Angiogenic cells can be rapidly mobilized and efficiently harvested from the blood following treatment with AMD3100

Rebecca M. Shepherd, Benjamin J. Capoccia, Steven M. Devine, John DiPersio, Kathryn M. Trinkaus, David Ingram, and Daniel C. Link

Circulating endothelial progenitor cells (EPCs) are thought to contribute to angiogenesis following vascular injury, stimulating interest in their ability to mediate therapeutic angiogenesis. However, the number of EPCs in the blood is low, limiting endogenous repair, and a method to rapidly mobilize EPCs has not been reported. In this study, healthy donors were mobilized sequentially with the CXCR4 antagonist, AMD3100, and G-CSF. The number of EPCs and circulating angiogenic cells (CACs) in the blood and pheresis product was determined and the angiogenic capacity of each cell population assessed. Compared with baseline, treatment with AMD3100 or G-CSF increased the number of blood CACs 10.0-fold $\pm$ 4.4-fold and 8.8-fold $\pm$ 3.7-fold, respectively. The number of EPCs in the blood increased 10.2-fold $\pm$ 3.3-fold and 21.8-fold $\pm$ 5.4-fold, respectively. On a per-cell basis, CACs harvested from G-CSF–mobilized blood displayed increased in vivo angiogenic potential compared with AMD3100-mobilized CACs. Mobilized EPCs displayed a greater proliferative capacity than EPCs isolated from baseline blood. Both CACs and EPCs were efficiently harvested by leukapheresis. Cryopreserved CACs but not EPCs retained functional activity after thawing. These data show that AMD3100 is a potent and rapid mobilizer of angiogenic cells and demonstrate the feasibility of obtaining and storing large numbers of angiogenic cells by leukapheresis. (Blood. 2006;108:3662-3667)

Introduction

There is compelling evidence that circulating angiogenic cells exist in humans that are able to home to sites of vascular injury and stimulate angiogenesis. This observation has led to tremendous interest in the translational potential of these cells to mediate therapeutic angiogenesis. At least 2 distinct angiogenic cell populations in the blood have been identified. Endothelial progenitor cells (EPCs; also termed endothelial outgrowth cells (EOCs)2 or endothelial cell colony-forming unit (CFU-EC)3) are true progenitor cells with high proliferative capacity and the ability to form large colonies of mature endothelial cells. Circulating angiogenic cells (CACs; also termed early EPCs8) are monocyte-like cells that appear to stimulate angiogenesis through secretion of growth factors such as vascular endothelial growth factor (VEGF).10,11 The number of CACs and EPCs in the blood at baseline is low, limiting their delivery to sites of ischemia and subsequent stimulation of angiogenesis. There is evidence that treatment with certain cytokines induces angiogenic cell mobilization from the bone marrow into the blood, potentially overcoming this limitation.12-15 In particular, granulocyte colony-stimulating factor (G-CSF) has been shown to mobilize EPCs in various animal models and humans.11,16-18 Based on these observations, several clinical trials of G-CSF–induced EPC mobilization following acute myocardial infarction have been performed.19-21 However, the kinetics of EPC mobilization by G-CSF may not be optimal to achieve maximal revascularization following vascular injury. Based on the kinetics of hematopoietic progenitor mobilization, peak EPC mobilization likely is not achieved until day 4 or 5 of G-CSF treatment.22

An alternative approach to increase angiogenic cell delivery to sites of ischemia is the direct injection of enriched cell populations of EPCs or CACs into the ischemic area. Because bone marrow is thought to be a reservoir for EPCs, many clinical trials have used autologous bone marrow cells as the source of angiogenic cells, requiring that patients undergo a bone marrow harvest procedure. Herein, we compare the ability of AMD3100, a novel mobilizing agent, with that of G-CSF to mobilize EPCs and CACs into the blood of healthy individuals. AMD3100, a CXCR4 antagonist, is an attractive mobilizing agent, because maximal mobilization (at least of hematopoietic progenitor cells) is achieved within 6 hours of administration.23 We show that treatment with AMD3100 or G-CSF markedly increases the number circulating EPCs and CACs. Moreover, these cells can be efficiently harvested by leukapheresis, providing a noninvasive method to obtain a large number of angiogenic cells.

Materials and methods

Clinical study

Healthy donors for allogeneic stem cell transplantation were recruited to a clinical trial evaluating the safety and efficacy of AMD3100 to mobilize hematopoietic stem cells. Informed consent was obtained according to the Declaration of Helsinki. The mobilization schema is shown in Figure 1. Donors received a single subcutaneous injection of AMD3100 (240 μg/kg/d) and 4 hours later underwent continuous-flow leukapheresis (3.5 to 4 times blood volume) using a Cobe Spectra (Gambro, Lakewood, CO). No
donor experienced more than Common Terminology Criteria for Adverse Events (CTCAE) 3.0 grade 1 toxicity after AMD3100 administration. Following a washout period of at least 7 days, the donors then received G-CSF (10 µg/kg/d subcutaneously for 5 days) and again underwent leukapheresis after the final dose of G-CSF. All donors received AMD3100 first and G-CSF second. Each pheresis product was cryopreserved in 10% dimethyl sulfoxide (DMSO) by controlled-rate freezing and stored in the vapor phase of liquid nitrogen.

Peripheral blood (70 mL) was collected prior to any treatment (baseline) and just prior to leukapheresis after AMD3100 or G-CSF mobilization. An aliquot of each fresh and cryopreserved pheresis product also was analyzed. Leukapheresis efficiency for CD34+ collection was calculated as previously described. The study was performed with the approval of the Human Studies Committee at Washington University School of Medicine.

Angiogenic cell culture

Cells were cultured in EGM2 media (Cambrex, Walkersville, MD) with 20% heat-inactivated fetal calf serum and 100 to 200 ng/mL recombinant human VEGF (Peprotech, Rocky Hill, NJ) in 6-well collagen I or fibronectin-coated plates (BD Biosciences, Bedford, MA) at a density of 10 × 10^5 mononuclear cells (MNCs) per well. The EPC replating assay was performed as a modification of the assay described by Ingram et al. Briefly, individual EPC colonies were harvested and replated in 1 well of a 6-well plate. Colonies that grew to confluence within 7 days were again harvested, and 10% of the cells were replated; cells were passed at least 3 times and up to 10 times.

Flow cytometry

Adherent cells were harvested from cultures by incubation with Cell Dissociation Buffer (Invitrogen, Grand Island, NY) for 20 minutes at 37°C, incubated with Fc Block (Miltenyi Biotec, Auburn, CA) to reduce nonspecific staining, and analyzed by flow cytometry as previously described. The following directly conjugated antibodies were used (all antibodies from BD Pharmingen, San Diego, CA, unless otherwise stated): phycoerythrin (PE)-conjugated CD45, CD40, CD60, and CD163 and fluorescein isothiocyanate (FITC)-conjugated CD31, CD34, and CD144 (vascular endothelial [VE]-cadherin; Novus Biologicals, Littleton, CO). Unlabeled CD105 was conjugated to Alexa Fluor 488 using a monoclonal fluorescein isothiocyanate (FITC)–conjugated CD31, CD64, and CD144 (BD PharMingen, San Diego, CA, unless otherwise stated): phycoerythrin (PE)-conjugated CD45, CD40, CD60, and CD163 and fluorescein isothiocyanate (FITC)-conjugated CD31, CD34, and CD144 (vascular endothelial [VE]-cadherin; Novus Biologicals, Littleton, CO). Unlabeled CD105 was conjugated to Alexa Fluor 488 using a monoclonal antibody labeling kit (Molecular Probes, Eugene OR). Isotype-matched antibodies were used as negative controls. Cells were analyzed on a FACSScan flow cytometer (Becton Dickinson, San Jose, CA). Cell sorting was performed using a MoFlo high-speed flow cytometer (Dako, Fort Collins, CO).

Mobilization of angiogenic cells

Mobilization of angiogenic cells was performed using a MoFlo high-speed flow cytometer (Dako, Fort Collins, CO).

Murine hind-limb ischemia model

Nonobese diabetic/severe combined immunodeficiency β2-microglobulin–deficient (NOD/SCID β2M) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Unilateral hind-limb ischemia was surgically induced as previously described. In brief, the femoral artery and vein were dissected away from the femoral nerve and ligated with 6-0 silk sutures. The distal section of the saphenous artery and vein and associated branches were also ligated and excised. The skin was closed with Nexaband veterinary glue (Abbott Animal Health, Abbott Park, IL). Cultured and sorted cells (5 × 10^5 unless stated otherwise) or saline alone were injected in the lateral tail vein 24 hours after surgical induction of hind-limb ischemia. Blood perfusion in the hind limb was monitored by laser Doppler imaging (MoorLD1-2; Moor Instruments, Axminster, United Kingdom). To control for ambient light and temperature, calculated perfusion was expressed as the flux ratio between the ischemic and nonischemic limbs. All experiments were approved by the Washington University Animal Studies Committee.

Statistical analysis

Data represent the mean ± SEM unless otherwise stated. CAC and EPC numbers in the blood were compared at baseline and after AMD3100 or G-CSF administration. Data from a total of 9 human donors are reported. Three did not progress to G-CSF; all 9 were measured at baseline and after AMD3100. Cell counts were analyzed on a log scale to approximate a Gaussian distribution and were compared using linear repeated measures models with unstructured covariance. The mobilization agent was treated as a fixed effect. Contrasts were used for pairwise comparisons of mean cell counts. For transplantation outcomes, similar linear repeated measures models were used with contrasts testing for differences on each day and between curves. Analyses were carried out using SAS version 9.1 (SAS, Cary, NC).

Results

Characterization of CACs and EPCs

Flow cytometry– and culture-based assays have been used to measure circulating EPCs. While able to select for cell populations enriched for EPCs, current flow cytometry–based methods are not able to definitively identify EPCs or CACs. Thus, in the present study we used a culture-based assay to quantify these cells. CACs were identified as adherent spindle-shaped cells present on days 4 to 7 of culture that stained for ulex-lectin, took up acetylated low-density lipoprotein (LDL), and expressed the monocyte markers CD45, CD14, CD64, and CD163 but were generally negative for the endothelial markers CD34, CD105 (endoglin), CD144 (VE-cadherin), and CD146 (M-CAM) (Table 1; Figure 2). EPCs were identified by the formation of discrete colonies of endothelial cells on days 14 to 28 of culture. Cells in these colonies stained with ulex-lectin, took up acetylated LDL, and expressed high levels of the endothelial antigens CD34, CD31 (PECAM-1), CD105, CD144, and CD146 but were negative for leukocyte antigens CD14 and CD45; the small fraction of CD14+ and CD45+ cells likely reflects residual CACs in the culture (Table 1; Figure 3).

Table 1. Flow cytometric analysis of CACs and EPCs

<table>
<thead>
<tr>
<th>Marker</th>
<th>CD14</th>
<th>CD45</th>
<th>CD31</th>
<th>CD34</th>
<th>CD105</th>
<th>CD144</th>
<th>CD146</th>
<th>CD163</th>
<th>CD64</th>
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<tbody>
<tr>
<td>CAC</td>
<td>–</td>
<td>–</td>
<td>High</td>
<td>–</td>
<td>Low</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EPC</td>
<td>–</td>
<td>–</td>
<td>High</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Cells were harvested on day 7 (CACs) or day 21 (EPCs) of culture, stained with the indicated antibodies, and analyzed by flow cytometry. Unless otherwise indicated, expression was categorized as present (+) or absent (−) based on isotype controls (see Figures 2-3). A small subpopulation of CACs was CD144+.

Figure 1. Clinical protocol. Healthy donors for allogeneic stem cell transplantation were treated with a single dose of AMD3100 (240 µg/kg) and, following a 1-week washout period, with 5 days of daily G-CSF (10 µg/kg/d). Leukapheresis was initiated 4 hours after AMD3100 and 5 days after G-CSF treatment. Peripheral blood was drawn prior to AMD3100 treatment (baseline) or immediately prior to each leukapheresis.
In the present study, CACs were quantified by determining the fraction of adherent cells on day 7 of culture that expressed CD45 and CD14. To confirm that these cells have angiogenic capacity in vivo, CD45⁻/H11001 CD14⁻/H11001 cells were sorted by flow cytometry from day-7 cultures and analyzed using a xenotransplantation model of acute vascular injury. Specifically, the purified CACs were infused intravenously into NOD/SCID β2M mice 24 hours after surgical induction of unilateral hind-limb ischemia. Perfusion in the ischemic limb was monitored by laser Doppler imaging and compared with perfusion in the nonischemic limb. In control mice treated with saline alone, a slow and partial improvement in blood flow to the ischemic limb was observed over 2 weeks (Figure 4). In mice treated with CACs, a dramatic improvement in perfusion was observed on day 10 after surgery that persisted through day 14.

**AMD3100 and G-CSF mobilize functional CACs and EPCs into the blood**

We next measured the level of CACs and EPCs in the blood after treatment with AMD3100 or G-CSF in healthy donors for allogeneic stem cell transplantation. At baseline, an average of $4.0 \pm 0.10 \times 10^4$ CACs per milliliter and $0.05 \pm 0.002$ EPCs per milliliter were detected in peripheral blood (Figure 5A-B). Treatment with AMD3100 or G-CSF resulted in a significant increase in the number of CACs in the blood, with fold increases from baseline of $10.0 \pm 4.4$ and $8.8 \pm 3.7$, respectively (Figure 5A). Of note, AMD3100 and G-CSF did not differ significantly in their ability to mobilize CACs. Likewise, both AMD3100 and G-CSF mobilized EPCs into the blood, with G-CSF inducing a statistically greater number of EPCs than AMD3100. Treatment with AMD3100 or G-CSF induced fold increases of $10.2 \pm 3.3$ or $21.8 \pm 5.4$ in circulating EPCs, respectively (Figure 5C).

The in vivo angiogenic capacity of CACs mobilized by AMD3100 or G-CSF was assessed using the xenotransplantation hind-limb ischemia model. CACs recovered from day-7 cultures of AMD3100- or G-CSF–mobilized blood mononuclear cells were infused intravenously into mice 24 hours after induction of hind-limb ischemia. Both AMD3100- and G-CSF–mobilized CACs significantly improved limb perfusion by day 10 and sustained an improvement through day 14 (Figure 5B). Interestingly, perfusion was modestly but significantly better in mice treated with G-CSF–mobilized versus AMD3100-mobilized CACs. Thus, on a per-cell basis, the in vivo angiogenic capacity G-CSF–mobilized CACs was superior to that of AMD3100-mobilized CACs.

**Figure 4. CACs demonstrate angiogenic potential in vivo.** CACs (400,000 cells) were infused intravenously into NOD/SCID β2M mice 24 hours after surgical induction of hind-limb ischemia (control, n = 5; CACs, n = 9). (A) Representative laser Doppler images at 14 days after surgery. (B) Perfusion in the ischemic hind limb relative to nonischemic hind limb was measured at the indicated time after surgery (*P < .001).
G-CSF–mobilized pheresis MNCs have greater in vivo angiogenic capacity compared with AMD3100-mobilized CACs. Comparable analyses were performed for EPCs. A relatively large number of EPCs were collected in the pheresis product after AMD3100 or G-CSF treatment (Table 2). Similar to CACs, the relative efficiency of EPC harvest by leukapheresis was 48.3% ± 18.3% (Figure 6A). Collectively, these data show that both CACs and EPCs can be efficiently harvested by leukapheresis from mobilized blood.

**Cryopreservation preserves functional CACs but not EPCs**

The viability and functional capacity of CACs and EPCs after cryopreservation with DMSO were examined. Pheresis products that had been cryopreserved were thawed after 3 to 6 months and cultured as previously described. CACs were readily recovered after cryopreservation at an efficiency of 54.6% ± 11.8% compared with fresh pheresis product (Figure 6C). Moreover, CACs recovered from the cryopreserved pheresis product were able to stimulate angiogenesis in mice after induction of hind-limb ischemia (Figure 6D). In contrast to CACs, EPCs were only rarely recovered from the cryopreserved pheresis product (Figure 6D). These data show that functional CACs but not EPCs can be recovered after cryopreservation with DMSO.

**Discussion**

There are emerging data that at least 2 distinct cell populations exist in human blood that are capable of stimulating angiogenesis. Consistent with previous reports, we show that EPCs are true progenitor cells with high proliferative capacity and the ability to form large colonies of mature endothelial cells. EPCs are extremely rare in peripheral blood, with a frequency of 1 EPC per 20 mL of blood. As described in prior studies, we show that CACs are adherent cells that appear early in culture (days 4 to 7), express monocyte markers, and are able to stimulate angiogenesis in vivo. While the mechanism by which CACs stimulate angiogenesis is unclear, there is evidence that they may act through a paracrine mechanism by secreting large amounts of angiogenic growth factors, including VEGF, at sites of vascular injury. There is considerable interest in agents that can mobilize angiogenic cells into the blood, thereby augmenting their delivery to sites of vascular injury and potentially enhancing revascularization. Cytokines known to mobilize these cells include granulocyte macrophage colony-stimulating factor (GM-CSF), VEGF, and placental growth factor (PlGF). G-CSF, the prototypical mobilizing cytokine, has been shown to mobilize angiogenic cells in nonhuman primates and rodents. In humans, G-CSF treatment increases the number of CD34+CD133+ cells, a population enriched for EPCs. These data have stimulated clinical trials of G-CSF following acute myocardial infarction. Several small randomized nonblinded trials suggested that G-CSF results in improvement in cardiac function. However, 2 recent randomized double-blinded trials of G-CSF in patients with acute myocardial infarction failed to show a significant improvement in cardiac function.

**Table 2. Leukapheresis product summary**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mice</th>
<th>CACs Total</th>
<th>No. per kilogram</th>
<th>EPCs Total</th>
<th>No. per kilogram</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMD3100</td>
<td>9</td>
<td>1.25 ± 0.10 × 10^9</td>
<td>1.47 ± 0.12 × 10^7</td>
<td>2.0 ± 0.22 × 10^3</td>
<td>21.0 ± 2.2</td>
</tr>
<tr>
<td>G-CSF</td>
<td>6</td>
<td>1.68 ± 0.18 × 10^9</td>
<td>2.02 ± 0.24 × 10^7</td>
<td>0.7 ± 0.07 × 10^3</td>
<td>7.9 ± 0.57</td>
</tr>
</tbody>
</table>

Data are reported as the total number of CACs or EPCs per pheresis product or as the number of CACs or EPCs per kilogram of donor mass.
In the present study, we compared EPC and CAC mobilization by G-CSF with AMD3100, a novel agent with rapid kinetics of hematopoietic stem cell mobilization. A crossover design was used to minimize the considerable individual variability in mobilization responses. Both G-CSF and AMD3100 markedly increased the number of circulating CACs and EPCs. Mobilization of CACs was comparable after G-CSF or AMD3100 treatment, with fold increases from baseline of 8.8 ± 3.7 and 10.0 ± 4.4, respectively. The functional capacity of CACs mobilized by these agents, as measured by their ability to stimulate angiogenesis in the murine hind-limb ischemia model, was preserved. Importantly, on a per-cell basis, the in vivo angiogenic activity of CACs harvested from G-CSF–mobilized blood was superior to that observed with AMD3100–mobilized CACs. Because G-CSF is known to regulate monocyte activation, this observation raises the possibility that G-CSF signals augment the angiogenic capacity of CACs. While both agents mobilized EPCs, higher levels of circulating EPCs were observed after G-CSF treatment, with fold increases of 10.2 ± 3.3 (AMD3100) and 21.8 ± 5.4 (G-CSF). Interestingly, EPCs mobilized by G-CSF or AMD3100 display a higher proliferative capacity than EPCs harvested from baseline blood, indicating that these agents mobilize a more primitive subset of EPCs. Although not directly assessed in this study, previous studies have shown that clonogenic EPCs can stimulate angiogenesis in the murine hind-limb ischemia model. Moreover, our preliminary data suggest that clonogenic EPCs, when placed in a fibronectin/collagen matrix and transplanted subcutaneously into immunodeficient mice, are able to form chimeric blood vessels with the murine vasculature. (Mervin C. Yoder, Laura Mead, Daniel Prater, Theresa R. Krier, Karim Mrovec, Fang Li, Rachel Krasich, Constance J. Temm, Josef T. Pichal, and D. L., manuscript submitted). Collectively, these data show that G-CSF and AMD3100 are potent mobilizers of functional CACs and EPCs. In the setting of acute vascular injury such as acute myocardial infarction, it may be important to rapidly deliver angiogenic cells to ischemic tissues. In this regard, the rapid kinetics of CAC and EPC mobilization by AMD3100 suggest that this agent may merit study in acute vascular injury syndromes.

Several clinical trials have used direct injection of enriched populations of angiogenic cells to stimulate revascularization. Although preliminary, there is evidence that this approach may have modest clinical benefit in patients with acute myocardial infarction or peripheral vascular disease. In most cases, bone marrow was the source of angiogenic cells, requiring an invasive procedure to collect the cells. Hernandez et al showed that EPCs could be recovered by leukapheresis; however, the angiogenic potential of these cells was not ascertained, and the recovery of CACs not addressed. In the present study, we show that large numbers of CACs and EPCs can be efficiently collected from mobilized peripheral blood by leukapheresis. The leukapheresed CACs retain their in vivo angiogenic potential and, in fact, on a per-cell basis are similar to freshly harvested CACs. Finally, we show that CACs but not EPCs can be cryopreserved and retain functional activity after thawing. Of note, we used a DMSO-based cryopreservation protocol; it is possible that other cryopreservation procedures may improve the recovery of EPCs.

In summary, this study shows that AMD3100 is a potent and rapid mobilizer of angiogenic cells, providing a promising strategy to stimulate therapeutic angiogenesis following acute vascular injury. This study also shows the feasibility of obtaining and storing large numbers of angiogenic cells from the blood of cytokine-treated individuals. Whether mobilized peripheral blood mononuclear cells are superior to bone marrow cells in stimulating angiogenesis will require further study. Ultimately, it will be important to definitively identify the cell type(s) that mediate angiogenesis in vivo and develop techniques to isolate them.

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Authorship
R.M.S. performed most of the studies and prepared the manuscript; B.J.C. performed and analyzed the in vivo characterization of
angiogenic cells; S.M.D. and J.D. were principal investigators of the clinical trial; K.M.T. performed all of the statistical analyses; D.I. developed and assisted with the EPC replating assay; and D.C.L. supervised all of the research and edited the manuscript. The authors declare no competing financial interests.

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References

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