Angiogenesis by Implantation of Peripheral Blood Mononuclear Cells and Platelets Into Ischemic Limbs

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Background—Peripheral blood mononuclear cells (PBMCs), platelets, and polymorphonuclear leukocytes (PMNs) contain various angiogenic factors and cytokines.

Methods and Results—Unilateral hindlimb ischemia was surgically induced in athymic nude rats, and fluorescence-labeled human blood cells (PBMCs [10^7] + platelets [10^8] or PBMCs [10^7] + platelets [10^8] + PMNs [10^8]) were intramuscularly implanted into the ischemic limbs. Laser Doppler imaging revealed markedly increased blood perfusion in PBMC + platelet–implanted limbs (44% increase, P < 0.001) compared with control implantation of human umbilical vein vascular endothelial cells. The addition of PMNs to PBMCs + platelets attenuated blood perfusion (27% decrease, P < 0.01). Neocapillary densities were increased by implantation of PBMCs + platelets or platelets alone (3.5-fold and 2.4-fold, respectively; P < 0.001), whereas PMNs inhibited (32%, P < 0.05) PBMC + platelet–mediated capillary formation. There was no incorporation of implanted PBMCs into neocapillaries, whereas PBMCs and platelets accumulated around arterioles after implantation. Cellular extract from PBMCs + platelets, in which vascular endothelial growth factor (VEGF), basic fibroblast growth factor, platelet-derived growth factor-AB, and transforming growth factor-β were detected, markedly stimulated tube formation of human umbilical vein vascular endothelial cells. Anti-VEGF neutralizing antibody markedly inhibited tubule formation and in vivo vessel formation. Neutrophil elastase inhibitor blocked the antiangiogenic action of PMNs, whereas inhibitors of oxygen metabolites had no effect.

Conclusions—This study demonstrated that implantation of PBMCs and platelets into ischemic limbs effectively induces collateral vessel formation by supplying angiogenic factors (mainly VEGF) and cytokines, suggesting that this cell therapy is useful as a novel strategy for therapeutic angiogenesis. (Circulation. 2002;106:2019-2025.)

Key Words: angiogenesis • vasculature • lymphocytes • blood cells • platelets

To induce angiogenesis, investigators have delivered vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), or hypoxia-inducible factor-1α/etoposide. Although early studies focused on endothelial cells, it has recently become clear that the subsequent stages of remodeling and stabilization are crucial for obtaining stable and functional vessels. Tight orchestration of monocytes/macrophages, endothelial cells, and smooth muscle cells/pericytes is critical for the development of mature blood vessels. Macrophages alter VEGF levels by producing the factor themselves as well as by secreting matrix metalloproteinases (MMPs), which release extracellular matrix–bound VEGF. The recruitment of pericytes by platelet-derived growth factor (PDGF)-BB produced by endothelial cells is critical for the stabilization and maturation of nascent vascular structures. VEGF often produces vessels that are hyperpermeable and leaky, but this effect can be tempered by the codelivery of angiopoietin-1. Given the investment of the formed mature vessels with periendothelial matrix and pericyte/smooth muscle cells, combination with various angiogenic growth factors may be preferable in future therapies.

Endothelial progenitor cells (EPCs) were discovered to participate in postnatal neovascularization after mobilization from bone marrow (BM). Kamihata et al11 and Shintani et al12 have shown that BM mononuclear cells (BM-MNCs) contain not only EPCs but also angiogenic factors and cytokines and that implantation of BM-MNCs into ischemic tissues augments collateral vessel formation. Peripheral blood mononuclear cells (PBMCs),13 polymorphonuclear leukocytes (PMNs),14 and platelets15,16 synthesize and release high levels of VEGF as well as PDGF and transforming growth factor (TGF)-β, known to be prerequisites for the investment of stable vessels with pericytes. PMNs have also been shown to release various angiogenic cytokines, including interleukin (IL)-1, IL-6, IL-8, and tumor necrosis factor-α,18 and MMPs19 and to enhance proliferation and tubule formation of endothelial cells in vitro.20 Although these reports have suggested angiogenic activities by implantation of
peripheral blood cells, their efficacy and availability for therapeutic angiogenesis remain undefined. In the present study, we tested whether implantation of PBMNCs, platelets, or PMNs can augment new vessel formation in hindlimb ischemia.

Methods

**PMMNCs, PMNs, Platelets, and BM-MNCs**

PBMNCs and PMNs were separately isolated from human peripheral blood by density gradient centrifugation (Polymerprep, Nycodens) and labeled with green fluorescence cell linker (PKH2-GL, Sigma Chemical Co). The purities of PBMNCs and PMNs were >99%, as determined by differential leukocyte scattergram analysis (XE-2100 Sysmex) (Figure 1A). Because PBMNCs could not be clearly separated from platelets by density gradient centrifugation because of their similar gravity, we examined the combined effect. The effect of platelets alone was examined by using commercially available human platelet concentrate (Nippon Red Cross). Endothelial lineage cells were subjected to fluorescence-activated cell sorter (FACS) analysis with the use of anti-human CD34 antibody (Beckman Coulter), Dil-acetylated LDL (DiI-acLDL) incorporation (Biogenes), and Ulex lectin binding (Sigma), as described previously. Human BM cells were aspirated from the ilea of patients with ischemic limbs undergoing angiogenic therapy by using BM-MNCs. CD34+ cells were separated from BM-MNCs by using CD45 magnetic microbeads (Miltenyi Biotec). The purity of enriched CD34+ cells was 88±2.7% (n=5) by FACS analysis. Patients consented to the experimental use of BM-MNCs and PBMNCs by document.

**Hindlimb Ischemia and Cell Implantation**

Unilateral hindlimb ischemia was induced by resecting the right femoral arteries and veins of immunodeficient nude rats (F344/N rnu/rnu) under anesthesia with sodium pentobarbital (50 mg/kg IP). Rats were then transplanted with PBMNCs (10^7 cells)+platelets (10^8), PMNCs (10^8)+platelets (10^9)+PMNs (10^8), or PMNs (10^8) intramuscularly into the ischemic thigh area (4 sites) with the use of a 27-gauge needle (n=10 for each implantation). Four weeks after surgery, skeletal muscles were isolated and snap-frozen in liquid nitrogen. The Institutional Animal Care and Use Committee approved all animal protocols.

**Immunohistochemistry**

Four pieces of ischemic tissues from the adductor and semimembranosus muscles were obtained. Frozen sections were stained with anti-factor VIII or anti-human specific glycoprotein (GP) Ib/IIa antibodies (not cross-reactive with rat GP Ib/IIa, DAKO) followed by incubation with TRITC-conjugated secondary antibody. Five fields from 2 muscle samples of each animal were randomly selected for capillary counts. To ensure that capillary densities were not overestimated as a consequence of myocyte atrophy or underestimated because of interstitial edema, the capillary/muscle fiber ratio was determined. To examine whether transplanted cells survived in the tissues, adjacent sections were subjected to alkaline phosphatase (AP) staining by the indoxyl-tetrazolium method. AP staining turns capillary endothelial cells a dark blue color only when they are viable and when the intracellular enzyme activity remains intact.

**Laser Doppler Analysis and Angiography**

We measured the ratio of the ischemic (right)/normal (left) limb blood flow by using a laser Doppler perfusion image (LDPI) analyzer (Moor Instruments). After blood flow was scanned twice, stored images were subjected to computer-assisted quantification, and the average flows of ischemic and nonischemic limbs were calculated. To minimize data variables due to ambient light and temperature, the LDPI index was expressed as the ratio of ischemic to nonischemic limb blood flow. Collateral formation was evaluated by using a Microfocus X-Ray Television Device (Hitec Co Ltd) on day 28. Longitudinal laparotomy was performed to introduce a catheter into the abdominal aorta, followed by injection of contrast medium (lipiodol). Angiography was performed for 2 seconds after the injection.

**Endothelial Tubule Formation**

We used a 2D coculture system of human umbilical vein endothelial cells (HUVECs, Cascade Biologics Inc) and human diploid fibroblasts (TCS Cell Works) to evaluate angiogenic activities by quantifying the tubule formation of HUVECs (Angiogenesis Kit, Kurabo). For experiments, the medium was changed to medium containing 2% FCS with or without cellular extracts for 4 days. HUVECs were immunostained with anti-human CD31 antibody with the use of 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium as the substrate for secondary antibody. Each culture was photographed at a magnification of x32 with a Nikon microscope equipped with an Olympus camera system. A grid of lines 5 mm
Angiogenic Cytokines and Factors in Blood Cells

<table>
<thead>
<tr>
<th></th>
<th>VEGF, pg</th>
<th>bFGF, pg</th>
<th>PDGF-AB, ng</th>
<th>HGF, ng</th>
<th>TGF-β, ng</th>
<th>TNF-α, pg</th>
<th>IL-1β, pg</th>
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<tbody>
<tr>
<td>PBMNCs</td>
<td>447±19</td>
<td>72±10</td>
<td>119±16</td>
<td>ND</td>
<td>89±16</td>
<td>31±4</td>
<td>20±4</td>
</tr>
<tr>
<td>Platelets</td>
<td>341±18</td>
<td>117±3</td>
<td>68±8</td>
<td>ND</td>
<td>90±3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PMNs</td>
<td>965±142</td>
<td>35±4</td>
<td>88±7</td>
<td>ND</td>
<td>12±5</td>
<td>24±4</td>
<td>93±11</td>
</tr>
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</table>

HGF indicates hepatocyte growth factor; ND, not detected. Concentrations of angiogenic factors and cytokines in cellular extract were determined by ELISA. Cellular extracts from PBMNCs (10⁷ cells), platelets (10⁹), or PMNs (10⁷) were obtained by freezing and thawing cells 3 times in medium (5 mL).

Apart was layered over the photograph, and the numbers of intersections of cords/tubes crossing the lines were measured. Neutralizing monoclonal antibodies for VEGF, bFGF, PDGF-BB (reactive to PDGF-AA and PDGF-BB), or TGF-β (R&D Systems) or enzyme inhibitors were preincubated with cell-conditioned medium for 60 minutes. The antibody concentrations were sufficient to neutralize angiogenic factors included in the cellular extracts (Table). Superoxide dismutase (SOD, Sigma), catalase (Sigma), and a neutrophil elastase inhibitor (AAPVCK, Enzyme System) were purchased from the sources shown.

Neutralizing Anti-VEGF Antibody and In Vivo Matrigel Plug Assay
Goat anti-human VEGF-specific neutralizing antibody that did not cross-react with rat VEGF (R&D Systems) was intraperitoneally injected (7.5 μg twice a week for 28 days). An in vivo Matrigel plug assay was used as a positive control to evaluate its neutralizing effect. Matrigel (300 μL) mixed with human VEGF (100 ng/mL) was implanted subcutaneously into the flank of rats treated with anti-VEGF antibody. On day 28, the plugs were removed and fixed, and paraffin was embedded. Staining was performed with hematoxylin and cosin (HE).

Statistical Analysis
Statistical analyses were performed by 1-way ANOVA, followed by pairwise contrasts using the Dunnett test. Data (mean±SEM) were considered significant at P<0.05.

Results
Isolation of PBMNCs, PMNs, and Platelets
Leukocyte scattergrams indicated the presence of lymphocytes, monocytes, and PMNs, including neutrophils and eosinocytes, in whole blood (Figure 1A). Neutrophils and eosinocytes were excluded from the PBMNC+platelet fraction (Figure 1B). Eosinocytes were not observed in the PBMNC+PMN+platelet fraction (Figure 1C), and lymphocytes and monocytes were not detected in the platelet fraction (Figure 1D). PBMNCs included 0.02±0.004% of CD34⁺ cells in PBMNCs (n=6) (Figure 1E), of which endothelial lineage cells assessed by Dil-acLDL uptake and Ulex lectin binding accounted for 14±1.6% (n=6, Figure 1F).

Laser Doppler Blood Perfusion
PBMNCs (10⁷ cells)+platelets (10⁹), PBMNCs (10⁷)+platelets (10⁹)+PMNs (10⁷), platelets (10⁹), or PMNs (10⁷) were implanted into ischemic hindlimbs. To analyze subcutaneous blood perfusion, LDPI analysis was performed (Figure 2). A greater degree of blood perfusion was observed in the ischemic limb of PBMNC+platelet-transplanted rats (44% increase at day 21, P<0.001) compared with HUVEC-transplanted control rats. Compared with PBMNC+platelet implantation, PMN+PBMNC+platelet implantation significantly attenuated blood perfusion on day 21 (27% decrease, P<0.01). Interestingly, implantation of platelets alone also augmented blood perfusion by 28% (P<0.001). Implantation of PMNs alone or saline injection as a control did not cause any significant increase (data not shown).

Angiography
All animals were subjected to iliac angiography on postoperative day 28. Representative angiograms (n=5) are shown in Figure 3, in which arrows indicate ligated ends of femoral arteries. Numerous collateral vessels developed in PBMNC+platelet-implanted or platelet-implanted limbs but not in HUVEC-transplanted control limbs.

| Image 308x121 to 536x442 |

Figure 2. LDPI analysis. A, Representative LDPI. Greater blood perfusion (red to yellow) was observed in PBMNC+platelet-transplanted limbs, in contrast to reduced perfusion (green to blue) in HUVEC-transplanted control limbs. B, LDPI analyses revealing significantly greater blood perfusion values in the PBMNC+platelet group than in other groups. Values are mean±SEM (n=10) at each time point. *P<0.05 and **P<0.01 vs control.
Immunohistochemical Analysis of New Vessel Formation

Immunohistochemical staining for anti–factor VIII (Figure 4) revealed the presence of numerous capillary endothelial cells in PBMNC+platelet-implanted or platelet-implanted rats; capillary/muscle fiber ratios for these rat groups were markedly increased (3.6-fold and 2.4-fold, respectively) compared with the ratio for the HUVEC-implanted control group. Implantation was significantly attenuated (32% decrease, \( P<0.05 \)) for PMN+PBMNC+platelet-implanted compared with PBMNC+platelet-implanted muscles. Similar results were also observed in AP staining for viable endothelial cells (Figure 4).

Although implanted PBMNCs (10^7 cells) were fluorescence-labeled and although CD34^+ cells were included in this fraction (\( \approx 2\times10^7 \) cells), as shown in Figure 1E, no incorporation of labeled cells into capillaries was observed. To confirm the lack of involvement of CD34^+ cells, different numbers of human marrow CD34^+ cells were implanted into ischemic limbs. When 10^6 or 10^5 labeled CD34^+ cells were implanted, some capillaries incorporated them, whereas implantation of 10^4 CD34^+ cells did not cause any detectable incorporation (Figure 5A). Significant increases in blood perfusion were observed in ischemic limbs in which 10^6 or 10^5 CD34^+ cells were implanted (Figure 5B).

We compared angiogenic actions between BM-MNCs and PBMNCs isolated from the same patients (\( n=7 \)). BM-MNCs contained \( \approx 100 \)-fold greater numbers of CD34^+ cells (2.4\( \pm \)0.4%) than did PBMNCs (0.02\( \pm \)0.01%). When the same numbers of mononuclear cells (10^7 cells) were implanted, the angiogenic effect of PBMNCs was \( \approx 72\% \) relative to that of BM-MNCs 21 days after implantation (Figure 5C).

Migration of Implanted PBMNCs and Platelets Toward Perivascular Regions

We examined the time-dependent tissue localization of PBMNCs and platelets after implantation (Figure 6). Implanted PBMNCs labeled by green fluorescence accumulated around arterioles 3 days after implantation. Platelets were immunostained by mouse anti-human specific GP IIb/IIIa antibody but not by control mouse IgG. Most platelets accumulated in perivascular and interstitial regions 3 days after implantation (arrows in Figure 6). On day 7, the accumulation of PBMNCs and platelets was markedly decreased. The same phenomenon was observed in all tested limbs (\( n=5 \)).

Roles of Angiogenic Factors in PBMNC+Platelet–Mediated Tubule Formation

Because VEGF, bFGF, PDGF-AB, and TGF-\( \beta \) were detected in cellular extracts from PBMNCs, platelets, and PMNs (Table), we analyzed what kinds of factors are responsible for angiogenic activities by testing tubule formation of HUVECs. Cell extracts from PBMNCs+platelets markedly increased CD31-positive tubule formation by 5.2-fold over the control extracts, whereas the addition of PMN extract significantly attenuated it (61%) (Figure 7A). Preincubation with anti-VEGF neutralizing antibody abolished tubule formation by
PBMNCs+platelets, whereas neutralizing antibodies for bFGF, PDGF-BB, or TGF-β showed no significant effects.

To study in vivo the role of VEGF released from implanted PBMNCs+platelets, we intraperitoneally injected the neutralizing anti-human VEGF antibody that did not cross-react with rat VEGF. An in vivo Matrigel plug assay was used as a positive control to evaluate its neutralizing effect. When the Matrigel including human VEGF was implanted subcutaneously into the flank of rats, angiogenesis was induced in the Matrigel. Without VEGF inclusion, no vessel formation was detected (Figure 7B). Intraperitoneal injection of neutralizing anti-VEGF antibody abolished angiogenesis in the Matrigel, including human VEGF (Figure 7B), suggesting the efficacy of this antibody to neutralize human VEGF. The increase in blood perfusion in PBMNC+platelet–implanted ischemic limbs was completely inhibited by this neutralizing antibody toward the HUVEC-implanted control level, whereas the injection of control IgG did not affect blood perfusion (Figure 7B).

**Mechanism for PMN-Mediated Antiangiogenic Action**

Neutrophils caused endothelial injury via cytotoxic products such as granule proteases and toxic oxygen radicals. We tested the effects of a neutrophil elastase inhibitor (AAPVCK), a scavenger enzyme of hydrogen peroxide (catalase), SOD, myeloperoxidase (azide), and a hydroxyl radical scavenger (mannitol). AAPVCK completely reversed the inhibitory effect of PMNs, whereas inhibitors of various oxygen metabolites had no effects on PMN-mediated action (Figure 7A).

**Discussion**

The main findings in the present study are that (1) implantation of PBMNCs and platelets into ischemic limbs effectively augments collateral vessel formation by supplying various angiogenic factors (VEGF, bFGF, PDGF, and TGF-β), in which VEGF plays a key role; (2) neutrophils attenuate
Matrigel including VEGF (100 ng/mL) shows endothelial cell–forming vessels (arrows). Intraperitoneal injection of goat anti-human VEGF antibody (7.5 μg twice a week for 28 days) abolished angiogenesis in the Matrigel, including VEGF, and reduced the increase of blood perfusion in PBMNC + platelet–implanted ischemic limbs toward the control level (n = 6 each). Control indicates HUVEC–implanted limbs; control IgG, intraperitoneal injection of goat IgG (7.5 μg twice a week for 28 days, n = 6). *P < 0.05 and **P < 0.001 vs control; †P < 0.01 vs tubule formation by PBMNC + platelets or PBMNCs + platelets + PMNs, respectively. B, Histological appearance of Matrigel plug sections and LDPI analysis of ischemic limbs 28 days after implantation are shown.

PBMCN-mediated angiogenesis via the release of lysosomal elastases rather than cytotoxic oxygen metabolites; and (3) implanted PBMCNs and platelets migrate toward arterioles and accumulate in the perivascular region to exert their angiogenic activities.

Preclinical studies have indicated that angiogenic growth factors promote the development of collateral arteries, a concept called therapeutic angiogenesis. Tight orchestration of the relative abundance of cell types, such as monocytes/macrophages, endothelial cells, and smooth muscle cells/pericytes, is critical to the development of mature blood vessels. Mononuclear cells alter local VEGF levels by producing the factor themselves as well as by secreting MMPs that release extracellular matrix–bound VEGF. The recruitment of pericytes by PDGF-BB is critical to the stabilization and maturation of nascent vascular structures. VEGF often produces vessels that are hyperpermeable and leaky, but this effect can be tempered by the codelivery of PDGF, TGF-β, or angiopoietin-1. Given the investment of the formed mature vessels with periendothelial matrix and pericyte/smooth muscle cells, combination with these angiogenic growth factors, as supplied by PBMCNs + platelets, may be preferable in future angiogenic therapies.

Kamihata et al. and Shintani et al. showed that EPCs included in BM-MNCs differentiated into endothelial cells of newly formed vessels. A randomized controlled clinical trial using BM-MNC implantation established the feasibility of this angiogenic cell therapy for patients with peripheral artery diseases. In the present study, we found that implantation of PBMCNs or platelets can effectively induce angiogenesis via the supply of angiogenic factors rather than the involvement of EPCs. We have shown that BM-MNCs not only contain EPCs but also supply angiogenic factors such as VEGF, bFGF, and angiopoietin-1. As shown in Figure 5, when angiogenic action was compared between the same numbers of BM-MNCs and PBMCNs (containing 2.4% and 0.02% CD34+ cells, respectively), the effect of PBMCNs was 72% relative to that of BM-MNCs. Taken together, these observations suggest that PBMCN implantation is a novel strategy for therapeutic angiogenesis, although the inclusion of EPCs induces greater neocapillary formation.

Angiogenesis plays a role in transporting inflammatory cells to the ischemic tissue or supplying nutrients and oxygen. Although PMNs have been shown to accumulate around neocapillaries in corneal diseases, PMN-mediated action on angiogenesis in vivo remains to be determined. Circulating neutrophils adhere to the vascular endothelium, and the activated neutrophils have the potential to mediate endothelial injury by releasing lysosomal proteinases or generating oxygen metabolites. In the present study, we clearly showed that PMNs have an inhibitory effect on angiogenesis in vivo and in vitro: neutrophil elastases rather than oxygen metabolites are the responsible antiangiogenesis molecules, consistent with the previous observation that neutrophil-derived elastase but not oxygen metabolites mediate endothelial cell detachment through the digestion of endothelial surface proteins. H2O2, a representative reactive oxygen species, was reported to stimulate tubule formation of endothelial cells at low concentrations but to inhibit it at high concentrations. However, we found that neither a scavenger enzyme of H2O2 (catalase) nor SOD had any significant effect on tubule formation. Given the ability of neutrophil elastase to activate MMPs, further studies may be needed to define the in vivo effects of oxygen metabolites or elastases on angiogenesis.

Implanted PBMCNs and platelets migrated toward arterioles and accumulated in the perivascular region. Chemotactic factors released from ischemic tissues or by implanted PBMCNs and platelets may play key roles. In fact, collagen-derived factors were reported to induce chemotaxis of plate-
lets when collagen was exposed with plasma moieties.\textsuperscript{30} VEGF released from ischemic tissues or implanted cells may cause extravasation of plasma moieties from arterioles, leading to the release of collagen-derived factors that promote chemotaxis.

In summary, the present study demonstrates that intramuscular implantation of human PBMCNs and platelets into ischemic limbs effectively induces collateral vessel formation mainly by supplying VEGF. In contrast, PMNs showed an antiangiogenic effect that was due to the release of neutrophil elastase. Although therapeutic angiogenesis using VEGF gene therapy was established in patients with peripheral artery diseases, remote angiogenic actions by elevation of circulating VEGF concentrations, such as neoangiogenesis in malignant tumors or atherosclerotic lesions, have been suggested.\textsuperscript{1,3} We showed that implanted PBMCNs accumulate in the perivascular region and probably release a variety of angiogenic factors locally and systemically. PBMCNs can be easily sorted from the peripheral blood of patients with peripheral artery diseases and can be autologously implanted into ischemic limbs. Although this novel angiogenic cell therapy seems to be feasible, remote angiogenic actions should be considered as possible side effects, and the clinical efficacy should be tested by a randomized controlled study.

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/content/127/23/e842.full.pdf

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Notice of Retraction

The American Heart Association (AHA) published an expression of concern1-3 simultaneously in Circulation, Circulation Research, and Hypertension, and we have now been notified by Kyoto Prefectural University of Medicine that certain data and figures in these 5 articles were falsified. Kyoto Prefectural University of Medicine sends their deepest apologies to the academic community at large.

The AHA is hereby retracting these articles:

References