represent the mean ± SD values of triplicate samples. F) Western blotting analysis of Sp1, Sp3, Sp4, and VEGF. Cells were treated with vehicle or high AA for 1 h, washed, cultured, and analyzed after 24 h. There were significant differences in the expression levels of these molecules between the vehicle-treated K562 and K562-HIF1α cells (*$P<0.01$, ** $P<0.0001$). There were significant differences in the expression levels of Sp1, Sp3, and Sp4 between the vehicle-treated and high AA-treated K562 or K562-HIF1α cells ($^{\dagger}P<0.01$, $^{\dagger\dagger}P<0.001$, $^{\dagger\dagger\dagger}P<0.0001$). There was a significant difference in the expression level of VEGF between the vehicle-treated and high AA-treated K562 ($^{\dagger\dagger\dagger}P<0.0001$), but not between the vehicle-treated and high AA-treated K562-HIF1α cells ($P>0.05$).
doi:10.1371/journal.pone.0062717.g005

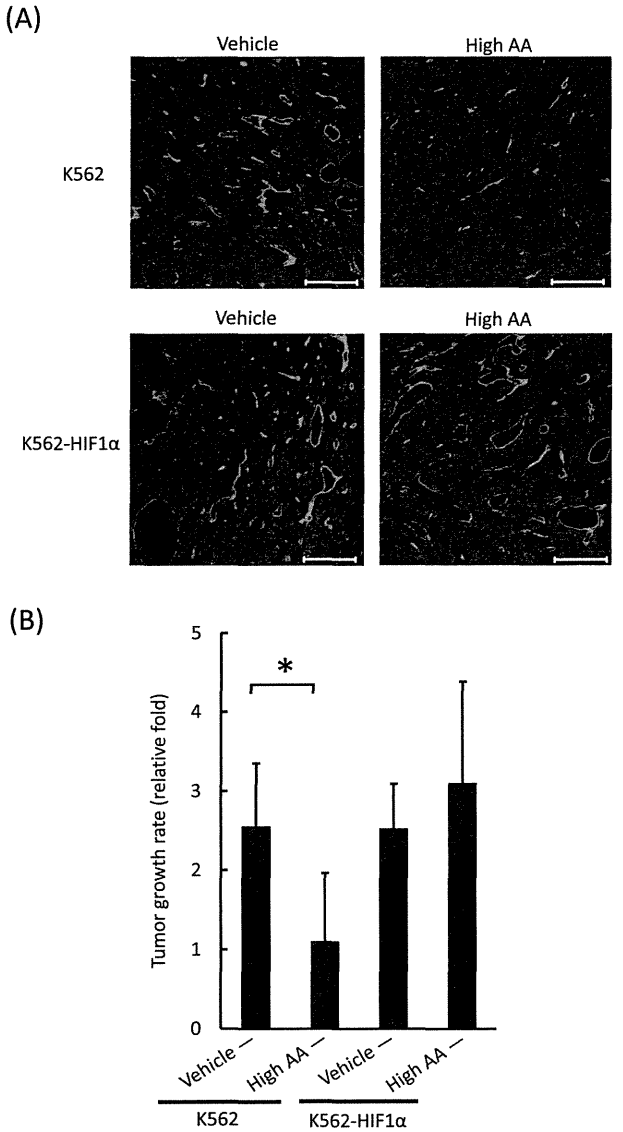

Figure 6. Effects of high AA on tumor growth in the presence or absence of overexpression of *HIF-1α*. A) Immunohistochemical analysis of tumor neoangiogenesis in vehicle-treated (left) and high AA-treated (right) mice transplanted with K562 (upper column) or K562-HIF1α cells (lower column). The green and blue signals represent CD31 and DAPI, respectively. The bars indicate 100 μm. Note that administration of high AA suppressed tumor neoangiogenesis in mice transplanted with K562 cells, but not in mice transplanted with K562-HIF1α cells. B) In the xenogeneic transplant model, high AA or vehicle was injected for 5 days. Administration of high AA significantly inhibited tumor growth of K562 cells (*$P<0.05$) but not of K562-HIF1α cells ($P>0.05$). Tumor growth rate was estimated using the following equation: tumor volume on day 4 after high AA treatment/tumor volume just before high AA treatment. The values represent the mean ± SD values for 4 mice.
doi:10.1371/journal.pone.0062717.g006

because the effects were caused largely by the H_2O_2 that was generated extracellularly by high AA (Figure 1B).

However, we found that *HIF-1α* overexpression completely abrogated the inhibitory effects of high AA on tumor growth and neoangiogenesis in vivo and significantly diminished the induction of apoptosis by high AA in the leukemic cells in vitro. HIF-1α regulates the expression of Bcl-2 family members such as Mcl-1, Bcl-x_L, and Bcl-2, which are essential for the growth and survival of leukemic cells because they prevent the induction of apoptosis by ROS [14,29,53–57]. Here, we demonstrated that high AA significantly suppressed expression of Mcl-1, Bcl-x_L, and Bcl-2, and induced apoptosis in K562 cells but not in K562 cells that overexpressed *HIF-1α*.

We further assessed the involvement of Sp1, Sp3, and Sp4 in the antileukemic effect of high AA because high AA exhibits anticancer activity towards colon cancer cells, which is due in part to downregulation of Sp transcription factors and Sp-regulated genes [58]. Similar results have been observed in bladder and pancreatic cancer cells treated with H_2O_2 or other ROS inducers [59–61]. Further, it has been reported that knockdown or downregulation of Sp1, Sp3, and Sp4 represses expression of Sp-regulated genes, including *VEGF* and *BCL-2*, inhibits cancer cell growth, and induces apoptosis [59–61]. In the present study, the expression levels of Sp1, Sp3, and Sp4 in K562-HIF1α cells were higher than those in K562 cells, suggesting some interaction between HIF-1α and Sp proteins. Further, the expression of Sp1, Sp3, and Sp4 was also downregulated by high AA in K562 cells, as was observed in colon cancer cells [58]. However, in K562-HIF1α cells, the expression of these factors was also downregulated by high AA, but the expression of VEGF and Bcl-2 was not. These results strongly suggest that marked inhibition of *HIF-1α* transcription and expression of HIF-1α-regulated molecules play a crucial role in the antileukemic effects of high AA along with the generation of H_2O_2. However, high AA do not specifically affect the transcription of *HIF-1α* because high AA block the activation of NF-κB, which acts as a transcription factor to regulate the expression of genes involved in the response of leukemic cells to extracellular signals such as *HIF-1α* [48,49]. Therefore, other molecular mechanisms might also play a role in the response to high AA treatment.

Because the use of high AA appears to be remarkably safe in clinical settings [40], it may provide an alternative option for cancer therapy. However, the anticancer effects of high AA vary among cancers or patients [7,9,41]. It is known that an increased number of leukemic cells, normal erythrocytes, or fibroblasts around leukemic cells inversely correlates with high AA-induced leukemic cell death because of increased catalase activity [62]. Therefore, the volume and localization of cancer cells should be considered to obtain more stable clinical effects of high AA. We think that it is reasonable to conclude that combinations with other drugs that compensate for H_2O_2 decomposition may also provide a new strategy for eliminating cancer cells [63].

Acknowledgments

The authors thank the members of the Research Center for Regenerative Medicine of Tokai University School of Medicine for the helpful

discussions and assistance. They also thank the members of the animal facility of Tokai University for the meticulous care of the experimental animals, the members of the Tokai Cord Blood Bank for their assistance, and Dr Eiichiro Nagata for kindly providing anti-Bcl-2 and anti-Bcl-x_L antibodies.

Author Contributions

Conceived and designed the experiments: HK MK. Performed the experiments: MK TU H. Matsuzawa YN. Analyzed the data: HK MK KA. Contributed reagents/materials/analysis tools: MS H. Matsushita KA. Wrote the paper: HK.

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Inhibition of Plasminogen Activator Inhibitor Type-1 Activity Enhances Rapid and Sustainable Hematopoietic Regeneration

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Key Words. Hematopoietic stem cells • Bone marrow stromal cells • Hematopoiesis • Stem cell transplantation • Tissue regeneration • Osteoblast

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Received August 12, 2013; accepted for publication October 3, 2013; first published online in STEM CELLS *EXPRESS* October 24, 2013.

1066-5099/2014/$30.00/0

http://dx.doi.org/10.1002/stem.1577

ABSTRACT

The prognosis of patients undergoing hematopoietic stem cell transplantation (HSCT) depends on the rapid recovery and sustained life-long hematopoiesis. The activation of the fibrinolytic pathway promotes hematopoietic regeneration; however, the role of plasminogen activator inhibitor-1 (PAI-1), a negative regulator of the fibrinolytic pathway, has not yet been elucidated. We herein demonstrate that bone marrow (BM) stromal cells, especially osteoblasts, produce PAI-1 in response to myeloablation, which negatively regulates the hematopoietic regeneration in the BM microenvironment. Total body irradiation in mice dramatically increased the local expression levels of fibrinolytic factors, including tissue-type plasminogen activator (tPA), plasmin, and PAI-1. Genetic disruption of the *PAI-1* gene, or pharmacological inhibition of PAI-1 activity, significantly improved the myeloablation-related mortality and promoted rapid hematopoietic recovery after HSCT through the induction of hematopoiesis-promoting factors. The ability of a PAI-1 inhibitor to enhance hematopoietic regeneration was abolished when *tPA*-deficient mice were used as recipients, thus indicating that PAI-1 represses tPA-dependent hematopoietic regeneration. The PAI-1 inhibitor not only accelerated the expansion of the donor HSCs during the early-stage of regeneration, but also supported long-term hematopoiesis. Our results indicate that the inhibition of PAI-1 activity could be a therapeutic approach to facilitate the rapid recovery and sustained hematopoiesis after HSCT. STEM CELLS *2014;32:946–958*

INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) is used as a therapy for patients who suffer from hematological malignancies. In general, such patients are myeloablated by chemotherapy and/or radiotherapy to eradicate the deranged host hematopoietic system, followed by transplantation of healthy donor-derived hematopoietic cells [1]. However, due to a low engraftment efficiency and delayed bone marrow (BM) reconstitution, these patients occasionally suffer from severe immunodeficiency, thus leading to an increased susceptibility to serious infectious diseases, and therefore, to a high-risk of transplant-related death [2]. The establishment of an efficient strategy to improve the recovery and sustain hematopoiesis is a goal in the treatment of patients undergoing HSCT.

The fibrinolytic pathway breaks down fibrin clots in the blood, and plasmin plays a central role in this process [3]. The proenzyme plasminogen (Plg) is produced from the liver and circulates in the blood stream. Under certain circumstances, such as wound healing, Plg is proteolytically converted into the active enzyme plasmin by tissue-type plasminogen activator (tPA), which is released from endothelial cells [4]. The blood also contains negative regulators of the fibrinolysis pathway, including plasminogen activator inhibitor-1 (PAI-1). The production of plasmin from plasminogen is thus regulated by a balance between activator molecules (e.g., tPA) and their inhibitors (e.g., PAI-1) [4, 5]. Therefore, inhibiting the PAI-1 activity is expected to accelerate the activation of the tPA-mediated fibrinolytic pathway.

Recently, Hattori and colleagues demonstrated that the fibrinolytic pathway regulates hematopoietic regeneration [6, 7]. They showed that the deletion of the Plg gene impaired the entry of quiescent HSCs into the cell cycle and delayed hematopoietic regeneration. In contrast, the activation of Plg by the exogenous administration of recombinant tPA promoted HSC proliferation and differentiation

through the potentiation of matrix metalloproteinase (MMP)-mediated release of c-kit ligand (c-kitL) from BM stromal cells. The fibrinolytic pathway thus plays a role in hematopoiesis.

We have recently developed a low molecular weight synthetic inhibitor of PAI-1, TM5275 (5-chloro-2[((2-[4-(diphenylmethyl) piperazine-1yl]-2-oxoethoxy)acetyl) amino]benzoate) [8, 9]. TM5275 binds selectively to the A β-sheet (s4A) position of the PAI-1 molecule, preventing the formation of the PAI-1/tPA complex, thereby preserving active tPA. Previous studies have demonstrated that TM5275, which was given orally, provided antithrombotic benefits without prolonging the bleeding time in rodent and monkey thrombosis models [10]. Given the potential importance of the fibrinolytic pathway in HSCT, in this study, we addressed whether the suppression of PAI-1 activity could affect the hematopoietic regeneration after transplantation in PAI-1 knockout (KO) mice and using the PAI-1 inhibitor, TM5275. Our study demonstrates that PAI-1 is a negative regulator of hematopoietic regeneration, and that suppression of the PAI-1 activity leads to both a rapid recovery and long-term maintenance of donor-derived hematopoiesis. Therefore, the inhibition of PAI-1 activity could be a therapeutic approach to facilitate rapid recovery and sustained hematopoiesis.

Materials and Methods

Animals

Eight- to twelve-week-old C57Bl/6J mice were purchased from CLEA Japan (Tokyo, Japan, www.clea-japan.com). *PAI-1*-deficient mice (B6.12952-Serpine1^{tm1Mg}/J) [11, 12] were purchased from Jackson Laboratory (Bar Harbor, ME, www.jax.org). *tPA*-deficient mice (B6.129S2-Plat^{tm1Mlg}/J) [13] were kindly provided by Dr. Koichi Hattori, University of Tokyo, Japan. All mice were housed in cages at the animal facility of Tokai University School of Medicine. All the protocols for animal experiments were approved by the Animal Care Committee of Tokai University, and animals were treated in accordance with the institutional guidelines.

Cell Transplantation

To distinguish donor (Ly5.1)- and recipient (Ly5.2)-derived BM cells, we used the Ly5.1/Ly5.2 congenic system and analyzed the reconstitution of hematopoietic cells by a fluorescence-activated cell sorting (FACS) analysis by gating Ly5.1$^+$ donor-derived cells. Before transplantation, the recipient mice were lethally irradiated (9 Gy) in an x-ray irradiator (MBR-1520R-3, Hitachi Medico, Tokyo, Japan, www.hitachi-power-solution.com). Ly5.1$^+$ BM mononucleic cells (MNCs, 2.5 $\times$ 10^6) were transplanted intravenously into the retro-orbital plexus of Ly5.2$^+$ congenic mice.

For the secondary transplantation, 1 $\times$ 10^6 donor-derived Ly5.1$^+$ BM MNCs from the primary recipients were retransplanted into Ly5.2$^+$ secondary recipients which had been irradiated with 9 Gy. To protect secondary recipients from radiation-related lethality, 5 $\times$ 10^5 Ly5.2$^+$ competitor cells were transplanted along with the Ly5.1$^+$ donor cells. To compare the proportion of long-term HSCs, Ly5.1$^+$ BM cells from the primary recipients were serially diluted and administered along with 5 $\times$ 10^5 Ly5.2$^+$ competitor cells into secondary recipients that had been irradiated with 9 Gy. At 12 weeks after secondary transplantation, the BM MNCs were collected and stained with APC-conjugated anti-Ly5.1, FITC-conjugated anti-B220, PE-conjugated anti-Gr-1 and anti-Mac-1 antibodies, and were analyzed by FACS LSRFortessa (BD Bioscience, San Jose, CA, www.bdbioscience.com). The proportion of donor cells was calculated from a total of 200,000 events. Successfully engrafted mice were defined as recipients that contained more than 1.0% Ly5.1$^+$ donor-derived cells with both lymphoid (B220$^+$) and myeloid (Gr-1$^+$/Mac-1$^+$) differentiation markers. In the radioprotection assay, we used 12 Gy irradiation, which results in 20% survival when 1 $\times$ 10^6 cells were transplanted (Supporting Information Fig. S1).

Administration of the PAI-1 Inhibitor or tPA

TM5275 is a specific inhibitor of PAI-1 molecules that had a half-maximal inhibition (IC_{50}) value of 6.95 μM in a tPA-dependent hydrolysis assay [8, 10]. It does not interfere with other serpin/serine protease systems, such as the alpha1-antitrypsin/trypsin and alpha2-antiplasmin/plasmin systems.

After BM transplantation, TM5275 (100 mg/kg) or vehicle (saline) was administered daily to mice via oral gavage using a feeding needle for 5 consecutive days. Recombinant tPA (10 mg/kg, Eisai, Tokyo, Japan, www.eisai.com) was administered to the mice daily by intraperitoneal injection for 5 consecutive days. The dosage is equivalent to that used in the clinical setting.

Immunohistochemistry

Isofluorane-anesthetized mice were perfused with 4% paraformaldehyde in phosphate buffered saline (PBS) through the left ventricle. The femur and tibia were removed, decalcified, embedded in OCT compound, and frozen in liquid nitrogen. Alternatively, the decalcified bones were embedded in paraffin. The deparaffinized sections were stained for tPA by incubation with a rabbit anti-mouse tPA polyclonal antibody (Santa Cruz Biotechnology, California, CA, www.scbt.com), for Plg/plasmin with a rabbit anti-human Plg/plasmin polyclonal antibody (Santa Cruz Biotechnology) or for PAI-1 with a rabbit anti-mouse PAI-1 polyclonal antibody (Abcam, Cambridge, MA, www.abcam.com), followed by visualization with a catalyzed signal amplification II system (Dako, California, CA, www.dako.com). The slides were then developed with diaminobenzidine and counterstained with methyl green. For the double immunohistochemical staining analysis, the bone sections were stained with PAI-1 or tPA antibodies, followed by costaining with either rat anti-mouse PECAM-1 (CD31) monoclonal antibodies (BD BioSciences), goat anti-mouse osteocalcin polyclonal antibodies (Santa Cruz Biotechnology), or goat anti-mouse alpha one chain of type I collagen (Col(I) α1) polyclonal antibodies (Santa Cruz Biotechnology). Serial sections of bone were stained with rabbit anti-mouse c-kit polyclonal antibodies (Santa Cruz Biotechnology) or rabbit anti-mouse proliferation cell nuclear antigen (PCNA) polyclonal antibodies (Abcam). Fluorescent immunohistochemistry was also performed with secondary antibodies as follows: Alexa Fluor 488 goat anti-rabbit IgG, Alexa Fluor 594 donkey anti-goat IgG or Alexa Fluor 594 goat anti-rabbit IgG secondary antibody (Life Technologies Corporation, Grand Island, NY, www.lifetechnologies.com), followed by counterstaining with 4′,6-diamidino-2-phenylindole. The endosteal region of the BM was defined as that within 12 cells from the endosteum [14, 15]. Images were captured using a HS All-in-one Fluorescence Microscope Biorevo 9000 (Keyence Corporation, Osaka, Japan, www.keyence.com) and analyzed by the BZ II analyzer software program (Keyence Corporation).

Evaluation of the tPA, Plasmin, PAI-1, MMP-9, and C-KitL Levels in Blood Plasma and BM Fluid

Plasma was prepared from peripheral blood (PB) with EDTA as an anticoagulant, and then the specimens were centrifuged at 11,600*g* for 10 minutes to completely remove platelets. BM liquid was collected as described previously [16]. Briefly, four long leg bones were perused with 1 ml of PBS containing 2 mM ethylenediaminetetraacetic acid (EDTA) and 0.5% bovine serum albumin (BSA). BM cells were removed by centrifugation at 350*g*, and the resulting supernatant was designated as BM liquid. The volume of the BM cavities of the bones was assumed to be 25 μl in this study, and the cytokines concentration were multiplied by this dilution factor (1,000/25 = 40×) and expressed per unit BM volume as described previously [16]. The concentrations of plasmin (Innovative Research, Novi, MI, www.innov-research.com), tPA (Innovative Research), active PAI-1 (Innovative Research), total MMP-9 (R&D System, MN, www.rndsystems.com), and stem cell factor (SCF) (c-kitL, R&D System) in the plasma and in BM fluid were determined by enzyme-linked immunoabsorbent assay (ELISA) kits according to the manufacturers' instructions.

PB Cell Counts

PB was collected and a complete blood count was determined using a Sysmex Hematology Analyzer (Sysmex Co., Kobe, Japan, www.sysmex.com).

Analysis of HSC and Cell Engraftment

On day 2 and 1, 3, and 15 weeks after the infusion of MNCs, the mice were euthanized, and the BM MNCs were collected from the femurs and tibiae. The BM MNCs were counted, and aliquots of cells were stained with various antibodies as noted below. Donor-derived hematopoietic cells were labeled with a PE-conjugated anti-Ly5.1 antibody (CD45.1, BD Biosciences) and biotin-conjugated antibody cocktail for lineage markers (CD5, CD11b, CD45R, Gr-1, 7-4, and Ter119; Miltenyi Biotec, Bergisch Gladbach, Germany, www.miltenyibiotec.com), followed by perinidin chlorophyll protein-cyanine 5.5 (PerCP-Cy5.5)-conjugated streptavidin (BD Biosciences). The labeled cells were divided into two aliquots, each of which was then mixed with either antibody cocktail A (APC-conjugated anti-mouse c-kit [CD117] antibody [eBioscience, San Diego, CA, www.ebioscience.com], PE-Cy7-conjugated anti-mouse Sca-1 [Ly6A/E] antibody [eBioscience], and FITC-conjugated anti-mouse CD34 antibody [eBioscience]) or with antibody cocktail B (APC-conjugated anti-mouse CD48 antibody [eBioscience] and PE-Cy7-conjugated anti-mouse CD150 antibody [eBioscience]). A flow cytometric analysis was performed on the FACS LSRFortessa (BD Bioscience) instruments using the FACS-Diva software program (BD Bioscience). Dead cells were gated out by staining with propidium iodide. The proportion of each lineage was calculated from 1,000,000 events.

Cell Proliferation and Cell Cycle Analysis

At 1 week post-transplantation, Ly5.1$^+$, lineage-negative, Sca-1-positive, c-kit-positive (LSK) cells were isolated and stained with anti-Ki67-FITC antibody according to the manufacturer's instructions (BD Bioscience).

Statistical Analysis

The data were analyzed using unpaired two-tailed Student's *t* tests or the Log-rank test for the survival analysis using the PRISM software program (GraphPad software, LA Jolla, CA, www.graphpad.com). For comparisons of more than three groups, a one-way ANOVA followed by Bonferroni post-tests was performed. A value of $p < .05$ was considered to be significant.

RESULTS

Irradiation Activates the Fibrinolytic Pathway and Increases the Expression of PAI-1 in the BM Microenvironment

To examine the effects of irradiation on the fibrinolytic system in the BM microenvironment, the levels of fibrinolytic factors, such as tPA, plasmin/Plg, and its inhibitor, PAI-1, in the BM fluid and in plasma were measured by ELISA. Two days after 9 Gy irradiation, the levels of tPA and plasmin in the BM fluid, as well as in the plasma, were significantly elevated (Fig. 1A). The level of active PAI-1, a negative regulator of the fibrinolytic system, was also elevated in the plasma and BM fluid of the irradiated mice (Fig. 1A). Of note, the increases of these fibrinolytic factors and PAI-1 were much more prominent in the BM fluid than in the blood, suggesting that the irradiation dramatically activates the fibrinolytic pathway and its inhibitor, PAI-1, in the hematopoietic microenvironment.

Increases in the expression levels of tPA, plasmin (or Plg), and PAI-1 in the BM microenvironment were also confirmed by means of immunohistochemical approaches (Fig. 1B, 1C). The irradiation severely destroyed the BM structure, which may have increased the chance of non-specific staining. Therefore, the specificity of the antibodies used in these immunostaining was verified in tPA KO mice or PAI-1 KO mice. No substantial material in the tissue was stained with the respective antibodies under the same assay conditions in these mice (Supporting Information Fig. S2).

Next, the cells responsible for producing the fibrinolytic factors and PAI-1 in the irradiated BM were identified. tPA was detected in vasculature-lining CD31$^+$ endothelial cells and was significantly elevated after irradiation (Fig. 1B, 1C and Supporting Information Fig. S3A). In agreement with previous reports [4, 5], PAI-1 was detected in megakaryocyte-like cells in untreated mice, but after irradiation, the PAI-1 producing megakaryocyte-like cells disappeared, and the PAI-1 levels in the endosteal region were increased. The tissue distributions of plasmin and Plg colocalized with that of PAI-1 after irradiation (Fig. 1B). Our subsequent double-staining studies revealed that the majority of PAI-1-expressing cells in the irradiated BM were both osteocalcin-positive and Col(I) α1-positive osteoblasts (Fig. 1C and Supporting Information Fig. S3B, S3C).

The activation of fibrinolytic factors and the fibrinolysis inhibitor, PAI-1, in nonhematopoietic cells in the hematopoietic niche shortly after irradiation was also conformed in in vitro studies. We observed that primary BM stromal cells, as well as the BM stromal cell line, HESS5, produced fibrinolytic factors and PAI-1 after irradiation (Fig. 1D and Supporting Information Fig. S4).

PAI-1 Is a Negative Regulator of Early-Phase Hematopoietic Regeneration

To investigate role of PAI-1 in the hematopoietic regeneration, we transplanted 2.5 × 10^6 Ly5.1$^+$ BM MNCs into Ly5.2$^+$

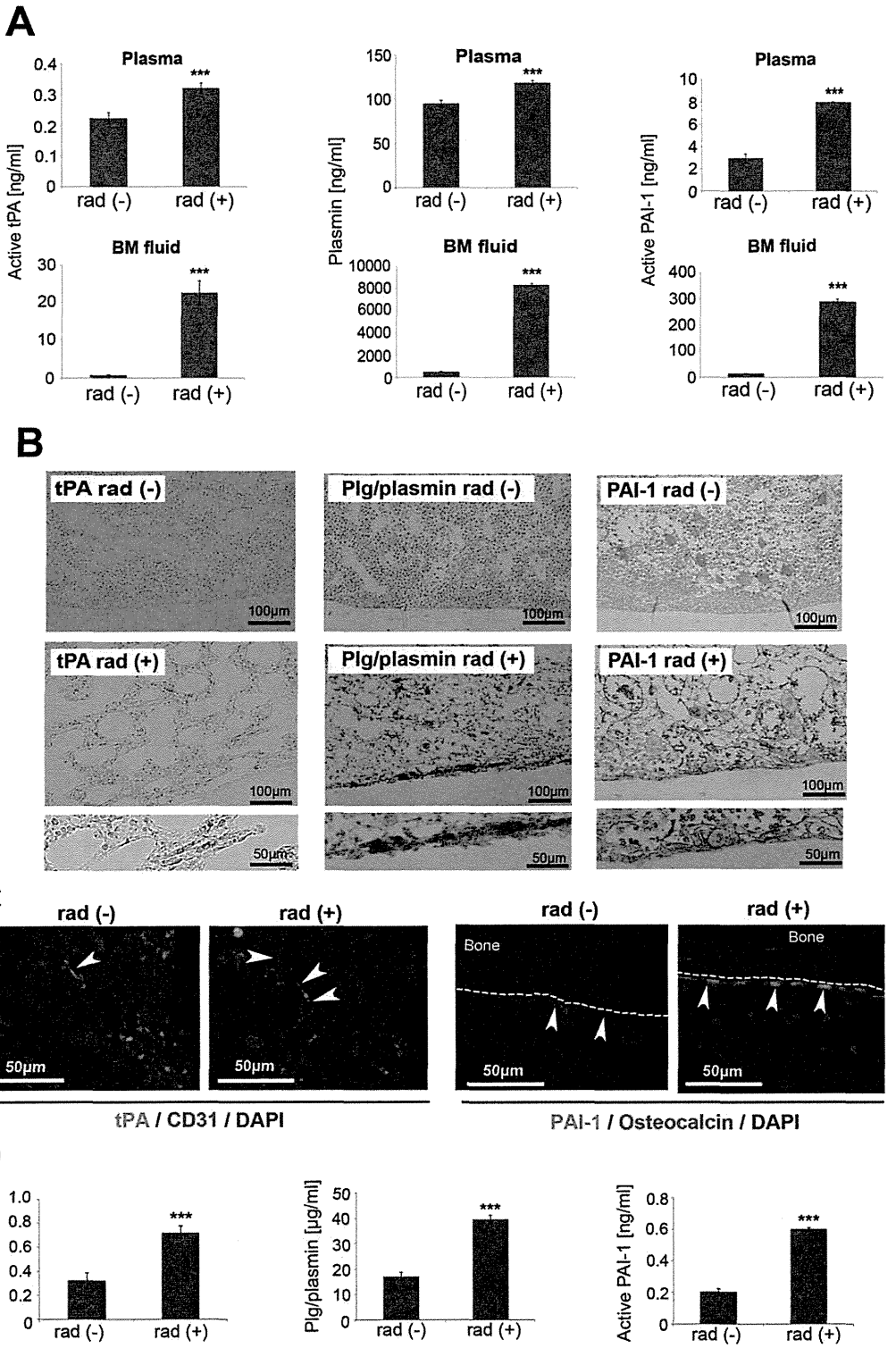

Figure 1. The fibrinolytic pathway and its inhibitor are activated after irradiation. Mice were treated with 9 Gy irradiation [rad (+)] or were untreated [rad (−)], and plasma and BM fluid were collected 2 days later. **(A):** The concentrations of active tPA, plasmin, and active PAI-1 in the plasma and BM fluid were determined by enzyme-linked immunoabsorbent assay (ELISA). Data from three independent experiments are shown ($n = 6$ for each condition; ***, $p < .001$) and are expressed as the means ± SD. **(B, C):** Representative images of immunohistochemical staining for tPA, Plg/plasmin, and PAI-1 in the femur BM. Cells at the endosteal region were intensely stained. (C): The sections were double-immunostained with antibodies for CD31 (red) and tPA (green) (left set), or osteocalcin (red) and PAI-1 (green) (right set). Nuclei were counterstained with DAPI (blue). Arrowheads indicate positive cells. **(D):** The irradiation-induced expression of fibrinolytic factors in cultured BM cells. Primary BM stromal cells in culture were exposed to 10 Gy irradiation. The culture media were collected 24 hours after irradiation, and the concentrations of active tPA, Plg/plasmin, and active PAI-1 were measured by ELISA. Data from three independent experiments are shown ($n = 6$ for each condition; ***, $p < .001$ vs. [rad (−)]) and are expressed as the means ± SD. Abbreviations: BM, bone marrow; DAPI, 4′,6-diamidino-2-phenylindole; PAI-1, plasminogen activator inhibitor-1; tPA, tissue-type plasminogen activator; Plg, plasminogen.

congenic mice, which had been myeloablated by 9 Gy irradiation. PAI-1 KO mice (Ly5.2$^+$) were used as HSCT recipients to monitor the effects of the PAI-1 inhibition on hematopoietic regeneration. The PAI-1 KO recipient mice did not exhibit the induction of active PAI-1, regardless of the transfer of wild-type (WT; PAI-1$^{+/+}$) hematopoietic cells, suggesting that donor-derived hematopoietic cells may not be involved in the regulation of the fibrinolytic pathway during hematopoietic regeneration (Fig. 2A).

A previous study by another group [6] reported that the activation of the fibrinolytic pathway by recombinant tPA promoted hematopoietic cell proliferation through the MMP-mediated release of c-kitL. We therefore evaluated the expression levels of active tPA and other hematopoietic regulatory factors (i.e., MMP-9 and c-kitL) in the PAI-1 KO mice. The results demonstrated a marked increase in the expression of these factors compared to the WT mice at each time point (Fig. 2B–2E).

We subsequently examined the efficiency of hematopoietic regeneration by a flow cytometric analysis (Supporting Information Fig. S5). Over 90% (93.96% $\pm$ 1.56%, $n = 36$) of the hematopoietic cells in the recipient BM were Ly5.1$^+$ donor-derived cells. The absolute number of BM MNCs (Fig. 2F) and the proportion of Ly5.1$^+$ donor-derived Lin$^-$ SLAM (CD150$^+$CD48$^-$) HSCs (Fig. 2G, 2H) were higher in the PAI-1 KO mice than in the WT mice during both the steady state and post-transplant periods. We also examined another HSC marker, CD34$^-$LSK (Lin$^-$Sca-1$^+$c-kit$^+$), and showed that the proportion of Ly5.1$^+$ CD34$^-$LSK cells was higher in the PAI-1 KO mice than in the WT mice (Fig. 2I, 2J), supporting our hypothesis that the induction of PAI-1 in the hematopoietic microenvironment inhibits hematopoietic regeneration. Collectively, our results demonstrated that radiation-induced myeloablation augments the expression levels of not only hematopoietic regeneration-enhancing factors, tPA and Plg/plasmin, but also simultaneously enhances the expression of their negative regulator, PAI-1.

The Expression of Fibrinolytic Factors Is Augmented by PAI-1 Inhibition During Hematopoietic Recovery

We tested our hypothesis that the pharmacological inhibition of PAI-1 can augment the endogenous tPA-mediated fibrinolytic pathway activity more efficiently than exogenous tPA administration, leading to more efficient hematopoietic reconstitution after BM transplantation. A PAI-1 inhibitor or recombinant tPA was thus administered to the WT irradiated mice, and the early phase of hematopoietic recovery and the changes in fibrinolytic factors and the fibrinolysis inhibitor in the plasma were monitored at several time points. The administration of a PAI-1 inhibitor resulted in almost complete suppression of the elevation of active PAI-1 after BM transplantation (Fig. 3A). The PAI-1 inhibitor significantly increased the plasma levels of active tPA and plasmin (Fig. 3B, 3C). Based on the fact that the half-life of recombinant tPA is only a few minutes in rodents [17], as well as the results in Figure 1, it is likely that the increased tPA present at 2 and 7 days post-transplantation reflects the local production of tPA in the BM generated by irradiation. Surprisingly, the PAI-1 inhibitor augmented the induction of fibrinolytic factors more strongly than did the direct administration of recombinant tPA. This may be explained, at least in part, by the fact high PAI-1 activity was maintained during recombinant tPA administration (Fig. 3A), which may limit its benefit on fibrinolytic factors.

We confirmed that administering a PAI-1 inhibitor to mice induced an increase in the plasma levels of hematopoiesis-promoting factors, such as MMP-9 and c-kitL (3- and 1.7-fold compared to the vehicle treatment, respectively) as shown in Figure 3D and 3E. These results demonstrate that the inhibition of PAI-1 effectively induces factors promoting hematopoiesis.

The Activation of the Fibrinolytic Pathway by PAI-1 Inhibition Enhances the Hematopoietic Reconstitution

The protection against BM damage after irradiation and/or chemotherapy is a primary factor that determines the survival rate of animals [18], which hinges on how rapidly the transplanted (i.e., following radioablation) or remaining (i.e., following chemotherapy) hematopoietic cells can reconstitute the crippled hematopoietic system. The survival rates of recipient mice after either a lethal dose of radiation (12 Gy) or 5-fluorouracil administration were investigated in the presence of either recombinant tPA or a PAI-1 inhibitor. The administration of tPA improved the survival rate after myeloablative treatment, but PAI-1 inhibitor treatment offered significantly more effective protection (Fig. 4A, 4B).

The benefits on the hematopoietic recovery were also confirmed in 9 Gy-irradiated mice, which had been transplanted with BM MNCs. The numbers of both white blood cells and platelets markedly increased after BM transplantation: the PAI-1 inhibitor treatment led to more successful hematopoietic recovery than did the treatment with recombinant tPA (Fig. 4C, 4D). These results demonstrate that the inhibition of PAI-1 promotes hematopoietic recovery and protects against myeloablation-induced mortality.

Suppressing the PAI-1 Activity Induces tPA-Mediated HSC Proliferation in the BM After HSCT

To further elucidate the mechanism(s) by which the PAI-1 inhibition improves the hematopoietic recovery, 2.5×10^6 Ly5.1$^+$ BM cells were transplanted into the 9 Gy-irradiated Ly5.2$^+$ congenic mice, and the effects of tPA or a PAI-1 inhibitor on the proliferation of HSCs were assessed. The absolute number of BM MNCs in the recipients indeed increased in the mice given a PAI-1 inhibitor (Fig. 5A). At 3 weeks after transplantation, both the proportion and the absolute number of phenotypically identified Ly5.1$^+$ donor-derived HSC compartments were significantly higher in the PAI-1 inhibitor-treated mice than in the control or the tPA-treated mice (Fig. 5B–5E). The proportion of mature myeloid and lymphoid Ly5.1$^+$ donor-derived cells in the recipient BM was equivalent in the vehicle-treated and PAI-1 inhibitor-treated recipients, suggesting that the PAI-1 inhibitor does not influence the differentiation of HSCs (Supporting Information Fig. S6). The ability of a PAI-1 inhibitor to induce hematopoietic regeneration, that is, upregulation of MMP and c-kitL production and enhancement of donor cell engraftment was completely negated when the tPA KO mouse was used as the recipient (Fig. 5F–5J), further supporting our hypothesis that the hematopoietic regeneration in our model was derived from the

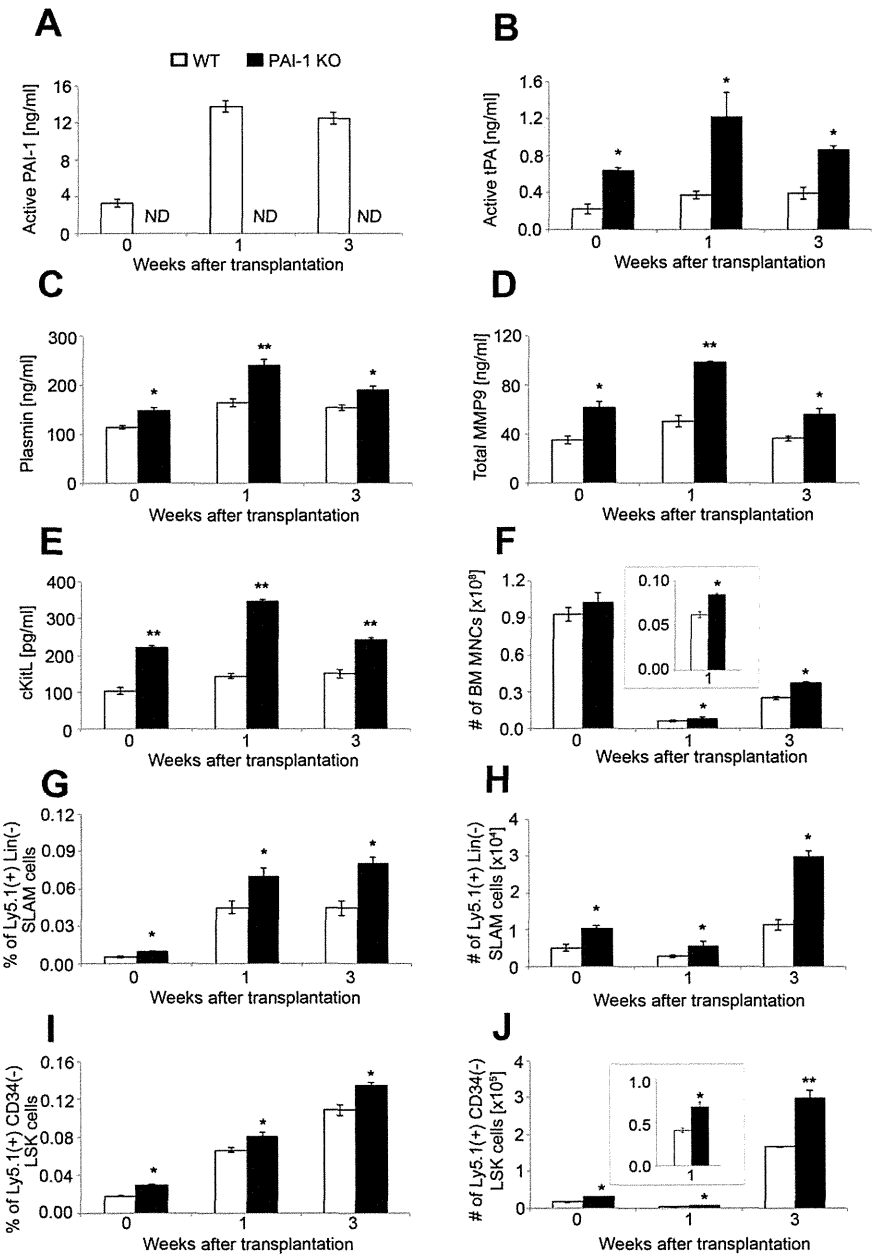

Figure 2. Hematopoietic regeneration is enhanced in PAI-1 KO mice. BM MNCs (2.5×10^6) from the Ly5.1$^+$ donor mice (PAI-1$^{+/+}$) were transplanted into WT (Ly5.2$^+$, PAI-1$^{+/+}$) or PAI-1 KO (Ly5.2$^+$, PAI-1$^{-/-}$) mice that had been irradiated with 9 Gy. Plasma was collected from the recipient mice 1 or 3 weeks after transplantation. **(A–E):** The levels of soluble factors in the plasma. The levels of active PAI-1 (A), active tPA (B), plasmin (C), total MMP-9 (D), and c-kitL (E) in the plasma were determined by enzyme-linked immunoabsorbent assay. Plasma samples from unirradiated (untransplanted) mice were used as controls (0 weeks). White and black bars represent WT and PAI-1KO recipient mice, respectively. The data from three independent experiments are shown ($n = 9$ for each condition). *, $p < .05$; **, $p < .01$ versus WT at the respective time points. ND, not detected. Data are expressed as the means ± SD. **(F–J):** BM MNCs were collected from four long leg bones (two femurs and two tibias) per mouse, pooled, and analyzed by fluorescence-activated cell sorting. The total number of BM MNCs (F), the proportion of Lin$^-$SLAM cells among the donor-derived Ly5.1$^+$ cells (G), the total number of Lin$^-$ SLAM cells (H), the proportion of CD34$^-$LSK cells among Ly5.1$^+$ cells (I), and the total number of CD34$^-$LSK cells (J) were calculated at zero, 1 and 3 weeks. The data from three independent experiments are shown ($n = 9$ for each condition). *, $p < .05$; **, $p < .01$ versus WT at the respective time points. Larger graph scales of the results at 1 week are included as inset graphs in (F) and (J). The data are expressed as the means ± SD. Abbreviations: BM, bone marrow; MNC, mononucleic cell; PAI-1, plasminogen activator inhibitor-1; tPA, tissue-type plasminogen activator; WT, wild type.

PAI-1 inhibition and subsequent tPA-mediated proliferation of HSCs during early hematopoietic reconstitution.

To confirm that the PAI-1 inhibitor induces HSC proliferation, the 9 Gy-irradiated Ly5.2$^+$ congenic mice were transplanted with 2.5×10^6 Ly5.1$^+$ BM cells and given a PAI-1 inhibitor for 5 consecutive days. The expression of Ki67 in the Ly5.1$^+$ donor-derived hematopoietic stem and progenitor cells was examined 1 week after HSCT. The Ki67$^+$ donor-derived LSK

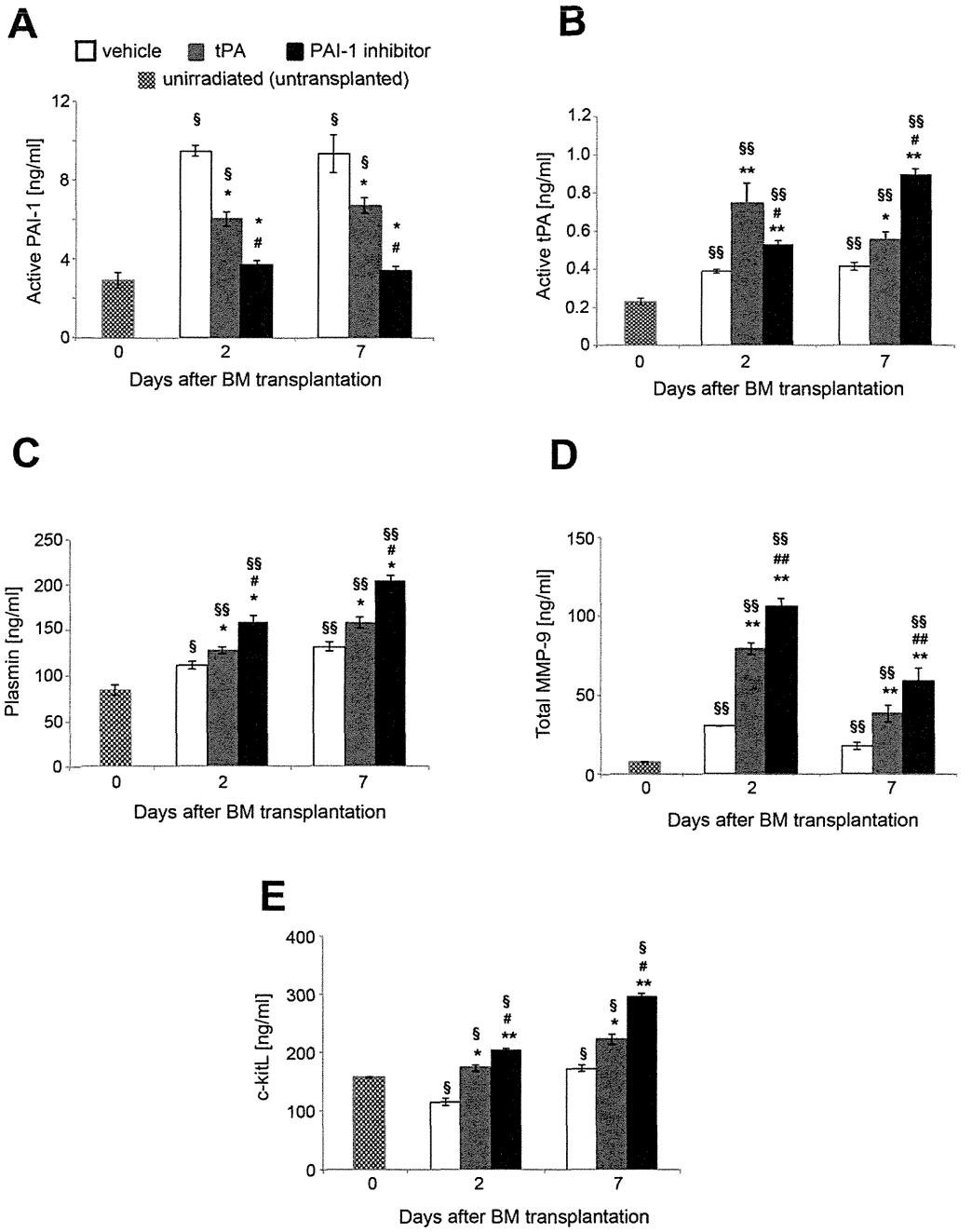

Figure 3. The PAI-1 inhibitor activates the fibrinolytic pathway during hematopoietic regeneration. BM MNCs from the Ly5.1$^+$ donor mice (2.5×10^6) were transplanted into the 9 Gy-irradiated Ly5.2$^+$ mice. Then, vehicle, tPA (10 mg/kg, i.p.), or a PAI-1 inhibitor (TM5275; 100 mg/kg, p.o.) was given daily to the recipient mice for 5 consecutive days. The plasma levels of active PAI-1 **(A)**, active tPA **(B)**, plasmin **(C)**, total MMP-9 **(D)**, and c-kitL **(E)** were measured by enzyme-linked immunoabsorbent assay. The bars in white, gray, and black represent the vehicle-, tPA-, and PAI-1 inhibitor-treated mice, respectively. Hatched bars represent the unirradiated (untransplanted) control mice. The data from four independent experiments are shown as the means ± SD ($n = 12$ for each condition). *, $p < .05$; **, $p < .01$, versus the vehicle group; $^{\#}$, $p < .05$; $^{\#\#}$, $p < .01$, versus the tPA group; §, $p < .05$; §§, $p < .01$, versus the unirradiated group. Abbreviations: BM, bone marrow; PAI-1, plasminogen activator inhibitor-1; tPA, tissue-type plasminogen activator; MMP-9, matrix metalloproteinase-9.

cells were detected at the highest level in the mice given the PAI-1 inhibitor (Fig. 6A), which correlated with the HSC proportion in the recipient. The inhibition of PAI-1 thus stimulates hematopoietic stem/progenitor cells (HSPCs) to enter into the cell cycle. The PCNA$^+$c-kit$^+$ proliferating HSPCs were preferentially located in the endosteal region of the BM (arbitrarily defined as within 12 cells of the endosteum) [14, 15] at 1 week after transplantation (69.4% ± 4.1%; $n = 1{,}811$ PCNA$^+$c-kit$^+$ cells in the vehicle-treated group and 76.7% ± 3.4%; $n = 2{,}983$ PCNA$^+$c-kit$^+$ cells in the PAI-1 inhibitor-treated group, $p < .01$), indicating that the proliferation of HSPCs in the PAI-1 inhibitor-treated recipients as regulated by their interaction with the niche (Fig. 6C).

In the early-stage of regeneration, the HSPCs underwent apoptosis at a higher rate in the vehicle-treated recipient,

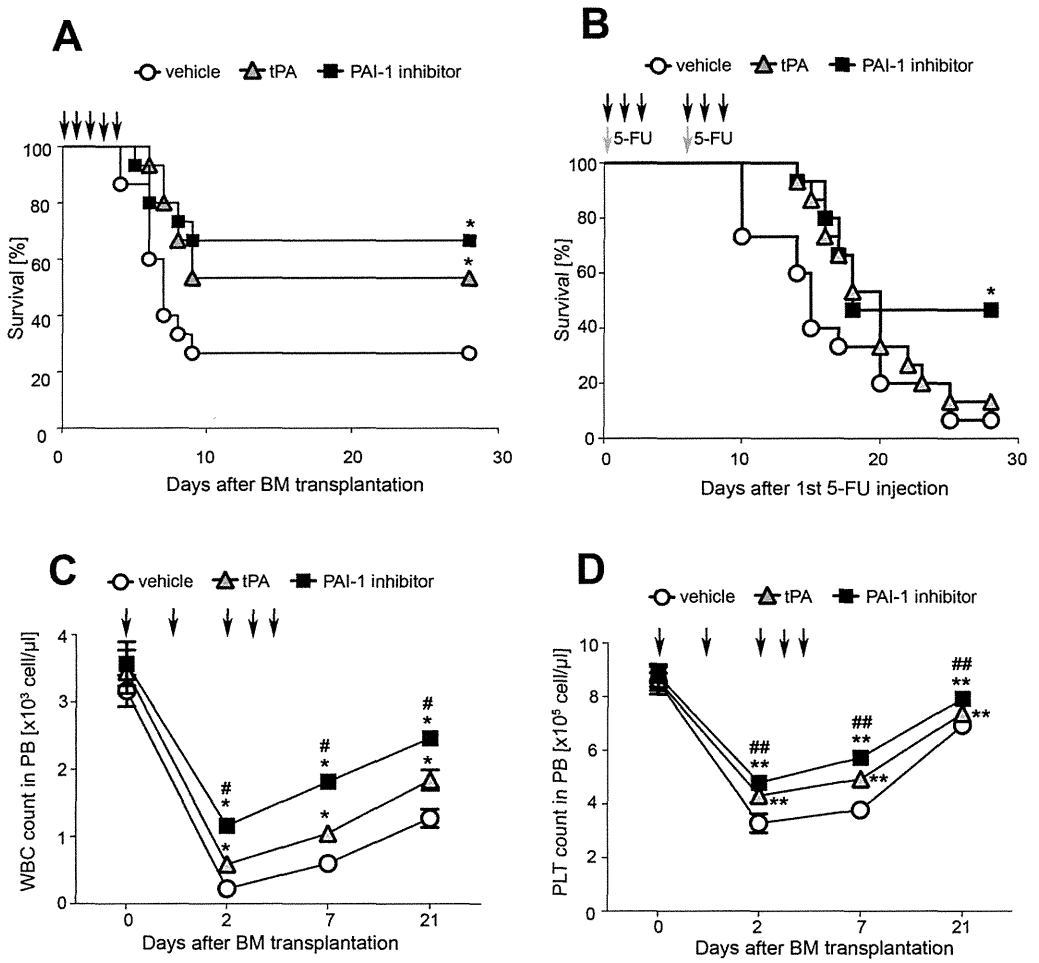

Figure 4. The PAI-1 inhibitor enhances the protection against myeloablation and promotes the rapid recovery of hematopoiesis. **(A):** The survival rate of mice after lethal-dose irradiation. Mice were exposed to 12 Gy of radiation and were transplanted with 1×10^6 BM MNCs. The mice were administered vehicle (white circle), tPA (10 mg/kg, i.p.; gray triangle), or a PAI-1 inhibitor (100 mg/kg, p.o.; black square) daily for 5 consecutive days (arrows). The data from three independent experiments are shown ($n = 15$ for each condition; *, $p < .05$ vs. the vehicle group). **(B):** The survival rate after 5-FU injection. The 5-FU (225 mg/kg, i.p.) was given twice (on days 0 and 7). The mice subsequently received vehicle (white circle), tPA (10 mg/kg, i.p.; gray triangle), or a PAI-1 inhibitor (100 mg/kg, p.o.; black square) for 3 consecutive days after the day of 5-FU injection (arrows). The data from three independent experiments are shown ($n = 15$ for each condition; *, $p < .05$ vs. both the vehicle group and the tPA group). **(C, D):** The hematopoietic recovery. Mice ($Ly5.2^+$) were exposed to 9 Gy of radiation and transplanted with 2.5×10^6 BM MNCs ($Ly5.1^+$). The numbers of white blood cells (WBC, C) and platelets (PLT, D) at the indicated time points were measured. The data from three independent experiments are shown as the means ± SD ($n = 6$ for each condition). *, $p < .05$; **, $p < .01$ versus the vehicle group, #, $p < .05$; ##, $p < .01$ versus the tPA group. Abbreviations: BM, bone marrow; PB, peripheral blood; PAI-1, plasminogen activator inhibitor-1; tPA, tissue-type plasminogen activator; 5-FU, 5-fluorouracil.

whereas the PAI-1 inhibitor prevented the apoptotic cell death of HSPCs (Supporting Information Fig. S7). Altogether, these findings indicate that the PAI-1 inhibitor enhances the proliferation of HSPCs and protects them from stress-induced apoptosis, leading to improve the hematopoietic regeneration.

PAI-1 Inhibition Potentiates the Self-Renewal Capacity of HSCs

The rapid proliferation of HSCs occasionally results in their exhaustion (i.e., loss of competence as HSCs), eventually leading to the failure of long-lasting hematopoiesis in the BM [19–21]. To examine whether the elevation of tPA activity, either by tPA- or PAI-1 inhibitor administration, induced transplanted HSCs to undergo exhaustion, the recipient BM was analyzed at 15 weeks in the $Ly5.2^+$ mice that had been transplanted with $Ly5.1^+$ donor BM cells and given a PAI-1 inhibitor. The results indicated that when the PAI-1 inhibitor was administered to mice, it increased not only the number of BM MNCs (Fig. 7A), but also the proportion, as well as the absolute number of phenotypic $Ly5.1^+$ HSCs compared to the vehicle- or recombinant tPA-treated group (Fig. 7B-7E), suggesting that the inhibition of PAI-1 activity by the PAI-1 inhibitor efficiently prolonged the survival of donor-derived HSCs in the BM.

The self-renewal capacity of HSCs was also examined in a secondary transplant performed 15 weeks after the primary transplant. Twelve weeks after the secondary transplant, the chimerism of the primary donor-derived $Ly5.1^+$ hematopoietic cells was fourfold higher in the PAI-1 inhibitor-treated group than

in the vehicle group (Fig. 7F). The inhibition of PAI-1 therefore enhances not only the rapid hematopoietic recovery in the early phase, but also the long-term self-renewal capacity of HSCs.

To compare the repopulating activity of long-term HSCs, a small number of primary Ly5.1$^+$ donor-derived hematopoietic cells after limiting dilution was transplanted into Ly5.2$^+$ mice (6×10^4 or 2×10^4 cells per mouse). Recipient Ly5.2$^+$ mice were thought to be successfully engrafted if Ly5.1$^+$ cells with the potential for multilineage differentiation were detected in excess of 1% of the total BM cells in the recipient (representative FACS profiles are shown in Supporting Information Fig. S8). The results demonstrated that the chimerism of Ly5.1$^+$ cells was higher in the PAI-1 inhibitor-treated group (1.26% ± 0.1% with 2×10^4 cells and 3.45% ± 1.09% with 6×10^4 cells) than in the vehicle-treated group (0.38% ± 0.26% with 2×10^4 cells and 1.04% ± 0.19% with 6×10^4 cells) (Fig. 7G). In addition, successful engraftment was achieved more often in the PAI-1 inhibitor-treated group (five of the five mice with 2×10^4 and 6×10^4 cells) than in the vehicle-treated group (one of the five mice with 2×10^4 cells and two of the five mice with 6×10^4 cells). Collectively, our results of the phenotypic (i.e., cell surface analysis) and repopulating (i.e., transplantation) assays revealed that inhibiting the PAI-1 activity during the early phase of reconstitution efficiently expands the long-term repopulating HSCs.

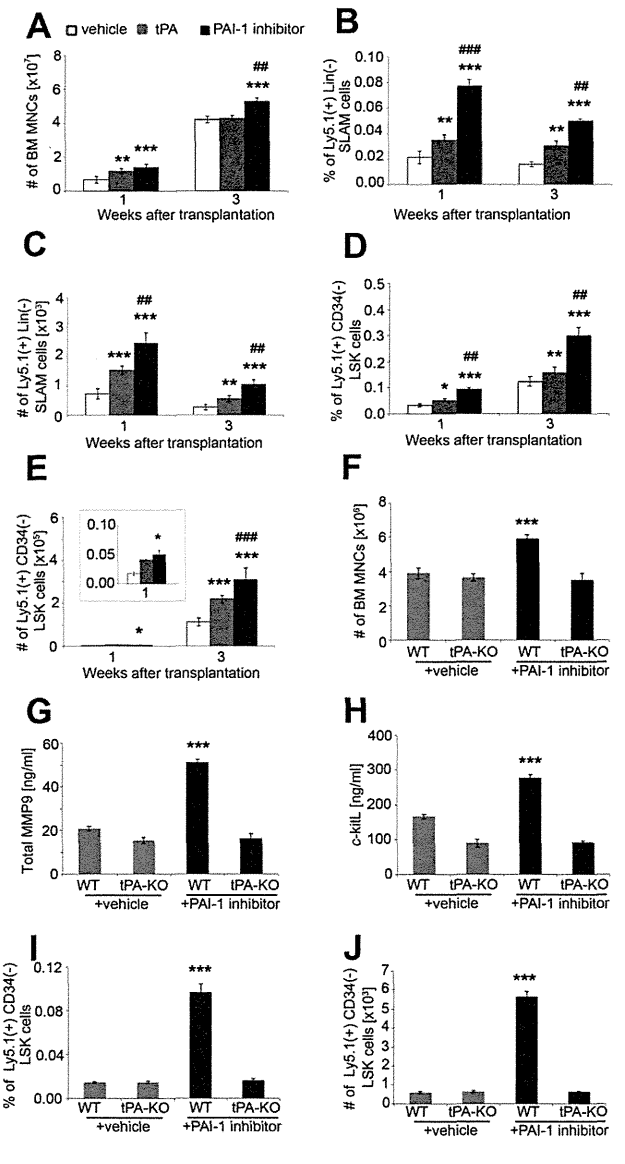

Figure 5. The PAI-1 inhibitor increases the proportion of hematopoietic stem cells (HSCs) during hematopoietic reconstitution. BM MNCs from the Ly5.1$^+$ donor mice (2.5×10^6) were transplanted into the 9 Gy-irradiated Ly5.2$^+$ mice, followed by repeated administration of saline (vehicle), tPA (10 mg/kg, i.p.), or the PAI-1 inhibitor (100 mg/kg, p.o.) for 5 consecutive days. **(A–E):** The results of the fluorescence-activated cell sorting (FACS) analysis of the HSCs in recipient mice. BM MNCs were obtained at the indicated time points from four long leg bones per mouse, and were pooled and subjected to a FACS analysis. (A): The total number of BM MNCs per recipient. (B): The proportion of Lin$^-$ SLAM cells among the donor-derived Ly5.1$^+$ cells, (C) the total number of Lin$^-$SLAM cells, (D) the proportion of CD34$^-$LSK cells among the donor-derived Ly5.1$^+$ cells, and (E) the total number of CD34$^-$LSK cells were calculated at 1 and 3 weeks. The bars in white, gray, and black represent the vehicle-, tPA-, and PAI-1 inhibitor-treated mice, respectively. The data from four independent experiments are expressed as the means ± SD ($n = 12$ for each condition). The inset graph in (E) is a magnification of the data at 1 week. *, $p < .05$; **, $p < .01$; ***, $p < .001$ versus the vehicle group, ##, $p < .01$; ###, $p < .001$ versus the tPA group. **(F–J):** In separate experiments, WT (Ly5.2$^+$, tPA$^{+/+}$) or tPA-KO (Ly5.2$^+$, tPA$^{-/-}$) mice were exposed to 9 Gy radiation and transplanted with 2.5×10^6 BM MNCs (Ly5.1$^+$, tPA$^{+/+}$). Either vehicle or a PAI-1 inhibitor was administered to the recipient mice daily for 5 consecutive days beginning the day after the BM infusion. Plasma and BM MNCs were collected 1 week after transplantation. (F): The total number of isolated BM MNCs per mouse at the indicated time points. The plasma levels of total MMP-9 (G) and c-kitL (H) in the recipient mice. Donor-derived Ly5.1$^+$ cells were gated, and the proportion of CD34$^-$LSK cells (I) and the total number of CD34$^-$LSK cells (J) in the recipient BM were analyzed. The data from three independent experiments are shown as the means ± SD ($n = 6$ for each condition). ***, $p < .001$ by one-way ANOVA. Abbreviations: BM, bone marrow; MNC, mononucleic cell; PAI-1, plasminogen activator inhibitor-1; tPA, tissue-type plasminogen activator; WT, wild type; MMP9, matrix metalloproteinase 9.

Discussion

This study was undertaken to elucidate the role of PAI-1, a negative regulator of the fibrinolytic pathway, in hematopoietic regeneration after irradiation-induced myeloablation. The results demonstrated the following: first, the expression levels of fibrinolytic factors, such as tPA and plasmin, are markedly augmented in endothelial and nonhematopoietic stromal cells, respectively, in the BM of mice after sublethal irradiation. Second, the PAI-1 expression is simultaneously upregulated in nonhematopoietic stromal cells, especially osteoblasts. Third, PAI-1 negatively regulates hematopoietic regeneration: the genetic deletion of PAI-1, as well as the administration of a PAI-1 inhibitor, activates the tPA-mediated fibrinolytic pathway, eventually accelerating hematopoietic regeneration. Finally, the inhibition of PAI-1 activity at an early phase of transplantation facilitates the recovery and maintenance of hematopoiesis (Supporting Information Fig. S9).

Previous studies have shown that the levels of fibrinolytic factors and their inhibitors are elevated in several tissues

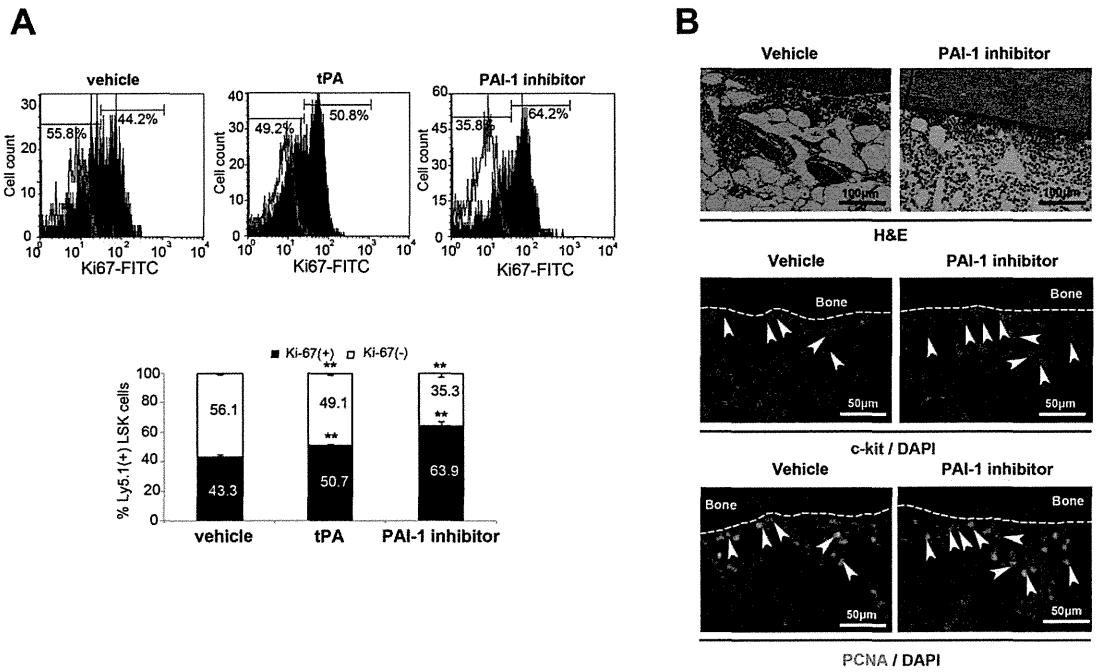

Figure 6. The PAI-1 inhibitor promotes hematopoietic stem cell (HSC) proliferation. Ly5.2$^+$ mice were exposed to 9 Gy radiation and transplanted with 2.5 $\times$ 10^6 Ly5.1$^+$ BM MNCs. Thereafter, vehicle or the PAI-1 inhibitor was administered daily to the mice for 5 consecutive days, and the mice were sacrificed on day 7 for the fluorescence-activated cell sorting (FACS) and BM histological analyses. **(A):** Representative FACS profiles of the Ki67-expression in the donor-derived Ly5.1$^+$ LSK cells. BM MNCs were collected, and the proportion of Ki67$^+$ cells among the Ly5.1$^+$ donor-derived HSCs was analyzed. Isotype-matched control IgG was used to identify the Ki67$^-$ cells. The proportion of Ki67$^+$ or Ki67$^-$ cells is shown above each histogram. The average proportion of Ki67$^+$ Ly5.1$^+$ LSK cells from three independent experiments (each with two mice) is shown in the right panel. **, $p < .01$ versus the vehicle group by one-way ANOVA followed by Bonferroni post-test. **(B):** Representative H&E staining (left panel set) or fluorescent immunostaining (c-kit [red] in the middle panel set; PCNA [green] in the right panel set) of serial sections of bone. Nuclei were counterstained with DAPI (blue). Arrowheads indicate positive cells. Abbreviations: FITC, fluorescein isothiocyanate; DAPI, 4′,6-diamidino-2-phenylindole; PAI-1, plasminogen activator inhibitor-1; H&E, hematoxylin and eosin; PCNA, proliferation cell nuclear antigen; tPA, tissue-type plasminogen activator.

following irradiation [22–24]. However, the dynamics of fibrinolytic system factors, particularly PAI-1, in hematopoietic regeneration of the BM have not been elucidated. This study revealed, for the first time, that irradiation has a substantial impact on the fibrinolytic system in the BM. Upon irradiation, the levels of fibrinolytic factors and PAI-1 in the BM fluid increased dramatically (~30-fold), in sharp contrast to the moderate increase in these factors in the plasma (1.8–3-fold). Of note, their levels in the BM fluid were 1 or 2 orders of magnitude higher than those in the plasma (e.g., 300 and 8 ng/ml of PAI-1 in the BM fluid and plasma, respectively), demonstrating that BM cells have a potent capacity to produce fibrinolytic factors and PAI-1 upon irradiation. In good agreement with these in vivo results, the results of the in vitro cultured cell experiments demonstrated that nonhematopoietic BM stromal cells readily produce tPA, plasmin, and PAI-1 after irradiation.

On closer inspection by immunohistochemistry, we identified nonhematopoietic cells, especially stromal cells, as the primary source of fibrinolytic factors and PAI-1. Our data in the irradiated PAI-1 KO mice (recipient), which have undetectable levels of PAI-1 in the plasma after transplantation of normal hematopoietic cells (PAI-1$^{+/+}$), suggested that the marked increase in PAI-1 after irradiation does not originate from donor hematopoietic cells. Nonhematopoietic stromal cells in the BM thus play a significant role in repairing the myeloablated hematopoietic microenvironment. This is not surprising, because the irradiation of mice with a lethal dose of radiation eradicates the hematopoietic cells and their progenitor cells, but not nonhematopoietic cells [25, 26].

We also elucidated the mechanism by which PAI-1 regulates the hematopoietic regeneration in the BM. A previous study by Hattori and colleagues demonstrated that activation of the fibrinolytic pathway by administration of recombinant tPA results in the conversion of the transmembrane form to the soluble form of c-kitL [6], a hematopoiesis-promoting factor. They suggested that activated plasmin subsequently transforms MMP-2/9 into active forms, which in turn release c-kitL from stromal cells. It is therefore likely that PAI-1 activity prevents the hematopoietic regeneration in the BM by inhibiting the fibrinolytic pathway and c-kitL release. Our results showing that the suppression of PAI-1 activity either by a pharmacological approach or by the genetic deletion of the PAI-1 gene can elevate the tPA/plasmin activity, promote c-kitL production, and lead to the rapid recovery of hematopoiesis after myeloablation. Although other products of fibrinolytic degradation may also promote engraftment, these results support the critical role of c-kitL production in the improvement of engraftment by PAI-1 inhibition.

Emerging evidence suggests that PAI-1 expression generally limits tissue repair by negatively regulating the fibrinolytic environment and by inhibiting cell migration [27]. For example, the skin wound healing process is accelerated in PAI-1-deficient mice [28]. PAI-1 KO mice are also protected against liver fibrosis [29] and radiation enteropathy [24]. In terms of tissue

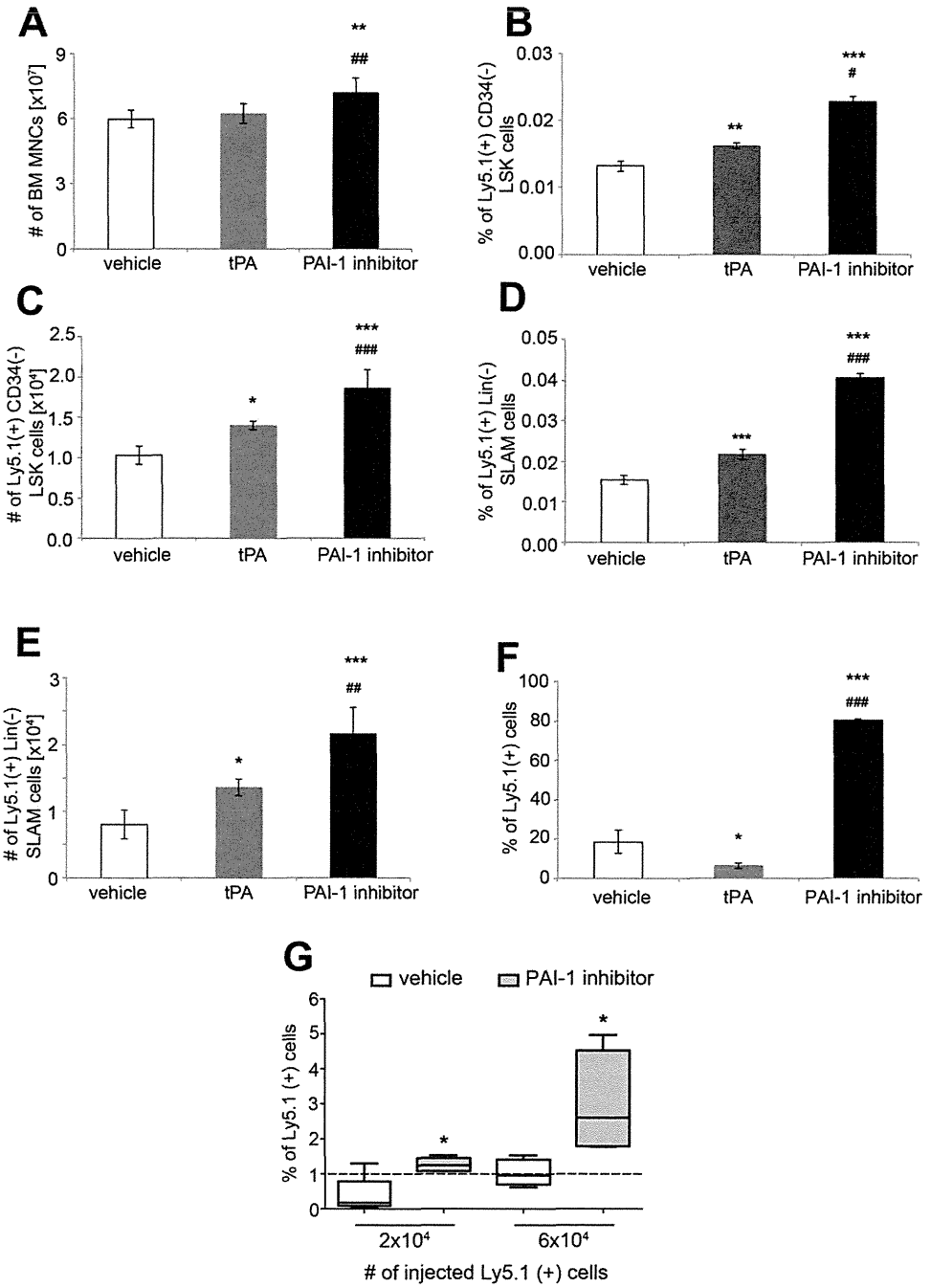

Figure 7. The PAI-1 inhibitor enhances the self-renewal capacity of hematopoietic stem cells. **(A–E):** The $Ly5.2^+$ mice were exposed to 9 Gy radiation, followed by transplantation with 2.5×10^6 $Ly5.1^+$ BM MNCs. They were administered the vehicle, tPA, or the PAI-1 inhibitor daily for 5 consecutive days. BM MNCs were collected 15 weeks after transplantation. BM MNCs were obtained from four long leg bones per mouse, and were pooled for the analysis of (A) the total number of donor-derived $Ly5.1^+$ BM MNCs, (B) the proportion of $CD34^-$LSK cells among $Ly5.1^+$ cells, (C) the total number of $CD34^-$LSK cells, (D) the proportion of Lin^-SLAM cells among $Ly5.1^+$ cells, (E) the total number of Lin^-SLAM cells. The data from four independent experiments are shown ($n = 12$ for each condition). *, $p < .05$; **, $p < .01$; ***, $p < .001$ versus the vehicle group; #, $p < .05$; ##, $p < .01$; ###, $p < .001$ versus the tPA group. The p value was calculated by one-way ANOVA followed by Bonferroni post-test. **(F):** The chimerism of donor-derived $Ly5.1^+$ cells in the secondary recipients. Donor-derived $Ly5.1^+$ BM MNCs (1×10^6) in the primary recipient mice were retransplanted, together with 5×10^5 $Ly5.2^+$ competitor cells, into the 9 Gy-irradiated $Ly5.2^+$ secondary recipient mice. BM cells were analyzed 12 weeks after the secondary transplantation. The data from three independent experiments are shown ($n = 10$ for each condition). *, $p < .05$; ***, $p < .001$ versus the vehicle group; ###, $p < .001$ versus the tPA group. **(G):** The chimerism of a small number of donor-derived $Ly5.1^+$ cells in the secondary recipients. A total of 2×10^4 or 6×10^4 $Ly5.1^+$ BM cells from the primary recipient mice, with 5×10^5 $Ly5.2^+$ competitor cells, were transplanted into the 9 Gy-irradiated $Ly5.2^+$ secondary recipient mice. Twelve weeks after the secondary transplantation, the chimerism of donor cells in the BM was evaluated. The boxes in white and gray represent the chimerism of donor-derived $Ly5.1^+$ cells in the vehicle- and PAI-1 inhibitor-treated mice, respectively. The horizontal lines in the box represent the median chimerism, and bars indicate the SD. The dashed line indicates the cut-off value (1%). Data from two independent experiments are shown ($n = 5$ for each condition). *, $p < .05$ versus the vehicle group. Abbreviations: BM, bone marrow; MNC, mononucleic cell; PAI-1, plasminogen activator inhibitor-1; tPA, tissue-type plasminogen activator.

regeneration, Galipeau and colleagues have demonstrated that mesenchymal stem cells (MSCs) derived from PAI-1 KO mice exhibited higher regenerative potential than those from WT mice [30]. Furthermore, chemical manipulation of the PAI-1 activity improves the engraftment of MSCs, defining PAI-1 as a negative regulator of transplanted stem cell survival in vivo [30]. This study clarified the active involvement of PAI-1 in the hematopoietic regeneration after irradiation.

Proper treatment of the initial stage of hematopoietic recovery and the prevention of premature HSC exhaustion could therefore significantly improve the clinical outcome of transplantation [1, 2]. In this regard, our study demonstrated that, despite a short period of administration, the suppression of the PAI-1 activity by a low molecular weight compound could induce both rapid hematopoietic regeneration through increased cycling of HSCs and expansion of the long-term HSCs. This opens a new avenue for improving HSCT. It should be emphasized that the PAI-1 inhibitor does not induce HSC exhaustion or malignancy in spite of its potent ability to increase the cycling of HSCs. The results of this study clearly demonstrated that c-kit$^+$ HSPCs in the group treated with the PAI-1 inhibitor preferentially localized to the BM niche, just like in the vehicle-treated group, suggesting that the interaction between the hematopoietic progenitor cells and niche is maintained even after the treatment with a PAI-1 inhibitor. This may be a plausible explanation for why the PAI-1 inhibitor does not induce HSC exhaustion.

Both the PAI-1 inhibitor and tPA theoretically activate the fibrinolytic pathway and the subsequent hematopoietic regeneration, but their effects in vivo in animals appear to be different. This is partly explained by the differences in the routes of administration between tPA and the PAI-1 inhibitor, as well as the doses, mechanisms of action, and/or half-lives of these agents. Recombinant tPA is administered intravenously (a large amount of tPA is given directly into the circulation), and immediately activates the fibrinolytic pathway, but its half-life is only a few minutes [17]. In contrast, the PAI-1 inhibitor was given orally, and was absorbed in the gut, entered into the circulation gradually, inhibited the PAI-1 moiety, and subsequently upregulated the tPA activity leading to its effects on the fibrinolytic pathway. The half-life of the PAI-1 inhibitor is much longer (6.5 hour) than that of tPA.

It is also important to note that tPA administration itself increased the PAI-1 level, suggesting a potential negative feedback effect in this pathway and limits to the therapeutic benefits of tPA for hematopoietic regeneration. In addition, the repopulating capacity of HSCs in tPA-treated mice showed a slight decrease, suggesting that tPA treatment may induce HSC exhaustion. It should also be mentioned that PAI-1 regulates not only tPA, but also other proteins (i.e., vitronectin, urokinase-type plasminogen activator (uPA), and low density lipoprotein receptor (LDLR)) [5, 31, 32] and thereby has an impact on broader biological systems.

Conclusion

In conclusion, our study provides the first direct evidence that PAI-1 is a negative regulator of hematopoietic regeneration, and that the inhibition of PAI-1 activity, either genetically or by a low molecular weight compound, significantly improves donor-derived hematopoiesis after transplantation. Our findings give new insights into the treatment of HSCT and for clinical transplantation medicine.

Acknowledgments

We appreciate the help of Dr. Koichi Hattori (Institute of Medical Science, University of Tokyo, Japan) for kindly providing the tPA KO mice. We thank Dr. Nobuo Watanabe (Tokai University School of Medicine, Japan) for helpful discussion and wrote the manuscript. We also thank the members of the Research Center for Regenerative Medicine of Tokai University, especially Tomomi Takanashi, Kozue Hiyama, and Tomoko Uno, for the technical support. We thank the members of the Animal Care Center of Tokai University for their meticulous care of the experimental animals. This work was supported by Japanese Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), from the Ministry of Health, Labor and Welfare (MHLW), from the National Institute of Biomedical Innovation (NIBIO), from the Japan Science and Technology Agency (JST), and from the Tokai University School of Medicine Research Aid. Special thanks are due to the MARA Education Foundation, Malaysia, for supporting the scholarship awarded to A.A.I.

Author Contributions

A.A.I.: collection and assembly of data, data analysis and interpretation, and manuscript writing; T.Y.: conception and design, data analysis and interpretation, manuscript writing, and financial support; M.O.: data analysis and interpretation; T.D.: provision of study material; C.v.Y.d.S.: manuscript writing; T.M.: provision of study material, data analysis and interpretation, and manuscript writing; K.A.: conception and design, data analysis and interpretation, financial support, and final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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Int J Hematol (2014) 99:175–183
DOI 10.1007/s12185-013-1494-6

ORIGINAL ARTICLE

ACE deletion polymorphism is associated with a high risk of non-infectious pulmonary complications after stem cell transplantation

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Received: 31 May 2013 / Revised: 8 December 2013 / Accepted: 9 December 2013 / Published online: 21 December 2013

Abstract Non-infectious pulmonary complication (NIPC) is a serious adverse event for allogeneic hematopoietic stem cell transplantation (allo-HSCT) patients. NIPC includes both categories of lung complications: early onset idiopathic pneumonia syndrome (IPS) (onset <120 days) and late-onset non-infectious pulmonary complications (LONIPCs). Both categories have high mortality and morbidity rates, and critical treatments are not available. The renin–angiotensin system plays a critical role in pulmonary fibrosis. We, therefore, studied the relationship between angiotensin-converting enzyme gene (ACE) insertion/deletion polymorphisms and NIPC incidence in 149 consecutive allo-HSCT recipients. A total of 12.1 % (18/149) of these patients were diagnosed with NIPC (IPS, 3; LONIPCs, 15). Eight NIPC patients died from respiratory failure (mortality rate, 44.4 %). Peripheral blood stem cell transplantation was associated with a significantly higher incidence of NIPC than bone marrow transplantation and cord blood transplantation by univariate analysis (HR 3.13, $P = 0.031$). The serum ACE levels differed significantly according to the ACE insertion/deletion polymorphism. Patients with an ACE D/D genotype occurred at a significantly higher frequency among NIPC patients than I/D and I/I patients (HR 9.03, $P < 0.0001$). Multivariate analysis confirmed that NIPC is associated with ACE D/D genotypes (HR 8.8, $P < 0.001$). Our data support a role for the renin–angiotensin system in the pathogenesis of NIPC after allo-HSCT, and may represent a therapeutic target for complications.

Keywords *ACE* genetic polymorphism · Non-infectious pulmonary complication · Idiopathic pneumonia syndrome · Lung injury after stem cell transplantation

Introduction

Pulmonary complications following allogeneic hematopoietic stem cell transplantation (HSCT) are frequently fatal. Idiopathic pneumonia syndrome (IPS) describes a subset of HSCT patients who have the signs and symptoms of pneumonia, as well as evidence of widespread alveolar injury in the absence of lower respiratory tract infection [1, 2]. Risk factors for developing IPS include previous infections, a pre-transplant condition, a low performance status prior to transplantation, a high dose of total-body irradiation (TBI), and the presence of graft-versus-host disease (GVHD) [3–5]. The median time of onset for IPS is 19 days (range 4–106 days) [1, 4, 6]. Late-onset non-infectious pulmonary complications (LONIPCs) are also recognized as major pulmonary events after HSCT [5, 7–9]. LONIPCs encompass a range of different events that occur later than 3 months after transplantation. These include interstitial pneumonia (IP), bronchiolitis obliterans syndrome (BOS), and lung fibrosis. The presence of extensive chronic GVHD is significantly associated with the

Electronic supplementary material The online version of this article (doi:10.1007/s12185-013-1494-6) contains supplementary material, which is available to authorized users.

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development of LONIPCs, with Sicca syndrome being the most significantly related [5]. There has been limited progress in understanding the pathogenesis of IPS and LONIPCs despite their clinical significance as complications after allogeneic HSCT.

Previous studies have reported that the renin-angiotensin system plays a key role in lung tissue fibrosis as well as in maintaining blood pressure [10–14]. In a clinical study by Morrison et al. [15], patients with pulmonary fibrosis showed significant disequilibrium in the frequency of insertion/deletion genetic polymorphisms in the angiotensin-converting enzyme gene (*ACE*) compared with normal controls. *ACE* insertion/deletion polymorphisms within intron 16 are well-described, and involve either the insertion or deletion of a 287-bp fragment. The D/D (deletion/deletion) polymorphism genotype is associated with significantly higher levels of ACE and angiotensin II (A-II) compared to the I/I (insertion/insertion) genotype, whereas individuals with the I/D (insertion/deletion) genotype have intermediate levels of both enzymes[16, 17]. Based on both basic and clinical data, we hypothesized that pulmonary complications after HSCT could also involve the renin-angiotensin system. Previously, we retrospectively analyzed the incidence of non-infectious pulmonary complication (NIPC) and *ACE* genetic polymorphisms in 118 Japanese patients who underwent HSCT from HLA identical sibling donors [18]. We showed that the D/D genotype is associated with a high frequency of NIPC [18]. The present study investigated a second, independent cohort comprising 149 consecutive patients, to validate our previous observations. To improve the accuracy of outcome analysis and to reveal relevant multifactorial interaction with NIPC in this cohort, the statistical methods used included time-to-event and multivariate analysis, an independent pulmonologist diagnosed NIPC without retrospective selection, and the patients were from a single institute.

Materials and methods

Study patients

A total of 149 consecutive patients, who received allogeneic HSCT from sibling, related, unrelated, and cord blood donors at Tokai University Hospital from September 2004 to February 2012, were reviewed for this study. Patients who underwent allogeneic HSCT within the previous year and for whom pre-transplant samples for extracting DNA were not available were excluded from the analysis. Patient characteristics are shown in Table 1. GVHD prophylaxis was administered with 43 cases receiving cyclosporine A (CsA) (starting dose of 1.5 mg/kg, twice daily by 3-h i.v. infusion from day −1) and 106 cases receiving tacrolimus (starting dose of 0.02 mg/kg, by continuous i.v. infusion from day −1) and a short course of methotrexate (15 mg/day i.v. on day 1 and 10 mg/day i.v. on days 3 and 6). CsA was used for HLA-matched sibling transplantations and tacrolimus was used for the other donor transplants. The background diseases were as follows: acute myeloid leukemia (AML) in 61 patients, acute lymphoblastic leukemia (ALL) in 30, non-Hodgkin lymphoma (NHL) in 27, myelodysplastic syndrome (MDS) in 14, aplastic anemia (AA) in eight, chronic myelogenous leukemia (CML) in six, primary myelofibrosis in two and multiple myeloma in one patient. The conventional regimen for myeloid malignancy is cyclophosphamide (CY; 60 mg/kg for 2 days) and total-body irradiation (TBI; 12 Gy divided with six fractions); $n = 66$, thoraco-abdominal irradiation (TAI; 8 Gy divided with four fractions); $n = 5$, or busulfan (BU; 0.8 mg/kg four times for 4 days) and CY (60 mg/kg for 2 days); $n = 8$. For lymphoid malignancy, the conventional regimen is CY + TBI 12 Gy with VP-16 (15 mg/kg for 2 days); $n = 10$. Reduced intensity stem cell transplantation (RIST) involved fludarabine (25 mg/m^2 for 5 days) and melphalan (70 mg/m^2 for 2 days); $n = 60$, with inclusion criteria defined as patients more than 55 years of age, with a low performance status, or with a high grade of HCT-CI score [19].

All procedures for dealing with the subjects were in agreement with the ethical standards of our institute. Genomic DNA from recipients was purified from peripheral blood or bone marrow obtained before transplantation. The *ACE* genotype was determined by PCR amplification of intron 16, based on a previously described method [20]. We measured ACE levels in 116 subjects whose serum was available. The samples were collected from patients prior to initiation of the conditioning regimen for HSCT, processed and aliquoted within 30 min of collection, and then kept frozen at −80 °C until further analysis. Serum ACE levels were measured by a College of American Pathologists-certified commercial laboratory (SRL, Inc., Tokyo, Japan) using an enzyme-linked immunosorbent assay (Immuno-biological Laboratories Co., Ltd., Takasaki, Japan), according to the standard procedures recommended by the manufacturer.

Definition of NIPC

NIPC includes IPS and LONIPCs, and patients were diagnosed according to previously published criteria, which include evidence of widespread alveolar injury in multilobar infiltrate, chest X-ray or computed tomography, and clinical symptoms of pneumonia, hypoxemia, and evidence of abnormal respiratory physiology such as restrictive impairment in a pulmonary function test [2].

Table 1 Patient characteristics

Variable	Non-NIPC ($n = 131$)	NIPC ($n = 18$)	*P*	ACE genotype			*P*
				I/I ($n = 54$)	I/D ($n = 64$)	D/D ($n = 31$)	
Age (years)	46 (16–70)	43 (17–61)	0.24[a]	46 (16–69)	44 (17–70)	43 (24–61)	0.39[b]
≤40	49	7		19	24	13	
>40	82	11	0.78	35	40	18	0.83
Sex							
Male	82	10		31	44	17	
Female	49	8	0.61	23	20	14	0.30
Donor source							
BMT	65	9	0.6	23	29	22	
PBSCT	36	9	0.031	18	19	8	
CBT	30	0	0.057	13	16	1	0.048
Donor relationship							
Related	39	9		18	21	9	
Unrelated	92	9	0.11	36	43	22	0.91
Sex disparity							
Female to male	67	9		23	35	18	
Others	64	9	0.84	31	29	13	0.29
Underlying diagnosis[c]							
Acute leukemia/MDS	92	13		37	45	23	
Others	39	5	0.78	17	19	8	0.86
HLA							
Full match	83	13		32	39	25	
Mismatch	48	5	0.60	22	25	6	0.10
Regimen							
Conventional	80	12		27	41	24	
RIST	51	6	0.78	27	23	7	0.038
Calcineurin inhibitors							
CsA	35	8		16	18	9	
Tac	96	10	0.12	38	46	22	0.98
TBI							
non-3 Gy	53	8		27	26	8	
12 Gy	78	10	0.75	27	38	23	0.092

BMT bone marrow transplantation, *PBSCT* peripheral blood stem cell transplantation, *CBT* cord blood transplantation, *CsA* cyclosporine, *Tac* tacrolimus, *RIST* reduced intensity stem cell transplantation, *NA* not applicable

[a] Calculated by student *t* test

[b] Calculated by one-way ANOVA

[c] Others included NHL, CML, MF, MM and AA

Invasive fungal infections were defined according to the European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) criteria [21]. To exclude lower respiratory tract infections, standard culture and staining methods for bacterial, viral, and protozoal pathogens were employed. Patients were tested weekly for cytomegalovirus (CMV) pp65 antigenemia, as well as serum DNA copy number analysis for herpes simplex virus, CMV, and Epstein–Barr virus by RT-PCR.

Pulmonary function tests (PFTs) were performed before transplantation and approximately 3 months after transplantation. The criteria used to label PFTs were as follows: percent forced vital capacity (FVC) of the predicted value (FVC % predicted) <80 % and forced expiratory volume at 1 s (FEV1)/FVC at least 70 % as a restricted pattern, FVC % predicted at least 80 % and FEV1/FVC <70 % as an obstructive pattern, and FVC % predicted <80 % and FEV1/FVC <70 % as a mixed pattern.

The clinical features, laboratory test results, respiratory physiology testing and imaging data were reviewed for all NIPC patients by an independent third pulmonologist.

Statistical analyses

Host factors of interest included patient age, sex, and their underlying diagnosis. Transplantation characteristics included level of TBI conditioning, conditioning regimen, hematopoietic stem cell source (bone marrow, peripheral blood, or cord blood), donor type (human leukocyte antigen HLA-matched or unmatched and related or unrelated donor), female to male transplantation, and type of calcineurin inhibitor. Death without signs of NIPC was considered a competing risk in the analysis of NIPC incidence. Similarly, death due to disease and death without signs of disease relapse were considered a competing risk in the analyses of transplantation-related mortality (TRM) and relapse, respectively. Acute GVHD (aGVHD) was diagnosed and graded according to standard criteria. Only grades II and higher of aGVHD were considered for the analysis of incidence. We analyzed the incidence of chronic GVHD (cGVHD) and LONIPCs beyond 100 days after transplantation because LONIPCs are associated with cGVHD [5, 22, 23].

The association between *ACE* polymorphism and the cumulative incidences of NIPC, TRM, and relapse was evaluated using a model by Fine and Gray for univariate and multivariate analyses [24]. We excluded nine patients with aplastic anemia from the analysis of relapse. Statistical significance was compared between NIPC and non-NIPC patients and also the genotype of *ACE* polymorphism for the analysis of categorical factors, whereas Fisher's exact test was performed for the 2 × 2 contingency tables for each individual allele (Table 1). The 2 × m analysis was conducted using the Chi-square test for trend. The relationships of age to incidence of NIPC and *ACE* polymorphism were evaluated by Student's *t* test and one-way ANOVA, respectively. The probability of OS was compared using the Mantel-Cox test.

Multivariate analysis was on the cumulative incidence of NIPC. Factors associated with a two-sided *P* value of <0.10 in the univariate analysis were included in a multivariate analysis. We used a backward-stepwise selection algorithm and retained only the statistically significant variables in the final model.

Because no cord blood transplantation (CBT) patients developed NIPC, CBT could not be treated as a single multivariate factor for the time-to-event analysis. A two-sided *P* value of <0.05 was considered statistically significant. Univariate and multivariate analysis was conducted using STATA v 12 and Prism v 5 software (StataCorp LP, TX and GraphPad Software, Inc. CA, USA, respectively).

Results

Clinical background and developing NIPC

Non-infectious pulmonary complication was initially diagnosed in 20 patients by three hematologists, but one pulmonologist did not agree with two of these diagnoses because infectious disease could not be ruled out upon final review. Therefore, a total of 12.1 % (18/149) of patients were finally diagnosed as NIPC. In the analysis of donor source, peripheral blood stem cell transplantation (PBSCT) patients had a significantly higher frequency of developing NIPC compared with bone marrow transplantation (BMT) and cord blood transplantation (CBT) patients ($P = 0.031$) (Table 1). There were no NIPC events among CBT patients, but the *P* value was over the threshold ($P = 0.057$) (Table 1). In addition, all the patients transplanted from unrelated donors were BMT and tended to have a lower frequency of NIPC ($P = 0.067$) (Table 1). Comparison of the three sources by the Mantel-Cox test reached statistical significance ($P = 0.040$) (Fig. 1). The univariate analysis indicated that age, sex, conditioning regimen, kind of calcineurin inhibitor, TBI, female donor to male recipient transplantation, HLA disparity, underlying diagnosis, and grades of acute GVHD was not significantly associated with the development of NIPC (Table 1). The Chi-square test for trend and Fisher's exact test for each variable also did not reach statistical significance.

The median time of onset was 245 days (range 20–1331 days) after stem cell transplantation. The clinical

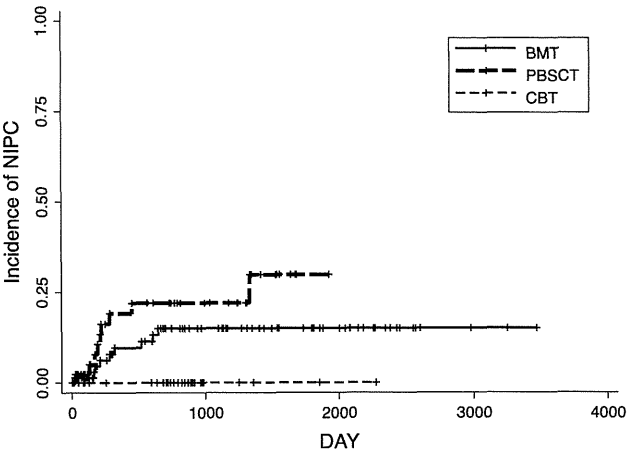

Fig. 1 Donor source and incidence of NIPC. Kaplan–Meier failure incidence curve of NIPC and donor source. Comparison of the three sources by the Mantel-Cox test reached statistical significance ($P = 0.040$)