L-NAME; 3 mmol/L in drinking water) to the wild-type mice reduced the NO metabolite production to a level comparable to the PYK2$^{-/-}$ mice (Figure 4B).

Ach-mediated vasodilatation is induced by the eNOS-NO system. We next examined whether Ach-mediated relaxation of the aorta is influenced by PYK2 deficiency. The dose-dependent relaxation of the isolated aorta constricted by norepinephrine was evaluated (Figure 4C). Ach (10 μmol/L) -mediated relaxation response was much weaker in the PYK2-deficient aorta than in the wild-type aorta (32±8% versus 59±5% relaxation; P<0.01), whereas norepinephrine (300 nmol/L) -mediated vasoconstriction in the PYK2-deficient aorta did not significantly differ from the wild-type aorta (190±30% versus 165±6% relative to 50 mmol/L KCl–mediated constriction; n=5). This result suggests that Ca^{2+} signaling transmitted mainly through voltage-dependent Ca^{2+} channel leading to the constriction of vascular smooth muscle cells is not impaired in the PYK2-deficient aorta.

To evaluate the NO dependency on Ach-mediated vasorelaxation, the effect of L-NAME was studied. Addition of L-NAME (10 μmol/L) markedly suppressed the Ach-mediated maximum relaxation of the wild-type aorta (from 59±5% to 17±2%; P<0.001; n=4), whereas in the PYK2-deficient aorta, the reduced relaxation response was suppressed further to a level comparable to L-NAME–treated

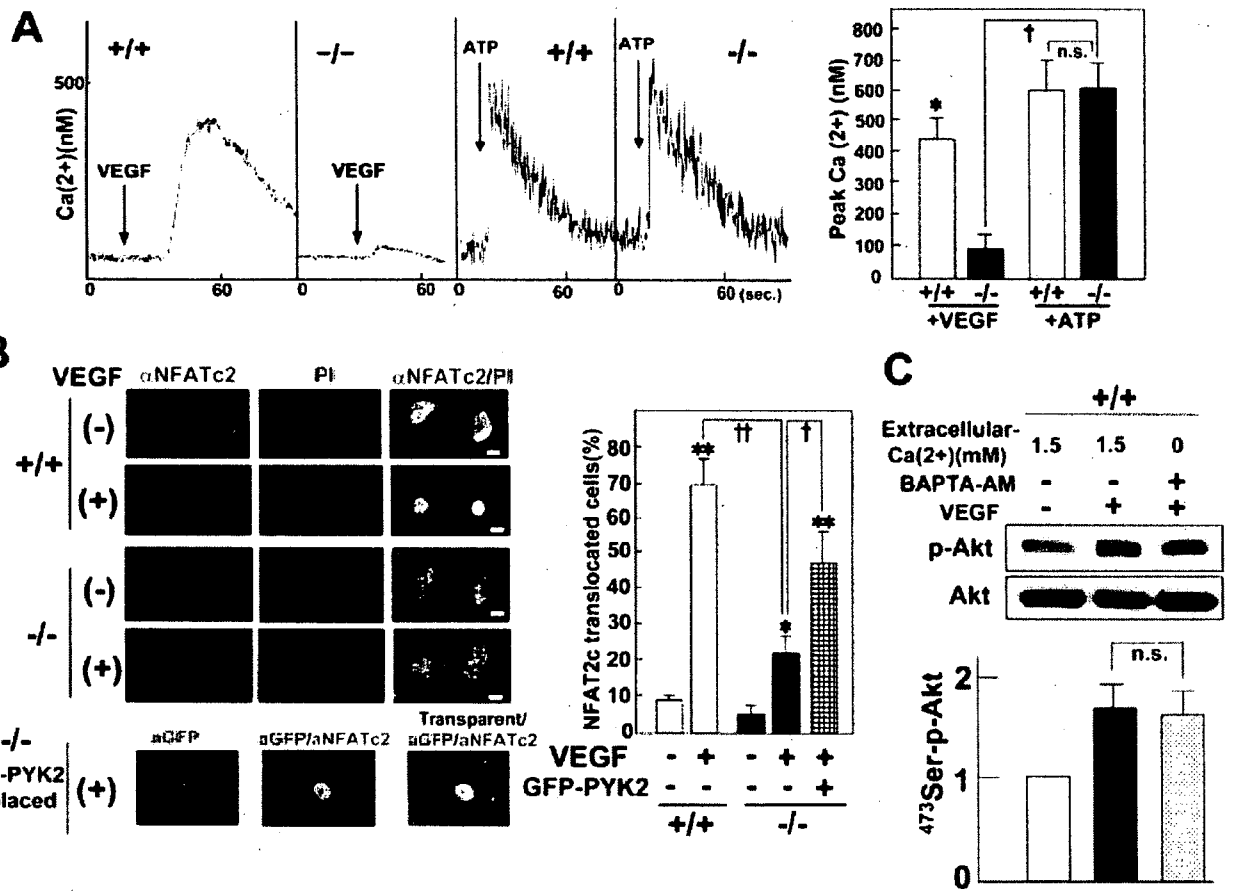

Figure 3. Impaired Ca^{2+} mobilization in PYK2-deficient mice. A, VEGF (100 ng/mL) - or ATP (1 mmol/L) -mediated cytoplasmic Ca^{2+} concentrations were measured in Fura-2–loaded ECs by a fluorescence microscope (n=12 each). †$P<0.001$, *$P<0.001$ vs VEGF-stimulated $PYK2^{-/-}$ cells. B, Top, ECs were fixed with 4% paraformaldehyde 30 minutes after VEGF stimulation, treated with 0.05% triton, and immunostained with anti-NFATc2 antibodies and propidium iodide (PI). Bottom, PYK2-deficient ECs were transfected with GFP-tagged PYK2 plasmid. Forty-eight hours after transfection, cells were stimulated with VEGF for 30 minutes and immunostained with anti-NFATc2 and anti-GFP antibodies. The number of cells in which NFATc2 was translocated to the nucleus was counted and shown relative to total plated cell numbers (n=4 in each experiment). *$P<0.05$, **$P<0.005$ vs nonstimulated control cells. †$P<0.05$, ††$P<0.01$. Scale bar=10 μm. C, After serum starvation (0.5% FBS) for 16 hours, cells were preincubated with BAPTA-AM (5 μmol/L) in the Ca^{2+}-free medium for 45 minutes and subsequently stimulated by VEGF for 5 minutes. Lysates were subject to immunoblot by antibodies against Akt and ^{473}Ser-phosphorylated Akt.

wild-type aorta (from 32±8 to 18±3%; $P<0.005$; n=4). These findings indicate that Ach-mediated vasorelaxation depends mainly on NO production, in which an involvement of PYK2-mediated NO signaling was estimated to be ≈64% [(59−32/59−17)×100] of the total NO-mediated vasodilation activated downstream of Ach. NO donor (nitroprusside) –induced vasorelaxation of the aorta was similar in both groups (82±4 versus 83±4%; n=5), suggesting that NO-mediated signal transduction for vasodilatation is not impaired in the PYK2-deficient aorta (Figure 4C).

NO-mediated vasodilatation requires cGMP as a second messenger. We therefore measured the amount of cGMP in the aorta. Basal cGMP production in the $PYK2^{-/-}$ mice was 41% lower than that in the wild-type mice. Ach increased aortic cGMP production 4.8-fold in the wild-type mice, whereas the increase in the $PYK2^{-/-}$ mice was 2.0-fold, significantly ($P<0.01$) lower than in the wild-type mice (Figure 4D).

Decrease in Neovessel Formation by PYK2 Deficiency

Angiogenesis in the ischemic tissue requires eNOS activation.[17] We analyzed the blood flow recovery and neovessel formation after hind-limb ischemia. The ratio of blood flow recovery assessed by laser Doppler imaging was significantly lower in the $PYK2^{-/-}$ mice than in the wild-type mice (50% versus 76% recovery at 3 weeks after ischemia; $P<0.01$; Figure 5A). Oral administration of L-NAME (3 mmol/L in drinking water) significantly reduced the recovery ratio in the wild-type (from 76% to 62%; $P<0.05$) and $PYK2^{-/-}$ (from 50% to 37%; $P<0.05$) mice (Figure 5A). Considering that the recovery ratio of the L-NAME–treated wild-type mice (62%) is close to that of the $PYK2^{-/-}$ mice (50%), the blood flow recovery after hind-limb ischemia is considered to be regulated mainly by PYK2-mediated NO signaling. We also counted the number of $CD31^+$ vessels in the ischemic muscle. There was no significant difference in the basal vessel

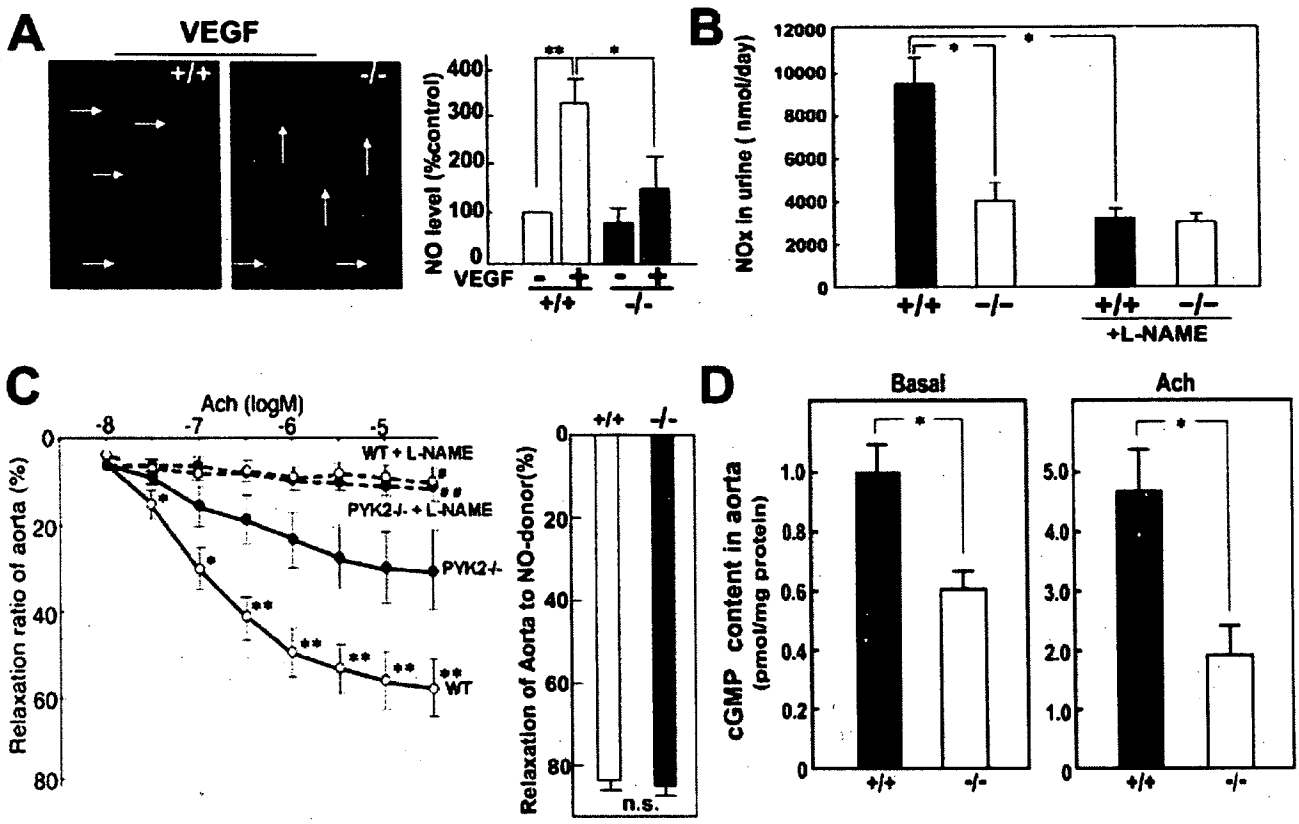

Figure 4. PYK2 effects on NO production and Ach-mediated vasodilatation. A, Measurement of the intracellular NO level. ECs were loaded with DAF-FM DA (10 μmol/L), and NO was visualized as green under laser microscopy. The average intensities in the ECs relative to the control group were evaluated. *P<0.01, **P<0.005 (n=8 each). B, NO production assessed by NO metabolites in the 24-hour urine sample was evaluated in the mice treated or untreated with L-NAME (3 mmol/L) for 7 days. Data are mean±SE (n=6). *P<0.005. C, Ach- and NO donor- (nitroprusside) mediated vasodilation in the aorta constricted by norepinephrine (100 nmol/L). Ach (10 nmol/L to 100 μmol/L) -mediated or nitroprusside (10 μmol/L) relaxation in the Tyrode's solution with or without L-NAME (10 μmol/L) was assessed by percent relaxation relative to papaverine (100 μmol/L) -mediated relaxation (100%). *P<0.05, **P<0.01 vs the same concentration of the PYK2$^{-/-}$ mice; #P<0.001 vs the maximum concentration of the wild-type mice; ##P<0.005 vs the maximum concentration of the PYK2$^{-/-}$ mice. D, cGMP contents in tissue lysates from untreated (basal) or Ach (1 μmol/L) -stimulated aorta were measured with an enzyme immunoassay kit. *P<0.01. Data are mean±SE (n=4 each); representative results are shown.

numbers surrounding the muscle fibers. The vessel number (per muscle fiber) in the wild-type mice increased 2.3-fold (P<0.005) after hind-limb ischemia, whereas the PYK2$^{-/-}$ mice showed no significant increase (Figure 5B).

We next examined whether PYK2 deficiency affects the mobilization or differentiation of EPCs. We found that 3 days after limb ischemia, the number of circulating CD45^{-}/Flk-1^{+} EPCs was significantly lower (36%; P<0.05) in the PYK2$^{-/-}$ mice than the wild-type mice (0.28±0.06% and 0.18±0.03% relative to total peripheral blood mononuclear cells, respectively; n=10 each; Figure 5C). Considering that the mobilization of EPCs was reportedly regulated by the eNOS function of EPCs in a VEGF-dependent manner,[18] the present study suggests that PYK2 deficiency attenuates VEGF-mediated EPC mobilization by impairing Ca^{2+}/eNOS signaling.

Impaired cGMP-Dependent Protein Kinase– and eNOS-Mediated Migration of PYK2-Deficient ECs

NO-mediated angiogenesis depends on the migration of ECs, in which cGMP-dependent protein kinase (GK) or eNOS plays a crucial role.[19] Because it was reported that the migration of macrophages was severely impaired in PYK2$^{-/-}$ mice,[11] the migration activity of aortic ECs was evaluated by the Boyden chamber assay. VEGF-mediated migration was 41% lower (P<0.01) in the PYK2-deficient ECs than wild-type ECs (Figure 6A). Pretreatment with L-NAME (3 mmol/L) or Rp-8-Br-cGMPS (1 μmol/L, a GK inhibitor) markedly inhibited the VEGF-mediated migration activities of wild-type ECs (50% and 45% inhibition, respectively; Figure 6A). L-Arginine (1 mmol/L) or GK activator 8-Bromo-cGMP (1 μmol/L) treatment restored the reduced migration of PYK2-deficient ECs to the wild-type level, whereas L-NAME or GK inhibitor (1 μmol/L) did not significantly affect their migration activities (Figure 6A).

The tubular formation activity and 3-dimensional angiogenesis in the Matrigel plug were significantly decreased in the PYK2-deficient ECs (59% and 38% decrease, respectively; Figure 6B and 6C). L-NAME diminished the tubular formation of wild-type ECs (39%; P<0.05), and L-arginine treatment improved the decreased activity of PYK2-deficient ECs (52%; P<0.01) (Figure 6B). These findings suggested that PYK2-mediated migration of ECs and angiogenic response are regulated mainly by NO and GK activation.

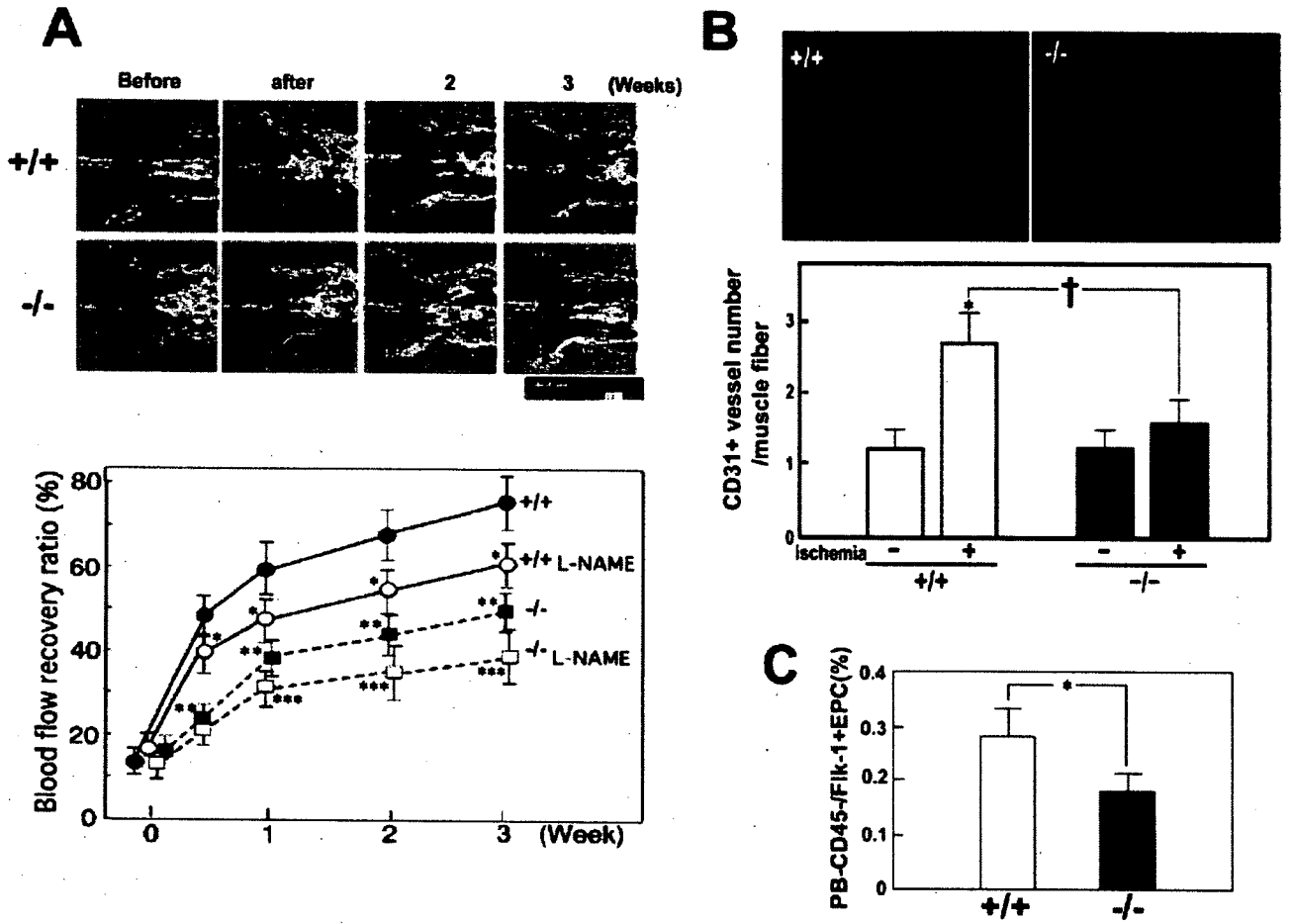

Figure 5. Reduced recovery of blood flow and neovessel formation in the ischemic hind limb of PYK2$^{-/-}$ mice. A, Reduced blood perfusion in ischemic limbs (green to blue) was observed in the PYK2$^{-/-}$ mice in contrast with perfusion (red to yellow) in the wild-type mice. Computer-assisted analyses revealed significantly lower blood perfusion values in PYK2$^{-/-}$ mice. Administration of L-NAME (3 mmol/L) in drinking water reduced the increased perfusion in both the wild-type and PYK2$^{-/-}$ mice. Values shown are mean±SE (n=8 each time point). *P<0.05, **P<0.01 vs wild-type mice; ***P<0.05 vs PYK2$^{-/-}$ mice. B, Hind-limb muscles were removed 14 days after ischemia, and ECs were immunostained with an anti-CD31 antibody. The number of CD31$^+$ vessels surrounding the muscle fiber is shown. Data are mean±SE (n=8 each). *P<0.005 vs ischemia (-) muscles; †P<0.005. C, Three days after hind-limb ischemia, peripheral blood was incubated with FITC-conjugated anti-CD45 and PE-conjugated anti-Flk-1 antibodies. Peripheral blood–derived mononuclear cells were analyzed by fluorescence-activated cell sorter after lysis of erythrocytes. The relative number of CD45$^-$/Flk-1$^+$ EPCs to total mononuclear cells was shown (n=10; *P<0.05).

Considering that GK affects the cytoskeleton structure,[20] PYK2 may modulate the cytoskeleton structure, leading to the regulation of cell migration and eventually angiogenesis. We therefore examined the effect of PYK2 on the F-actin structure of ECs. In the basal condition, we observed the stress fibers in 55±5% of the total wild-type ECs attaching on a fibronectin-coated dish and in 76±5% of PYK2-deficient ECs (P<0.05) (Figure 7A). VEGF stimulation markedly decreased the number of stress fiber–positive wild-type cells (from 55±5% to 15±4%; P<0.01) and 64±6% of the total attaching wild-type cells exhibited the accumulation of F-actin at the plasma membrane, whereas in the PYK2-deficient ECs, 72±8% of cells still had the apparent stress fibers and the cells showing the F-actin accumulation at the plasma membrane was only 23±3%. Pretreatment with L-NAME inhibited VEGF-mediated F-actin accumulation at the plasma membrane (P<0.01) and increased the stress fiber formation (P<0.05) in the wild-type cells, whereas the addition of L-arginine to PYK2-deficient ECs attenuated the stress fiber formation (P<0.01) and enhanced the VEGF-mediated F-actin accumulation at the plasma membrane (P<0.05).

Because F-actin structure was regulated by Rho-family small GTPases, we evaluated the activities of RhoA and Rac1 by pull-down assay using GST-RBD[21] and GST-PBD,[22] respectively (Figure 7B). The amount of GTP-bound RhoA was decreased significantly in the wild-type ECs 5 minutes after VEGF treatment, whereas in the PYK2-deficient ECs, they were reduced more extensively than in the wild-type ECs. In contrast, the amount of GTP-bound Rac1 was increased markedly in the wild-type ECs, whereas its increase was abolished by the PYK2 deficiency. Considering that Rac1 plays a pivotal role in F-actin reorganization[23] and PYK2 promotes Rac-mediated JNK activation,[24] lack of VEGF-mediated Rac1 activation may be associated with the altered F-actin structure in the PYK2-deficient cells. Further-

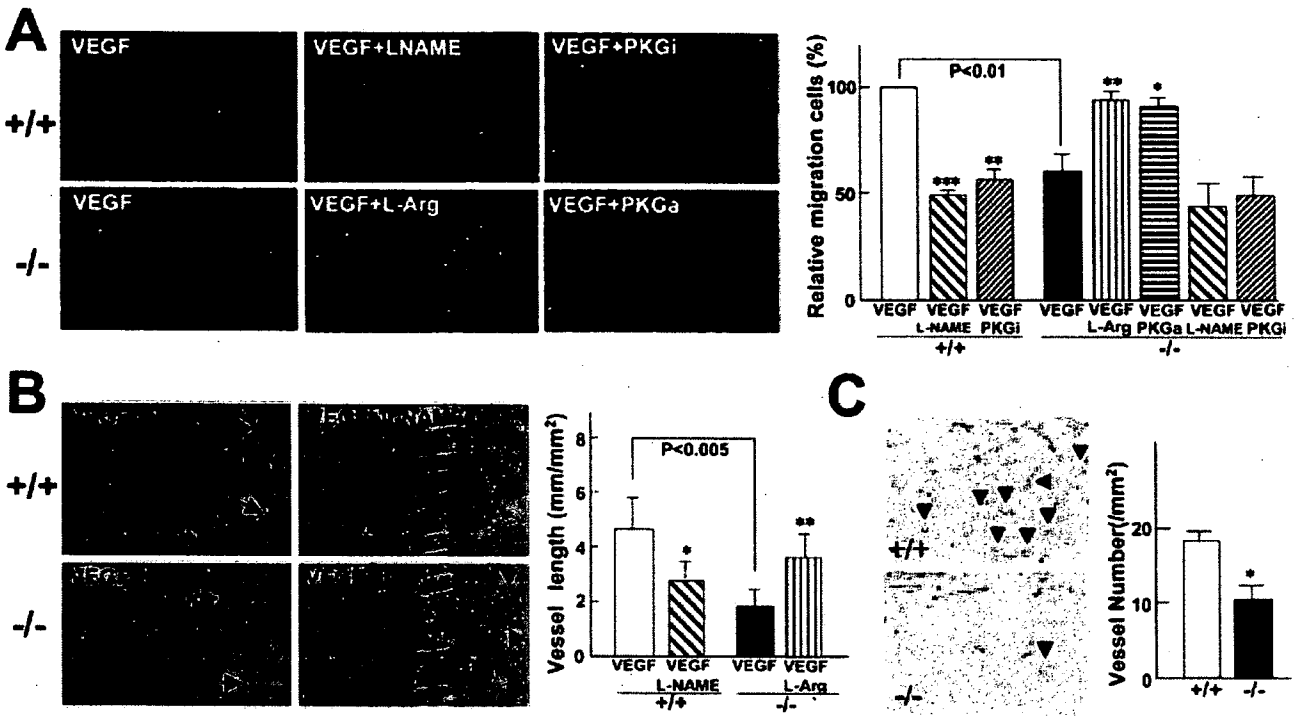

Figure 6. Decrease in GK- and eNOS-dependent migration of PYK2-deficient ECs. A, VEGF-mediated migration of ECs was evaluated with GK inhibitor (PKGi; 1 μmol/L Rp-8-Br-cGMPS), L-NAME (3 mmol/L), GK activator (PKGa; 1 μmol/L 8-Bromo-cGMP), and L-arginine (L-Arg; 1 mmol/L) using the modified Boyden chamber assay. The migrated cells were counted and arbitrarily expressed relative to VEGF-migrated wild-type ECs (100%). *$P<0.01$, **$P<0.005$, ***$P<0.001$ vs VEGF-treated control cells (n=5). B, Tubular formation of ECs was assessed with or without L-NAME (3 mmol/L) or L-Arg (1 mmol/L). Arrows indicates tubular formation. Total vessel lengths in the microscopic fields were evaluated (n=4 each). *$P<0.05$, **$P<0.01$ vs VEGF-treated control cells. C, Histological appearance (hematoxylin-eosin staining) of the Matrigel plug sections showing EC-forming vessels (arrowheads). The vessels were counted (n=6). *$P<0.01$.

more, treatment with L-NAME attenuated VEGF-mediated Rac1 activation in the wild-type ECs, whereas addition of L-arginine to the PYK2-deficient ECs restored the response, indicating that PYK2-mediated Rac1 activation is NO dependent (Figure 7B).

Taniyama et al[25] showed that in the angiotensin II–stimulated cells (vascular smooth muscle cells), Ca^{2+}-activated PYK2 acts as a scaffold for Src-dependent phosphorylation of 3-phosphoinositide-dependent protein kinase, the activator of Akt, whereas VEGF-induced Akt activation appears to be Ca^{2+} independent (Figure 3C). We therefore evaluated the target of PYK2 in the VEGF-mediated signaling pathways leading to Akt activation or Ca^{2+} mobilization. We found that VEGF stimulation causes PYK2 association with Src in the wild-type ECs, leading to the phosphorylation of Src (Figure 7C) and Akt (Figures 2B and 7C). PYK2 deficiency significantly inhibited VEGF-mediated Src and Akt activation, and inhibition of Src activity by PP1 blocked Akt and PYK2 phosphorylation (Figures 2B and 7C). Immunoprecipitation experiments indicated that Src, but not PYK2, is closely associated with the p85 subunit of PI3K and that the Src/PI3K complex binds to PYK2 in response to VEGF (Figure 7C, bottom).

To study PYK2-mediated Ca^{2+} signaling after stimulation with VEGF, we studied PLCγ1 activation, known to cause an increase in Ca^{2+} level.[26] We found that VEGF-mediated Src association with PLCγ1 and phosphorylation of ^{783}Tyr-PLCγ1 (both basal and VEGF induced) were significantly decreased in the PYK2-deficient cells and that the treatment with the Src inhibitor PP1 abolished VEGF-induced PLCγ1 phosphorylation (Figure 7D).

Taken together, it is likely that the direct target of PYK2 is Src and that Src-bound PI3-kinase and Src-bound PLCγ are involved in activation of Akt and Ca^{2+} mobilization, respectively.

Discussion

Tyrosine kinases have been assumed to be the upstream molecule for PI3K-Akt-eNOS or Ca^{2+}-eNOS pathways. eNOS is activated by Akt, and intracellular Ca^{2+} upregulates eNOS activity, raising the possibility that Ca^{2+}-dependent tyrosine kinase PYK2 is a possible eNOS activator. This study provides the first evidence that (1) PYK2 deficiency attenuates VEGF-mediated eNOS phosphorylation associated with decreased Akt activation and intracellular Ca^{2+} mobilization, (2) PYK2 is associated with the Src/PI3K complex and inhibition of Src blocked Akt phosphorylation, (3) Ach-mediated vasodilation of the aorta was diminished by decreased cGMP production in PYK2$^{-/-}$ mice, and (4) PYK2 plays a central role in VEGF- or ischemia-mediated eNOS activation followed by angiogenic response in which VEGF-induced EPC mobilization, VEGF-dependent migration, actin cytoskeletal reorganization associated with reduced Rac1 activation were markedly inhibited in the PYK2-deficient

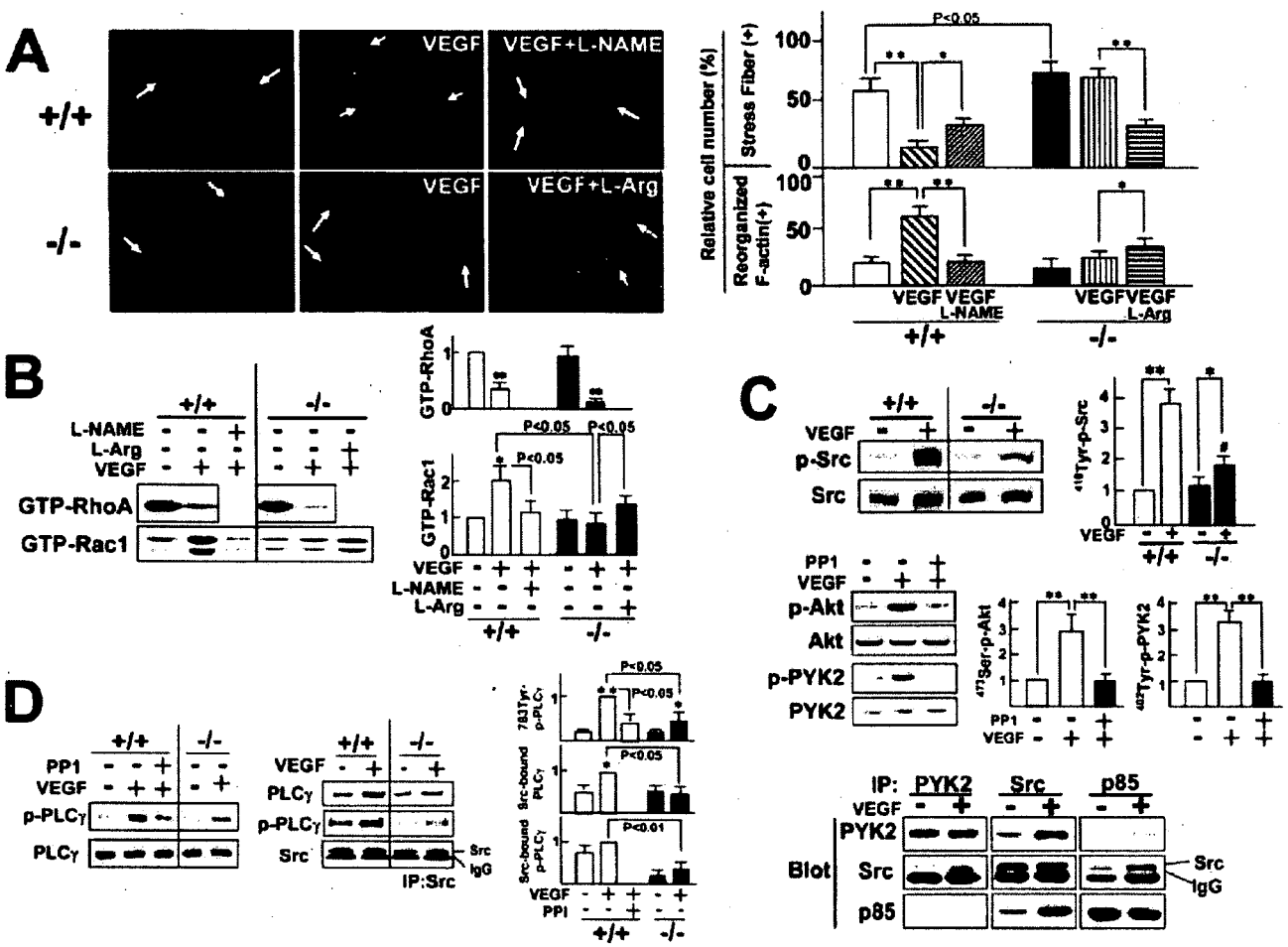

Figure 7. Impaired reorganization of F-actin in PYK2-deficient cells. A, F-actin structure of ECs. ECs were cultured on the fibronectin-coated glass chamber. After 16 hours of starvation (0.5% serum) with or without preincubation by L-arginine (1 mmol/L) or L-NAME (3 mmol/L), ECs were stimulated with VEGF (100 ng/mL) for 30 minutes, fixed with 4% paraformaldehyde, and permeabilized with 0.02% triton, and F-actin was stained with TRITC-labeled phalloidin. Representative staining was shown (n=7 each). Yellow arrows indicate the stress fiber; white arrows, accumulated F-actin at the plasma membrane. The cells with stress fiber or accumulated F-actin at the plasma membrane were counted, and the ratio (%) relative to total attaching cells was shown. *$P<0.05$, **$P<0.01$. B, Measurement of the VEGF-induced RhoA:GTP/Rac1:GTP. After 16 hours of starvation with or without L-NAME or L-arginine, ECs were incubated by VEGF for 5 minutes. Cell lysates were incubated with GST-RBD (Rho-binding domain) and GST-PBD (p21-binding domain) bound to glutathione beads as described in the Methods section of the online Data Supplement. The amount of RhoA:GTP and Rac1:GTP complex was determined by immunoblot with anti-RhoA and anti-Rac1 antibodies. *$P<0.05$, **$P<0.01$ (n=4 each) vs non-stimulated group. C, After 16 hours of starvation with or without PP1, ECs were incubated with VEGF for 5 minutes. Top, Cell lysates were subjected to Western blotting with antibodies against anti-^{416}Tyr-phosphorylated-Src or Src. Middle, Cell lysates of the wild-type ECs were analyzed by Western blotting with antibodies against Akt, ^{473}Ser-phosphorylated Akt, PYK2, and ^{402}Tyr-phosphorylated PYK2. Bottom, Cell lysates of the wild-type ECs were immunoprecipitated (IP), followed by Western blotting using antibodies against anti-PYK2, Src, and p85 subunit of PI3-kinase. *$P<0.05$, **$P<0.005$ (n=5 each experiment). D, After 16 hours of starvation with or without PP1, ECs were stimulated with VEGF for 5 minutes. Cell lysates were subjected to immunoblotting with antibodies against PLCγ1 or ^{783}Tyr-phosphorylated PLCγ1, plus immunoprecipitation with anti-Src antibody, followed by immunoblot with antibodies against PLCγ1, ^{783}Tyr-phosphorylated PLCγ1, or Src. *$P<0.05$, **$P<0.005$ (n=4 each) vs nonstimulated groups. #$P<0.05$ vs VEGF-stimulated wild-type ECs.

ECs. Cell migration reportedly requires the recycled mobilization of actin from the old focal adhesion toward the plasma membrane of the leading edge to form the lamellipodia and new focal contact,[27] suggesting that impaired reorganization of the F-actin at the plasma membrane plausibly causes attenuated migration of the PYK2-deficient ECs.

eNOS activation is dependent on an increase in $[Ca^{2+}]i$ and the binding of Ca^{2+}/calmodulin to the enzyme, leading to conformational change to displace the autoinhibitory loop.[28] If Ca^{2+} mobilization is totally abolished, administration of the eNOS substrate arginine could not restore eNOS activity. However, VEGF-induced increase in $[Ca^{2+}]i$ and subsequent translocation of NFATc2 in PYK2-deficient ECs remain ≈23% and ≈32%, respectively, of the wild-type cells (Figure 3). This increase in $[Ca^{2+}]i$ could cause the conformational change in eNOS, resulting in the recovery in eNOS activation after arginine administration.

The present study showed that PYK2 deficiency attenuates VEGF-mediated association of Src with PLCγ1 and phosphorylation of PLCγ1. VEGF was shown to stimulate the

association of VEGF receptor-2 with Src, and subsequent Src activation was a requisite for VEGF-mediated PLCγ1 activation.[29] Taken together, it is conceivable that the lack of intracellular Ca^{2+} mobilization in VEGF-stimulated PYK2-deficient cells is due to the inhibition of Src-associated PLCγ activation.

Src and PYK2 are mutually activated in a stepwise manner. Association of Src-SH2-domain with ^{402}Tyr-phosphorylated-PYK2 leads to conformational change in Src to release the internal autoinhibition, resulting in the upregulation of its activity. Conversely, activated Src phosphorylates ^{881}Tyr-PYK2, leading to the downstream Grb2/Ras/MAPK pathway.[10] In response to VEGF receptor-2 stimulation, Src binds toward the phosphorylated ^{1212}Tyr of VEGF receptor-2 with its SH2 domain, leading to Src activation.[30] In addition, VEGF promotes association of VEGF receptor-2 with integrin ($\alpha V\beta 3$) and transmits integrin-dependent cell biological responses.[31] Activation of integrin ($\alpha V\beta 3$) induces phosphorylation of ^{402}Tyr-PYK2 and its association with integrin $\beta 3$.[32] Integrin-activated PYK2 is associated with Src[33] and involved in VEGF-mediated cell migration.[34] Furthermore, integrin ($\alpha V\beta 5$) plays a crucial role in angiogenesis,[35] and inhibition of integrin ($\alpha V\beta 5$) disrupted VEGF-mediated and Src-dependent angiogenesis.[36] Thus, PYK2/Src complex is likely to integrate integrin with the VEGF receptor-2 signaling system.

Fluid shear stress–mediated activation of eNOS is dependent on Ca^{2+} mobilized through a mechanical stress–activated Ca^{2+} channel on the plasma membrane, whereas a Ca^{2+}-independent system has been reported recently. Fleming et al[37] demonstrated that shear stress elicits Src-mediated phosphorylation of platelet EC adhesion molecule-1 (PECAM-1) at the cell-to-cell contact, which is crucial for subsequent activation of Akt and eNOS. Furthermore, Tzima et al[38] showed that PECAM-1 forms a mechanosensory complex with VEGF receptor-2, leading to activation of PI3-kinase. These findings suggest that VEGF receptor-2 is involved in Src/PECAM-1–mediated Akt/eNOS activation in Ca^{2+}-independent manner. Unlike PECAM-1, PYK2 is localized at the cell-to–extracellular-matrix contact region.[8,33] VEGF promotes the association of VEGF receptor-2 with integrin and integrin-dependent cell biological responses.[31] Thus, the PYK2/Src complex transmits the VEGF signals in association with extracellular matrix/integrins, suggesting that the Ca^{2+}-independent Src/PECAM-1 system is unlikely to be involved in PYK2-mediated Src activation.

Because the blood flow recovery ratio in the ischemic limbs was increased ≈5-fold on day 7 compared with the day 0 control level (Figure 5A), hemodynamic shear stress also could be proportionally elevated in the newly formed vessels. Shear stress was shown to elicit the phosphorylation of ^{1177}Ser-eNOS by activating both Akt and PKA.[28] As shown in Figure 2A, Akt, AMPK, and PKA showed peak phosphorylation on day 1 and at 2 hours, respectively, and then PKA and AMPK reversed to the baseline level on day 7, whereas moderate activation of Akt was observed on day 7 (210% increase compared with the basal level), suggesting that Akt, rather than PKA and AMPK, is involved in the eNOS activation 7 days after limb ischemia. However, further studies are required to define the involvement of other kinases associated with flow shear stress.

Conclusions

This analysis of $PYK2^{-/-}$ mice demonstrates the critical role of PYK2 in Akt/NO signals activated by vasoactive substances or ischemic stress that modulates the vascular tonus or angiogenesis. These findings indicate that PYK2 can operate as a modulator for extracellular versatile stimuli, leading to eNOS activation, and is closely involved in the receptor- or ischemia-activated NO signaling events and thus regulates the cytoskeleton structure, vasoreactive function, or angiogenic response.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Although endothelial dysfunction causes atherosclerosis or vascular aging, drugs to improve endothelial functions have not been developed. Nitric oxide (NO) plays pivotal roles in the maintenance of endothelial function and vascular homeostasis, including vasodilatation, antiinflammatory effect, anticoagulation, antiproliferative effect of vascular smooth muscle cells, angiogenesis, and vasculogenesis. NO is produced by endothelial NO synthase (eNOS); therefore, a drug that upregulates eNOS function may improve endothelial function. For this purpose, it is important to clarify the molecular mechanism to activate eNOS. In the present study, we showed that tyrosine kinase PYK2 plays a crucial role in eNOS activation. Both PYK2 and eNOS are activated by hemodynamic mechanical stress, ischemic stress, and stimulation with endothelial growth factors, and PYK2 transmits calcium and Akt signaling pathways, both of which activate eNOS, suggesting that the drug developed to activate PYK2 may be feasible therapy for maintaining vascular homeostasis in various stress conditions. However, PYK2 also is involved in angiotensin II–mediated signaling to induce atherosclerosis, including vasoconstriction, vascular inflammation, and proliferation of vascular smooth muscle cells, indicating that PYK2 has dual effects on vascular function. The role of PYK2 in the maintenance of vascular homeostasis should be investigated further.

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New Targeted Angiogenic Strategy: Bursting Bubbles

Tomosaburo Takahashi and Hiroaki Matsubara

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New Targeted Angiogenic Strategy
Bursting Bubbles

Tomosaburo Takahashi, Hiroaki Matsubara

Although recent procedural advances in revascularization such as percutaneous coronary intervention and coronary artery bypass grafting improve quality of life and prognosis of the patients with ischemic diseases, these is still a subset of patients who are refractory to these conventional therapies and have poor prognosis. Therapeutic angiogenesis might offer a novel approach to these patients. Therapeutic angiogenesis involves an intervention to induce the formation of new blood vessels to restore the arterial blood and oxygen supply to ischemic tissues.[1] Here, the term "angiogenesis" represents the process of new blood vessel formation in general (although the same term is also used to describe a more specific biological process in which the new capillaries sprout from preexisting vessels).

Evolving knowledge of mechanisms of new blood vessel formation has raised the expectations for therapeutic angiogenesis as a treatment option. Recent studies have identified many angiogenic growth factors, vascular transcription factors, and the cells involved in neovascularization.[1,2] Current potential strategies for therapeutic angiogenesis include delivering an angiogenic factor as a protein or a gene, and supplying cells which themselves are vascular progenitors or are releasing angiogenic factors. These strategies have worked in animal studies and in initial small scale open-labeled clinical trials. However, in larger, double-blinded controlled trials, therapeutic angiogenesis approaches have failed to show clinical benefits.[1,2] Why? Perhaps there are subtle differences in angiogenesis between animals and humans, or the ischemic pathophysiology of animal models and human diseases are dissimilar. Another possibility is technical difficulties in translating the biology into the practice.

In this issue of *Circulation Research*, Leon-Poi and colleagues report that targeted delivery of vascular endothelial growth factor (VEGF) gene using ultrasound-mediated destruction of cationic lipid microbubbles restores microvascular blood flow in a rat model of chronic hindlimb ischemia.[3] Ultrasound-targeted microbubble destruction uses ultrasound contrast agents, mostly perfluorocarbon bubbles stabilized with albumin or a lipid shell. When insonified at high acoustic power, these agents oscillate, resulting in microbubble disintegration.[4,5] This microbubble destruction has been shown to induce biophysical effects in the vicinity of contrast agents, and the therapeutic use of this phenomenon has been proposed for delivery of genes or drugs, and direct mechanical effects. One of the advantages of this method is that these bubbles cross the pulmonary circulation, so that the agents can be administrated intravenously, and reach any part of the body with arterial blood supply. And, at the desired sites, the agents can be activated or delivered by ultrasound. Leon-Poi et al coated the microbubbles with VEGF-expressing plasmid, intravenously administrated these bubbles, and then transferred the gene at the site of ischemia with ultrasound in hindlimb ischemia model.[3] This model of ischemia is chronic, because the gene was transferred 14 days after common iliac artery ligation.

Leon-Poi et al observed a significant increase in tissue perfusion mainly through the process of arteriogenesis, rather than angiogenesis in a narrow sense.[3] Arteriogenesis refers to the maturation or de novo synthesis of collateral vessels. The effect of VEGF gene transfer with this method on arteriogenesis rather than angiogenesis is intriguing, as remodeling or development of collaterals could be much more effective than an increase in capillary bed to restore the blood flow in the setting of flow-limiting proximal conduit artery lesions. It will be an important issue to determine whether the preferential effect on arteriogenesis is associated with the method of gene delivery, ultrasonic destruction of microbubble destruction (Figure).

Although the gene delivery is an important application of ultrasound-mediated microbubble destruction,[4–6] use of this method is not limited to gene therapy. Acoustic cavitation leads to microbubble oscillation and collapse. Electron microscopic analysis showed transient pore formation on cell membrane immediately after microbubble destruction, called sonoporation.[4,5] These mechanical effects facilitate entry of gene into the cells. At the same time, these mechanical forces affect nearby cells and tissues in vicinity of microbubble destruction. For example, microbubble destruction can facilitate thrombolysis in combination with thrombolytic agents such as urokinase and tissue plasminogen activator.[7,8] Furthermore, ultrasound-mediated microbubble destruction itself can be angiogenic, as ultrasound-mediated microbubble destruction has been shown to be able to induce capillary rupture and increase the density of arterioles in ischemic muscle with local recruitment of VEGF producing inflammatory cells.[9,10] Although capillary rupture with higher energies of ultrasound is just a step from adverse tissue damage, even with ultrasound without capillary rupture, ultrasound-mediated microbubble destruction has direct effects on vasculature and surrounding tissues. These effects can be

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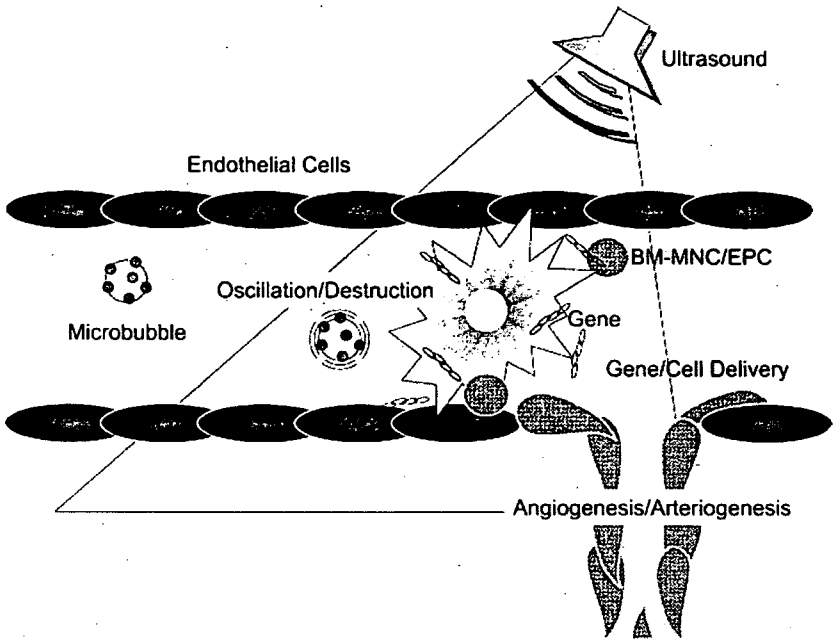

Schematic diagram of ultrasound-mediated microbubble destruction. Microbubbles enclosing therapeutic agents such as genes or drugs reach the local sites through circulation. At the desired site, ultrasound is applied, which oscillates and collapses the microbubbles, leading to direct biophysical effects on tissue or the delivery of therapeutic agents.

used for potential therapies, especially when combined with other modalities. One such example is microbubble destruction in combination with cell therapy. We have shown that targeted delivery of bone marrow-derived mononuclear cells by ultrasound-mediated microbubble destruction significantly enhances angiogenic response both in an ischemic hindlimb model and in a δ-sarcoglycan deficient cardiomyopathy model.[11,12] In the ischemic hindlimb model, ultrasound-mediated microbubble destruction induces platelet activation on the surface of endothelium, and subsequent induction of adhesion molecules on endothelium, which in turn stimulates recruitment of angiogenic mononuclear cells and enhances new vessel formation.[11] Ultrasound-mediated targeted cardiac delivery of marrow-derived mononuclear cells also efficiently enhances regional blood flow in myopathic hamsters, leading to improvement of cardiac function.[12]

Recent proof-of-principal studies show that ultrasound-mediated microbubble destruction has great potential to target various substrates including genes, proteins, drugs and cells to the desired sites. However, before this strategy is adopted in the clinic, many issues have to be resolved. Which patients are ideal subjects? What tissues are ideal target locations? What proteins and cells are ideal substrates to be delivered by microbubbles? Furthermore, technical issues of ultrasound-mediated microbubble destruction such as microbubble composition and ultrasound application must be refined. More collaboration between clinicians, biologist, chemists and engineers is needed to bring this exciting technique into the clinic.

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Disclosures

None.

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KEY WORDS: therapeutic angiogenesis ■ gene therapy ■ ultrasound ■ microbubbles

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Intracoronary Transplantation of Non-Expanded Peripheral Blood-Derived Mononuclear Cells Promotes Improvement of Cardiac Function in Patients With Acute Myocardial Infarction

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Background Transplantation of non-expanded peripheral blood mononuclear cells (PBMNCs) enhances neovessel formation in ischemic myocardium and limbs by releasing angiogenic factors. This study was designed to examine whether intracoronary transplantation of PBMNCs improves cardiac function after acute myocardial infarction (AMI).

Methods and Results After successful percutaneous coronary intervention (PCI) for a ST-elevation AMI with occlusion of proximal left anterior descending coronary artery within 24 h, patients were assigned to either a control group or the PBMNC group that received intracoronary infusion of PBMNCs within 5 days after PCI. PBMNCs were obtained from patients by COBE spectra-apheresis and concentrated to 10 ml, 3.3 ml of which was infused via over-the-wire catheter. The primary endpoint was the global left ventricular ejection fraction (LVEF) change from baseline to 6 months' follow-up. The data showed that the absolute increase in LVEF was 7.4% in the control group and 13.4% (p=0.037 vs control) in the PBMNC group. Cell therapy resulted in a greater tendency of ΔRegional ejection fraction (EF) or significant improvement in the wall motion score index and Tc-99m-tetrofosmin perfusion defect score associated with the infarct area, compared with controls. Moreover, intracoronary administration of PBMNCs did not exacerbate either left ventricular (LV) end-diastolic and end-systolic volume expansion or high-risk arrhythmia, without any adverse clinical events.

Conclusion Intracoronary infusion of non-expanded PBMNCs promotes improvement of LV systolic function. This less invasive and more feasible approach to collecting endothelial progenitor cells may provide a novel therapeutic option for improving cardiac function after AMI. (*Circ J* 2007; **71:** 1199–1207)

Key Words: Acute myocardial infarction; Angiogenesis; Cardiac function; Peripheral blood-derived mononuclear cells

Differentiation of mesodermal cells to angioblasts and subsequent endothelial differentiation was believed to exclusively occur in embryonic development; but this dogma was overturned when human adult peripheral blood mononuclear cells (PBMNCs) were demonstrated to differentiate into the endothelial lineage.[2] These cells named "endothelial progenitor cells" (EPCs) expressed endothelial markers, and were incorporated into the sites of ischemia.[3,4] We have recently demonstrated that bone marrow mononuclear cells (BMMNCs) contain EPCs in the CD34$^+$ cell fraction and various proangiogenic factors, such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and angiopoietin 1 in the CD34$^-$ cell fraction, and that implantation of BMMNCs into the site of ischemia enhances angiogenesis via harmonic supply of EPCs and angiogenic factors.[5,6] This technique has been used clinically and developed as a useful therapeutic option for human critical limb ischemia.[7]

The concept of the heart as an organ composed of terminally differentiated myocytes incapable of regeneration is also being challenged.[8–10] Although attempts to replace necrotic tissue by transplanting other cells (eg, fetal cardiac myocytes or skeletal myoblasts) succeeded in reconstituting heart muscle, these cells failed to completely integrate structurally and to display characteristic physiological function.[11–13] In contrast, bone marrow cells (BMCs) have the ability to differentiate into various tissue and are likely to

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regenerate myocardium by inducing myogenesis and angiogenesis, as shown by improved cardiac function and myocardial perfusion in recent accumulating evidence from animals and humans[14–17] In particular, cardiac transfer of BMC-derived stem/progenitor cells can have a favorable impact in patients with acute myocardial infarction (AMI).[18,19] Clinical efficacy of intracoronary transplantation of BMCs after AMI has been the focus of recent large scale, randomized, and controlled trials. The potential benefit of intracoronary injection of BMCs for left ventricular (LV) function was reported in the randomized Bone marrow transfer to enhance ST-elevation infarct regeneration (BOOST) trial[20] and in the Reinfusion of Enriched Progenitor Cells and Infarct Remodeling in Acute Myocardial Infarction (REPAIR-AMI) trial.[21] In contrast, intracoronary injection of BMCs after AMI did not significantly improve LV function in the Autologous Stem-Cell Transplantation in Acute Myocardial Infarction (ASTAMI) trial[22] or in the trial reported by Janssens et al.[23] Thus, the latest randomized clinical studies for transplantation of BMCs against AMI retain discrepancies that must be resolved in future trials.

We have previously reported that NOGA-catheter based implantation of non-expanded PBMNCs alone can significantly improve systolic function in ischemic hibernating myocardium of pigs,[24] and that intramuscular injection of human PBMNCs markedly increases regional blood flow in hindlimb ischemia by releasing potent angiogenic factors such as VEGF and bFGF.[25] Moreover, it has been demonstrated that EPCs are indeed mobilized in patients with AMI, peak at 7 days after the onset,[26] and that stromal-cell-derived factor-1 (SDF-1), an important stem cell homing factor, is expressed in the myocardium immediately after AMI.[27] Because the invasiveness of BMC collection in the acute phase of AMI limits its clinical application, we hypothesized that transplantation of non-expanded PBMNCs would even improve the cardiac function in patients with AMI. In this context, we started a clinical trial named the "Japan Trial for Therapeutic Angiogenesis by Cell Transplantation of Peripheral Blood-derived Mononuclear Cells for Acute Myocadial Infarction (TACT-PB-AMI)" in 2004. The primary aim of our study was to examine whether intracoronary injection of non-expanded PBMNCs results in an improvement in LV function, as measured by LV ejection fraction (LVEF), after AMI. Additional objectives were to test the feasibility and safety of this treatment, as well as to assess the effectiveness on regional wall motion, cardiac volumes, and arrhythmias.

Methods

Patients and Study Protocol

Patients between 18 and 80 years of age were eligible for inclusion in the study if they had a first acute ST-elevation myocardial infarction with occlusion of the proximal left anterior descending (LAD) coronary artery and a creatine kinase (CK) level >1,000 IU, which was successfully treated by percutaneous coronary intervention (PCI) within 24 h. According to previous observations,[28,29] CK values were serially measured every 4 h for 24 h after the onset of AMI. Exclusion criteria were the presence of cardiogenic shock requiring intravenous pressors or intra-aortic balloon counterpulsation, pulmonary edema, advanced hepatic or renal dysfunction, evidence of malignant diseases, or unwillingness to participate. Because the patients were best suited for an evaluation of LV function by angiographic imaging, we decided to include only patients with anterior wall infarction. This study protocol was approved by the Ethics Review Board of Kyoto Prefectural University School of Medicine, and written informed consent was given by each patient.

The study was designed as an open-label and non-randomized clinical trial. Briefly, after successful PCI (TIMI III), patients were assigned to either the control (PCI alone) group or non-expanded PBMNC group that received intracoronary infusion of PBMNCs within 5 days after PCI. Intracoronary cell transplantation was performed by over-the-wire balloon catheter. Neither collection of PBMNCs nor sham injection was performed in the control group. The primary endpoint was the global LVEF change from baseline to 6 months' follow-up.

Catheterization Procedure for Progenitor Cell Transplantation

A mean of 2.5±0.5 days after the AMI, an over-the wire balloon catheter was advanced into the infarct-related artery (eg, LAD). To allow for adhesion and transmigration of the infused cells through the endothelium, the balloon was inflated inside the stent previously implanted during the acute reperfusion procedure with low pressure to block blood flow for 3 min while 3.3 ml of the PBMNCs suspension was infused distally to the occluding balloon through the central port of the balloon catheter, as previously described.[19] This maneuver was repeated 3 times to accommodate infusion of the total 10-ml cell suspension, interrupted by 3 min of reflow by deflating the balloon to minimize extensive ischemia. After completion of intracoronary cell transplantation, coronary angiography was repeated to ascertain vessel patency and unimpeded flow of contrast material.

Preparation of Progenitor Cells

A cell separator apheresis system with computer software (COBE Spectra, software version 6.1, Gambro BCT, Lakewood, Co, USA) was used to collect all PBMNC products via the standard MNC program. Acid citrate dextrose-A (ACD-A, Baxter Healthcare Corporation, Deerfield, IL, USA) was used as the anticoagulant at a whole body-to-ACD ratio of 20–25:1 in combination with 2,000 IU heparin sulfate. Apheresis was performed through central venous access from the femoral vein in all patients in the PBMNC group. With the aim of processing the largest amount of blood in the shortest possible time, apheresis procedures were performed with the highest possible but still tolerable blood flow rate, such as 55 ml/min. No more than 2.5-fold of the donor's blood volume was processed on a single day. We usually obtain PBMNCs ($\approx 5\times10^9$ cells) from patients by COBE spectra-apheresis and concentrate them to 10 ml by density gradient centrifugation (Kubota 9810, Japan).

After PBMNCs were harvested by COBE Spectra, 100 μl of the cell suspension was diluted by PBS(–) containing 0.5% bovine serum albumin (Fraction V, Sigma, St Louis, MO, USA) used for flow cytometric analysis. The cells were stained with allophycocyanin-conjugated (APC)-anti-human CD34 (Becton Dickinson, San Jose, CA, USA) and phycoerythrin-conjugated (PE)-anti-human VEGFR2 (KDR) (R&D Systems, Minneapolis, MN, USA).

Appropriate isotype controls were used for each staining procedure; 1×10^5 cells were gated within the lymphocyte region on forward-scatter vs side-scatter plots using a FACS Calibur (BD Bioscience). Next, the percentages of cells in

Table 1 Clinical Characteristics of the Study Population

	Control group (n=36)	PBMNC group (n=18)	p value
Age, years	60.4±11.3	61.8±8.7	0.65
Male sex, no. (%)	32 (88.9)	15 (83.3)	0.57
Hypertension, no. (%)	16 (44.4)	11 (61.1)	0.25
Hyperlipidemia, no. (%)	20 (55.6)	12 (66.7)	0.43
Diabetes, no. (%)	12 (33.3)	5 (27.8)	0.68
Smoking, no. (%)	20 (55.6)	12 (66.7)	0.43
PAD, no. (%)	3 (8.3)	1 (5.6)	0.71
Killip class on admission	1.20±0.48	1.22±0.43	0.66
Infarct segment	6.3±0.5	6.6±0.5	0.06
Vessel diameter, mm	3.24±0.38	3.22±0.31	0.89
Peak creatine kinase, IU/dl	3,764±2,506	4,255±1,615	0.47
Time to revascularization, h	6.1±5.7	5.2±2.4	0.49
Mean transplanted cells, no.	–	$4.92\pm2.82\times10^9$	
Medication			
ACEI, no. (%)	26 (72.2)	15 (83.3)	0.37
ARB, no. (%)	11 (30.6)	5 (27.8)	0.83
β-blocker, no. (%)	19 (52.8)	10 (55.6)	0.85
Diuretics, no. (%)	6 (16.7)	3 (16.7)	1.00
Statins, no. (%)	22 (61.1)	11 (61.1)	1.00

Values are expressed as mean±SD.
PBMNC, peripheral blood mononuclear cell; PAD, peripheral arterial disease; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin-receptor blocker.

each population described below were calculated using CELLQuest software (BD Bioscience).

LV Angiography

LV angiograms were obtained according to standard acquisition guidelines immediately after PCI and at 6 months' follow-up. LVEF and LV volumes were calculated by the area–length method, and regional wall motion was determined with the use of the centerline chord method.

Measurement of Other Parameters

For the assessment of regional LV wall motion, echocardiography was carried out before cell transplantation and at 6 months' follow-up. Two-dimensional resting echocardiography was performed in the 4 standard views (parasternal long-axis and short-axis views and apical 4- and 2-chamber views) and regional LV wall motion analysis was performed as described by the Committee on the Standards of the American Society of Echocardiography, dividing the left ventricle into 16 segments and scoring wall motion as 1=normal, 2=hypokinesis, 3=akinesis, 4=dyskinesis for each segment. The wall motion score index (WMSI) was calculated as the sum of the scores of the segments divided by the number of the segments evaluated at the day of cell transplantation and 6 months' follow-up.

We performed resting Tc-99m (^{99m}Tc)-tetrofosmin gated single photon emission computed tomography (SPECT) before hospital discharge and at 6 months' follow-up. In all patients, 592 MBq of ^{99m}Tc-tetrofosmin was intravenously injected at rest. Immediately after the injection, each patient drank a glass of milk to accelerate tracer clearance from the hepatobiliary system. Data acquisition for SPECT imaging was performed at 30 min after ^{99m}Tc-tetrofosmin injection, using a rotating digital gamma camera (Picker PRISM IRIX) equipped with a low energy, high resolution, and parallel-hole collimator. Reconstructed transaxial images were reoriented in the vertical long-axis and short-axis of the LV. The basal and midventricular segments on short-axis views of the LV myocardium were divided into 8 segments each, and 16 segments were taken. An apical region on the vertical long axis was also taken, and a total of 17 segments were analyzed. The ^{99m}Tc-tetrofosmin perfusion defects were visually evaluated by 2 experienced observers, who had no knowledge of the patient's clinical information, with a 5-point grading system (0=normal, 1=mildly decreased uptake, 2=moderately decreased uptake, 3=severely decreased uptake, 4=defect). The grading was decided on by consensus between the 2 observers, and the sum of the scores for all segments was used as the defect score.

To assess whether intracoronary cell transplantation was associated with proarrhythmic effects, we performed 24-h Holter recording for all patients before hospital discharge and at 6 months' follow-up, and estimated the Holter Lawn class by calculating premature ventricular complexes and ventricular tachycardias.

Follow-up Examinations

Six months after progenitor cell therapy, cardiac catheterization was repeated; left ventriculography was performed with identical projections and adequate contrast opacification for quantitative analysis according to standard guidelines and coronary angiograms were analyzed for the presence of restenosis in the infarct-related artery. Echocardiography, Holter ECG, and perfusion scintigraphy were also repeated after 6 months.

Statistical Analysis

Continuous variables are presented as means±SD. Control and cell therapy groups were compared using the chi-square test for discrete variables and unpaired Student's t-test for continuous variables according to standard statistical methods. Statistical significance was assumed at a value of $p<0.05$. All statistical analysis was performed with SPSS (Version 9.0, SPSS Inc, Chicago, IL, USA).

Results

Baseline Characteristics and Procedural Results of Cell Infusion

The clinical characteristics of the study population are

Table 2 Clinical Characteristics of the Patients' Measurements of Hemodynamics at the Time of AMI (Baseline) and 6-Months' Follow-up

	Control group (n=36)	*PBMNC group (n=18)*	*p value*
LVSP (mmHg)			
Baseline	*121.7±13.0*	*120.3±13.9*	*0.73*
6 months	*125.0±19.2*	*126.9±8.7*	*0.67*
p value (baseline vs 6 months)	*0.47*	*0.10*	
LVDP (mmHg)			
Baseline	*3.9±4.8*	*4.6±5.1*	*0.67*
6 months	*3.1±2.4*	*4.1±3.5*	*0.27*
p value (baseline vs 6 months)	*0.4*	*0.73*	
LVEDP (mmHg)			
Baseline	*20.1±6.1*	*19.8±4.8*	*0.89*
6 months	*13.6±3.7*	*14.3±5.3*	*0.57*
p value (baseline vs 6 months)	*<0.0001*	*<0.0001*	
AOMP (mmHg)			
Baseline	*90.3±13.4*	*88.4±9.7*	*0.61*
6 months	*89.5±14.2*	*90.2±9.7*	*0.85*
p value (baseline vs 6 months)	*0.84*	*0.58*	
HR (beats/min)			
Baseline	*88.3±7.7*	*88.0±10.9*	*0.91*
6 months	*65.8±6.8*	*65.0±7.2*	*0.69*
p value (baseline vs 6 months)	*<0.0001*	*<0.0001*	

Values are mean±SD.
AMI, acute myocardial infarction; LVSP, left ventricular systolic pressure; LVDP, left ventricular diastolic pressure; LVEDP, left ventricular end-diastolic pressure; AOMP, aortic mean pressure; HR, heart rate. Other abbreviation see in Table 1.

Table 3 Clinical Characteristics of Patients' Baseline Cardiac Function

	Control group (n=36)	*PBMNC group (n=18)*	*p value*
LVEF (%)	*48.8±11.3*	*43.8±12.5*	*0.20*
EDVI (ml/m²)	*60.9±14.8*	*70.0±15.0*	*0.07*
ESVI (ml/m²)	*32.0±13.1*	*39.9±14.9*	*0.054*
Regional EF (%)			
Segment #2	*11.7±6.1*	*12.3±7.0*	*0.77*
Segment #3	*8.4±6.5*	*5.5±5.8*	*0.11*

LVEF, left ventricular ejection fraction; EDVI, end-diastolic volume index; ESVI, end-systolic volume index; EF, ejection fraction. Other abbreviation see in Table 1.

shown in Table 1. The PBMNC group and control group were well matched with respect to baseline characteristics and procedural characteristics, such as age, sex, and coronary risk factors, Killip class, infarct segment, and vessel diameter. Although particularly important factors influencing cardiac function are considered to be peak CK and time to revascularization, there were no significant differences in these factors between the 2 groups. All patients were treated with aspirin (100 mg/day), ticlopidine (200 mg/day for at least 4 weeks after PCI) or cilostazol (200 mg/day at least 4 weeks after PCI), statin, β-blocker, and angiotensin-converting enzyme inhibitor (ACEI) or angiotensin-receptor blocker (ARB) during the hospitalization for AMI and continued until the 6-months' follow-up examination, unless these agents were contraindicated. There were no significant differences between the control and PBMNC groups in the administration of ACEIs, ARBs, β-blockers or statins (Table 1).

No patient had either bleeding complications through the central venous access from the femoral vein or systemic blood pressure fall during the apheresis procedures. Although a transient further ST elevation associated with balloon occlusion was seen in the infarct-related ECG leads in most of the patients receiving PBMNCs, there were no serious symptomatic complaints or circulatory disturbances during or after cell transplantation. Neither intracoronary infusion nor the stop-flow procedure was performed in the control group. There were no fatal cardiac events during the follow-up period, and no patient in either group had any clinical manifestation of heart failure.

Endothelial Progenitors in AMI Patients

FACS analysis showed that the percentage of $CD34^+$ (0.12±0.2) or $CD34/KDR^+$ (0.05±0.1) cells in the PBMNCs from AMI patients tended to be higher (2–10-fold, but not statistically significant), compared with healthy volunteers ($CD34^+$; 0.06±0.1, $CD34/KDR^+$; 0.003±0.001), and that these cells also possess the characteristics of EPCs, as demonstrated by DiI-acetylated LDL uptake and lectin binding.

Hemodynamics and LV Function by Angiography

Table 2 shows the hemodynamic measurements in the control and PBMNC groups at the time of AMI (baseline) and at 6-months' follow-up. Although LV end-diastolic pressure (LVEDP) and heart rate (HR) were significantly reduced from baseline to 6-months' follow-up in both groups, there were no significant differences in LV systolic pressure (LVSP), LV diastolic pressure (LVDP), or aortic mean pressure (AOMP). Moreover, there were no statistically significant differences in LVSP, LVDP, LVEDP, AOMP, or HR between groups at either baseline or 6-months' follow-up.

Table 3 shows the clinical characteristics of baseline LV cardiac function. There were no significant differences in

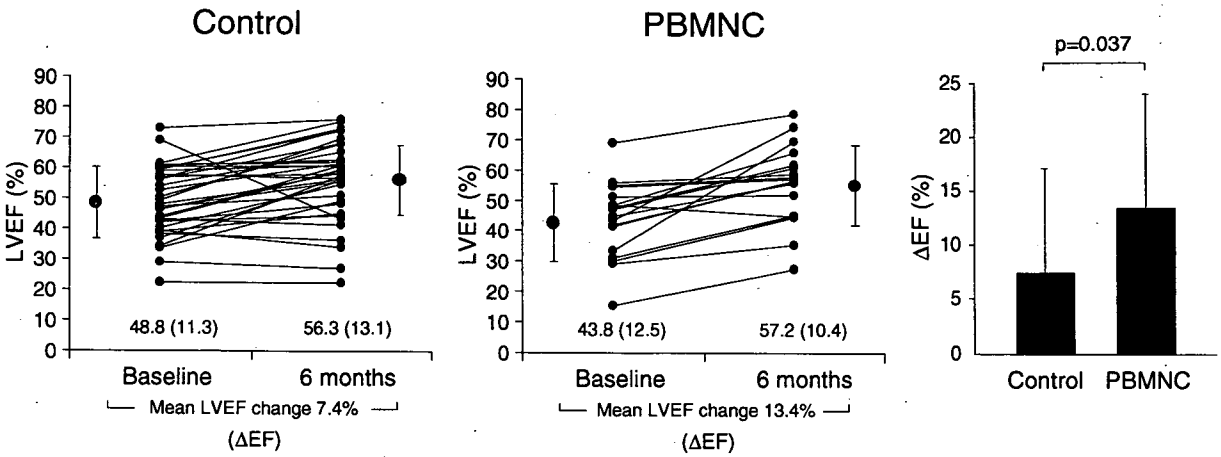

Fig 1. Global left ventricular ejection fraction (LVEF) at baseline and 6 months' follow-up, and the absolute increase in LVEF (Δejection fraction (EF)) in the control and peripheral blood mononuclear cell (PBMNC) groups. Small dots show data for individual patients; large dots show mean values. Vertical bars show SD.

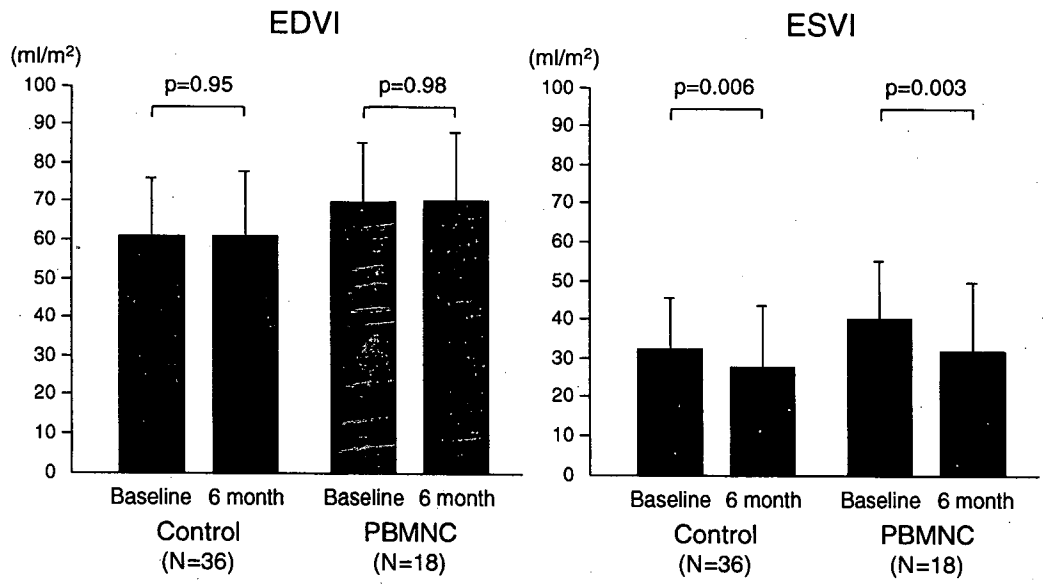

Fig 2. End-diastolic volume index (EDVI) and end-systolic volume index (ESVI) at baseline and 6 months' follow-up. Data are mean ± SD. PBMNC, peripheral blood mononuclear cell.

LVEF, end-diastolic volume index (EDVI), end-systolic volume index (ESVI), or regional ejection fraction (EF) between the control and PBMNC groups. Fig 1 illustrates LV function as assessed by cineventriculography at baseline and 6-months' follow-up. In the control group, LVEF was 48.8% at baseline and gradually increased to 56.3% after 6 months. In contrast, LVEF was 43.8% at baseline and increased to 57.2% after 6 months in the PBMNC group. Although the baseline measurement of LVEF did not differ significantly between the 2 groups, the absolute increase in LVEF (ΔEF) was 7.4% in the control group and 13.4% in the PBMNC group. Our data therefore show that cell transplantation significantly improved LVEF, and there was a modest but significant increase in global LVEF, even in the patients with PCI alone (ie, controls). It is notable that the ΔEF value in thePBMNC group was significantly greater than that in the control group (PCI alone) (Fig 1).

There were no significant difference in EDVI between baseline and 6 months' follow-up in either control or PBMNC group (Fig 2) and also no significant difference in the absolute difference in EDVI (ΔEDVI) between the control and PBMNC groups (p=0.90). ESVI was significantly decreased from baseline to 6 months' follow-up in both groups, such that the absolute difference in ESVI (ΔESVI) was –4.3% in the control group and –8.2% in the PBMNC group. Thus, although there was no statistically significant difference in ΔESVI value between the 2 groups (p=0.09), the ΔESVI value tended to be lower in the PBMNC group, compared with controls. Selective analysis of the infarcted zone showed that baseline measurements of regional wall motion (regional EF), that is, segments #2 and #3 of the AHA classification, did not differ significantly between the control and PBMNC groups (Table 3). Although regional wall motion in the infarct area was significantly improved from baseline to 6 months' follow-up in both groups, the absolute value of regional EF (ΔRegional EF)

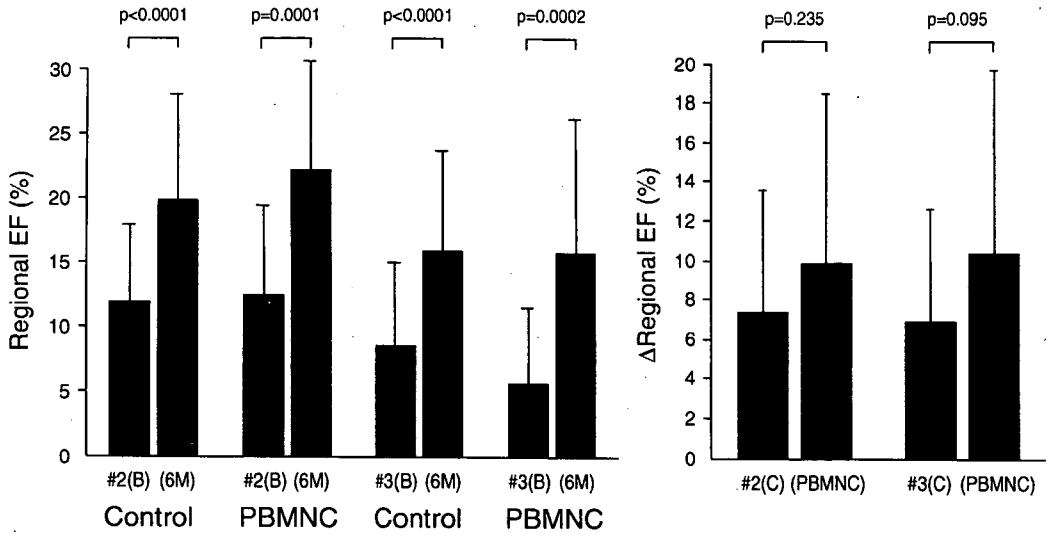

Fig 3. Regional ejection fraction (EF) at baseline and 6 months' follow-up, and the absolute increase in regional EF (ΔRegional EF) in the control and peripheral blood mononuclear cell (PBMNC) groups. Regional EF in the infarct area (segments #2 and #3) was estimated as described in Methods. Data are mean±SD.

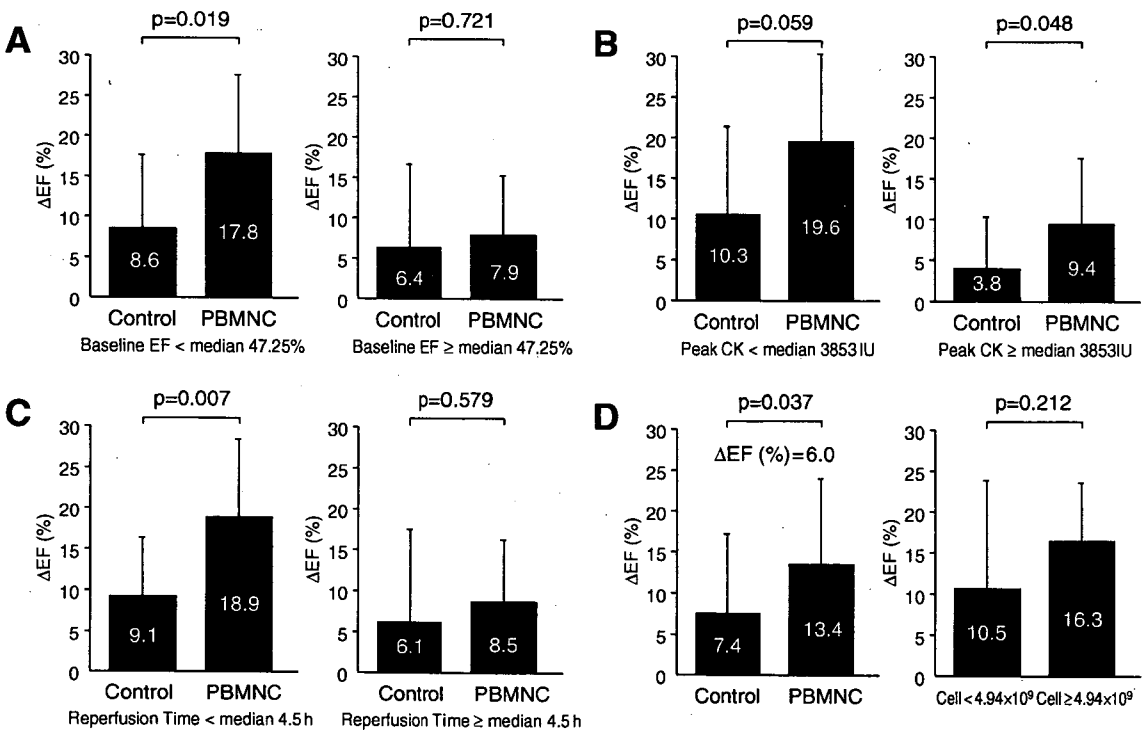

Fig 4. Impact of baseline ejection fraction (EF), peak creatine kinase (CK), reperfusion time, and transplanted cell number on cardiac function. All patients were divided into 2 groups by median baseline EF (A), median CK (B), median reperfusion time (C), and median transplanted cell number (D), and the treatment effect of peripheral blood mononuclear cell (PBMNC) infusion on the absolute increase in left ventricular EF (ΔEF) was analyzed. Data are mean±SD.3

tended to be greater in PBMNC group, compared with control (Fig 3).

Effects of Cell Transplantation on Other Parameters

Resting echocardiography indicated that cell transplantation significantly decreased the WMSI from baseline (1.67±0.18) to 6 months' follow-up (1.50±0.34) and this improvement in regional wall motion was especially seen in the infarct-related area of 15 of 18 patients. Resting ^{99m}Tc-tetrofosmin gated SPECT also showed that cell transplantation significantly decreased the perfusion defect score from baseline (20.4±9.0) to 6 months' follow-up (14.1±9.4), and this improvement in regional myocardial perfusion was seen in the infarct-related area of 14 of 18 patients. In con-

trast, cell transplantation did not significantly exacerbate but rather tend to decrease the Holter Lawn Class (data not shown).

Impact of Baseline Parameters and on Cardiac Function

We observed that cell transplantation significantly improved the ΔEF value, compared with controls (Fig 1). We further analyzed the effect of baseline parameters on the absolute increase in LVEF. In order to avoid our "arbitrary decision" on subgroup analysis, the total patient population was dichotomized according to the "median values" of baseline EF, peak CK, reperfusion time, and transplanted cell number at baseline, as previously reported[21] We then reanalyzed the data for addressing the clinical relevance of PBMNC administration.

We first examined the impact of baseline EF on cardiac function. When we divided all the patients into 2 groups by median baseline EF 47.25%, there was a significant interaction between the treatment effect of PBMNC infusion and the baseline EF. Among patients with a baseline EF below the median value, patients in the PBMNC group had an absolute increase in LVEF (ΔEF value) that was 2-fold that of the control group (Fig 4A) (absolute difference, 9.2%; 95% confidence interval (CI), 5.3 to 13.1). In contrast, among patients with a baseline EF at or above the median, the absolute difference between the 2 groups was only 1.5% (absolute difference, 1.5%; 95% CI, −2.1 to 5.1), suggesting that cell transplantation preferentially improved LV function in patients with relatively depressed contractility.

We next examined the impact of peak CK on cardiac function. When we divided all the patients into 2 groups by median peak CK 3,853 IU/dl, there was again a significant interaction between the treatment effect of PBMNC infusion and the peak CK. Among patients with a baseline peak CK at or above the median value, those in the PBMNC group had an absolute ΔEF value that was more than 2-fold the value for the control group (Fig 4B) (absolute difference, 5.6%; 95% CI, 2.8 to 8.4). Among patients with a baseline peak CK below the median, those in the PBMNC group also had an absolute ΔEF value that was ≈2-fold that in the control group, although there was not a significant difference in the ΔEF value between the 2 groups. The data suggest that cell transplantation preferentially improved LV function irrespective of infarct size.

We also examined the impact of reperfusion time on cardiac function. When we divided all the patients into 2 groups by a median reperfusion time of 4.5 h, there was again a significant interaction between the treatment effect of PBMNC infusion and the reperfusion time. Among patients with a baseline reperfusion time below the median value, those in the PBMNC group had an absolute ΔEF value that was almost 2-fold that in the control group (Fig 4C) (absolute difference, 9.8%; 95% CI, 6.1 to 13.5). In contrast, among patients with a baseline reperfusion time at or above the median, the absolute difference between the 2 groups was only 2.4% (absolute difference, 2.4%; 95% CI, −1.6 to 13.8), suggesting that cell transplantation preferentially improved LV function in patients with relatively early reperfusion.

We further examined the impact of transplanted cell number on cardiac function. When we divided the patients receiving cell therapy into 2 groups by a median cell number of 4.94×10^9, there was no significant interaction between the treatment effect of PBMNC infusion and the number of transplanted cells, suggesting that cell number did not significantly affect LV function, at least in our study (Fig 4D).

Clinical Manifestations and Adverse Effects

The occurrence of individual major adverse cardiac events of death, recurrence of myocardial infarction, or rehospitalization for heart failure did not differ significantly between the control and PBMNC groups. The rate of in-stent restenosis at the culprit lesion in patients who received PBMNC transplantation was 22.2%, which was not significantly different from that in the control patients (p=0.21).

Discussion

The major finding of the present study is that the intracoronary administration of non-expanded PBMNCs significantly enhanced the recovery of LV contractile function in patients optimally treated for AMI. After 6 months, the absolute increase in LVEF (ΔEF) was significantly higher in the PBMNC group than in controls. The enhanced recovery of LV contractile function after the administration of PBMNCs appeared to be related to a reduction in regional LV dysfunction within the territory of the infarct, because cell therapy resulted in a greater tendency of ΔRegional EF or significant improvement of WMSI and ^{99m}Tc-tetrofosmin perfusion defect score associated with the infarct area, compared with controls. Moreover, intracoronary administration of PBMNCs did not exacerbate LV expansion or high-risk arrhythmia after the infarction. Taken together, our findings indicate that when combined with optimal reperfusion therapy and standard medical treatment, intracoronary administration of PBMNCs is able to enhance the recovery of global and regional LV function after AMI.

Our results of subgroup analysis also provide some meaningful suggestions in the choice of patients for cell therapy against AMI; cell transplantation preferentially improved LV function in patients with relatively depressed contractility, irrespective of infarct size, and with relatively early reperfusion. Thus, patients with relatively early reperfusion and depressed LV contractile function had better improvement in contractile function after the intracoronary administration of PBMNCs. Our data therefore suggest that PBMNC transplantation may rescue dying myocytes that were severely stunned in the infarct border zone, irrespective of the infarct size.

Several lines of evidence suggest that the level of circulating CD34$^+$ EPCs is predictive of future cardiovascular events[30] and that bone marrow-derived CD34$^+$ cells could be important for cardiovascular repair[31] In the present study, we used a mean of 4.92×10^9 PBMNCs containing $\approx6\times10^6$ CD34$^+$ cells for intracoronary injection and obtained an increase of 6% in ΔEF value. In the BOOST and REPAIR-AMI trials, $\approx2.5\times10^9$ unfractionated BMCs and $\approx2.4\times10^8$ Ficoll-separated BMCs (≈2–3×10^6 CD34$^+$ cells) were transplanted, with increases of 6% and 2.5% in ΔEF values, respectively. In contrast, in Janssens's report and the ASTAMI trial, $\approx3\times10^8$ Ficoll-separated BMCs ($\approx2.8\times10^6$ CD34$^+$ cells) and $\approx7\times10^7$ Ficoll-separated BMCs ($\approx0.7\times10^6$ CD34$^+$ cells), respectively, were used, and there was no significant increase in ΔEF value. These data therefore indicate that the total number of injected cells or CD34$^+$ cells does not always correlate with the improvement in cardiac performance after cell transplantation, although trans-

planted cell numbers appear to have been relatively low in the ASTAMI trial. Indeed, cell number did not significantly affect LV function in our study (Fig4). Importantly, our study results also suggests that PBMNCs, which were even not culture-expanded, show great capability as a comparable cell source to BMCs.

In view of BMCs homing into the heart, the microenvironment (eg, niche) within the infarct tissue and the timing of cell delivery may be important for the incorporation of BMCs. Recent observations indicate that after intracoronary transfer only 1.3–2.6% of ^{18}F-FDG-labelled unselected BMMNCs were detected in the infarcted heart, whereas most cells homed into the liver and spleen within ≈1 h after intracoronary delivery[32] Therefore, the findings do not support the likelihood that progenitor cells home into jeopardized myocardium and transdifferentiate into cardiac myocytes capable of generating active force development in scar tissue, but rather suggest other potential mechanisms through angiogenesis and reduced apoptosis. Indeed, recent articles have shed light on the potential of BMCs to differentiate into hematopoietic and endothelial lineages able to secrete proangiogenic factors[33] rather than transdifferentiation into other cell lineages such as cardiac myocytes[34,35] These subsets of mature hematopoietic cells, either derived from bone marrow or peripheral blood, may cooperate with transplanted or resident cardiac and endothelial stem/progenitor cells to enhance their capacity for tissue repair through angiogenesis, anti-apoptosis, and myocyte proliferation after ischemic injury[36,37]

The most potential advantage of our method is its feasibility and safety in collecting PBMNCs from patients with depressed cardiac performance and bleeding tendency, by administration of enough antiplatelet agents, such as aspirin and ticlopidine. Although previous studies emphasize the feasibility and safety of BMCs aspiration, this maneuver is always accompanied by the risk of s serious bleeding accident from bone in patients receiving antiplatelet therapy. There are also a few other reports indicating a benefit of intracoronary infusion of granulocyte colony-stimulating factor (G-CSF) mobilized-PBMNCs for AMI[38–40] However, this procedure still involves several possible adverse effects of G-CSF, including serious thrombosis, bone pain, fever, and aggravation of in-stent restenosis[38–40] In contrast to those previous studies, we could easily collect ≈5×10^9 cells PBMNCs, avoiding contamination with neutrophils, within 2 h without any hemodynamic or bleeding problems. We could concentrate the collected PBMNCs to 10 ml by density gradient centrifugation aseptically through bag to bag, instead of by Ficoll gradient sedimentation methods. Our present data, therefore, show for the first time that intracoronary infusion of non-expanded PBMNCs alone can promote improvement of LV function without any bleeding accident or G-CSF-related serious adverse effects. Because we can easily obtain levels of ≈6×10^6 $CD34^+$ cells, which is higher than in either the BOOST or REPAIR-AMI trials, we never need another laboratory to expand the PBMNCs, giving substantial merit that this protocol can be easily accepted in any hospital worldwide.

A major limitation of our study is that evaluation of the present regeneration therapy was not randomized, double-blind, and controlled. Moreover, cardiac function was not assessed with state-of-the-art imaging modalities, such as magnetic resonance imaging (MRI), and LV angiography was used exclusively for the serial assessment of LV function. Although angiography is well suited to delineate regional contractile function for AMI by LAD occlusion, the use of MRI to assess global LV function would have more precisely depicted changes in the distorted geometry of the infarcted hearts. Although we choose contemporary controls, the control group does not reproduce the exact conditions of the cell therapy group to which the cells were transferred, including PBMNC collection and a placebo intracoronary injection. Therefore, the true benefit of cell transfer can not be fully appreciated and further research is needed to address these issues.

In conclusion, intracoronary infusion of PBMNCs in patients with AMI is associated with improved global LV contractile function; cell therapy preferentially improves LV function in patients with early reperfusion, but relatively depressed contractility after AMI, prevents end-diastolic and end-systolic LV volume expansion, and has not increased any adverse clinical events so far. Transplantation of PBMNCs might be an effective and novel therapeutic option for AMI, if cell transfer occurs expeditiously and in appropriate subjects. This less invasive and more feasible approach to collecting EPCs may be a novel therapeutic option for improving cardiac function after AMI.

Acknowledgments

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