PLASMIN THERAPY ENHANCES MOBILIZATION OF HPCs AFTER G-CSF

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ABSTRACT

The role of proteinases in the mobilization of hematopoietic progenitor cells (HPCs) after G-CSF remains unclear. Here, we report that genetic loss of the plasminogen activator inhibitor PAI-1 or of the plasmin inhibitor α2-antiplasmin increases HPC mobilization in response to G-CSF. Moreover, thrombolytic agents such as tenecteplase and micro-plasmin enhance HPC mobilization in mice and humans. Taken together, these findings identify a novel role for plasmin in augmenting HPC mobilization in response to G-CSF.
INTRODUCTION

Autologous or allogeneic G-CSF-mobilized peripheral blood is an important source of hematopoietic progenitor cells (HPCs) for subsequent transplantation in various hematological diseases, but the therapeutic success to mobilize sufficient numbers of CD34+ cells is limited in a number of patients, in part due to their refractoriness or poor response to G-CSF 1. Therefore, a better understanding of the underlying mechanisms regulating HPC mobilization in response to G-CSF might offer novel therapeutic opportunities for HPC mobilization.

Studies in mice genetically deficient for G-CSF or its receptor (G-CSF-R) have shown that G-CSF does not mobilize HPCs by binding to its receptor on these cells but, instead, by binding to G-CSF-R+ resident cells within the bone marrow (BM) microenvironment and stimulating HPC mobilization via paracrine signals 2. Other studies underscored an important role for BM-derived proteinases, such as neutrophil elastase and MMP-9, which inactivate adhesive signals anchoring HPCs in the BM 3-6. However, loss of these proteinases did not necessarily impair HPC mobilization after G-CSF 7,8.

Using gene deficient mice, we recently documented a role for the proteinase plasmin in HPC mobilization in response to G-CSF 9. Plasmin is generated from its zymogen plasminogen via proteolytic cleavage by two plasminogen activators (PA), i.e. tissue-type PA (tPA) and urokinase-type PA (uPA), whereas inactivation of plasmin occurs by α2-antiplasmin (AP) and its generation is prevented by PAI-1 (which neutralizes PA activity) (reviewed in 10). Apart from regulating hemostasis, plasmin also cleaves cell-surface receptors, activates matrix metalloproteases (MMPs), remolds the extracellular matrix and liberates matrix-bound growth factors, explaining why this proteinase system has such a pleiotropic role in tissue healing, regeneration and malignancy 10. A recent report showed that administration of recombinant human tPA promotes the hematopoietic recovery of myeloablated mice, but the authors did not investigate its effects on HPC mobilization following G-CSF therapy 11. Here, we investigated whether plasmin could enhance HPC mobilization in response to G-CSF.
EXPERIMENTAL METHODS

Animal studies

All experiments were performed according to the guidelines for care and use of laboratory animals approved by the institutional ethical animal care committee.

Patient study

The protocol of this non-randomized clinical study was approved by the local medical ethical committee (ML2526) and was performed in accordance with the Declaration of Helsinki.

Please see supplement for full Experimental Methods section.

RESULTS AND DISCUSSION

GENETIC LOSS OF PAI-1 OR α2-ANTIPLASMIN INCREASES HPC MOBILIZATION

We first analyzed mobilization of HPCs in response to G-CSF in mice lacking PAI-1 (PAI-1^-/-) or α2-antiplasmin (AP^-/-), the primary inhibitors of PAs and plasmin, respectively. No genotypic differences in circulating HPCs were detectable in steady-state conditions (not shown). After G-CSF treatment (200 µg/kg/d s.c. for 5 days), circulating HPCs were detectable in the peripheral blood of the respective wild type (WT) controls, as analyzed by counting the number of CFU-Cs (Figure 1A) and CFU-Ss (Figure 1B) per 10^5 peripheral blood mononuclear cells (PB-MNCs). Notably, PAI-1^-/- or AP^-/- mice mobilized more HPCs in the peripheral blood (Figure 1A,B). Indeed, compared to their respective WT controls, the number of circulating CFU-Cs and CFU-Ss was increased by ~1.4-fold and ~2.2-fold in PAI-1^-/- mice, and by ~2.6-fold and ~2.5-fold in AP^-/- mice, respectively (Figure 1A,B). Thus, genetic loss of PAI-1 or AP enhances HPC mobilization after G-CSF.

THROMBOLYTIC AGENTS ENHANCE HPC MOBILIZATION IN MICE

The results above indicate that plasmin augments G-CSF-mediated mobilization. We therefore evaluated whether thrombolytic compounds, that generate or increase plasmin,
and which are currently being used in the clinic (tenecteplase, recombinant human tPA) or are in clinical development (microplasmin, staphylokinase) might be useful to stimulate HPC mobilization. For the mouse study, we used three thrombolytics: recombinant human tPA (rtPA), tenecteplase (TNK; a rtPA mutant which has a prolonged half-life and is used for treatment of acute cardio- and cerebro-vascular syndromes), and micro-plasmin (µPli; a plasmin variant lacking the five aminoterminal kringle domains, which has an improved safety profile (less bleeding) and is easier to produce as recombinant protein than plasmin \(^{12}\)). Since mice are well known to be ~10-fold less responsive to these thrombolytic agents, especially when given as intraperitoneal bolus injection (discussed further below; \(^{13-15}\)), their dose was adapted accordingly. In steady-state conditions, administration of the thrombolytic compounds TNK (daily intraperitoneal bolus injection of 100 mg/kg) or µPli (100 µg/day, continuously over a period of 5 days via osmotic minipumps) failed to induce HPC mobilization in WT mice (not shown), raising the question whether these agents might only enhance HPC mobilization in conjunction with G-CSF. Indeed, when co-administered with G-CSF (200 µg/kg/d s.c. for 5 days), TNK enhanced the mobilization of CFU-Cs and CFU-Ss by ~1.7-fold and ~2.6-fold, respectively, compared to G-CSF alone (Figure 1C,D). Co-administration of µPli and G-CSF also stimulated mobilization of CFU-Cs and CFU-Ss by ~1.5-fold and ~2.8-fold, respectively (Figure 1C,D). These cells were capable of reconstituting hematopoiesis, as transplantation of these cells increased the survival of lethally irradiated WT recipient mice (Figure 1E).

It is well known that human rtPA has a short half-life in mice (i.e., in the order of minutes \(^{16}\)), resulting from its rapid sequestration by mouse PAI-1 and a low efficiency to convert mouse plasminogen to plasmin (>40-fold lower than for human plasminogen \(^{13-15}\)); we therefore used a 10-fold higher dose than normally administered to humans \(^{13-15}\). Nonetheless, daily intraperitoneal bolus injection of human rtPA (100 mg/kg) failed to enhance HPC mobilization in steady-state conditions or following G-CSF administration in mice (not shown). Our data thus differ from recent findings by Heissig \textit{et al.} that a 100-fold lower dose of human rtPA in mice increases HPC mobilization in steady-state conditions and after chemo-myeloablation \(^{11}\); the precise reason for this discrepancy remains unexplained.
Thus, TNK or µPli enhances HPC mobilization after G-CSF.

**THROMBOLYTIC AGENTS ENHANCE HPC MOBILIZATION IN HUMANS**

We also evaluated whether thrombolytic agents would be capable of inducing HPC mobilization in humans as well. Our mouse results indicate that thrombolytic agents only enhance HPC mobilization, when these cells are already primed by another mobilization stimulus (such as G-CSF). However, ethical reasons precluded us from testing whether thrombolytic agents enhance HPC mobilization in response to G-CSF in healthy volunteers. We therefore studied the effect of thrombolytic agents in the setting of acute myocardial ischemia, as this is a well-known stimulus for HPC mobilization. Therefore, peripheral blood samples were collected before and 24 hours after thrombolytic treatment (TNK or staphylokinase) of patients, who were admitted to the Coronary Care Unit with ST-segment elevation myocardial infarction (STEMI). As control group, blood samples were collected from STEMI patients before and 24 hours after percutaneous coronary intervention (PCI), since this procedure does not induce HPC mobilization at this early time point.

Mobilization of HPCs was determined by quantifying CFU-Cs using methylcellulose culture assays and the percentage of CD34+ cells using flow cytometry. A pilot group of 11 patients was analyzed: 6 received first-line PCI (PCI group), while 5 received thrombolytic therapy (Thrombolysis group: 3 treated with TNK, 2 treated with staphylokinase); additional characteristics of the study group are listed in Supplement Table 1. At the time of admission (i.e. before onset of treatment), both the PCI and Thrombolysis groups had comparable numbers of circulating HPCs (CFU-C/10^6 MNCs: 28 ± 6 versus 19 ± 10; % of CD34+ cells: 1.45 ± 0.22 versus 0.71 ± 0.34; P=NS). After 24 hours, PCI treatment failed to mobilize HPCs. Indeed, when expressed as a percentage of baseline, post-PCI values were: 90 ± 30% for the CFU-Cs, and 105 ± 13% for the CD34+ cells. This lack of mobilization after PCI is consistent with previous findings that PCI only mobilizes progenitors at later timepoints, i.e. at 3-7 days after treatment. In contrast, thrombolytic therapy significantly increased HPC mobilization at 24 hours after treatment. Compared to the pre-treatment levels, corresponding values after thrombolytic therapy were: 690 ± 230% for the CFU-Cs (P<0.05).
and 385 ± 75% for the CD34+ cells ($P<0.005$). Taken together, even though only a small number of patients was analyzed in a non-randomized study design (and larger cohorts will have to be analyzed in the future), these findings suggest that thrombolytic agents enhance the mobilization of HPCs in STEMI patients at 24 hours after myocardial infarction.

**CONCLUSIONS**

The main finding of this study is that plasmin augments HPC mobilization following G-CSF. Our findings that fibrinolytic agents stimulate HPC mobilization warrant further exploration of their therapeutic potential in individuals who mobilize poorly in response to G-CSF. Especially the use of micro-plasmin might be recommended in perspective of its reduced risk to induce bleeding.²
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CONTRIBUTIONS OF THE AUTHORS

Marc Tjwa: designed and performed experiments, analyzed data, participated in discussion and manuscript writing
Stefan Janssens: organized and supervised the collection of patient samples
Peter Carmeliet: designed and analyzed data, participated in discussion and manuscript writing, scientific direction

These authors have no conflict of interest to disclose.
REFERENCES

FIGURE LEGENDS

Figure 1: Plasmin enhances HPC mobilization after G-CSF.
A, B, Compared to WT mice, AP⁻/⁻ mice had more circulating CFU-Cs (A) and CFU-Ss (B) at 5 days after G-CSF. *: P<0.05 versus WT (N=10-15). C, D, Compared to vehicle, treatment with 100 mg/kg tenecteplase (TNK, daily i.p.) or 100 µg/day microplasmin (µPli, osmotic minipump) during 5 days increased the number of circulating CFU-Cs (C) and CFU-Ss (D), mobilized in response to G-CSF. Vehicle groups behaved similarly (not shown) and were therefore pooled together. *: P<0.05 versus WT (N=10-15). E, More lethally irradiated syngeneic WT recipients survived when they were transplanted with 1 x 10⁵ PB-MNCs from G-CSF-treated WT mice, receiving TNK or µPli than vehicle (P<0.05; Cox regression; N=13-20).
Figure 1
Plasmin therapy enhances mobilization of HPCs after G-CSF

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