CXCR4 blockade augments bone marrow progenitor cell recruitment to the neovasculature and reduces mortality after myocardial infarction

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We hypothesized that a small molecule CXCR4 antagonist, AMD3100 (AMD), could augment the mobilization of bone marrow (BM)-derived endothelial progenitor cells (EPCs), thereby enhancing neovascularization and functional recovery after myocardial infarction. Single-dose AMD injection administered after the onset of myocardial infarction increased circulating EPC counts and myocardial vascularity, reduced fibrosis, and improved cardiac function and survival. In mice transplanted with traceable BM cells, AMD increased BM-derived cell incorporation in the ischemic border zone. In contrast, continuous infusion of AMD, although increasing EPCs in the circulation, worsened outcome by blocking EPC incorporation. In addition to its effects as a CXCR4 antagonist, AMD also up-regulated VEGF and matrix metalloproteinase 9 (MMP-9) expression, and the benefits of AMD were not observed in the absence of MMP-9 expression in the BM. These findings suggest that AMD3100 preserves cardiac function after myocardial infarction by enhancing BM-EPC-mediated neovascularization, and that these benefits require MMP-9 expression in the BM, but not in the ischemic region. Our results indicate that AMD3100 could be a potentially useful therapy for the treatment of myocardial infarction.

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For most of the past two decades, the role of the microvasculature in recovery from myocardial infarction (MI) has been overshadowed by the intense focus on epicardial revascularization (1). However, results in animal models and early findings from therapeutic interventions in humans suggest that the microvasculature may be a viable target for cardiovascular therapy. In animals, the preservation or restoration of microvessels correlates with better left ventricular (LV) function after MI, and therapies designed to enhance the microvascular circulation have been shown to preserve cardiac function and integrity (2). Most compelling, however, are clinical trial data suggesting that protection or restoration of the myocardial microvasculature may provide benefits beyond those achieved with current state-of-the-art treatments (3–7), and that strategies designed to restore microvascular integrity (8) can improve long-term outcomes (9).

Studies in humans and animals have shown that a subpopulation of mononuclear cells (MNCs) with enhanced potential for differentiation into the endothelial lineage (i.e., endothelial progenitor cells or EPCs) are mobilized endogenously from the bone marrow (BM) in response to MI and become incorporated into sites of new vessel growth in the ischemic tissue (10–13). Stromal cell-derived factor 1 α (SDF-1 α) is a chemotactic factor for EPCs in humans; SDF-1 α induces EPC migration in vitro (14), and the binding of SDF-1 α to CXCR4-chemokine receptor 4 (CXCR4) is believed to prevent the release of EPCs from the BM. The SDF-1 α /CXCR4 axis also regulates EPC proliferation and survival (15, 16), as well as several intracellular signaling pathways

(17, 18). Thus, SDF-1 α and CXCR4 are key regulators of EPC mobilization and recruitment.

The pharmacological CXCR4 antagonist AMD3100 rapidly mobilizes EPCs from the BM by reversibly blocking the interactions between SDF-1 α and CXCR4 (19–22), and the combined administration of AMD3100 and the angiogenic cytokine G-CSF enhanced angiogenesis in a mouse model of acute hind limb ischemia by, at least in part, increasing the mobilization of BM MNCs (23). Here, we investigated whether AMD3100 enhances EPC mobilization and the incorporation of mobilized EPCs into sites of neovascularization after MI, and whether this enhancement leads to greater preservation of myocardial function and integrity.

Results

Acute CXCR4 Antagonism with AMD3100 Immediately After MI Increases Peripheral Blood EPC Counts for 1 to 2 Weeks. Because there are no unique markers for EPC identity, EPC counts were assessed both by culture assay and via flow cytometry analyses for coexpression of stem cell antigen 1 (Sca1) and fetal-liver kinase 1 (Flk1). In nonischemic mice, circulating EPC levels peaked 1 to 3 h after AMD3100 administration, and the number of c-kit-positive cells also increased (Fig. S1). The effect of AMD3100 on EPC mobilization after MI was evaluated by administering a single injection of AMD3100 or saline to mice 1 h after surgically induced MI. When assessed via culture assay, circulating EPC counts were significantly higher 7 and 14 d after MI in AMD3100-treated mice than in saline-treated mice (Fig. 1A). Circulating EPC counts increased significantly from before MI to day 7 in both groups; however, only AMD3100-treated mice maintained a statistically significant increase through day 14. Flow-cytometry analyses indicated that circulating Sca1/Flk1 double-positive cell counts peaked on day 3 after MI in both groups, and were significantly higher in AMD3100-treated mice than in the saline-treatment group on day 7 (Fig. S2A).

Acute CXCR4 Antagonism with AMD3100 Improves Cardiac Function After MI. The potential influence of AMD3100 on cardiac performance after MI was evaluated by comparing echocardiographic and hemodynamic assessments in mice administered

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Fig. 1. A single injection of AMD3100 after MI mobilizes EPCs, improves survival, reduces adverse cardiac remodeling, and increases capillary density. Mice received single s.c. injections of AMD3100 (AMD) or saline 1 h after MI. (A) Peripheral blood (PB) EPC counts were evaluated via the EPC culture assay and costaining for acLDL uptake and BS-1 lectin ($n = 5$). (B–D) To ensure suitable mortality for statistical analyses, MI was induced at the most proximal site of the coronary artery. (B) Survival rate of mice treated with AMD (solid line, $n = 48$) or saline (broken line, $n = 23$); subsequent assessments were performed in mice that survived through day 28. (C) Fibrosis was quantified as the ratio of the length of fibrosis to the LV circumference (AMD: $n = 12$; saline: $n = 5$); fibrotic tissue appears blue-green. (D) Capillary density was evaluated by staining the vasculature via *in vivo* BS-1 lectin perfusion before killing, and then quantifying the number of BS-1 lectin-positive (brown) vessels (AMD: $n = 12$; saline: $n = 5$). HPF, high-power field. *, $P < 0.05$; **, $P < 0.03$.

AMD3100 or saline after MI. LV fractional shortening (FS) and diastolic and systolic dimensions did not differ significantly between groups before MI or 7 d afterward, but FS was significantly greater, and LV dimensions were significantly smaller, in AMD3100-treated mice at days 14 (FS: AMD3100, $34 \pm 1\%$; saline, $25 \pm 1\%$; $P < 0.01$) and 28 (FS: AMD3100, $28 \pm 1\%$; saline, $22 \pm 1\%$; $P < 0.05$) (Table S1). Heart rates in both groups were similar at all time points, but compared to saline-treated mice, mice treated with AMD3100 exhibited higher LV developed pressures, higher positive and negative dP/dt, higher systolic pressures, and lower end diastolic pressures on days 14 and 28 after MI.

Acute CXCR4 Antagonism with AMD3100 Improves Survival, Reduces Adverse Cardiac Remodeling, and Increases Capillary Density After MI.

To determine whether AMD3100 could enhance survival after MI, surgical ligation was performed at the most proximal site of the left coronary artery, thereby inducing larger-sized infarcts that are associated with significant mortality; functional vascular structures were stained via systemic infusion of BS-1 lectin for 10 min before killing on day 28. The 28-day survival rate was significantly higher ($P < 0.03$) in mice treated with AMD3100 (58%) than in the saline-treatment group (30%) (Fig. 1B), and heart sections from AMD3100-treated mice displayed less LV dilation and significantly less LV fibrosis (Fig. 1C), cardiomyocyte hypertrophy, and myosin heavy chain β (β -MHC) expression (Fig. S2B and C), which signals adverse cardiac remodeling, whereas capillary density (Fig. 1D) and total endothelial cell mass (measured via the expression of platelet/endothelial cell adhesion molecule mRNA) (Fig. S2D) were significantly greater in AMD3100-treated mice.

Acute CXCR4 Antagonism with AMD3100 Increases BM-Derived EPC Incorporation in Ischemic Myocardial Tissue. The potential relationship between AMD3100-mediated increases in EPC mobilization and the enhanced capillary density observed in AMD3100-

treated mice was investigated by inducing MI in WT mice transplanted with BM from transgenic mice that expressed LacZ under control of the endothelial-specific Tie2 promoter (24). Seven and 14 days after MI, the number of incorporated X-gal-positive cells was significantly higher in the AMD3100-treatment group than in mice treated with saline (day 7: AMD3100, $3,776 \pm 111$ cells/mm²; saline: $2,231 \pm 317$ cells/mm²; $P < 0.05$. day 14: AMD3100, $1,906 \pm 137$ cells/mm²; saline, $1,441 \pm 56$ cells/mm²; $P < 0.05$) (Fig. 2A and B). At day 28, β -galactosidase (β -gal) activity in the ischemic myocardial tissue was significantly higher in mice treated with AMD3100 than in saline-treated mice (Fig. S3A and B), and the number of cells stained positively for both β -gal and the endothelial marker CD31 was significantly greater in tissue from the AMD3100-treatment group. (Fig. 2C). These findings were corroborated in female WT mice transplanted with BM from male transgenic mice that expressed GFP; 28 days after MI and AMD3100 treatment, mice were killed, and heart sections were assessed for the presence of the Y chromosome (via FISH) and for expression of GFP and the endothelial cell marker von Willbrand factor; Y chromosome-containing cells with overlapping GFP and von Willbrand factor expression were identified, indicating that EPCs mobilized from the transplanted BM had been incorporated in the vascular endothelium (Fig. S3C).

Acute CXCR4 Antagonism with AMD3100 Increases Inflammatory Cell Mobilization but Attenuates Inflammatory Cell Recruitment After MI.

Because BM cells contribute to the inflammatory response after MI, the mRNA expression of CD68, a monocyte/macrophage marker, was evaluated in mice treated with AMD3100 or saline after MI. Three and 7 days after MI, CD68 expression was significantly greater in peripheral blood (PB) MNCs from AMD3100-treated mice than in PB MNCs from mice treated with saline (Fig. S4A); however, CD68 levels in the ischemic border zone were significantly lower in AMD3100-treated mice on day 3 and did not differ significantly between groups on day 7 (Fig. S4B).

Continuous CXCR4 Blockade Increases Adverse Cardiac Remodeling, Reduces Capillary Density, and Impairs BM EPC Incorporation After MI.

The data described above suggest that blockade of the CXCR4/SDF-1 axis with AMD3100 increases EPC recruitment to the ischemic myocardium. This finding seems incompatible with previous reports demonstrating that SDF-1 administration can enhance the recovery of ischemic tissue and, consequently,

Fig. 2. A single injection of AMD3100 after MI increases BM EPC incorporation into ischemic tissue. WT mice transplanted with Tie2-LacZ BM received single s.c. injections of AMD3100 (AMD) or saline 1 h after MI. (A) BM EPCs were identified in the LV by X-gal staining (blue-green) for LacZ expression, and (B) the number of BM EPCs in the ischemic border zone was quantified ($n = 4$). (C) BM EPC incorporation in the ischemic border zone on day 28 was evaluated by staining for β -gal (green) and CD31 (red) expression and quantifying the number of double-positive cells (yellow) ($n = 3$). HPF, high-power field. *, $P < 0.05$; ***, $P < 0.01$. (Scale bar: 100 μ m.)

that interactions between SDF-1 and CXCR4 contribute to ischemic tissue repair (25, 26). However, our results also indicate that EPC mobilization endures for days to weeks after AMD3100 administration, so we determined whether the benefits associated with AMD3100 injection were also observed when AMD3100 was infused continuously (2.5 mg per week) over a 2-week period from an implanted osmotic pump. Fourteen days after MI, hearts harvested from mice treated with AMD3100 infusions displayed prominent LV aneurysms (Fig. S5) accompanied by wall thinning, LV dilation, and significantly greater LV fibrosis ($50.5 \pm 5.6\%$) than was observed in hearts from mice administered single AMD3100 injections ($26.0 \pm 4.3\%$) (Fig. 3A). Furthermore, both capillary density and EPC incorporation (in mice transplanted with Tie2-LacZ BM) were significantly lower in the AMD3100-infusion group than in mice treated with single AMD3100 injections or with saline infusions (Fig. 3B and C).

Therapeutic Effect of AMD3100 After MI Is Dependent on BM Matrix Metalloproteinase 9 Expression. Because matrix metalloproteinase 9 (MMP-9) is believed to participate in SDF-1 α /CXCR4-regulated progenitor cell mobilization (27), we investigated whether a single injection of AMD3100 after MI altered MMP-9 expression in PB and BM MNCs. MMP-9 expression peaked 1 d after MI (Fig. 4A) and was significantly lower in the AMD3100-treatment group than in saline-treated mice at this early time point, which is consistent with the acute antagonism of CXCR4 by AMD3100. However, on day 7, MMP-9 mRNA expression (Fig. 4A) and activity (in BM MNCs and the spleen) (Fig. S6A) were significantly higher in AMD3100-treated mice. MMP-2 mRNA expression was similar in BM MNCs from both groups (Fig. S6B).

To determine whether the benefits associated with AMD3100 treatment were dependent on MMP-9 expression, we evaluated EPC mobilization after MI in MMP-9 knockout (KO) mice, and we compared capillary density, total endothelial cell mass, and LV fibrosis after MI in WT and MMP-9 KO mice. Treatment with AMD3100 did not significantly alter EPC mobilization in MMP-9 KO mice (Fig. 4B), and MMP-9 KO mice did not display the enhanced capillary density (Fig. 4C and Fig. S6C) or the decline in

LV fibrosis (Fig. 4D) observed with AMD3100 treatment in WT mice. These findings indicate that MMP-9 expression was essential for AMD3100-induced EPC mobilization and cardiac preservation after MI.

Whether the cardioprotective effects of AMD3100 require MMP-9 activity in the BM or in the ischemic tissue was determined by performing experiments in MMP-9 KO mice transplanted with BM from WT mice (MMP-9 KO/WT_{BM}) and in WT mice transplanted with MMP-9 KO BM (WT/MMP-9 KO_{BM}). In MMP-9 KO/WT_{BM} mice, AMD3100 treatment was associated with significantly higher PB EPC counts at day 7 after MI and with significantly greater LV FS at day 14 (AMD3100: $26.2 \pm 6.2\%$; saline: $20.7 \pm 4.8\%$; $P < 0.03$) and day 28 (AMD3100: $28.6 \pm 6.5\%$; saline: $20.4 \pm 6.4\%$; $P < 0.01$) (Fig. 5A and C); however, these effects were not observed in WT/MMP-9 KO_{BM} mice at any time point (Fig. 5B and D). Similarly, LV fibrosis and capillary density at day 28 were significantly improved with AMD3100 treatment in MMP-9 KO/WT_{BM} mice but not in WT/MMP-9 KO_{BM} mice (Fig. 5E and F). Thus, the cardioprotective effects of AMD3100 appeared to be mediated by BM MMP-9 expression.

AMD3100-Induced MMP-9 Expression Is Mediated by VEGF. Because previous reports indicate that MMP-9 expression in stromal, endothelial (28), smooth-muscle (29, 30), and tumor cells (31–33) is regulated by VEGF, we investigated whether VEGF contributes to the AMD3100-induced up-regulation of MMP-9 expression in MNCs after MI. VEGF mRNA expression was significantly elevated in MNCs isolated from the PB of AMD3100-treated mice 7 d after MI (Fig. 6A), and in BM MNCs on days 3 and 7 (Fig. 6B). Furthermore, administration of a VEGF-neutralizing antibody blocked the AMD3100-induced up-regulation of MMP-9 in both PB and BM MNCs (Figs. 6C and D). Collectively, these observations suggest that VEGF contributed to the AMD3100-induced up-regulation of MMP-9 in MNCs after MI and, by extension, to the cardioprotective effects associated with AMD3100.

Discussion

The results presented here indicate that administration of a single s.c. dose of AMD3100 after MI mobilized EPCs from the BM

Fig. 3. Continuous AMD3100 infusion increases adverse cardiac remodeling, decreases capillary density, and impairs BM EPC incorporation. (A and B) WT mice or (C) WT mice transplanted with Tie2-LacZ BM received single injections of AMD3100 (AMD) or were implanted with osmotic pumps that continuously infused AMD3100 or saline for 2 weeks after MI. (A) Fibrosis was quantified as the ratio of the length of fibrosis to the LV circumference ($n = 5$); fibrotic tissue appears blue. (B) Capillary density was evaluated by staining the vasculature via in vivo BS-1 lectin perfusion before killing, and then quantifying the number of BS-1-lectin-positive vessels (green) ($n = 5$). (C) BM EPC incorporation in the ischemic border zone on day 14 after MI was quantified as the number of β -gal-positive cells (red) ($n = 3$). HPF, high-power field; i, ischemic tissue; n, nonischemic tissue. *, $P < 0.05$; ***, $P < 0.01$.

Fig. 4. The beneficial effects associated with a single injection of AMD3100 are dependent on MMP-9 expression. (A) WT mice, (B) MMP-9 knockout (KO) mice, or (C and D) WT and MMP-9 KO mice received single s.c. injections of AMD3100 (AMD) or saline 1 h after MI. (A) MMP-9 expression in PB or BM MNCs was evaluated via quantitative RT-PCR; measurements were normalized to 18S rRNA and expressed as the fold-change from levels before MI ($n = 4$). (B) PB EPC counts were determined in MMP-9 KO mice via flow-cytometry analyses for coexpression of Sca1 and Flk1 ($n = 4$). (C) Capillary density on day 28 after MI was evaluated by staining the vasculature via in vivo BS-1 lectin perfusion before killing, and then quantifying the number of BS-1 lectin-positive (brown) vessels ($n = 5$). (D) Fibrosis was quantified as the ratio of the length of fibrosis to the LV circumference; fibrotic tissue appears blue-gray ($n = 5$). *, $P < 0.05$; NS, not significant.

to the PB, and that the mobilized EPCs were recruited to the infarcted myocardium, where they contributed to neovascularization. The enhanced EPC recruitment was accompanied by reduced LV fibrosis, greater preservation of cardiac function, and a higher survival rate, and the benefits associated with AMD3100 treatment were dependent on MMP-9 expression in BM cells, but not in the ischemic tissue. Together, these observations emphasize the importance of BM-derived cell mobilization and recruitment during recovery from MI and suggest that AMD3100 could be a potentially useful therapeutic agent.

In nonischemic mice, EPC mobilization peaked 1 to 3 h after AMD3100 administration, which is consistent with the plasma half-life of AMD3100 (2–3 h). In addition, it is notable that the number of circulating c-kit-positive cells was also increased by AMD3100. However, when administered after MI, AMD3100 was associated with significantly higher circulating EPC and macrophage counts 3 to 7 d later, long after the acute effects of AMD3100 administration should have dissipated. Furthermore, AMD3100 administration was associated with a significant loss

Fig. 5. The beneficial effects associated with a single injection of AMD3100 are dependent on BM MMP-9 expression, but not on MMP-9 expression in the ischemic tissue. MMP-9 KO mice transplanted with WT BM and WT mice transplanted with MMP-9 KO BM received single s.c. injections of AMD3100 (AMD) or saline 1 h after MI. (A and B) PB EPC counts were evaluated via flow-cytometry analysis for coexpression of Sca1 and Flk1. (A) $n = 3$ at all time points. (B) Before MI, $n = 9$; day 1, $n = 3$ for AMD and 4 for saline; day 3, $n = 3$; day 7, $n = 4$ for AMD and 3 for saline; day 28, $n = 4$. (C and D) LV fractional shortening was evaluated echocardiographically. (C) Before MI, $n = 3$; day 7, $n = 5$; day 14, $n = 10$; day 28, $n = 11$ for AMD and 9 for saline. (D) Before MI, $n = 5$; day 7, $n = 4$; day 14, $n = 10$; day 28, $n = 10$ for AMD and 11 for saline. (E) Fibrosis on day 28 after MI was quantified as the ratio of the length of fibrosis to the LV circumference. MMP-9 KO/WT_{BM}, $n = 7$ for saline and 10 for AMD; WT/MMP-9 KO_{BM}, $n = 11$ for saline and $n = 6$ for AMD. (F) Capillary density on day 28 was evaluated by staining the vasculature via in vivo BS-1 lectin perfusion before killing, and then quantifying the number of BS-1 lectin-positive vessels. MMP-9 KO/WT_{BM}, $n = 6$ for saline and 7 for AMD; WT/MMP-9 KO_{BM}, $n = 14$ for saline and 7 for AMD. HPF, high-power field. *, $P < 0.05$; **, $P < 0.03$; ***, $P < 0.01$; NS, not significant.

of MMP-9 expression on day 1 after MI, which is consistent with previous reports linking SDF-1 administration to increases in MMP-9 expression (27), but MMP-9 expression was elevated in AMD3100-treated mice on days 3 and 7. Thus, the pathways activated by AMD3100 administration appear to be more persistent in the setting of MI than in noninfarcted animals, and the downstream consequences of these pathways can differ substantially from the acute effects associated with AMD3100 treatment.

Seven days after MI, significantly more BM-derived EPCs were identified in the ischemic border zone of mice treated with AMD3100 than in mice administered saline; the number of recruited EPCs declined on day 14, but the difference between treatment groups remained significant. These findings are consistent with our observations in mice administered systemic injections of EPCs without AMD3100 treatment: recruitment began shortly after MI, peaked on day 7, and continued through day 10 (34). Thus, CXCR4 antagonism appears to enhance EPC incorporation without altering the time course of EPC recruitment. AMD3100 administration also mobilized inflammatory cells from the BM, but macrophage recruitment to the ischemic border zone was impaired in AMD3100-treated mice, which suggests that AMD3100 does not worsen inflammation after MI.

The enhanced EPC recruitment observed in AMD3100-treated mice may be attributable to elevated systemic EPC levels, higher

Fig. 6. AMD3100 administration immediately after MI increases VEGF expression in PB and BM MNCs 3 to 7 d later. VEGF activity is required for AMD3100-induced MMP-9 expression. (A and B) Mice received single s.c. injections of AMD3100 (AMD) or saline 1 h after MI and then VEGF mRNA expression in (A) PB and (B) BM MNCs was measured via quantitative RT-PCR, normalized to endogenous 18S rRNA expression, and expressed as the fold-change from levels before MI. *, $P < 0.05$; $n = 5$. (C and D) AMD3100- and saline-treated mice received i.p. injections of a VEGF-neutralizing antibody (antiVEGF) or an IgG antibody after surgically induced MI. Five days later, MMP-9 mRNA expression was measured in (C) PB and (D) BM MNCs. mRNA measurements were normalized to endogenous 18S rRNA expression and expressed as the fold-change from measurements obtained in uninjured, untreated mice. *, $P < 0.05$; ***, $P < 0.01$; $n = 5$.

levels of MMP-9 expression, or both. Previous reports indicate that MMP-9 activation induces the recruitment and mobilization of hematopoietic stem cells and other undifferentiated cells, including EPCs (27); furthermore, SDF-1 α stimulates MMP-9 secretion from EPCs, and the migration of EPCs toward SDF-1 α is dependent on the production of MMP-9 (35). Here, we showed that the beneficial effects of AMD3100 treatment are intact in MMP-9 KO mice transplanted with WT BM, but not in WT mice transplanted with MMP-9 KO BM. Thus, the benefits associated with AMD3100 administration, including (presumably) the enhancement of EPC recruitment and mobilization, appear to require MMP-9 expression in BM cells but not in the ischemic tissue.

Previous reports indicate that MMP-9 expression in a variety of cells is regulated by VEGF (28–33), and the results described here suggest that AMD3100 administration after MI increased VEGF mRNA expression in BM and PB MNCs. Furthermore, coadministration of an anti-VEGF antibody abolished the AMD3100-induced increase in MMP-9 expression. Collectively, these observations suggest that the elevated MMP-9 levels in MNCs from AMD3100-treated mice evolved through an increase in VEGF expression. VEGF governs many components of the angiogenic response, including the mobilization and recruitment of BM cells, so the benefits associated with AMD3100 administration are likely generated, at least in part, by increased VEGF activity. Our data also show that the mobilization and recruitment of MNC subpopulations by AMD3100 is selective, with reduced macrophage recruitment into the ischemic myocardium. These findings are consistent with a recent report by Pitchford et al. (36) showing that VEGF pretreatment enhanced EPC mobilization by AMD3100 while inhibiting hematopoietic progenitor cell release.

The findings presented here raise questions about the mechanism responsible for the profound and long-term (compared with the half-life of the drug) effects of AMD3100 on VEGF and MMP-9 expression. The fact that this effect is observed in the setting of ischemia and not in nonischemic animals suggests the triggering of a cascade of signaling events that is primed by ischemia, perhaps via activation of the sympathetic nervous system (37). Indeed, the ability of AMD3100 to augment progenitor cell mobilization initiated by other factors, such as G-CSF (38), was, at least in part, the basis for its approval by the United States Food and Drug Administration. Furthermore, the changes noted in capillary density occur within a week after MI/AMD3100 administration and persist for at least 4 weeks. Given the dynamic relationship between capillary and arteriolar density, one could speculate that increased “downstream” or outflow could trigger the expansion of arteriolar growth. Thus, some of the increase in total endothelial cell mass may be attributable to the expansion of resistance vessels.

The benefits associated with a single injection of AMD3100 after MI were not observed with prolonged AMD3100 treatment. Instead, capillary density in the ischemic border zone was significantly lower in mice administered continuous AMD3100 infusions for 2 weeks after MI, and the decline in vascularity was accompanied by LV wall thinning and aneurysm formation. Continuous AMD3100 administration was also associated with lower numbers of incorporated BM-derived EPCs. In a previous report, the incorporation of exogenous, systemically administered BM cells declined when the cells were pretreated with AMD3100; this and other observations led the authors to conclude that SDF-1 is crucially involved in the recruitment of BM-derived cells to infarcted myocardium (39). Thus, the decline in BM cell incorporation observed with continuous AMD3100 treatment likely evolved from prolonged disruption of SDF-1–CXCR4 binding. Continuous AMD3100 infusion has also been linked to declines in VEGF-induced neovascularization (40).

In summary, the results presented here demonstrate that a single dose of AMD3100 after MI enhances the preservation of cardiac function and integrity after MI. Furthermore, many of the benefits associated with AMD3100 administration, including enhanced BM cell mobilization and recruitment, occur several days after the acute effects of AMD3100 have dissipated. These delayed effects appear to be attributable to VEGF-induced increases in MMP-9 expression, a previously unknown mechanism associated with this pharmacological agent.

Together, these findings provide enticing evidence regarding the potential use of AMD3100 as a therapeutic agent in the setting of MI. Beneficial effects were documented when the drug was administered at least 1 h after the onset of injury, and these benefits were noted despite permanent coronary-artery occlusion. In this regard, it is notable that AMD3100 is now approved in the United States for use as a mobilization agent and, therefore, has accumulating evidence for safety in human use.

Materials and Methods

Animal Models and Treatments. Mice were obtained from The Jackson Laboratories and are listed in *SI Materials and Methods*. MI was induced as described previously (41), and the BM transplantation procedure is summarized in *SI Materials and Methods*. Treatment was administered 1 h after closure of the chest wall. For evaluation of a single AMD3100 injection, AMD3100 (125 μ g in 100 μ L; Sigma-Aldrich Co.) or an equal volume of saline was injected subcutaneously. For evaluation of continuous AMD3100 administration, mice were implanted with miniosmotic pumps (DURECT Corporation) that infused 5 mg AMD3100 or an equivalent volume of saline over a period of 2 weeks (40), and a third treatment group received a single s.c. injection of AMD3100 (125 μ g in 100 μ L). Administration of the VEGF-neutralizing antibodies (50 μ g anti-mouse VEGF, R&D Systems Inc.) (42) and the control IgG antibodies (50 μ g Goat IgG, Jackson ImmunoResearch Laboratories Inc.) was performed via i.p. injection immediately after MI surgery and repeated 2 and 4 d later. All surgical procedures and animal care pro-

protocols were approved by the Northwestern University Animal Care and Use Committee.

Assessments. All evaluations were performed via standardized protocols as summarized in *SI Materials and Methods*. Cell counts were determined via the EPC culture assay and flow cytometry analysis. LV function was evaluated via echocardiography. Hemodynamic measurements were obtained with a 1.0-French high-fidelity pressure catheter (Millar Instruments, Inc.) inserted through the left carotid artery and into the LV. Histological assessments were performed in frozen or paraffin-embedded heart sections. Capillaries were identified by injecting mice with BS-1 lectin (Vector Laboratories) 10 min before killing. Incorporated Tie2-LacZ BM-derived cells were identified via X-gal staining, anti- β -gal and anti-CD31 costaining, or by optical-density measurements of homogenized tissue extracts; in female WT mice transplanted with BM from male GFP-expressing mice,

incorporated BM-derived cells were identified via FISH, anti-GFP staining, and anti-von Willbrand factor staining. The primers and probes used for RT-PCR analyses are shown in [Table S2](#).

Statistics. All values were expressed as mean $\pm$ SEM. Comparisons between two groups were evaluated with the unpaired *t* test, and comparisons among three or four groups were evaluated with one-factor ANOVA followed by the unpaired *t* test. Assessments at multiple time points were evaluated via repeated measures analysis followed by the unpaired *t* test. Survival was evaluated via Kaplan-Meier analysis and the Fisher's exact test. *P* < 0.05 was considered statistically significant.

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- Topol EJ (2003) Current status and future prospects for acute myocardial infarction therapy. *Circulation* 108 (Suppl 1):III6–III13.
- Kawamoto A, et al. (2003) Intramyocardial transplantation of autologous endothelial progenitor cells for therapeutic neovascularization of myocardial ischemia. *Circulation* 107:461–468.
- Gibson CM, et al.; TIMI Study Group (2002) Relationship of the TIMI myocardial perfusion grades, flow grades, frame count, and percutaneous coronary intervention to long-term outcomes after thrombolytic administration in acute myocardial infarction. *Circulation* 105:1909–1913.
- Wong GC, et al. (2002) Elevations in troponin T and I are associated with abnormal tissue level perfusion: A TACTICS-TIMI 18 substudy. Treat Angina with Aggrastat and Determine Cost of Therapy with an Invasive or Conservative Strategy-Thrombolysis in Myocardial Infarction. *Circulation* 106:202–207.
- Yip HK, et al. (2003) Effect of the PercuSurge GuardWire device on the integrity of microvasculature and clinical outcomes during primary transradial coronary intervention in acute myocardial infarction. *Am J Cardiol* 92:1331–1335.
- Britten MB, et al. (2003) Infarct remodeling after intracoronary progenitor cell treatment in patients with acute myocardial infarction (TOPCARE-AMI): Mechanistic insights from serial contrast-enhanced magnetic resonance imaging. *Circulation* 108: 2212–2218.
- Moreno R, Hernández-Antolín R, Alfonso F, Macaya C (2003) Diabetes mellitus and acute myocardial infarction: More data supporting a poorer microvasculature reperfusion. *Am Heart J* 146:E6.
- Erbs S, et al. (2007) Restoration of microvascular function in the infarct-related artery by intracoronary transplantation of bone marrow progenitor cells in patients with acute myocardial infarction: The Doppler Substudy of the Reinfusion of Enriched Progenitor Cells and Infarct Remodeling in Acute Myocardial Infarction (REPAIR-AMI) trial. *Circulation* 116:366–374.
- Assmus B, et al. (2002) Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI). *Circulation* 106:3009–3017.
- Shintani S, et al. (2001) Mobilization of endothelial progenitor cells in patients with acute myocardial infarction. *Circulation* 103:2776–2779.
- Brenner W, et al. (2004) ¹¹¹In-labeled CD34+ hematopoietic progenitor cells in a rat myocardial infarction model. *J Nucl Med* 45:512–518.
- Asahara T, et al. (1999) Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ Res* 85:221–228.
- Li B, et al. (2006) VEGF and PIGF promote adult vasculogenesis by enhancing EPC recruitment and vessel formation at the site of tumor neovascularization. *FASEB J* 20: 1495–1497.
- Aiuti A, Webb IJ, Bleul C, Springer T, Gutierrez-Ramos JC (1997) The chemokine SDF-1 is a chemoattractant for human CD34+ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34+ progenitors to peripheral blood. *J Exp Med* 185:111–120.
- Wang JF, Liu ZY, Groopman JE (1998) The alpha-chemokine receptor CXCR4 is expressed on the megakaryocytic lineage from progenitor to platelets and modulates migration and adhesion. *Blood* 92:756–764.
- Lataillade JJ, et al. (2000) Chemokine SDF-1 enhances circulating CD34(+) cell proliferation in synergy with cytokines: Possible role in progenitor survival. *Blood* 95: 756–768.
- Majka M, et al. (2000) Stromal-derived factor 1 and thrombopoietin regulate distinct aspects of human megakaryopoiesis. *Blood* 96:4142–4151.
- Ganju RK, et al. (1998) The alpha-chemokine, stromal cell-derived factor-1alpha, binds to the transmembrane G-protein-coupled CXCR-4 receptor and activates multiple signal transduction pathways. *J Biol Chem* 273:23169–23175.
- Hendrix CW, et al. (2000) Pharmacokinetics and safety of AMD-3100, a novel antagonist of the CXCR-4 chemokine receptor, in human volunteers. *Antimicrob Agents Chemother* 44:1667–1673.
- Broxmeyer HE, et al. (2005) Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist. *J Exp Med* 201: 1307–1318.
- Liles WC, et al. (2003) Mobilization of hematopoietic progenitor cells in healthy volunteers by AMD3100, a CXCR4 antagonist. *Blood* 102:2728–2730.
- Devine SM, et al. (2004) Rapid mobilization of CD34+ cells following administration of the CXCR4 antagonist AMD3100 to patients with multiple myeloma and non-Hodgkin's lymphoma. *J Clin Oncol* 22:1095–1102.
- Capoccia BJ, Shepherd RM, Link DC (2006) G-CSF and AMD3100 mobilize monocytes into the blood that stimulate angiogenesis in vivo through a paracrine mechanism. *Blood* 108:2438–2445.
- Sato TN, et al. (1995) Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature* 376:70–74.
- Askari AT, et al. (2003) Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy. *Lancet* 362:697–703.
- Yamaguchi J, et al. (2003) Stromal cell-derived factor-1 effects on ex vivo expanded endothelial progenitor cell recruitment for ischemic neovascularization. *Circulation* 107:1322–1328.
- Heissig B, et al. (2002) Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. *Cell* 109:625–637.
- Heissig B, et al. (2005) Low-dose irradiation promotes tissue revascularization through VEGF release from mast cells and MMP-9-mediated progenitor cell mobilization. *J Exp Med* 202:739–750.
- Yao JS, et al. (2004) Minocycline exerts multiple inhibitory effects on vascular endothelial growth factor-induced smooth muscle cell migration: The role of ERK1/2, PI3K, and matrix metalloproteinases. *Circ Res* 95:364–371.
- Wang H, Keiser JA (1998) Vascular endothelial growth factor upregulates the expression of matrix metalloproteinases in vascular smooth muscle cells: Role of flt-1. *Circ Res* 83:832–840.
- Owen JL, et al. (2003) Up-regulation of matrix metalloproteinase-9 in T lymphocytes of mammary tumor bearers: Role of vascular endothelial growth factor. *J Immunol* 171:4340–4351.
- Hiratsuka S, et al. (2002) MMP9 induction by vascular endothelial growth factor receptor-1 is involved in lung-specific metastasis. *Cancer Cell* 2:289–300.
- Dias S, et al. (2000) Autocrine stimulation of VEGFR-2 activates human leukemic cell growth and migration. *J Clin Invest* 106:511–521.
- Hamada H, et al. (2006) Estrogen receptors alpha and beta mediate contribution of bone marrow-derived endothelial progenitor cells to functional recovery after myocardial infarction. *Circulation* 114:2261–2270.
- Janowska-Wieczorek A, Marquez LA, Dobrowsky A, Ratajczak MZ, Cabuhat ML (2000) Differential MMP and TIMP production by human marrow and peripheral blood CD34(+) cells in response to chemokines. *Exp Hematol* 28:1274–1285.
- Pitchford SC, Furze RC, Jones CP, Wengner AM, Rankin SM (2009) Differential mobilization of subsets of progenitor cells from the bone marrow. *Cell Stem Cell* 4: 62–72.
- Katayama Y, et al. (2006) Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow. *Cell* 124:407–421.
- Flomenberg N, et al. (2005) The use of AMD3100 plus G-CSF for autologous hematopoietic progenitor cell mobilization is superior to G-CSF alone. *Blood* 106: 1867–1874.
- Abbott JD, et al. (2004) Stromal cell-derived factor-1alpha plays a critical role in stem cell recruitment to the heart after myocardial infarction but is not sufficient to induce homing in the absence of injury. *Circulation* 110:3300–3305.
- Grunewald M, et al. (2006) VEGF-induced adult neovascularization: Recruitment, retention, and role of accessory cells. *Cell* 124:175–189.
- Iwakura A, et al. (2006) Estradiol enhances recovery after myocardial infarction by augmenting incorporation of bone marrow-derived endothelial progenitor cells into sites of ischemia-induced neovascularization via endothelial nitric oxide synthase-mediated activation of matrix metalloproteinase-9. *Circulation* 113:1605–1614.
- Silvestre JS, et al. (2003) Vascular endothelial growth factor-B promotes in vivo angiogenesis. *Circ Res* 93:114–123.