Primining With Angiopoietin-1 Augments the Vasculogenic Potential of the Peripheral Blood Stem Cells Mobilized With Granulocyte Colony-Stimulating Factor Through a Novel Tie2/Ets-1 Pathway

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Background—The low engraftment rate of stem/progenitor cells infused via the intracoronary route to the ischemic myocardium is one of the most important factors limiting the efficacy of cell therapy. We investigated the concept of priming peripheral blood stem cells enriched by granulocyte colony-stimulating factor mobilization and apheresis (mobPBSCs) with angiopoietin-1 (Ang1), to enhance the engraftment into the ischemic tissue and neovasculogenic potential.

Methods and Results—The expression of Tie2, the Ang1 receptor, was significantly higher in mobPBSCs than naïve peripheral blood mononuclear cells (19.2±3.0% versus 1.2±0.8%; P<0.001 for mobPBSCs from acute myocardial infarction (AMI) patients with granulocyte colony-stimulating factor treatment for 3 days versus peripheral blood mononuclear cells from AMI patients versus peripheral blood mononuclear cells from stable angina patients). After 4 hours of cartilage oligomeric matrix protein (COMP)-Ang1 stimulation, mobPBSCs committed to the endothelial lineage with the induction of CD31 and VE-cadherin expression, mediated by Tie2/Ets-1 pathway. Priming of mobPBSCs with COMP-Ang1 induced the expression of α4β1 and α5β1 integrins, which are also Ets-1 downstream molecules, leading to enhanced adhesion to endothelial cells or fibronectin. In a rabbit ear ischemia/reperfusion model, priming of mobPBSCs with COMP-Ang1 improved first-pass engraftment to the distal vascular bed after intraarterial delivery. In a murine ischemic hind-limb model, intravascular delivery of primed mobPBSCs enhanced both engraftment and neovascularization.

Conclusions—The short-term priming with COMP-Ang1 may be a feasible and promising option to activate mobPBSCs by enhancing differentiation and adhesiveness and to improve the efficacy of cell therapy for ischemic diseases. (Circulation. 2009;120:2240-2250.)

Key Words: angiogenesis ■ cell adhesion molecules ■ growth substances ■ ischemia ■ myocardial infarction

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hurdles in improving the efficacy of cell therapy.6,7 Accordingly, we aimed to find a new method of augmenting neovascularization by overcoming the poor engraftment of mobPBSCs into ischemic tissue and enhancing its endothelial lineage commitment.

Clinical Perspective on p 2250

Experimental studies have reported that ex vivo genetic modification and pretreatment with small molecules enhanced cellular function.8,9 Relative to the safety concern of genetic manipulation and the clinical applicability of small molecules, it would be an attractive method to augment engraftment and induce lineage commitment of mobPBSCs by the short-term, protein-based, ex vivo stimulation of stem/progenitor cells. This short-term stimulation for boosting the neovasculogenic potential of mobPBSC is called priming.

Angiopoietin-1 (Ang1) is a growth factor binding to the Tie2 receptor expressed on endothelial cells (ECs) and hematopoietic stem cells. Through Tie2 signaling, Ang1 plays an essential role in postnatal angiogenesis by mediating vessel maturation and maintaining vessel integrity.10 However, little is known about its role in the engraftment and lineage commitment of stem cells and in the modulation of its downstream pathway.

In the present study, we found that mobPBSCs highly expressed Tie2 compared with naïve peripheral blood mononuclear cells (PBMCs) obtained from patients with AMI. Therefore, we investigated the effects of priming naïve PBMCs and mobPBSCs using Ang1 in vitro and in vivo. The short-term Ang1 stimulation of mobPBSCs, but not naïve PBMCs, augmented endothelial lineage commitment and engraftment through the Tie2/Ets-1 signaling pathway and eventually enhanced the neovasculogenic potential in the ischemic tissue.

Methods

All experiments dealing with humans or human products were conducted with informed consent and approved by the Institutional Review Board of Seoul National University Hospital. All animal experiments were performed after receiving approval from the Institutional Animal Care and Use Committee of the Clinical Research Institute of the Seoul National University Hospital and complied with the National Research Council Guidelines for the Care and Use of Laboratory Animals.

Statistical Analysis

One-way ANOVA with Tukey posthoc analysis was used to compare ≥3 groups with regard to continuous response variables, and the

<table>
<thead>
<tr>
<th>Variables</th>
<th>mobPBSC_AMI (n=33), %</th>
<th>PBMC_AMI (n=15), %</th>
<th>PBMC_SA (n=20), %</th>
<th>P</th>
</tr>
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<tr>
<td>AC133</td>
<td>2.3±6.6</td>
<td>0.3±0.2</td>
<td>0.2±0.1</td>
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<tr>
<td>CD34</td>
<td>17.6±12.8†</td>
<td>1.1±0.9†</td>
<td>0.6±0.8†</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>KDR</td>
<td>29.4±21.7†</td>
<td>0.6±0.4†</td>
<td>0.2±0.1†</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CD31</td>
<td>68.2±25.4†</td>
<td>38.0±13.8†</td>
<td>33.5±10.4†</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data presented are mean±SD.

*Non-significant difference between groups based on the Tamhane method.
†Non-significant difference between groups based on the Tamhane method.
‡Non-significant difference between groups based on the Tamhane method.

Table. Flow Cytometry of mobPBSCs and PBMCs

Figure 1. Induction of Tie2 expression on mobPBSCs. A, RT-PCR showed that the expression of Tie2 mRNA in mobPBSCs was significantly higher than that in PBMC_SA and PBMC_AMI. B, Flow cytometry analysis for Tie2 expression showed results similar to the RT-PCR data.

 Pearson χ² test was used to analyze categorical data. In the Table, the differences between 3 groups were analyzed with the Welch test for one-way ANOVA with the Tamhane method because of unequal within-group variances. In the experiment for in vivo perfusion recovery, repeated-measures ANOVA with Scheffé contrasts was used to quantify the main effects of 4 groups and time and significant interactions. The calculation was performed with SAS version 9.1 (SAS Institute, Cary, NC). The other calculations were performed with SPSS version 13.0 (SPSS Inc, Chicago, IL), and values of P<0.05 were considered statistically significant. Detailed experimental methods are described in the online-only Data Supplement.

Results

mobPBSCs Highly Express Tie2 Compared With PBMCs

We obtained mobPBSCs that were PBMCs after 3 days of G-CSF subcutaneous injection from 33 AMI patients enrolled in the Myocardial Regeneration in Acute and Old MI With G-CSF Mobilization and Intra-Coronary Stem Cell Infusion (MAGIC-Cell) program. We also selected PBMCs from 15 AMI patients without G-CSF treatment (PBMC_AMI) as a control for mobilized PBSCs from AMI patients. In addition, we selected PBMCs from 20 patients with stable angina (PBMC_SA) as another control for the disease of AMI. The characteristics of the patients are shown in Table I of the online-only Data Supplement.

We characterized mobPBSCs and PBMCs by flow cytometry analysis. We found that mobPBSCs contained more cells positive for KDR, CD34, and CD133, which are major surrogate markers of endothelial progenitor cells (EPCs), than nonmobilized PBMCs did (the Table). Interestingly, reverse-transcription polymerase chain reaction (RT-PCR) and flow cytometry showed significantly greater Tie2 expression in mobPBSCs than in PBMCs from AMI or SA patients (Figure 1A and 1B; quantitative RT-PCR data are given in Figure I of the online-only Data Supplement). These results encouraged us
to investigate the utility of the Tie2 agonist cartilage oligomeric matrix protein (COMP)-Ang1 (an Ang1 recombinant chimera) as a priming agent.

**Long-Term Stimulation of **

We tested the effect of COMP-Ang1 on the commitment of naïve PBMCs and mobPBSCs, both from AMI patients, into endothelial lineage cells. They were seeded in EPC culture media containing COMP-Ang1 (400 ng/mL), and 5 to 7 days later, clusters of attached cells were observed. The cluster number of naïve PBMCs did not change even after COMP-Ang1 treatment for 7 days compared with vehicle (n=12; \( P=0.65 \); Figure 2A). However, the cluster number of mobPBSCs after COMP-Ang1 treatment for 7 days increased ≈2-fold compared with vehicle (94±18% increase; n=12; \( P<0.01 \); Figure 2B). These clusters were composed of spindle-shaped cells similar to EPCs. To determine the phenotype of the increased clusters in mobPBSC group, we performed immunocytochemical staining using antibodies for CD31, CD34, VE-cadherin, Tie2, CXCR4, and KDR. Clusters were stained positive 7 days after cultivation with COMP-Ang1 (Figure 2C).

**Figure 2.** Long-term stimulation of mobPBSCs with COMP-Ang1 increases the number of endothelial lineage cell clusters, which is not observed in naïve PBMCs. A, After 7 days of culture in EPC culture media, naïve PBMCs showed cell clusters composed of spindle-shaped cells. The number of clusters was not affected by continuous treatment with COMP-Ang1 (400 ng/mL) for 7 days vs vehicle. Scale bar=400 \( \mu \)m. B, However, the number of clusters of mobPBSCs increased after continuous treatment with COMP-Ang1 vs treatment with vehicle alone (n=12; \( *P<0.01 \)). Scale bar=400 \( \mu \)m. C, Immunocytochemical staining of the clusters from mobPBSCs revealed that they were committed to endothelial lineage. Scale bar=100 \( \mu \)m.

**Short-Term Stimulation of mobPBSCs With COMP-Ang1 Increases Endothelial Lineage Commitment Through Tie2/Ets-1 Signaling**

Among the positively stained surface markers of the clusters, KDR, VE-cadherin, and Tie2 are all known to contain Ets-1 transcription factor binding sites in their promoter regions. Thus, we investigated whether the effect of COMP-Ang1 on the lineage commitment of mobPBSCs is mediated via the transcriptional regulation of Ets-1. The gene expression of Ets-1 did not change significantly after the stimulation of naïve PBMCs with COMP-Ang1 (Figure 3A). In mobPBSCs, however, expression of Ets-1 mRNA increased 2 hours after stimulation with COMP-Ang1 (Figure 3B; quantitative RT-PCR data are given in Figure II of the online-only Data Supplement). Furthermore, we pretreated mobPBSCs with anti-Tie2 neutralizing antibody 1 hour before COMP-Ang1 treatment and found that blockage of Tie2 receptor reduced the expression of Ets-1 mRNA (Figure 3C; quantitative RT-PCR data are given in Figure III of the online-only Data Supplement).

Next, we examined whether short-term stimulation of naïve PBMCs or mobPBSCs with COMP-Ang1 might induce...
endothelial lineage commitment. In naïve PBMCs, COMP-Ang1 treatment for 4 hours did not affect the expression of endothelial specific genes such as CD31 and VE-cadherin compared with vehicle treatment (Figure 3D and 3E). In mobilized PBSCs, however, COMP-Ang1 treatment resulted in stronger expression of CD31 and VE-cadherin, which was reversed by antisense Ets-1 oligodeoxynucleotides (Figure 3F; quantitative RT-PCR data are given in Figure IV of the online-only Data Supplement). These results were confirmed at the protein level by flow cytometry analysis (Figure 3G) in which short-term treatment with COMP-Ang1 increased the expression of CD31 and VE-cadherin by 51 ± 10% and 118 ± 13%, which was abrogated by transfection of mobilized PBSCs with antisense Ets-1 oligodeoxynucleotides by 56 ± 7% and 46 ± 5%.

**Figure 3.** Priming of mobilized PBSCs with COMP-Ang1 increases endothelial lineage commitment through Ets-1 and Tie2 signaling, which is not observed in naïve PBMCs. A, Temporal expression of Ets-1 in naïve PBMCs treated with COMP-Ang1 was evaluated by RT-PCR. Treatment with COMP-Ang1 did not significantly change Ets-1 expression. M indicates the molecular weight marker. B, RT-PCR addressed that treatment of mobilized PBSCs with COMP-Ang1 increased Ets-1 expression within 2 hours and sustained the expression for 10 hours. C, Ets-1 expression was mediated through Tie2 receptor. RT-PCR showed that Ets-1 mRNA expression increased in mobilized PBSCs treated with COMP-Ang1 for 4 hours, whereas 1-hour pretreatment with anti-Tie2 neutralizing antibody before COMP-Ang1 treatment prevented Ets-1 upregulation. D, RT-PCR showed that the expression of CD31 and VE-cadherin mRNA in naïve PBMCs did not change after treatment with COMP-Ang1 for 4 hours. E, Flow cytometry analysis showed results similar to RT-PCR. In naïve PBMCs, COMP-Ang1 did not affect the expression of CD31 or VE-cadherin. F, However, COMP-Ang1 stimulated the commitment of mobilized PBSCs to endothelial lineage, which was mediated by Ets-1 signaling. RT-PCR showed that the expression of CD31 and VE-cadherin mRNA was elevated after COMP-Ang1 stimulation for 4 hours. However, these upregulations were prevented by the prior transfection of antisense Ets-1 oligodeoxynucleotides. G, Flow cytometry analysis confirmed the expression at the protein level.

**Priming of mobilized PBSCs With COMP-Ang1 Activates α4β1 and α5β1 Integrins**

As shown above, the short-term stimulation of mobilized PBSCs with COMP-Ang1 induced Ets-1 expression. Moreover, a previous report showed that β3 integrin was downstream of Ets-1 activation. Therefore, we postulated that other integrins that contain an Ets-1 binding motif in their promoter region may be activated by COMP-Ang1 such as α4β1 and α5β1 integrins, both of which are known to play key roles in the homing and retention of stem/progenitor cells in bone marrow. Thus, we measured the expression of these integrins after short-term treatment with COMP-Ang1. The stimulation of naïve PBMCs with COMP-Ang1 for 4 hours did not change the mRNA and protein expression of α4, α5, and β1 integrin (Figure 4A and 4B). However, stimulation of mobilized PBSCs with COMP-Ang1 increased the expression of α4, α5, and β1 integrin (flow cytometry analysis: 107 ± 39%, 51 ± 24%, and 78 ± 21% increase for α4, α5, and β1 integrin, respectively; Figure 4C and 4D; quantitative RT-PCR data are given in Figure V of the online-only Data Supplement), and antisense Ets-1 oligodeoxynucleotides attenuated this expression (flow cytometry analysis: 45 ± 3%, 29 ± 7%, and 36 ± 9% decrease for α4, α5, and β1 integrin, respectively). We also investigated the expression of β2 integrin, which is another key molecule for EPC homing, but its mRNA and protein expression did not change significantly after COMP-Ang1 treatment in both the naïve PBMC and mobilized PBSC groups even though it was highly expressed at baseline.

To correlate these findings with functional changes in mobilized PBSCs, we performed in vitro adhesion assays between α4β1 on mobilized PBSCs and vascular cell adhesion molecule-1 on human umbilical vein ECs (HUVECs) and between α5β1 on mobilized PBSCs and fibronectin. COMP-Ang1 stimulation for 4 hours increased mobilized PBSC adhesion to HUVECs (150 ± 19% increase) and fibronectin (152 ± 9% increase; Figure 4E and 4F), whereas pretreatment with anti-Tie2 neutralizing antibody or anti-integrin antibody (anti-α4 or -α5) before COMP-Ang1 stimulation abrogated the increased adhesion, suggesting a Tie2-dependent increase in cell-to-cell and cell-to-matrix adhesion after COMP-Ang1 stimulation.
Priming of \textsuperscript{mob}PBSCs With COMP-Ang1 Increases Matrigel Tube Formation and Incorporation Ability of \textsuperscript{mob}PBSCs

Next, we investigated whether short-term stimulation of \textsuperscript{mob}PBSCs with COMP-Ang1 might induce vasculogenesis even though there was no continuous stimulation with the growth factor. When primed \textsuperscript{mob}PBSCs were cocultured with HUVECs, \textsuperscript{mob}PBSCs and HUVECs produced significantly more complete tubes as determined by tube number, area, and length after 3 days (59±5%, 35±10%, and 37±5% increase for tube number, area, and length, respectively; Figure 5A). Again, pretreatment with anti-Tie2 neutralizing antibody before COMP-Ang1 stimulation abrogated tube formation of \textsuperscript{mob}PBSCs treated with COMP-Ang1 (44±8%, 35±11%, and 36±9% decrease for tube number, area, and length, respectively). In addition, more \textsuperscript{mob}PBSCs were incorporated into

![Figure 4](http://circ.ahajournals.org/)

**Figure 4.** Activation of \( \alpha4 \) and \( \alpha5 \) integrins in \textsuperscript{mob}PBSCs by COMP-Ang1, which is not observed in naïve PBMCs. A, RT-PCR showed that the stimulation of naïve PBMCs with COMP-Ang1 for 4 hours did not significantly change the expression of \( \alpha4 \), \( \alpha5 \), \( \beta1 \), and \( \beta2 \) integrin. B, Flow cytometry analysis showed results similar to RT-PCR. C, RT-PCR showed that the stimulation of \textsuperscript{mob}PBSCs with COMP-Ang1 for 4 hours increased the expression of \( \alpha4 \), \( \alpha5 \), and \( \beta1 \) integrin, but this was prevented by prior transfection with antisense Ets-1 oligodeoxynucleotides. However, \( \beta2 \) integrin mRNA expression was not affected by COMP-Ang1 or by antisense Ets-1 oligodeoxynucleotide transfection. D, Flow cytometry analysis confirmed the above results at the protein level. E, COMP-Ang1 stimulation for 4 hours induced \textsuperscript{mob}PBSC adhesion to HUVEC monolayers (n=4 each; \(*P<0.01\) vs vehicle). Pretreatment of anti-Tie2 neutralizing antibody or anti-\( \alpha4 \) integrin (a known countermolecule for vascular cell adhesion molecule-1) antibody prevented this adhesion (\#P<0.01 vs COMP-Ang1). F, COMP-Ang1 stimulation induced \textsuperscript{mob}PBSC adhesion to fibronectin (n=4 each; \(*P<0.01\) vs control). However, pretreatment with anti-Tie2 neutralizing antibody or anti-\( \alpha5 \) integrin (a known countermolecule for fibronectin) antibody prevented this adhesion (\#P<0.01 vs COMP-Ang1).
Matrigel tubes when primed with COMP-Ang1, which was retarded when pretreated with anti-Tie2 neutralizing antibody (207±28% increase by priming and 66±2% decrease by Tie2 pre-blocking; Figure 5B).

Priming of moPBSCs With COMP-Ang1 Enhances the First-Pass Engraftment Into the Distal Vascular Bed of a Rabbit Ear Ischemia/Reperfusion Model After Intraarterial Delivery

Next, we investigated whether short-term stimulation with COMP-Ang1 may enhance the engraftment of PBMCs in the distal ischemic tissue after intraarterial delivery. For this examination, we developed a rabbit ear model of ischemia and reperfusion (Figure 6A). Scintigrams of ischemic rabbit ear after infusion of naïve PBMCs and unprimed moPBSCs showed scanty engraftment in the ischemic ear (Figure 6B and 6C). In contrast, moPBSCs primed with COMP-Ang1 showed markedly increased engraftment by 53±33% (n=6; P<0.01), which was reversed by pretreatment of moPBSCs with anti-Tie2 neutralizing antibody before COMP-Ang1 priming. Specific examinations of the tissue with immunofluorescent staining showed the same results (Figure VI of the online-only Data Supplement).

Priming of moPBSCs With COMP-Ang1 Enhances Engraftment and Neovascularization of the Ischemic Limb After Arterial Infusion

We next investigated whether injection of COMP-Ang1–primed moPBSCs enhanced neovascularization in vivo using a hind-limb ischemia model. COMP-Ang1–primed moPBSCs from patients were administered via intracardiac puncture into athymic nude mice after the induction of hind-limb ischemia. COMP-Ang1–primed moPBSC infusion improved limb perfusion compared with unprimed moPBSC infusion (perfusion ratio of ischemic to nonischemic limbs at day 21, 49.3±13.8% versus 34.9±3.0%; n=10 each, P<0.01 for primed versus unprimed moPBSC infusion), which was reversed by pretreatment with anti-Tie2 neutralizing antibody (perfusion ratio at day 21, 33.6±7.4%; n=10; P<0.05 versus...
COMP-Ang1–primed group; Figure 7A). Moreover, we assessed the neovascularogenic effect of PBMC_AMI. The naïve PBMCs were inferior to even unprimed mobPBSCs in terms of blood flow recovery (perfusion ratio ischemic to nonischemic limbs at day 21, 22.6% vs 34.9%; n = 10 each; \( P < 0.05 \) for naïve PBMCs versus unprimed mobPBSC infusion).

Histologically, we observed more capillaries in the ischemic limb that received COMP-Ang1–primed cells (192 ± 15% increase; \( n = 6 \); \( P < 0.01 \); Figure 7B and 7C). Next, to determine the contribution of engrafted cells to neovascularization, we checked cellular engraftment using carboxyfluorescein succinimidyl ester (CFSE)–labeled mobPBSCs and performed immunohistochemical staining with human-specific and vessel markers. More CFSE-labeled mobPBSCs (Figure 7D and 7E) and human-specific von Willebrand factor– and HLA-positive cells (Figure 7F and Figure VII of the online-only Data Supplement) were detected in the ischemic hind limb treated with COMP-Ang1–primed cells than with the unprimed cells. Furthermore, confocal analysis of immunofluorescent staining demonstrated that there were more cells double positive for human HLA and CD31 (Figure 7G) in the COMP-Ang1 priming group. Because cell-to-cell adhesion through binding of α4β1 integrins with vascular cell adhesion molecule-1 plays a greater role in the homing of injected PBSCs than cell-to-fibronectin adhesion,\(^{14}\) we examined the expression of α4 integrin. We found that α4-positive cells increased in the ischemic hind limb treated with COMP-Ang1–primed mobPBSCs. The cells positive for α4 integrin were colocalized with human-specific HLA and engrafted into areas of vascular regeneration. Pretreatment of anti-Tie2 neutralizing antibody reversed this phenomenon (Figure 7H).

**Systemic Administration of G-CSF Does Not Enhance Vasculogenesis in Mouse Ischemic Hind Limb but COMP-Ang1 Does**

We also compared the effects of systemic administration of COMP-Ang1 and G-CSF on the vasculogenesis in the mouse ischemic hind-limb model. On day 3 after the intravenous injection of recombinant adenovirus-expressing COMP-Ang1 or LacZ (vehicle), C57BL/6 mice underwent unilateral femoral artery excision (\( n = 6 \) in each group). Then, G-CSF or sterile saline was injected subcutaneously for 3 days (vehicle group, Ade-LacZ plus saline injection; COMP-Ang1 group, Ade-COMP-Ang1 plus saline injection; G-CSF group, Ade-LacZ plus G-CSF injection; combined cytokine group, Ade-COMP-Ang1 plus G-CSF injection).

When followed up at 14 days, the systemic administration of G-CSF did not improve neovascularization compared with vehicle, whereas COMP-Ang1 significantly improved perfusion. The combined cytokine group showed a result similar to the COMP-Ang1 alone group, suggesting no positive interaction between systemic administration of G-CSF and COMP-Ang1 (the perfusion ratio of ischemic to nonischemic limbs at day 14, 59.1 ± 3.6% versus 71.4 ± 4.8% versus 56.8 ± 4.1% versus 71.8 ± 3.6%; \( n = 6 \) each; \( P < 0.01 \) for vehicle versus COMP-Ang1 versus G-CSF versus combined cytokine; Figure 8A and 8B). In the histological analysis, systemic administration of G-CSF did not increase capillary density, whereas COMP-Ang1 or combined cytokines increased it compared with vehicle (78 ± 10% increase by COMP-Ang1 and 67 ± 10% increase by combined cytokines; \( n = 6 \) each; \( P < 0.01 \) versus vehicle by 1-way ANOVA with Tukey analysis; Figure 8C).

**Discussion**

Ang1 has been reported to be important for blood vessel formation in the embryonic period, and blood vessels in the Ang1–/– mice had fewer ECs and showed defects in the association of the endothelium with the extracellular matrix and vessel rupture.\(^{15}\) This phenomenon suggests the possibility that Ang1 contributes to the differentiation and homing of primitive stem cells that participate in new vessel formation. The recently developed synthetic COMP-Ang1, an Ang1
Figure 7. Effect of \( \text{mobPBSCs} \) primed with COMP-Ang1 on neovascularization in a murine model of hind-limb ischemia. A, Perfusion ratio of ischemic to nonischemic limb measured by laser Doppler perfusion image analyzer. Repeated-measures ANOVA with Scheffé contrast showed a significant perfusion improvement in the COMP-Ang1–primed mobPBSC group (\( P<0.05 \)). Data are mean±SEM. \( n=10 \) in each group. †\( P<0.05 \) vs unprimed mobPBSC group. B and C, Treatment with COMP-Ang1–primed mobPBSCs generated more capillaries in the ischemic limbs than unprimed mobPBSCs (\( n=6 \) each; *\( P<0.01 \) vs control; #\( P<0.01 \) vs COMP-Ang1). Red indicates BS-1 lectin for murine vessels; blue, DAPI for nuclei. D and E, CFSE-labeled mobPBSCs (green) primed with COMP-Ang1 were observed more...
variant that is both soluble and stable, is known to be more potent than native Ang1 in activating Tie2 in ECs and to augment new vessel formation in various disease models. The rationale for using COMP-Ang1 as a priming agent was our new finding that mbPBSCs express high levels of Tie2. We hypothesized that because of the high expression of the Tie2 receptor in mbPBSCs, these cells would be excellent candidates to embrace the various beneficial effects of COMP-Ang1. Our data showed that endothelial lineage commitment and functional upregulation of integrins occurred within 4 hours of treatment in the mbPBSC group, which means that priming of mbPBSCs can be performed in a short-term and simple manner and may be a clinically feasible method.

Neovascularization can be mediated by the vasculogenic potential of mbPBSCs, which can differentiate and acquire endothelial phenotype. G-CSF is well known to potently mobilize hematopoietic stem cells from bone marrow. Moreover, AC133CD34 cells, subsets of mbPBSCs, are considered to have the potential to differentiate to ECs and the ability to home to ischemic sites, resulting in neovascularization in adults. Thus, we hypothesized that enhancement of the endothelial commitment of mbPBSCs could augment neovascularization in ischemic tissue. In this study, the culture of mbPBSCs with COMP-Ang1 induced more EPC clusters compared with vehicle. In addition, priming of mbPBSCs with COMP-Ang1 for 4 hours enhanced the endothelial commitment of mbPBSCs and vasculogenesis in the

Figure 7 (Continued). Frequently in the ischemic muscle at day 21. n=6 in each group. *P<0.01 vs control; †P<0.01 vs COMP-Ang1. F, Immunohistochemical staining showed that human-specific von Willebrand factor (vWF)–positive cells were significantly increased in ischemic limb of a nude mouse treated with COMP-Ang1–primed mbPBSCs from patients. Scale bars=50 μm. G, Confocal analysis showed that COMP-Ang1–primed mbPBSCs from patients were committed to the endothelial lineage in nude mouse ischemic hind limb. Green indicates CD31; red, anti-HLA; and blue, DAPI. Scale bars=20 μm. H, Representative confocal images indicated that COMP-Ang1–primed mbPBSCs from patients were engrafted to nude mouse ischemic muscle through the activation of α4 integrins. Green indicates α4 integrins; red, anti-HLA; and blue, DAPI. Scale bars=20 μm.

Figure 8. In vivo vasculogenic effects of systemic administration of COMP-Ang1, G-CSF, or combination in a hind-limb ischemia model of C57BL/6 mice. A, Representative limb perfusion images obtained by laser Doppler perfusion imager. B, The quantified ratios of the perfusion of ischemic and nonischemic limbs. Repeated-measures ANOVA with Scheffé contrast showed an improvement in perfusion in the COMP-Ang1 group and combined cytokine group (P<0.05). However, it did not show the difference between the 2 groups at day 14. †P<0.05 vs vehicle group. C, Quantitative analysis of capillary density. Systemic administration of COMP-Ang1 enhanced the vasculogenesis of the ischemic limb vs the vehicle group; systemic administration of G-CSF did not. Combination treatment showed results comparable to COMP-Ang1, suggesting no positive interaction between COMP-Ang1 and G-CSF. Data are mean±SEM. n=6 in each group. *P<0.01 vs control; †P<0.01 vs COMP-Ang1.
Matrigel. In a nude mouse hind-limb model, animals treated with \(^{\text{m}0}\)PBSCs primed by COMP-Ang1 showed more capillaries that were composed of injected \(^{\text{m}0}\)PBSCs than animals treated with unprimed \(^{\text{m}0}\)PBSCs. To the best of our knowledge, this is the first report to demonstrate the effect of COMP-Ang1 on \(^{\text{m}0}\)PBSC commitment to endothelial lineage cells.

The direct physical incorporation of \(^{\text{m}0}\)PBSCs into the ischemic site is another important process for neovascularization.\(^{20}\) However, the mechanisms whereby \(^{\text{m}0}\)PBSCs home to sites of ischemia are unclear. Before stem/progenitor cells are mobilized to the circulation, they interact with the bone marrow microenvironment.\(^{21}\) Ang1 is released from the microenvironment and contributes to the retention of PBSCs in the marrow by Tie2 and integrin signaling.\(^{22,23}\) Here, we hypothesized that the interaction between Ang1 and Tie2 and subsequent activation of integrins would be important not only for retention in bone marrow but also for stem-cell homing to injured myocardium. In this study, the transcription of \(\alpha_4,\ \alpha_5,\) and \(\beta_1\) integrins was upregulated after short-term COMP-Ang1 treatment. Moreover, COMP-Ang1-primed \(^{\text{m}0}\)PBSCs were incorporated more abundantly into the tube formation process. In addition, COMP-Ang1 priming increased first-pass engraftment of \(^{\text{m}0}\)PBSCs in the distal vascular bed after intraarterial delivery in a rabbit ear model. In a murine hind-limb ischemia model, we showed greater engraftment of injected human \(^{\text{m}0}\)PBSCs treated with COMP-Ang1 in areas of new vessel growth, and this phenomenon was mediated by the expression of \(\alpha_4\) integrin in response to COMP-Ang1. These findings suggest that priming with Ang1 would play an important role in the initial engraftment of \(^{\text{m}0}\)PBSCs to ischemic tissue by upregulating those integrins and would help to overcome the poor engraftment of \(^{\text{m}0}\)PBSCs, which would be useful for clinical settings when cells are delivered through artery.

Among the homing mechanisms of stem/progenitor cells, the interaction between stromal cell--derived factor 1 and CXCR4 also has been known to play an important role in homing to injured myocardium.\(^{24}\) Recently, Honold et al\(^{25}\) reported that in the situation of mobilization with G-CSF, CXCR4 on the surface of mobilized EPCs may be degraded, leading to the decreased efficiency of homing to ischemic tissue driven by stromal cell--derived factor 1. However, our results showed that Ang1-primed \(^{\text{m}0}\)PBSCs were very effective in promoting neovascularization even if they were mobilized by G-CSF. The reason for the difference between 2 studies may be explained as follows. First, we selected patients with AMI rather than with chronic ischemic heart disease. Second, freshly isolated \(^{\text{m}0}\)PBSCs were different from EPCs obtained several days after ex vivo cultivation. Third, they did not present the total amount of CXCR4 in EPCs, even though some might be disrupted after G-CSF mobilization. We found that the total number of CXCR4 receptors in \(^{\text{m}0}\)PBSCs was \(\approx 3\)-fold higher than in nonmobilized PBMCs (CXCR4-positive fraction in flow cytometry analysis: \(34.2\pm 16.7\%\) versus \(11.7\pm 8.8\%\); \(P<0.01\) for \(^{\text{m}0}\)PBSCs versus PBMC_AMI; Figure VIII of the online-only Data Supplement). Therefore, the total number of the functionally active CXCR4 receptors in \(^{\text{m}0}\)PBSCs may not be lower or even higher than that of naïve PBMCs. Finally, this new priming strategy with COMP-Ang1 for G-CSF-mobilized stem/progenitor cells, including EPCs, may turn on the other homing mechanism mediated by integrin--vascular cell adhesion molecule-1 or integrin-fibronectin, which can overcome the possible impaired interaction between stromal cell--derived factor 1 and CXCR4.

Another novel finding of the present study is that the effect of COMP-Ang1 is mediated through Tie2/Ets-1 signaling. Ets-1 is the prototype of the Ets family transcription factors and is well known to be expressed in ECs during angiogenesis.\(^{11}\) However, few studies have addressed the effect of Ets-1 transcription factor in \(^{\text{m}0}\)PBSCs on adult vasculogenesis. Previous reports documented that Ets-1 increased the expression of some matrix metalloproteinases, urokinase-type plasminogen activator, and integrin \(\beta_3\) in ECs.\(^{26}\) Other reports showed that VE-cadherin and endothelium-specific receptor-type tyrosine kinases such as Flt-1, KDR, Tie1, and Tie2 contained the Ets binding motif in their promoter enhancer regions in ECs, and Ets family transcription factors were suggested to stimulate their promoter activities.\(^{14}\) In the present study, the transcription of Ets-1 in \(^{\text{m}0}\)PBSCs significantly increased after COMP-Ang1 treatment, which was reversed after Tie2 blocking, indicating that Ets-1 is downstream of the Tie2 signaling. In addition, CD31 and VE-cadherin, markers of endothelial commitment, which were upregulated after the treatment of \(^{\text{m}0}\)PBSCs with COMP-Ang1, were downregulated after antisense Ets-1 treatment, suggesting that the endothelial commitment of \(^{\text{m}0}\)PBSCs by COMP-Ang1 is dependent on Ets-1 signaling. Relative to the expression of various integrins, Ets-1 blockade reversed the upregulation of \(\alpha_4,\ \alpha_5,\) and \(\beta_1\) integrins by COMP-Ang1, suggesting that the expression of integrins is also under the regulation of Ets-1 signaling. Therefore, Ets-1 may be an important mediator of the proangiogenic effects of priming of \(^{\text{m}0}\)PBSCs with COMP-Ang1.

Conclusions

Our results show that priming of \(^{\text{m}0}\)PBSCs with COMP-Ang1, via the Tie2/Ets-1 pathway, significantly induces their endothelial commitment and upregulates their expression of integrins, leading to enhancement of engraftment even after intravascular delivery and new vessel formation in the ischemic tissue (Figure IX of the online-only Data Supplement). These data suggest that short-term priming of stem/progenitor cells with COMP-Ang1 can be a feasible and promising option to functionally augment \(^{\text{m}0}\)PBSCs and to enhance the therapeutic efficacy of cell therapy in clinical situations.

Sources of Funding

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Disclosures

None.
CLINICAL PERSPECTIVE

Although intracoronary cell infusion is the most popular method of cell delivery for cardiovascular disease because of its convenience, its efficacy is limited by the poor engraftment rate to ischemic myocardium after intracoronary delivery. We found that peripheral blood stem cells mobilized by granulocyte colony-stimulating factor (mPBSCs) expressed high levels of the Tie2 receptor compared with naïve peripheral blood mononuclear cells, suggesting that angiopoietin-1 could be a very specific priming agent for mPBSCs. Primed cells with angiopoietin-1 increased the expression of adhesion levels of the Tie2 receptor compared with naïve peripheral blood mononuclear cells, suggesting that angiopoietin-1 could be a very specific priming agent for mPBSCs. Primed cells with angiopoietin-1 increased the expression of adhesion levels of the Tie2 receptor compared with naïve peripheral blood mononuclear cells, suggesting that angiopoietin-1 could be a very specific priming agent for mPBSCs. Primed cells with angiopoietin-1 increased the expression of adhesion levels of the Tie2 receptor compared with naïve peripheral blood mononuclear cells, suggesting that angiopoietin-1 could be a very specific priming agent for mPBSCs. Primed cells with angiopoietin-1 increased the expression of adhesion levels of the Tie2 receptor compared with naïve peripheral blood mononuclear cells, suggesting that angiopoietin-1 could be a very specific priming agent for mPBSCs. Primed cells with angiopoietin-1 increased the expression of adhesion levels of the Tie2 receptor compared with naïve peripheral blood mononuclear cells, suggesting that angiopoietin-1 could be a very specific priming agent for mPBSCs.

### References


Primed With Angiopoietin-1 Augments the Vasculogenic Potential of the Peripheral Blood Stem Cells Mobilized With Granulocyte Colony-Stimulating Factor Through a Novel Tie2/Ets-1 Pathway
Min-Seok Kim, Choon-Soo Lee, Jin Hur, Hyun-Jai Cho, Soo-In Jun, Tae-Youn Kim, Sae-Won Lee, Jung-Won Suh, Kyung-Woo Park, Hae-Young Lee, Hyun-Jae Kang, Dong-Soo Lee, Gou-Young Koh, Hironori Nakagami, Ryuichi Morishita, Young-Bae Park and Hyo-Soo Kim

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SUPPLEMENTAL MATERIAL

Expanded Methods

The collection of $^{\text{mob}}$PBSCs and PBMCs:

Peripheral blood stem cells enriched by G-CSF mobilization and apheresis ($^{\text{mob}}$PBSCs): Between December 2004 and July 2006, 33 patients with AMI were enrolled in the MAGIC-Cell program.\(^1\) AMI was defined within 14 days from the onset of a new ST-segment elevating infarction. After revascularization, patients underwent daily subcutaneous injections of G-CSF (Dong-A Pharmaceutical, Seoul, Korea) at 10 $\mu$g/kg body weight for 3 days. $^{\text{mob}}$PBSCs were obtained using a COBE spectra apheresis system (COBE BCT. Inc., Lakewood, CO, USA) using the mononuclear cell collection method. Obtained cells were immediately used for experiments with or without COMP-Ang1-priming for 4 hours (2–10 hours depending on the experiments). In experiments to test the endothelial commitment by Ang1-priming, cells were cultured for 7 days to evaluate the expression of endothelial-specific markers.

Peripheral blood mononuclear cells (PBMCs): Peripheral blood (20 mL) was obtained from 15 patients with acute myocardial infarction (PBMC_AMI) or 20 patients with stable angina (PBMC_SA) with informed consent. The mononuclear cells were fractionated from other components of peripheral blood by centrifugation on Histopaque 1077 (Sigma, St. Louis, MO)
as described previously. Baseline clinical characteristics of enrolled patients were described in Supplementary Table 1.

\( \text{mob} \text{PBSCs (rabbit)}: \) male New Zealand White rabbits (3-3.5kg; Yonam Laboratory Animals, Korea) received G-CSF (Dong-A Pharmaceutical, Seoul, Korea) subcutaneously at 100 μg for 3 days. And then, peripheral blood (70 cc) mononuclear cells were isolated by Histopaque 1077 (Sigma) density gradient centrifugation and resuspended in PBS for radioisotope labeling. They were used for ischemic rabbit ear experiments to evaluate the first-pass engraftment efficiency to distal vascular bed after intra-arterial delivery.

**Flow cytometry analysis**

Antigen analysis was performed on \( \text{mob} \text{PBSCs} \). Single cell suspensions were analyzed on a FACS Caliber flow cytometer (Becton Dickinson). To identify the composition of \( \text{mob} \text{PBSCs} \) and PBMCs, FITC-conjugated anti-CD34 (DAKO), anti-CD31 (BD Pharmingen), and anti-VE-cadherin (Bender MedSystems) antibodies and PE-conjugated anti-CD31 (BD Pharmingen), anti-AC133 (Miltenyi Biotec), anti-KDR, anti-Tie2, and anti-CXCR4 (above all R&D Systems) antibodies were used. To examine the effect of COMP-Ang1 on the commitment and engraftment of \( \text{mob} \text{PBSCs} \), \( \text{mob} \text{PBSCs} \) stimulated with COMP-Ang1 (400
ng/ml) for 4 hours in EBM media were used, and FITC-conjugated anti-CD31 (BD Pharmingen) and anti-VE-Cadherin (Bender MedSystems) and PE-conjugated anti-integrin α4 (R&D Systems) antibodies were used. In addition, PE-Cy5-conjugated anti-integrin β1 (eBioscience) antibodies and APC-conjugated anti-integrin α5 (R&D Systems) and PE-conjugated anti-integrin β2 (Santa Cruz Biotechnology) were used. Isotype-matched IgG was used as a control.

**Cell culture and immunocytochemical staining**

PBMCs_AMI (naïve PBMCs) and mobPBSCs were resuspended using the EGM-2 BulletKit system (EGM-2 MV, Clonetics) consisting of endothelial basal medium, hEGF, hFGF-B, IGF-1, ascorbic acid, heparin, and 5% fetal bovine serum (FBS) as described previously. Some of them were cultured with COMP-Ang1 (400 ng/ml) to evaluate the capacity to generate endothelial progenitor cells and to commit to endothelial lineage. After 7 days in culture, we noted morphological appearances and performed immunocytochemical stainings in mobPBSCs to determine phenotypes. To identify the endothelial phenotype, we used FITC-conjugated anti-CD31 (BD Pharmingen), anti-CD34 (DAKO) and anti-VE-Cadherin (Bender MedSystems) antibodies and PE-conjugated anti-Tie2, anti-CXCR4, and anti-KDR (R&D Systems).
Systems) antibodies. To distinguish specific antibody binding from nonspecific staining, we used isotype control antibodies (BD bioscience).

Reverse transcriptase-polymerase chain reaction

Total cellular RNA was isolated from $1 \times 10^6$ naïve PBMCs and $^{\text{mob}}$PBSCs which had been incubated with COMP-Ang1 (400 ng/ml for 4 hours). To detect Ets-1 expression, RNA was isolated in a time-dependent manner after COMP-Ang1 stimulation. To determine whether Ets-1 expression by COMP-Ang1 is mediated by Tie2 receptor, we pretreated $^{\text{mob}}$PBSCs with anti-hTie2 IgG for 1 hour. To confirm the expressions of CD31, VE-cadherin, and integrin series genes in mobilized PBSCs, we harvested RNA after 4 hours of COMP-Ang1 stimulation (400 ng/ml) using TRIZOL reagent (Life Technologies, Gaithersburg, MD). cDNA was acquired using a Reverse Transcription system (TaqMan Reverse Transcription Reagents, Applied Biosystems) using 1µl of total mRNA. PCR reaction mixes contained 1µM primers, 1.5 mM MgCl$_2$, 200 µM dNTP (deoxynucleotide triphosphate), reaction buffer, 2.5 U Taq DNA polymerase (Genenmed), and 1µl of cDNA in a final volume of 20 µl. The PCR conditions used involved 30 amplification cycles [denaturation at 94°C (30sec), annealing at different temperatures for each primer (for 30sec), and extension at 72°C (30sec)], and a final extension
at 72°C (5min)]. PCR products were size fractionated on 1.5 agarose gel. GAPDH was used as an internal control. Primer sets and annealing temperatures were described in Supplementary Table 2.

**Quantitative reverse transcriptase-polymerase chain reaction**

The mRNA expression was quantified by real-time RT-PCR in 7500 Real-Time PCR System (Applied Biosystems) according to the manufacturer's protocols (comparative CT method). Real-time reactions were carried out in 96-well plates in triplicate with a 20-μl final volume and then normalized to GADPH RNA. Relative quantitative real-time RT-PCR for Tie2, Ets-1, CD31, VE-cadherin, and GAPDH was performed using TaqMan protocol. [TaqMan probes for Tie2 (Hs 00945155); Ets-1 (Hs 00901425); CD31 (Hs 01065282); GAPDH (Hs 99999905)]. The PCR conditions were as follows; After initial activation of uracyl-N-glycosylase at 50 °C (2 min), AmpliTaq Gold was activated at 95 °C (10min). The subsequent PCR condition consisted of 40 cycles of denaturation at 95 °C (15sec) and annealing extension at 60 °C (1 min) per cycle. Quantitative real-time RT-PCR for integrins was performed using SYBRGreen protocol. [The primers for Integrin α4 (Gene Id: 3676, symbol: ITGA4, Bioneer); Integrin α5 (Gene Id: 3678, symbol: ITGA5, Bioneer); Integrin β1 (Gene Id: 3688, symbol: ITGb1,
Bioneer). The PCR conditions used involved 1 cycle of pre-denaturation at 94°C (5min) and 40 amplification cycles [denaturation at 95°C (10sec), annealing at different temperatures for each primer at 58°C (25sec), and extension at 72°C (30sec)]. The expression level of target gene was represented as relative expression.

**Transfection of anti-sense Ets-1 oligodeoxynucleotides**

Because a high efficacy of transfection with hemagglutinating virus of Japan (HVJ)–coated liposomes was previously reported, we used the HVJ-liposome method to achieve a high level of transfection. Briefly 15 μM of anti-sense oligodeoxynucleotides (ODN) for human Ets-1 were added to 1ml of BSS containing total 10mg lipids and HVJ. PBSCs were seeded onto 12-well plates and transfected with antisense Ets-1 ODN using HVJ-liposomes (100ul) as described previously.

**Adhesion assays and Matrigel tube formation**

**mob** PBSCs adhesion onto human umbilical vein endothelial cells (HUVECs) or fibronectin (Sigma) were measured under static conditions as described previously. 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled PBSCs, 1×10^5 cells, were
incubated for 1 hour. The non-adhesive \textsuperscript{mob}PBSCs were removed by washing with PBS for 3 times. The total number of \textsuperscript{mob}PBSCs binding to HUVECs or fibronectin was counted from 10 random fields under a 100 magnification field of fluorescent microscope. Each set of experiments were repeated at least 3 times. Neutralizing antibodies against Tie-2 (R&D Systems), integrin $\alpha 4$ (R&D Systems), integrin $\alpha 5$ (Chemicon) and integrin $\beta 1$ (R&D Systems) were added 1 hour before adhesion assays.

A Matrigel network formation assay was performed to assess the effect of the COMP-Ang1 on the ability of \textsuperscript{mob}PBSCs to integrate into vascular structures. Growth factor-reduced Matrigel (Becton, Dickinson and Company) was added to chamber slides. After 1 hour of incubation at room temperature, $2 \times 10^4$ \textsuperscript{mob}PBSCs were added to the chamber slide with 500 µL EBM-2 media with 2% FBS. Twelve hours later, four representative fields were examined and the average total areas of complete tubes formed by cells per unit area were compared using Image-Pro Plus® (MediaCybernetics).

**Evaluation of \textsuperscript{mob}PBSC engraftment to distal vascular bed after intra-arterial infusion using rabbit ear ischemia/reperfusion model**

To quantify the engraftment of \textsuperscript{mob}PBSCs to ischemic tissue after bolus infusion of the cells
through artery, we used ischemic rabbit ear model which is a simulation for clinical situation, such as, intracoronary infusion of the cells for the vasculogenesis in patients with myocardial infarction. We evaluated the engraftment efficiency by two methods; (1) direct visualization of CFSE-labeled $^{\text{mob}}$PBSCs in histological sections of rabbit ear vessels which were harvested immediately after injecting $^{\text{mob}}$PBSCs, and (2) measurements of $^{\text{mob}}$PBSC specific radioactivity before and after injection into a central artery.

In detail, $^{\text{mob}}$PBSCs were collected from rabbit peripheral blood. Before transplanting cells, $^{\text{mob}}$PBSCs in suspension were washed with PBS and incubated with 5 µM CFSE in serum-free basal medium for 10 minutes at 37°C or with Tc-99m HMPAO at a level of 1mCi per $1 \times 10^7$ $^{\text{mob}}$PBSCs for 60 minutes. After washing, $^{\text{mob}}$PBSCs were resuspended in EBM-2 medium. Cell labeling and quantitation using Tc-99m HMPAO is a well-established method that produces stable labeling over 15 hours and ≤1% covariance at count levels of more than 10,000 cpm. Other rabbits were prepared for ischemic vessel formation. Their ear was sheared completely for visualization of artery. Proximal portion of ear artery was ligated by 3-0 nylon for 3 hours for ischemic condition. And then, vessel was reperfused and radiolabeled $^{\text{mob}}$PBSCs were injected into a central artery at the same time. Ears were harvested immediately after the injection. The perfusion decline on the laser Doppler imaging of each ear after ligation was
about 55% in all animals, similar to each other (data not shown). The engraftment of $^{\text{mob}}$PBSCs was visualized using a gamma camera and a pin-hole collimator, and the radioactivities of harvested ears were measured using a gamma counter after correcting for radioactive decay.

**Mouse hindlimb ischemia model**

The neovascularization of $^{\text{mob}}$PBSCs primed with COMP-Ang1 was investigated using a hindlimb ischemia model as in our previous reports.\textsuperscript{2,3,6} In brief, athymic nude mice (Daehan Biolink, Korea) were injected subcutaneously with G-CSF (50ug/kg) for 3 days, and then they anesthetized with 50 μL intra-peritoneal injection of a 1:4 mixture of ketamine & xylazine. Hindlimb ischemia was produced by femoral artery excision and was followed the delivery of $^{\text{mob}}$PBSCs. A total of $1 \times 10^7$ CFSE-labeled $^{\text{mob}}$PBSCs in 100 μL of PBS were injected by intra-cardiac puncture immediately after the operation. Before injection, $^{\text{mob}}$PBSCs were treated with (1) vehicle only (n=10), (2) COMP-Ang1 (400 ng/ml) for 4 hours (n=10), or (3) anti-Tie2 neutralizing antibody for 1 hour followed by COMP-Ang1, and then followed by serial laser Doppler perfusion imaging (LDPI, Moor Instruments) for 21 days. Mice were euthanized and hindlimbs were harvested to analyze neovascularization.

In addition, the systemic effect of COMP-Ang1 for the vasculogenesis with or without G-CSF
pretreatment was assessed using C57BL/6 mice hindlimb ischemia model (male, 4 weeks old). Recombinant adenovirus-expressing COMP-Ang1 and LacZ were constructed using previously reported methods. $1 \times 10^9$ pfu Ade-COMP-Ang1 or Ade-LacZ was diluted in 50 μL of sterile 0.9% NaCl and administered via tail-vein injection. On 3 days after adenoviral treatment, hindlimb ischemia was made using above mentioned method (n=6 in each group). And then G-CSF (100 μg/ml) or sterile saline was injected subcutaneously for 3 days. In previous data, circulating serum levels of COMP-Ang1 increased at 3 days after treatment, peaked at 5 days. Thus, we could evaluate the effect of G-CSF pretreatment before the effect of COMP-Ang1 was fully blown up.

**Immunofluorescent and immunohistochemical staining**

Immunofluorescent staining: For capillary density measurement, four frozen sections of each group were stained with TRITC-labeled BS-1 lectin (Sigma) antibody. We also performed immunofluorescent staining using anti-human CD31 (BD Pharmingen), anti-human integrin α4 (R&D Systems), anti-HLA-ABC (Chemicon) as primary antibodies, and FITC (green)-conjugated or PE (red)-conjugated antibodies as secondary antibodies to characterize the transplanted cells in vivo. Figures were obtained using a confocal microscopy or a fluorescent
inverted microscopy.

**Immunohistochemical staining**: Immunohistochemistry was performed as previously described. The primary antibodies used were anti-SDF-1 (1:200, R & D systems) for confirming its expression in ischemic tissue staining, and anti-human HLA (1:200, Santa Cruz Biotechnology) and anti-human vWF (1:200, DAKO) antibodies for detecting administrated **PBSCs**.
References


Supplementary Figure legends

Supplementary Figure 1.
Quantitative RT-PCR showed greater Tie2 expression in \textsuperscript{mob}PBSCs than in PBMCs from patients with AMI or SA (Relative quantitation of Tie2 transcriptional expression, 293±16% increase in \textsuperscript{mob}PBSCs compared with PBMC_SA, *p<0.01).

Supplementary Figure 2.
Quantitative RT-PCR showed that treatment with COMP-Ang1 increased Ets-1 expression within 2 hours and sustained the expression for 10 hours (*p<0.01; #p<0.05 compared with vehicle).

Supplementary Figure 3.
Quantitative RT-PCR showed that Ets-1 mRNA expression increased in \textsuperscript{mob}PBSCs treated with COMP-Ang1 for 4 hours (297±17% increase by COMP-Ang1), whereas 1 hour pretreatment with anti-Tie2 neutralizing antibody prior to the COMP-Ang1 treatment prevented Ets-1 upregulation (88±2% decrease by Tie2 pre-blocking). *p<0.01 vs. control; #p<0.01 vs. COMP-Ang1.

Supplementary Figure 4.
Quantitative RT-PCR presented that the stimulation of \textsuperscript{mob}PBSCs with COMP-Ang1 for 4
hours increased the mRNA expression of CD31 and VE-cadherin (Relative quantitation, 105±3% and 128±26% increase for CD31 and VE-cadherin, respectively), and antisense Ets-1 ODN attenuated these expression (57±3% and 57±7% decrease for CD31 and VE-cadherin, respectively). *p<0.01 vs. control; #p<0.01 vs. COMP-Ang1.

Supplementary Figure 5.
Quantitative RT-PCR presented that the stimulation of \textsuperscript{mob}PBSCs with COMP-Ang1 for 4 hours increased the mRNA expression of α4, α5, and β1 integrin (Relative quantitation, 43±4%, 114±17%, and 75±10% increase for α4, α5, and β1 integrin, respectively), and antisense Ets-1 ODN attenuated these expression (52±5%, 29±5%, and 62±3% decrease for α4, α5, and β1 integrin, respectively). *p<0.05 vs. control; #p<0.05 vs. COMP-Ang1.

Supplementary Figure 6.
Confocal images with immunofluorescent staining showed that CFSE-stained (green) \textsuperscript{mob}PBSCs treated with COMP-Ang1 engrafted in the harvested ear vessels more than untreated \textsuperscript{mob}PBSCs did. Pretreatment of \textsuperscript{mob}PBSCs with anti-Tie2 neutralizing antibody abrogated this engraftment. PECAM-1 (red) for vessels and DAPI (blue) for nuclei. Scale bars, 50 μm.

Supplementary Figure 7.
Immunohistochemical staining showed that human-specific HLA-positive cells were
significantly increased in ischemic limb of nude mouse treated with COMP-Ang1-primed
\textsuperscript{mob}PBSCs from patients. Scale bars, 50 μm. *p<0.01 vs. control; #p<0.01 vs. COMP-Ang1.

**Supplementary Figure 8.**

Flow cytometry analysis for CXCR4 expression in naïve PBMCs collected from patients with AMI (PBMC\_AMI) and \textsuperscript{mob}PBSCs collected from AMI patients with G-CSF treatment. CXC4R expression was greater on \textsuperscript{mob}PBSCs than on naïve PBMCs (34.2±16.7\% vs. 11.7±8.8\%, p<0.01 for \textsuperscript{mob}PBSCs vs. PBMC\_AMI).

**Supplementary Figure 9.**

Schematic illustration of priming \textsuperscript{mob}PBSCs with COMP-Ang1. Note that this priming procedure does not include the systemic administration of COMP-Ang1 which is used as the ex vivo agent to prime the cells before infusion.
### Supplementary Table 1. Baseline Patient Characteristics

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WBC, cells/㎖

8578±2800  11110±4014  6455±1355  <0.001

C-reactive protein,  
mg/L

2.9±2.9  3.3±6.2  0.5±0.3  0.062

\text{mob} \text{PBSC\_AMI} = \text{mobilized peripheral blood stem cell in this study were obtained after G-CSF subcutaneous injection to AMI patients}

\text{PBMC\_AMI} = \text{Peripheral blood mononuclear cell obtained from AMI patients}

\text{PBMC\_SA} = \text{Peripheral blood mononuclear cell obtained from stable angina patients}

\text{ACEI} = \text{angiotensin converting enzyme inhibitor, ARB} = \text{angiotensin receptor blocker}

Data presented are number of patients (percent) or mean±SD
<table>
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<th>Genes</th>
<th>Sense(S) /Antisense(AS)</th>
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Supplementary Figure 3

Supplementary Figure 4

Supplementary Figure 5
Supplementary Figure 6

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</tbody>
</table>

**PECAM-1**

**mobPBSC (CFSE-labeled)**

**DAPI**

**mobPBSC**

**Merge**
Supplementary Figure 7

mobPBSC

vehicle | COMP-Ang1/anti-IgG | COMP-Ang1/anti-Tie2

anti-HLA

[Images showing HLA-positive cell counts for vehicle, COMP-Ang1/anti-IgG, and COMP-Ang1/anti-Tie2 conditions.]

HLA-positive cells (relative to vehicle, %)

- Vehicle
- COMP-Ang1/anti-IgG
- COMP-Ang1/anti-Tie2

* Statistically significant difference

# Non-significant difference
Supplementary Figure 8

CXCR4

PBMC_AMI

11.7±8.8%

mobPBSC

34.2±16.7%
Supplementary Figure 9

[Diagram depicting the process of bone marrow stem cell mobilization, PBSC collection, ex vivo Ang-1 priming, and subsequent differentiation into endothelial lineage commitment and engraftment into ischemic tissues.]