Plasminogen Regulates Stromal Cell–Derived Factor-1/CXCR4–Mediated Hematopoietic Stem Cell Mobilization by Activation of Matrix Metalloproteinase-9

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Objective—Granulocyte colony-stimulating factor (G-CSF) is a widespread therapeutic agent for stimulation of hematopoietic progenitor and stem cell (HPSC) mobilization from bone marrow (BM). Plasminogen (Plg) has been shown to be critical for HPSC mobilization. Here, we investigated the role of Plg in G-CSF-induced HPSC mobilization and the underlying mechanism.

Methods and Results—By using gene-targeted mice, our data show that Plg is required for G-CSF-induced HPSC egress to sinusoidal capillaries in BM and subsequent mobilization to peripheral circulation. G-CSF induced Plg-dependent activation of matrix metalloproteinase-9 (MMP-9) in BM, and MMP-9 neutralization or deficiency suppressed HPSC migration and mobilization. Reconstitution of MMP-9 activity by BM transplantation after lentiviral overexpression rescued HPSC mobilization in Plg-deficient mice, indicating that MMP-9 activation is required for Plg-mediated HPSC mobilization. Interestingly, after G-CSF simulation, Plg downregulated stromal cell–derived factor-1 in BM and spatiotemporally regulated the expression of C-X-C chemokine receptor type 4 (CXCR4) on mobilized HPSCs, and reconstitution of MMP-9 activity in Plg-deficient mice reversed CXCR4 expression on HPSCs in plasma and BM, suggesting that CXCR4 serves as a new downstream signal of Plg/MMP-9 in HPSC mobilization.

Conclusion—Our data elucidated a novel mechanism that Plg regulates MMP-9-dependent CXCR4 expression to facilitate HPSC mobilization in response to G-CSF. (Arterioscler Thromb Vasc Biol. 2011;31:2035-2043.)

Key Words: Granulocyte colony stimulating factor ■ MMP-9 ■ plasminogen ■ SDF-1/CXCR4 ■ hematopoietic progenitor and stem cells

In multiple pathological settings, including stroke and myocardial infarction, hematopoietic progenitor and stem cells (HPSCs) are mobilized from the bone marrow (BM) to sites of injury to promote tissue repair and regeneration.1 Stimulation of HPSC mobilization by cytokine challenge has emerged as an important therapeutic strategy for treatment of ischemic heart disease.1,2 Granulocyte colony-stimulating factor (G-CSF) is the most commonly used mobilizing agent; however, impaired response to G-CSF is observed in 25% of patients and 10% to 20% of healthy donors.3–5 Therefore, a better understanding of the underlying mechanisms regulating G-CSF-induced HPSC mobilization may offer novel approaches for strengthening stem cell–mediated therapeutics.

Central to the regulation of HPSC mobilization is proteinase-mediated inactivation of cytokine signals, eg, c-kit/c-kit ligand (c-kitL) and stromal cell–derived factor-1 (SDF-1)/C-X-C chemokine receptor type 4 (CXCR4), that anchors HPSCs in the BM microenvironment;6 the inactivation of these cytokine signals allows HPSCs to proliferate, migrate to the sinusoid capillaries, and eventually enter the peripheral blood. Plasminogen (Plg), activated by tissue-type and urokinase-type Plg activator (uPA) to plasmin, is a critical mediator for HPSC mobilization from BM to the circulation.7–10 The underlying mechanism is poorly understood, but it is likely related to regulation of these cytokine signals. Previous studies8 show that Plg regulates HPSC mobilization through plasmin cleavage of uPA receptor (uPAR) to release soluble uPAR to facilitate HPSC migration. Interestingly, uPA is not required for G-CSF-induced HPSC mobilization,11 indicating that uPAR works as a downstream target of plasmin rather than a Plg activator receptor for plasmin generation during G-CSF-induced HPSC mobilization. However, a 3- to 4-fold lower inhibition in HPSC mobilization was observed in uPAR−/− mice compared with Plg−/− mice,8 suggesting there are unexplored mechanisms for Plg regulation of HPSC mobilization other than merely via uPAR cleavage. Although c-kitL is inactivated by Plg-mediated activation of matrix metalloproteinase-9 (MMP-9) during 5-fluorouracil (5-FU)-induced HPSC mobilization,7 c-kitL does not seem to be involved in G-CSF-induced HPSC mobilization.
because G-CSF does not affect its level.12 SDF-1/CXCR4 signal is a major chemotactic signal for stem cell mobilization13–15; however, whether SDF-1/CXCR-4 signal contributes to Plg-mediated HPSC mobilization by G-CSF is unknown.

In addition to adhesion and chemotaxis signals, HPSC mobilization is also subjected to regulation by proteolytic enzyme-mediated matrix degradation.6,10 We have shown that MMP-9 acts downstream of Plg to regulate inflammatory cell migration.16 Consistently, MMP-9 is required for Plg-regulated hematopoietic regeneration after 5-FU-induced myeloablation;7 however, its role in Plg-regulated HPSC mobilization by G-CSF remains elusive. Although there is evidence for roles of Plg, MMP-9, and SDF-1/CXCR4 in HPSC mobilization from the BM, the interaction of these pathways has not been investigated.

In the present study, we investigate the roles of SDF-1/CXCR4 and MMP-9 in Plg-mediated HPSC mobilization by G-CSF in vivo. Our findings establish a novel mechanism by which Plg regulates SDF-1/CXCR4 expression to modulate HPSC mobilization through MMP-9 activation.

**Materials and Methods**

**Mice**

The Plg−/− and MMP-9−/− mice were on the C57BL/6J background. All animal procedures were performed in accordance with protocols approved by the institutional animal care and use committee of the Cleveland Clinic (see expanded methods in the supplemental data, available online at http://atvb.ahajournals.org).

**Stem Cell Mobilization**

Mice received a daily subcutaneous injection of mouse G-CSF (200 µg/kg body weight, Peprotech Inc) for 5 consecutive days. For some experiments, on day 4 immediately after the G-CSF injection, mouse proform of MMP-9 (1 µg) or the active form of human MMP-9 (actMMP-9) (1 µg) (Calbiochem) were intravenously injected into the retroorbital sinus. Five days after G-CSF treatment, blood was drawn from the retroorbital sinus, and the number of white blood cells from BM was counted with a hematocytometer. Blood, BM, femurs, and tibia were harvested from 6- to 8-week-old Plg−/− and Plg+/− mice, and BM cells were isolated from BM cells by immunomagnetic cell sorting (Miltenyi Biotec) and labeled with fluorescein isothiocyanate–anti-mouse CXCR4 antibody (clone 2B11, BD Pharmingen), which recognizes 0 to 63 amino acids of the N terminus of CXCR4. CXCR4 expression on cells was expressed as mean fluorescence intensity. The fluorescence-activated cell sorting analysis was performed using a modified Boyden chamber (see expanded methods in the supplemental data).

**Flow Cytometry**

For analysis of HPSCs (Lin− c-kit+), blood cells from G-CSF-treated mice were incubated with a cocktail of biotin-conjugated lineage-specific monoclonal antibodies (CD5, CD45R, CD11b, Gr-1, TER119, and 7/4) (Miltenyi Biotec) and labeled with fluorescein isothiocyanate–anti-biotin IgG together with phycoerythrin–anti-c-kit (BD Pharmingen). For analysis of CXCR4 in HPSCs, cells were labeled with lineage cocktail antibodies, phycoerythrin–anti-c-kit, and fluorescein isothiocyanate–anti-mouse CXCR4 antibody (clone 2B11, BD Pharmingen), which recognizes 2 to 38 amino acids of the N terminus of CXCR4. Flow cytometry was performed on a FACScalibur or FACScanto II (BD Biosciences). Isotype control (cBioscience) antibodies were used to exclude false-positive cells.

**Zymography of MMP-9**

BM and blood plasma were subjected to electrophoresis and gelatin zymography as described previously.16 The intensities of the bands were quantified using ImageJ software.

**SDM-1 ELISA**

SDF-1 concentration in BM and blood plasma was determined by using a SDF-1 ELISA kit (R&D Systems) following the manufacturer’s instructions.

**Isolation of BM-Derived Lin− c-kit+ Cells and Chemotaxis Assay**

Lin− c-kit+ cells were isolated from BM cells by immunomagnetic separation using MACs beads (Miltenyi Biotec). A migration assay was performed using a modified Boyden chamber (see expanded methods in the supplemental data).

**Lentivirus Production**

Murine MMP-9 cDNAs, wild-type (WT), or Gly100Leu mutant (a gift from Dr Elaine W. Raines, University of Washington) were amplified by polymerase chain reaction and subcloned into pLVX (Clontech). All clones were verified by double enzyme digestion and sequencing. Lentivirus was generated by transfection of HEK293T cells with pLVX-MMP-9 and packaging vectors following the Lenti-X system instructions (Clontech). Virus titers were determined by p24 ELISA following the manufacturer’s instructions (Clontech).

**BM Transduction and Transplantation**

BM cells were harvested from 6- to 8-week-old Plg−/− donor mice and cultured in Dulbecco’s modified Eagle’s medium containing 15% FBS (Invitrogen) for 24 hours at 37°C. Cells were transduced with lentivirus (multiplicity of infection = 5) in the culture medium containing 8 µl/L polybrene (Sigma) overnight. Age- and sex-matched Plg−/− or Plg+/− recipient mice were lethally irradiated (single dose of 10.5 Gy) and injected with 2×10⁶ infected BM cells via the retroorbital sinus. Four weeks later, mice were injected with G-CSF for 5 consecutive days. BM and blood cells were collected for fluorescence-activated cell sorting analysis.

**Histological Studies**

The femur and tibia were harvested on day 4 after G-CSF injection. Bone sections were stained with hematoxylin/eosin. For immunohistochemistry, the antibodies used included c-kit (clone c-19, Santa Cruz Biotechnology) or CXCR4 (TP503, Torrey Pines Biolabs), which recognizes 2 to 38 amino acids of the N terminus of CXCR4. A peroxidase DAB detection system was applied according to the manufacturer’s instructions (Vector Labs). No background staining was seen with either an irrelevant isotype-matched monoclonal antibody or in the absence of a primary antibody.

**Statistical Analysis**

All the data in the text and figures are expressed as mean±SEM and were analyzed using a t test and ANOVA with a Newman-Keuls post test. A probability value <0.05 was considered significant.

**Results**

Plg Is Required for Mobilization of HPSCs From BM to the Circulation

To understand the mechanism underlying stem cell mobilization, we investigated whether Plg is required for egress of cells from BM after cytokine challenge. Mice were injected with G-CSF for 5 days, and a 4-fold increase in white blood cell counts was detected in Plg−/− mice but not in Plg+/− mice (Figure 1A). G-CSF significantly increased HPSC (Lin− c-kit+) cell number by 8-fold in Plg+/− mice, whereas Plg deficiency blocked this increase (Figure 1B). The difference in HPSC number of Plg+/− and Plg−/− mice (33.9±8.0 versus 17.0±3.5×10³/mL) remained significant after longer G-CSF treatment (6 days). These data confirm a previous study showing that Plg is necessary for HPSC mobilization from BM to the circulation.

Furthermore, our data show that...
administration of aprotinin, a Plg inhibitor targeting the catalytic site, inhibits G-CSF-induced HPSC mobilization, suggesting an important role of plasmin proteolytic function in Plg-dependent HPSC mobilization by G-CSF (Supplemental Figure 1A and IB).

**HPSC Mobilization Is Impaired in the BM in Plg$^{-/-}$ Mice After G-CSF Injection**

A critical process for HPSC mobilization is the release of cells from the endosteal niche and migration to the sinusoidal capillaries in BM. To test whether Plg regulates HPSC egress to the sinusoidal capillaries, the femur and tibia bone sections were stained with hematoxylin/eosin or immunostained with c-kit antibody. Sinusoids (white circles), a specific circulation system in BM, were characterized by hematoxylin/eosin staining in Plg$^{+/+}$ and Plg$^{-/-}$ mice (Figure 1C). Before G-CSF treatment, hematopoietic cells (dark blue) were present in BM matrix, and no cells were observed in sinusoids of either Plg$^{+/+}$ or Plg$^{-/-}$ mice. Notably, a few sinusoids containing a thrombus associated with a large number of erythrocytes were observed in Plg$^{-/-}$ mice (yellow arrows in Figure 1C), a typical feature of Plg deficiency.17,18 Four days after G-CSF injection, in Plg$^{+/+}$ mice, the dilated lumen of the sinusoids was filled with numerous hematopoietic cells, confirmed as c-kit$^{+}$ HPSCs (Figure 1D). In Plg$^{-/-}$ mice, G-CSF did not induce dilatation of the sinusoidal lumen (Figure 1C). Some sinusoids were filled with erythrocytes associated with thrombi, but not HPSCs, as characterized by their morphology and negative c-kit antigen expression (Figure 1D). These results substantiate the dependence of G-CSF-induced HPSC mobilization on Plg and suggest that Plg regulates HPSC entry to sinusoids in BM to modulate cell mobilization.

**G-CSF Induces a Plg-Dependent MMP-9 Activation and HPSC Mobilization Is Impaired in MMP-9$^{-/-}$ Mice**

Matrix metalloproteinases, including MMP-3, MMP-9, and MMP-13, serve as downstream targets of plasmin activity,19 and MMP-9 has been shown to be critical for HPSC recruitment.12,20–22 To test whether MMP-9 is involved in Plg-mediated HPSC mobilization, Plg-regulated proteolysis in BM was investigated by gelatin zymography. No detectable MMP-9 activity was observed in the BM of either Plg$^{+/+}$ or Plg$^{-/-}$ mice before G-CSF injection, but G-CSF significantly induced MMP-9 activation in both blood and BM of Plg$^{-/-}$ mice (Figure 2A to 2D). In contrast, Plg deficiency significantly attenuated G-CSF-stimulated MMP-9 activation by 40%, but not proMMP-9 expression. Consistently, aprotinin significantly inhibited MMP-9 activation both in plasma and BM (Supplemental Figure IC and ID). Together, these results indicate that G-CSF induces Plg-dependent MMP-9 activation during HPSC mobilization.

Extensive studies have established the essential role of MMP-9 in stem cell mobilization; however, the results of HPSC mobilization generated from MMP-9$^{-/-}$ mice are still controversial.23,24 To investigate whether MMP-9 is required for HPSC mobilization by G-CSF stimulation, stem cell mobilization was assessed in MMP-9$^{-/-}$ mice (yel-

**MMP-9 Activation Is Required for Plg-Regulated HPSC Mobilization From BM to the Peripheral Blood**

MMP-9 is required for Plg-regulated hematopoietic regeneration after 5-FU-induced myeloablation; however, its role in
Plg-regulated HPSC mobilization by G-CSF remains unknown. BM-derived HPSCs (Lin− c-kit+) express both MMP-9 and Plg activators such as uPAR,7,8 implying a proteolytic Plg/MMP-9 activation system by HPSCs. To test whether MMP-9 activation is required for Plg-regulated HPSC migration, we determined the migratory capacity of BM-derived Lin− c-kit+ cells in response to SDF-1 gradient. Lin− c-kit+ cells were isolated from the BM with a purity of >90% (Supplemental Figure II). Plg is mainly synthesized by liver and released into the circulation at a high level (200 μg/mL). Because no plasmin activity was detected in HPSCs (Lin− c-kit+ cells) isolated from Plg+/+ or Plg−/− mice (Supplemental Figure III), in our in vitro migration system, Lin− c-kit+ cells isolated from Plg+/+ mice were used, and an exogenous Plg protein was introduced to test the effect of Plg on HPSC migration. Lin− c-kit+ cells were seeded and exposed to SDF-1 gradient in a modified Boyden chamber.

Plg (20 μg/mL) was added into the upper chamber, and MMP-9 in medium was neutralized with an antibody that is able to efficiently block MMP-9 activation, as we previously showed.16 Zymography results confirm in vitro MMP-9 activation by Plg and the inhibition of MMP-9 activity with this antibody (Figure 3A, bottom panel). Plg significantly stimulated Lin− c-kit+ cells migration in response to SDF-1 (Figure 3A, top panel). However, MMP-9 neutralization successfully abolished this stimulation, indicating a requirement of MMP-9 activation in Plg-stimulated HPSC migration in vitro.

To assess whether MMP-9 activation is necessary for Plg-regulated HPSC mobilization in vivo, MMP-9 activity in Plg−/− mice was restored by administration of actMMP-9, and HPSC mobilization was measured. ActMMP-9, rather than proMMP-9, almost fully rescued HPSC mobilization in Plg−/− mice (Figure 3B) to a level comparable with the circulating HPSC number in Plg+/+ mice, thus suggesting a necessary role of Plg-induced MMP-9 activation in HPSC mobilization. To further verify this, BM cells were isolated and lentivirus transduced ex vivo to express WT or mutated MMP-9 (Gly100Leu), followed by BM transplantation to lethally irradiated recipients. The Gly100Leu mutation efficiently weakens the interaction between the prodomain and the catalytic subunit, inducing the autolytic cleavage and production of active MMP-9.26 Transduction BM cells with the Gly100Leu mutant increased MMP-9 activity by 3-fold in BM plasma after BM transplantation (Figure 3C). Lentiviral treatment induced an increase in basal HPSC mobilization in both Plg+/+ and Plg−/− mice, likely because of a nonspecific effect of transduction (Figure 3D). Importantly, overexpression of MMP-9Gly100Leu, but not of MMP-9WT, rescued HPSC mobilization in Plg−/− mice, thus confirming that Plg-required MMP-9 activation is necessary for HPSC mobilization induced by G-CSF.

Plg Regulates Expression of BM SDF-1 but Not c-kitL During HPSC Mobilization

c-kit/c-kitL and SDF-1/CXCR4 are the major cytokine signals for chemotaxis in cytokine-induced HPSC mobilization.6 Recently c-kitL has been shown to be a downstream target of Plg/MMP-9 during myeloablation-induced HPSC mobilization.7 Our results indicate that G-CSF did not affect c-kitL expression in the plasma of Plg+/+ or Plg−/− mice 5 days after G-CSF injection (Figure 4A), suggesting no involvement of c-kitL in G-CSF-induced HPSC mobilization, which is consistent with a previous study.12

SDF-1 is a key chemotaxant for HPSC mobilization from BM. Previous studies15 have reported that G-CSF induces a significant decrease in SDF-1 in BM to a level lower than that in blood, therefore generating a gradient between BM and peripheral blood to induce HPSC egress into blood. Consistently, G-CSF treatment induced a SDF-1 gradient in Plg+/+ mice, as indicated by a lower concentration of 0.39±0.08 ng/mL in BM compared with unaffected
Figure 3. Matrix metalloproteinase-9 (MMP-9) activation is required for plasminogen (Plg)-regulated hematopoietic progenitor and stem cell (HPSC) mobilization. A, HPSCs (Lin–c-kit+ cells) were isolated from bone marrow (BM) of Plg+/+ mice and subjected to in vitro cell migration assay with stromal cell–derived factor-1 (SDF-1) as the chemoattractant. MMP-9 activity in culture medium of cell migration chambers was analyzed by gelatin zymography. Culture medium of cells isolated from MMP-9–/– mice treated with Plg was loaded as a negative control. Top: number of migrated cells. Bottom: zymograph. B, Plg+/+ and Plg−/− mice were treated daily with granulocyte colony-stimulating factor (G-CSF) for 5 days and intravenously administered PBS, proform of MMP-9, or the active form of MMP-9 (actMMP-9) on day 4. HPSCs (Lin–c-kit+ cells) in peripheral blood from G-CSF-treated Plg+/+ and Plg−/− mice were analyzed by fluorescence-activated cell sorting (FACS) (n=5 to 8). C and D, BM cells isolated from Plg−/− mice and lentivirally transduced to overexpress wild-type (WT) (proMMP-9), or Gly100Leu MMP-9 (actMMP-9). Transduced BM cells were transplanted (BMT) into lethally irradiated recipient Plg+/+ or Plg−/− mice. Four weeks later, BMT mice were injected daily with G-CSF for 5 days (n=4). C, BMT mouse BM plasma was subjected to gelatin zymography. Mouse proMMP-9 (105 kDa) and actMMP-9 (91 and 88 kDa), as well as proMMP-2 (72 and 69 kDa), were identified by molecular weight relative to markers. D, HPSCs (Lin–c-kit+ cells) in blood isolated from BMT mice with G-CSF injection were analyzed by FACS. Bars indicate mean±SEM. *P<0.05. NS (not significant) indicates P>0.05.

Concentration of 0.65±0.07 ng/mL in plasma, 5 days after injection (Figure 4B and 4C). After G-CSF injection, a slight increase in BM SDF-1 (0.74±0.04 ng/mL) compared with no change and lower concentration in blood SDF-1 (0.54±0.06 ng/mL) (Figure 4B and 4C) were observed in Plg−/− mice, preventing the formation of a SDF-1 gradient between the BM and blood. In addition, G-CSF did not affect SDF-1 in plasma of either Plg−/− or Plg+/− mice, therefore suggesting a BM-specific regulation of SDF-1 by Plg. Together, these results indicate that Plg may regulate G-CSF-induced HPSC mobilization through modulation of SDF-1, but not c-kitL. Interestingly, SDF-1 baseline (without G-CSF treatment) was lower in Plg+/− mice (Figure 4B) compared with Plg+/+ mice, but no increase in cell number of circulating HPSCs was observed, suggesting that SDF-1 gradient alone may not be sufficient to induce HPSC mobilization.

Plg Regulates Expression of CXCR4 During HPSC Mobilization

CXCR4, the major receptor of SDF-1, is critical for G-CSF-induced stem cell mobilization. CXCR4 interaction with SDF-1 requires an N-terminal region on CXCR4 that can be proteolytically cleaved to inactivate the CXCR4/SDF-1 function.27,28 We investigated whether Plg regulates the expression of functional, intact CXCR4 in mobilizing HPSCs by using an antibody specifically recognizing the ligand binding site of CXCR4 (N-terminal 0 to 63 amino acids). In Plg−/− mice, our data show that G-CSF induced an oscillation of CXCR4 expression on HPSCs mobilized from BM to the circulation consistent with previous work.13 Namely, G-CSF gradually increased CXCR4 expression on BM HPSCs (Figure 4D), indicating that high CXCR4 expression may favor HPSCs mobilizing from BM to the peripheral blood, where SDF-1 is higher. Five days after daily G-CSF injection, CXCR4 expression on circulating HPSCs was significantly decreased (by 67%) compared with CXCR4 expression on BM HPSCs (Figure 4D). This indicated that CXCR4 expression was reduced after HPSC mobilization to the circulation, and this reduction may be necessary for preventing HPSCs from migrating back to the BM from the blood. However, both Plg deficiency (Figure 4D) and plasmin inhibition by aprotinin (Supplemental Figure IV) completely abrogated this oscillation, with neither an increase in CXCR4 expression in the BM nor downregulation in circulation, indicating that Plg, particularly its enzyme activity, is essential for the regulation of CXCR4 expression during egress of HPSCs from BM to the peripheral blood.

Furthermore, CXCR4 immunostaining indicated that in control mice, functional CXCR4+ cells were selectively distributed in the endosteal region in the vicinity of trabecular bone, where the most primitive HPSCs reside (Figure 4E). After G-CSF stimulation, CXCR4 expression was enhanced on BM cells, and the majority of CXCR4+ cells were present in the sinusoid lumen, suggesting that HPSCs with high expression of intact CXCR4 were recruited from the endosteal site to the sinusoids. In contrast, after G-CSF injection, the CXCR4 expression on BM cells of Plg−/− mice was much less than that of WT mice, indicating that Plg deficiency inhibited G-CSF-induced CXCR4 expression in BM and resulted in fewer hematopoietic cells in sinusoids. These data suggest that Plg is required for the regulation of CXCR4 in G-CSF-induced stem cell mobilization.

Plg Regulates CXCR4 Expression Through MMP-9 Activation During G-CSF-Induced HPSC Mobilization

Previous studies have shown that MMP-9 degrades extracellular matrix in BM and cleaves cytokines and their receptors (c-kit/c-kitL, SDF-1/CXCR4, and vascular cell adhesion molecule-1/αβ integrin) to promote HSC egress from BM to the circulation.29,30 We investigated whether MMP-9 regulates CXCR4 expression in Plg-mediated HPSC mobilization by using MMP-9−/− mice. MMP-9 deficiency reversed G-CSF-induced upregulation of CXCR4 expression in BM HPSCs and downregulation of CXCR4 expression in circulating HPSCs (Figure 5A). In G-CSF treated mice, BM
transplantation with lentiviral overexpression of Gly100Leu MMP-9 reconstitution of MMP-9 activity in Plg/−/− mice significantly increased CXCR4 expression on BM HPSCs and decreased CXCR4 expression in circulating HPSCs (Figure 5B and 5C). Compared with the vector alone, this led to the successful rescue of HPSC mobilization in Plg/−/− mice (Figure 3D), indicating that CXCR4 expression is regulated by MMP-9 during HPSC mobilization by G-CSF. In summary, these results reveal that Plg regulates G-CSF-induced HPSC mobilization through MMP-9-mediated CXCR4 expression.

Discussion

G-CSF is a cytokine produced by a variety of cells, and clinically the recombinant form is used as a therapeutic agent to enhance HPSC mobilization in chemotherapy recovery, HPSC transplantation, and ischemic cerebral and heart damage. In the BM, G-CSF and other mobilizing agents induce changes in the release of proteases and modulation of SDF-1/CXCR4, c-kit ligand (c-kitL) in blood was measured by ELISA (n=6 to 7), B to D, Mice were injected with daily G-CSF or PBS for 1 or 5 days, SDF-1 in bone marrow (BM) and blood were measured by ELISA, and CXCR4 expression on HPSCs in BM and blood analyzed by fluorescence-activated cell sorting and expressed as mean fluorescence intensity (MFI). B and C, Plasma and BM SDF-1 levels determined by ELISA (n=6 to 7). (D) CXCR4 expression (n=4). Bars indicate mean±SEM. *P<0.05, **P<0.01. NS (not significant) indicates P>0.05. E, Mice were injected daily with G-CSF for 4 days, and bone sections of femurs and tibias subjected to CXCR4 immunohistochemistry staining. Brown indicates CXCR4+ cells. Shown are representative sections of 3 mice per treatment. Magnification: ×200 or ×600, as indicated.

G-CSF is a cytokine produced by a variety of cells, and clinically the recombinant form is used as a therapeutic agent to enhance HPSC mobilization in chemotherapy recovery, HPSC transplantation, and ischemic cerebral and heart damage. In the BM, G-CSF and other mobilizing agents induce changes in the release of proteases and modulation of SDF-1/CXCR4, c-kit ligand (c-kitL), and adhesive molecules to enhance HPSC mobilization. The present study highlights a role of Plg in the G-CSF-induced HPSC mobilization and identifies a novel underlying mechanism via MMP-9-mediated SDF-1/CXCR4 expression. Our findings show that MMP-9 activation is required for Plg-mediated HPSC mobilization by G-CSF. Furthermore, Plg regulates HPSC mobilization through SDF-1/CXCR4, the major chemotaxis signaling pathway in stem cell mobilization. Interestingly, CXCR4 works downstream of Plg-dependent MMP-9 activation during G-CSF-induced HPSC mobilization. Thus, our data elucidate the molecular mechanism of Plg-mediated HPSC mobilization by activation of MMP-9 and regulation of SDF-1/CXCR4 signals.

Consistent with a recent study by Tjwa et al, our data identify a critical role of Plg in HPSC mobilization by G-CSF. Their mechanistic study shows that cleavage of uPAR is involved in the Plg-regulated HPSC mobilization by G-CSF. However, uPAR deficiency inhibits much less HPSC mobilization in response to G-CSF than Plg deficiency, suggesting the presence of other unidentified pathways for Plg-mediated HPSC mobilization. The novel mechanism revealed by our study indicates uPAR is not the only signal responsible for Plg-mediated HPSC mobilization. The newly identified MMP-9-mediated SDF-1/CXCR4 signal seems to be a critical mediator for G-CSF-induced HPSC mobilization, as indicated by marked suppression in HPSC mobilization by Plg and MMP-9 deficiency and significant rescue of HPSC mobilization in Plg−/− mice by overexpression of active MMP-9 in BM cells.

Proteolytic enzymes such as neutrophil elastase, cathepsin G, and MMP-9 play important roles in HPSC mobilization. Previous studies show that an elevated MMP-9 in BM in clinical and experimental animal therapies is induced by G-CSF, as well as by other stem cell recruitment.
agents, such as interleukin-8, 5-FU, and GROβ. Genetic loss of MMP-9 in mice severely impairs HPSC recruitment to blood by G-CSF. Neutralization with anti-MMP-9 antibody inhibits interleukin-8- or GROβ-induced HPSC mobilization by 90% and G-CSF-induced mobilization by 40% in mice, indicating that MMP-9 is critical for cytokine-induced stem cell mobilization. Controversially, other studies show that MMP-9 deficiency in mice fails to affect stem cell mobilization by cytokines, likely because of differences in mouse background and dose of G-CSF treatment. (1) MMP-9−/− mice with different backgrounds, including C57BL/6 (in our study), 129,20,23,24 and FVB21 were used. Our previous studies indicate that different mouse strains have different phenotypes, eg, inflammatory cell recruitment, even though all of them are WT. (2) Mice were treated with G-CSF at different doses, such as daily at 50 μg/kg per day, daily at 250 μg/kg per day, or twice daily at 250 μg/kg per day. Our study found that MMP-9 deficiency significantly inhibited HPSC mobilization by G-CSF, suggesting a critical role of MMP-9 in stem cell mobilization. Incomplete inhibition of HPSC mobilization may be due to the following reasons: (1) MMP-9 is a crucial but not the sole factor required for G-CSF-induced stem cell mobilization, and (2) other MMPs may compensate for the function of MMP-9 for HPSC mobilization in MMP-9 knockout mice. Our data for the first time establishes a necessary role of MMP-9 activation in Plg-regulated stem cell mobilization by a rescue experiment that reconstitution MMP-9 activity in Plg−/− mice restores HPSC mobilization in our G-CSF model. During stem cell mobilization, protease activation cleaves cytokines that regulate HPSC migration, including c-kitL and SDF-1/CXCR4 in BM. Accumulating evidence indicates that activation of the CXCR4 is critical for MMP-9-mediated HPSC mobilization. Recent studies show that G-CSF injection fails to induce stem cell mobilization in CXCR4−/− mice, regardless of the fact that G-CSF markedly stimulates MMP-9 activity in BM, thus suggesting that MMP-9-mediated HPSC mobilization may be dependent on CXCR4. Furthermore, transplantation of CXCR4+ vascular endothelial growth factor receptor+ cells into MMP-9−/− mice rescues the severe defects in ischemic revascularization. However, the direct evidence for MMP-9 regulating CXCR4 expression in HPSC mobilization is lacking. In our study, the results show that both G-CSF treatment and Plg deficiency do not affect c-kitL level, a verified downstream signal of Plg/MMP-9 in stem cell mobilization. However, G-CSF induces a Plg/MMP-9-dependent CXCR4 expression in BM, and actMMP-9 treatment in Plg−/− mice restores the CXCR4 expression to the level in G-CSF-treated WT mice, suggesting that CXCR4, instead of c-kitL, acts as a new downstream signal of Plg/MMP-9 in G-CSF-induced HPSC mobilization.

SDF-1/CXCR4 is critical for cell chemotaxis in BM during HPSC mobilization, and CXCR4 is expressed on the majority of circulating G-CSF-mobilized HPSCs. To date, the precise role of CXCR4 in HPSC mobilization has not been well defined. Previous studies show that acute inhibition of CXCR4 with the nonpeptide antagonist AMD3100 promotes HPSC mobilization, where chronic inhibition with AMD3100 or neutralizing antibody impairs mobilization. Previous studies suggest that G-CSF induces a spatiotemporal regulation of CXCR4 expression to facilitate HPSC mobilization: CXCR4 expression is transiently decreased
immediately after G-CSF injection, which may facilitate HPSC detachment from stromal cells, but gradually increases and peaks during stem cell mobilization in BM and may stimulate cell chemotaxis in response to a SDF-1 gradient. CXCR4 expression on mobilized cells in the circulation is significantly lower than that in BM, which may prevent cells from homing back to the BM. Our results show this mobilization-favorable oscillation of CXCR4 expression can be abolished by Plg/MMP-9 deficiency, indicating that Plg/MMP-9 may spatiotemporally regulate CXCR4 expression to facilitate stem cell mobilization. Of great interest, whether MMP-9 regulates CXCR4 expression directly or indirectly remains to be determined.

In conclusion, we elucidate a distinct mechanism underlying Plg-mediated stem cell mobilization in response to G-CSF. In this newly identified pathway, Plg regulates MMP-9 activation and SDF-1/CXCR4 signaling, particularly MMP-9-dependent CXCR4 expression, to modulate HPSC mobilization, contributing a better understanding of the role of Plg in G-CSF-induced stem cell mobilization. Targeting these pathways may offer a therapeutic opportunity to enhance G-CSF-induced stem cell mobilization for treatment of ischemic disease.

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Disclosures

None.

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Supplemental Methods and Materials

Mice
The Plg-deficient mice were previously generated and backcrossed into the C57BL/6J background \(^1\). The Plg heterozygous mice were bred and offspring genotyped by PCR as previously described \(^2\). MMP-9\(^{-/-}\) mice in a C57BL/6J background were generously provided by Dr. Robert Senior, Washington University, St. Louis. Mice were bred, housed in sterilized isolator cages, maintained on a 14 hour light / 10 hour dark cycle, and provided with sterilized food and water \textit{ad libitum} in the Biological Resource Unit of the Cleveland Clinic Lerner Research Institute. Both male and female Plg\(^{+/+}\) and Plg\(^{-/-}\) mice were tested between 6 and 9 weeks of age. All animal experiments were performed in accord with protocols approved by the Institutional Animal Care and Use Committee.

Tissue harvest
At different time points after G-CSF treatment, mice were sacrificed and whole blood was collected with 4\% sodium citrate as anticoagulant. Whole blood (50\,ul) was subjected to flow cytometry analysis (FACS) to determine Lin\(^{-}\)c-kit\(^{+}\) cells. The remaining blood was centrifuged and the supernatant was collected as plasma. The femurs and tibias were removed, flushed with 500 \,\mu l PBS and centrifuged. The supernatant solution was collected as BM plasma and cell pellets were collected as BM cells. BM cells were subjected to FACS to determine Lin\(^{-}\)c-kit\(^{+}\) cells. For histology studies, femurs and tibias were isolated, fixed and decalcified by Decalcifier I solution (Surgipath Medical Inc.) and
followed by washing with PBS. Bones were paraffin-embedded, sectioned (5µm) and subjected to histological studies.

**Aprotinin treatment**

Aprotinin (Sigma) was dissolved in sterilized phosphate-buffered saline at 5mg/ml. Two hours before G-CSF injection, aprotinin was administered subcutaneously (s.c.) daily at 0.5 mg per mouse for 5 days. Mice were sacrificed 5 days after both aprotinin and G-CSF injection. The number of WBC in the peripheral blood was counted by hematocytometer. BM cells were collected as mentioned above. Lin⁺ c-kit⁺ BM cells were analyzed by FACS after labeled with a cocktail of biotin-conjugated lineage-specific monoclonal antibodies (CD5, CD45R, CD11b, Gr-1, TER119 and 7/4) (Miltenyi Biotec.) and followed with FITC-anti-biotin IgG and PE-anti-c-kit (BD Pharmingen). Plasma and BM plasma were subjected to gelatin zymography to measure MMP-9 activity. The intensities of the bands were quantified using ImageJ software.

**Isolation of BM Lin⁻ c-kit⁺ Cells and Chemotaxis Assay**

Lin⁻ c-kit⁺ cells were isolated by immunomagnetic separation using a sequence of negative and positive selection using MACs beads (Miltenyi Biotec). The Lin⁺ cells were magnetically labeled with a cocktail of biotin-conjugated antibodies/Anti-Biotin microBeads specific for the following hematopoietic lineages: CD5, CD45R (B220), CD11b, anti-Ly-6G (GR-1, 7-4 and Ter-19) (Lineage Cell Depletion Kit, Miltenyi Biotec). This magnetic cell fraction containing the Lin⁺ cells was eluted using an autoMACs Separator. Subsequently, the remaining Lin⁻ cells were directly labeled with c-kit MicroBeads and sorted,
with the positive fraction containing the Lin− c-kit+ cells. After separation, Lin−c-kit+ cells were subjected to FACS analysis to detect c-kit expression, and over 94% cells were positive for c-kit. Isolated Lin− ckit+ cells were cultured in IMDM medium (Invitrogen) containing 20% FBS and 100 ng/ml mouse stem cell factor, 20 ng/ml mouse IL-6, and 10 ng/ml mouse IL-3 (PeproTech Inc) at 37°C in a humidified atmosphere of air containing 5% CO2. Before the experiment, Lin−c-kit+ cells were washed with PBS and suspended in 0.1% BSA/DMEM plus 100 ng/ml mouse stem cell factor, 20 ng/ml mouse IL-6, and 10 ng/ml mouse IL-3. Cell suspensions were added to filter inserts with 8-µm pores (BD Pharmingen) in a 24-well chamber (2.5 × 10^5 cells per well). Chemotaxis was initiated by addition of human SDF-1α (100 ng/ml, R&D Systems) in the lower chamber, followed by incubation at 37°C for 24 h. Mouse plasminogen (10 µg/ml), isolated on a lysine-sepharose column, was added to the upper chamber, and MMP-9 neutralizing antibody or mouse IgG (20 µg/ml, Calbiochem) was added to upper chamber. Cells remaining on the upper surface of inserts were removed and the membrane was fixed and stained with 1% toluidine blue. The number of migrated cells was determined in 6 random high-power fields (×200) for each filter in triplicate in three separate experiments.

**Plasmin Activity (casein zymography)**

Plasmin activity in Lin−c-kit+ cell-conditioned medium was examined by casein zymography. Lin−c-kit+ cell were isolated from the BM of Plg+/+ and Plg−/−. Cell suspensions were added to filter inserts with 8-µm pores (BD Pharmingen) in a 24-well chamber (2.5 × 10^5 cells per well) with 500 µl IMDM media and *in vitro*
migration assay was performed as mentioned above. After 24h, migrated Lin\(^-\)c-kit\(^+\) cells were counted and culture media were collected. 200 \(\mu\)l culture media were concentrated and subjected to electrophoresis with 10% pre-stained casein zymogram gels (Invitrogen). Mouse plasin (20 ng) was loaded as a control. After renaturing and developing the gels according to manufacturer’s instructions, intensity of the caseinolytic bands was quantified using ImageJ software.

**Supplemental Results**

**Plasmin enzyme activity is required for G-CSF-regulated HPSC mobilization, MMP-9 activation and CXCR4 expression.**

Two sites within plasmin(ogen) are pivotal to its biological functions: (i) the catalytic site of plasmin, which mediates proteolysis; and (ii) the lysine-binding site (LBS), which mediates binding to substrates, receptors and inhibitors. Aprotinin, a plasmin catalytic site inhibitor, was administered (s.c. 0.5 mg/mice) to the Plg\(^{+/+}\) mice to test whether plasmin enzyme activity is required in HPSC mobilization. Previous studies\(^3\),\(^4\) have shown that the level of aprotinin administered was sufficient to inhibit plasmin activity and nontoxic. Our data showed that after aprotinin injection, both WBC and HPSC number (Supplemental data Figure 1A,B) in the blood was robustly decreased WBC and HPSC number in the blood of G-CSF-treated mice suggesting that proteolytic function of plasminogen is required for G-CSF-induced HPSC mobilization. It has been reported that aprotinin is able to inhibit Plg-induced MMP-9 activation *in vitro*\(^5\),\(^6\). Our data showed after G-CSF treatment, MMP-9
activity is significantly inhibited by aprotinin in both plasma and BM (Supplemental data Figure 1C,D) indicating that plasmin catalytic site may be required for regulation of MMP-9 activation during HPSC mobilization. In addition, aprotinin induced a decrease in CXCR4 expression on BM HPSC and an increase in CXCR4 expression on circulating HPSC, to a degree similar to that in Plg−/− mice (Supplemental data Figure 4). Therefore, these data confirm that Plg regulates HPSC mobilization through MMP-9 activation and CXCR4 expression. Also, these results suggest that the protease-activating enzyme activity of Plg plays a critical role in regulation of G-CSF-induced HPSC mobilization.

**Lin∗kit∗ cells purity evaluation**

To test the purity of isolated Lin∗c-kit∗ cell, isolated cells were incubated with a cocktail of biotin-conjugated lineage-specific monoclonal antibodies (CD5, CD45R, CD11b, Gr-1, TER119 and 7/4) and labeled with FITC-anti-biotin IgG together with PE-anti-c-kit. Labeled cells were subjected to FACS analysis. Our data (Supplemental data Figure 2) show a high purity of isolated HPSC, as indicated by that 93.6% cells are lineage-negative and c-kit-positive cells.

**There is no difference in the migration capacity of Lin∗c-kit∗ cells isolated from Plg−/− and Plg+/+ mice.**

To test whether Lin∗c-kit∗ cells express plasminogen and whether the migration capacity is different in HPSC from Plg−/− or Plg+/+ mice, Lin∗c-kit∗ cells in Plg−/− or Plg+/+ mice were isolated and subjected to *in vitro* migration assay as mentioned above. Culture media were collected from migration transwell, centrifuged and
subjected to casein zymography to test plasmin activity. Our data (Supplement data Figure 3) showed that there is no difference in migration capacity of Lin\(^c\)-kit\(^+\) cells isolated from either Plg\(^+\)/+ mice or Plg\(^-\)/- mice. Casein zymography shows no detectable plasmin activity in the culture media of Lin\(^c\)-kit\(^+\) cells with or without SDF-1 treatment. These data suggest that plasminogen deficiency does not cause intrinsic migratory defects on HPSC.

Reference List


Supplemental Figure 1. Plasmin enzyme activity is required for HPSC mobilization by G-CSF. Plg<sup>+/+</sup> mice were treated with aprotinin (s.c. daily, 0.5 mg/mice) and G-CSF (s.c. daily, 200 µg/kg) for 5 days (n = 6). (A) White blood cells (WBC) in peripheral blood counted. (B) Blood cells stained, and Lin<sup>-</sup> c-kit<sup>+</sup> hematopoietic stem cells analyzed by FACS. (C and D) The protease activity in plasma and BM analyzed by gelatin zymography 5 days after injection. (C) Representative pictures of zymographs. (D) Quantified data represent 3 independent assays. Bars indicate mean ± SEM. *P<0.05, **P<0.01; NS, P>0.05.
Supplemental Figure 2. Identification of BM-derived HPSC. BM cells were isolated from femur and tabia of mouse, and subjected to immunomagnetic separation by MACs for Lineage depletion and c-Kit selection. Cells were stained with control isotype IgG or c-kit and Lineage antibodies, followed by FACS analysis. Representative results shown are from 3 separated assays.
Supplemental Figure 3. Lin\(^{-}\) c-kit\(^{+}\) cells isolated from Plg\(^{+/+}\) and Plg\(^{-/-}\) mice have similar in vitro migration ability in response to SDF-1. HPSC (Lin\(^{-}\) c-kit\(^{+}\) cells) were isolated from BM of Plg\(^{+/+}\) and Plg\(^{-/-}\) mice, and subjected to in vitro cell migration assay with SDF-1 as the chemoattractant. (A) Migrated cells counted. (B) Plasmin activity in culture medium of cell migration chambers analyzed by casein zymography. Mouse plasmin (20 ng) standard loaded as a positive control. Bars indicate mean ± SEM. NS, not significant.
Supplemental Figure 4. Plasmin enzyme activity regulates CXCR4 expression on HPSC during mobilization. Plg^{+/+} mice were treated with aprotinin (s.c. daily 0.5 mg/mice) and G-CSF (s.c. daily) for 5 days (n = 6). CXCR4 expression on HPSC in BM and blood was analyzed by FACS and expressed as mean fluorescence intensity (MFI). Bars indicate mean ± SEM. *P < 0.05.