Serpina1 is a potent inhibitor of IL-8-induced hematopoietic stem cell mobilization

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Here, we report that cytokine-induced (granulocyte colony-stimulating factor and IL-8) hematopoietic stem cell (HSC) and hematopoietic progenitor cell (HPC) mobilization is completely inhibited after low-dose (0.5 Gy) total-body irradiation (TBI). Because neutrophil granular proteases are regulatory mediators in cytokine-induced HSC/HPC mobilization, we considered a possible role for protease inhibitors in the induction of HSC/HPC mobilization. Bone marrow (BM) extracellular extracts that were obtained from murine femurs after 0.5 Gy of TBI contained an inhibitor of elastase. Also, after low-dose TBI, both Serpina1 mRNA and protein concentrations were increased in BM extracts, compared with extracts that were obtained from controls. The inhibitory activity in BM extracts of irradiated mice was reversed by addition of an Ab directed against Serpina1. To further study a possible *in vivo* role of Serpina1 in HSC/HPC mobilization, we administered Serpina1 before IL-8 injection. This administration resulted in an almost complete inhibition of HSC/HPC mobilization, whereas heat-inactivated Serpina1 had no effect. These results indicate that low-dose TBI inhibits cytokine-induced HSC/HPC mobilization and induces Serpina1 in the BM. Because exogenous administration of Serpina1 inhibits mobilization, we propose that radiation-induced Serpina1 is responsible for the inhibition of HSC/HPC mobilization. Also, we hypothesize that cytokine-induced HSC/HPC mobilization is determined by a critical balance between serine proteases and serine protease inhibitors.

protease | protease inhibitors | bone marrow | adhesion molecules

Since a number of years ago, cytokine-mobilized blood stem cells have become the primary source of hematopoietic stem cells (HSCs) and hematopoietic progenitor cells (HPCs) for transplantation in humans (1–3). In mice, many cytokines and chemokines induce HSC/HPC mobilization after prolonged administration, such as granulocyte colony-stimulating factor (G-CSF), whereas only few cytokines and chemokines induce HSC/HPC mobilization upon a single injection, such as IL-8 (4–6). Despite the wide applications of mobilized HSC/HPC, the mechanisms that contribute to the release of HSC/HPC into the peripheral blood are not completely understood. However, evidence has accumulated that HSC/HPC mobilization is a multistep process in which multiple cell types and molecules are involved.

Under steady-state conditions, HSC/HPC are retained in the bone marrow (BM) microenvironment by cytoadhesive molecules. Adhesion blocking experiments have revealed that the very late antigen (VLA)-4/vascular cell adhesion molecule 1 (VCAM-1), VLA-5/fibronectin, and β_2 integrins/intercellular adhesion molecule 1 (ICAM-1) pathways have a role in the attachment of $CD34^+$ cells to stromal cells (7, 8). Interruption of c-Kit/stem cell factor (SCF), CXCR-4/stromal-derived factor 1 (SDF-1 and CXCL12) and VLA-4/VCAM-1 has been shown to be involved in HSC/HPC mobilization (9–15), demonstrating a major role for disrupting adhesive interactions between hematopoietic stem cells and the BM stromal microenvironment during HSC/HPC mobilization.

Previous studies from our laboratory have demonstrated that a single injection of the CXC chemokine IL-8, which is a chemoattractant and activator of neutrophils, induces rapid (15–30 min) mobilization of HPC and repopulating HSC (6, 16). Neutrophils have been shown to be indispensable for this process, because IL-8-induced HSC/HPC mobilization is abolished in mice that are rendered neutropenic after administration of a depleting anti-GR-1 Ab (17). Also, neutralizing Abs against the β_2 integrins lymphocyte function-associated molecule 1 (LFA-1) and Mac-1 (CD11b) prevented IL-8-induced HSC/HPC mobilization (18). This effect was not due to direct targeting of hematopoietic progenitor cells and stem cells, because β_2 integrins are not expressed on the surface of hematopoietic progenitor cells and stem cells (19, 20). β_2 integrins are critically involved in firm attachment of neutrophils to the endothelium, which forms a crucial step in enabling degranulation after activation by IL-8. These observations indicate that β_2 integrin Abs abolished IL-8-induced HSC/HPC mobilization by preventing adhesion, attachment, and degranulation of neutrophils. The crucial role of neutrophils was demonstrated also by the observation that HSC/HPC mobilization in response to IL-8 in neutropenic hosts was restored upon the infusion of purified neutrophils (17). Also, proteases that are present in the granules of neutrophils have been shown to be essential for G-CSF- and IL-8-induced HSC/HPC mobilization (12, 13, 21).

Here, we report that low-dose total-body irradiation (TBI) inhibits IL-8-induced HSC/HPC mobilization, which is a phenomenon that is associated with the induction of the protease inhibitor Serpina1 (also known as α_1-antitrypsin or α_1-proteinase inhibitor) in the BM. We show that Serpina1 is a potent inhibitor of HSC/HPC mobilization after exogenous administration, and we propose that radiation-induced Serpina1 is responsible for the inhibition of mobilization observed after low-dose TBI.

Results

Hematopoietic Stem Cell Mobilization Is Inhibited at 24 h After 0.5-Gy TBI. To study the effect of low-dose irradiation on HSC/HPC mobilization, cohorts of mice received 0.5 Gy of TBI and were injected with IL-8 at 24 h after TBI. IL-8 induced HSC/HPC mobilization to the peripheral blood was significantly impaired in irradiated mice compared with sham-irradiated controls

Conflict of interest statement: No conflicts declared.

Abbreviations: HSC, hematopoietic stem cell; HPC, hematopoietic progenitor cell; TBI, total-body irradiation; BM, bone marrow; VLA, very late antigen; VCAM-1, vascular cell adhesion molecule 1; G-CSF, granulocyte colony-stimulating factor; CFU-GM, granulocyte macrophage colony-forming units; BMEE, BM extracellular extracts; MMP-9, matrix metalloproteinase 9; α_2-MG, α_2-macroglobulin.

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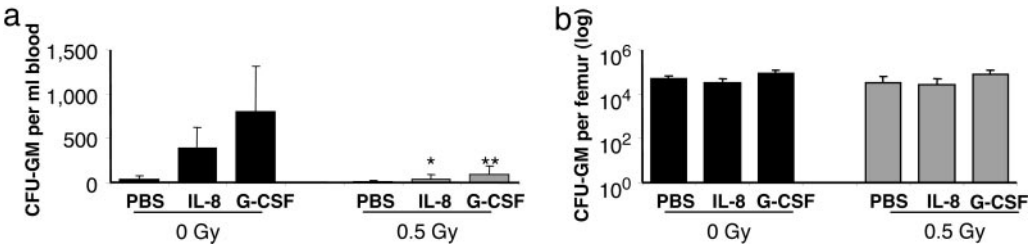

Fig. 1. IL-8-induced and G-CSF-induced HSC/HPC mobilization is inhibited after low-dose TBI. Mice received 0.5 Gy of TBI or were sham-irradiated and subsequently mobilized with IL-8 (30 μg per mouse; $n = 12$), G-CSF ($n = 10$), or PBS ($n = 11$) as a control. Colony-forming capacity of peripheral blood (*a*) and BM (*b*) are shown. Results are shown as mean ± SD. *, $P < 0.0001$ vs. 0 Gy/IL-8; **, $P < 0.0001$ vs. 0 Gy/G-CSF.

[388.1 ± 233.3 vs. 37.1 ± 51.5 granulocyte macrophage colony-forming units (CFU-GM) for IL-8 vs. 0.5 Gy/IL-8 respectively; $P < 0.0001$; Fig. 1*a*], whereas the number of progenitor cells in the BM was similar in all groups ($P > 0.5$; Fig. 1*b*). HSC/HPC mobilization after 0.5 Gy of TBI induced by three daily injections of G-CSF (initiated at 24 h after TBI) was also inhibited significantly in irradiated (0.5 Gy) mice, compared with sham-irradiated controls (800 ± 513.6 vs. 91.9 ± 96.7 CFU-GM for G-CSF vs. 0.5 Gy/G-CSF, respectively; $P < 0.0001$; Fig. 1*a*). No effect of low-dose TBI on neutrophil numbers in peripheral blood and BM was observed (data not shown).

BM Extracellular Extracts (BMEE) Inhibit Elastase Activity. To determine the mechanism by which cytokine-induced HSC/HPC mobilization is inhibited after 0.5 Gy of TBI, cohorts of mice received low-dose (0.5 Gy) TBI or were sham-irradiated. At 24 h after TBI, the mice were killed and BMEE were harvested. Subsequently, elastase activity was evaluated in BMEE by using a chromogenic substrate-conversion assay, which was designed specifically to detect elastase activity. No elastase activity was found in BMEE from irradiated mice (Fig. 2*a*). After addition of 3.125 or 6.25 μg/ml elastase to the BMEE obtained from low-dose (0.5 Gy)-irradiated mice, elastase activity was significantly ($P < 0.05$) reduced (60.3% and 44.1%, respectively) compared with BMEE obtained from sham-irradiated control mice (Fig. 2*a*), indicating the presence of an elastase inhibitor in BMEE after low-dose TBI.

Inhibition of Elastase Activity Can Be Reversed by Serpina1-Neutralizing Abs. To assess whether the inhibition of elastase activity in BMEE obtained from low-dose-irradiated mice can be explained by the presence of Serpina1 in the BMEE, neutralizing anti-Serpina1 Abs were incubated with BMEE obtained from low-dose (0.5 Gy)-irradiated mice ($n = 2$), and the activity of exogenous elastase was evaluated. As expected, after the addition of 3.125 μg/ml elastase to the BMEE of 0.5-Gy-irradiated mice, elastase activity was inhibited compared with BMEE obtained from unirradiated mice. However, after the addition of anti-Serpina1 Abs, elastase activity was restored (Fig. 2*b*), indicating that Serpina1 was responsible for the inhibitory activity in the BMEE obtained from low-dose-irradiated mice.

Induction of Serpina1 mRNA and Protein by Low-Dose TBI. Because Abs against Serpina1 abolished the inhibitory effect of BMEE from irradiated mice, Serpina1 was considered as the primary candidate serine protease inhibitor to inhibit elastase activity in our *in vitro* system. To investigate whether Serpina1 mRNA is induced after low-dose (0.5 Gy) TBI, mRNA was isolated from 0.5-Gy-irradiated mice ($n = 2$) at 24 h after TBI. Relative to HPRT expression, Serpina1 mRNA was increased 7-fold at 24 h after 0.5-Gy TBI (Fig. 3*a*) and 50-fold after 3 Gy (data not shown). Relative to the more abundantly expressed housekeeping genes β-actin and GAPDH, a 20-fold and 7-fold increase of Serpina1 mRNA was found, respectively, after 0.5-Gy TBI. These results indicate that Serpina1 mRNA concentrations are increased after low-dose TBI.

To assess whether the up-regulation of Serpina1 mRNA also resulted in an increase in Serpina1 protein concentration, the presence of Serpina1 protein in BMEE was investigated by Western blotting. We loaded 10 μg of protein derived from BMEE, harvested from 0.5-Gy- or sham-irradiated mice, on an acryl/bisacrylamide gel and Serpina1 was visualized by chemiluminescence (Fig. 3*a*). Next, the bands of each lane were quantitated and related to sham-irradiated controls on the same blot. The relative amount of Serpina1 protein was increased significantly by 28% in BMEE from low-dose-irradiated (0.5 Gy) mice compared with sham-irradiated controls (Fig. 3*b*; $P < 0.0001$). Also, upon 8.0-Gy TBI, the relative concentration of Serpina1 protein was increased by 63% compared with sham-irradiated controls (data not shown). These results indicate that Serpina1 protein is induced upon low-dose TBI.

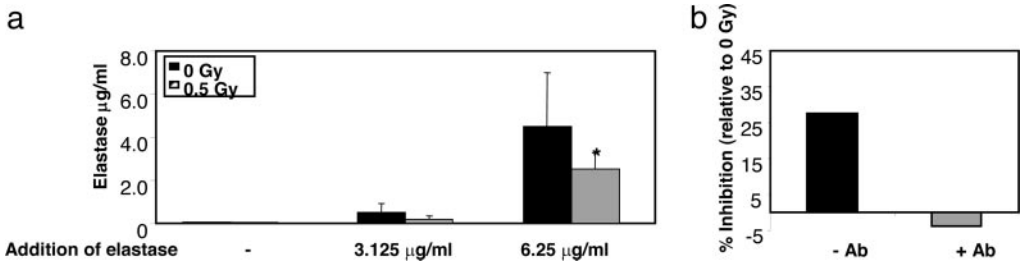

Fig. 2. BMEE inhibit elastase activity. (*a*) Inhibition of elastase activity by BMEE obtained from irradiated mice. Low-dose (0.5 Gy)-irradiated mice ($n = 9$) were killed 24 h after TBI and BMEE were obtained. Elastase activity was measured in BMEE both in the absence and presence of additional elastase. *, $P < 0.05$ compared with BMEE obtained from sham-irradiated controls. (*b*) Elastase activity can be restored by anti-Serpina1 Abs. BMEE obtained from low-dose (0.5 Gy)-irradiated mice ($n = 2$) were incubated with 3.125 μg/ml elastase in the absence and presence of anti-Serpina1 Abs (solid and hatched bars, respectively). Subsequently, elastase activity was assessed by a chromogenic substrate assay. Results are related to the elastase activity in BMEE obtained from sham-irradiated controls.

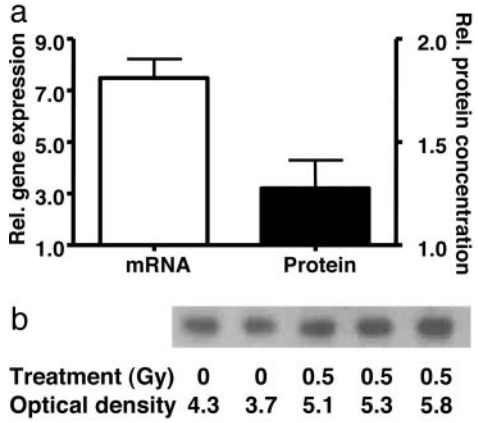

Fig. 3. Induction of Serpina1 mRNA and protein after low-dose TBI. (*a*) Real-time PCR for Serpina1 mRNA analysis of total BM cells at 24 h after 0.5-Gy TBI (white bar; $n = 2$). Serpina1 mRNA is increased 7-fold at 24 h after 0.5-Gy TBI relative to HPRT expression (left *y* axis). BM supernatants obtained from 0.5-Gy-irradiated (black bar; $n = 7$) mice were analyzed for the presence of Serpina1 protein by Western blotting. The relative concentration of Serpina1 protein was assessed by OD measurement of each band and related to sham-irradiated mice of the same blot (right *y* axis). (*b*) We analyzed 10 μg of total protein from BM supernatants obtained from sham-irradiated and 0.5-Gy-irradiated mice for the presence of Serpina1 protein by Western blotting. The relative concentration of Serpina1 was assessed by OD measurement of each band.

IL-8-Induced Mobilization Is Inhibited by Serpina1. To further study a possible *in vivo* role of Serpina1 in IL-8-induced HSC/HPC mobilization, mice ($n = 14$) were treated with Serpina1 (300 μg per mouse) at 2 h and at 5 min before IL-8 administration, and the frequency of CFU-GM in peripheral blood was evaluated. Administration of Serpina1 before IL-8 injection completely prevented IL-8-induced HSC/HPC mobilization (PBS/IL-8, 685.1 ± 522.1, $n = 7$; Serpina1/PBS, 31.5 ± 51.9, $n = 9$; and Serpina1/IL-8, 131.1 ± 180.9, $n = 14$ CFU-GM per ml of blood; $P < 0.05$; Fig. 4*a*), whereas heat-incativated Serpina1 did not inhibit HSC/HPC mobilization (779.3 ± 363.0; $n = 4$). Also, Serpina1 alone did not induce HSC/HPC mobilization nor did it affect GM-CSF-induced colony formation *in vitro* (data not shown). Also, the number of colony-forming cells in the BM remained unaltered, indicating that Serpina1 administration did not affect colony-forming cells *in vivo* (Fig. 4*b*).

Discussion

In this article, we show that low-dose (0.5 Gy) TBI inhibits IL-8- and G-CSF-induced HSC/HPC mobilization. The mechanism that underlies the inhibition of HSC/HPC mobilization was reversible because HSC/HPC mobilization was restored at 5 days after low-dose TBI (data not shown). Also, neither the progenitor cell content of the BM compartment nor the number of peripheral blood lymphocytes was affected by this low dose of irradiation. Because the release of proteases, including neutrophil elastase and cathepsin G represents a final pathway in the induction of cytokine-induced HSC/HPC mobilization (10, 12, 13), we investigated a possible role of serine proteases or serine protease inhibitors in this process. The inhibition of HSC/HPC mobilization was partially reversed upon transfusion of neutrophils obtained from unirradiated donor mice, which may indicate that neutrophils or neutrophil-derived proteases are involved in this process (data not shown). Also, BMEE obtained from low-dose (0.5 Gy)-irradiated mice were found to inhibit the bioactivity of elastase in a substrate-conversion assay. Moreover, addition of anti-Serpina1 Abs to BMEE reversed the inhibition,

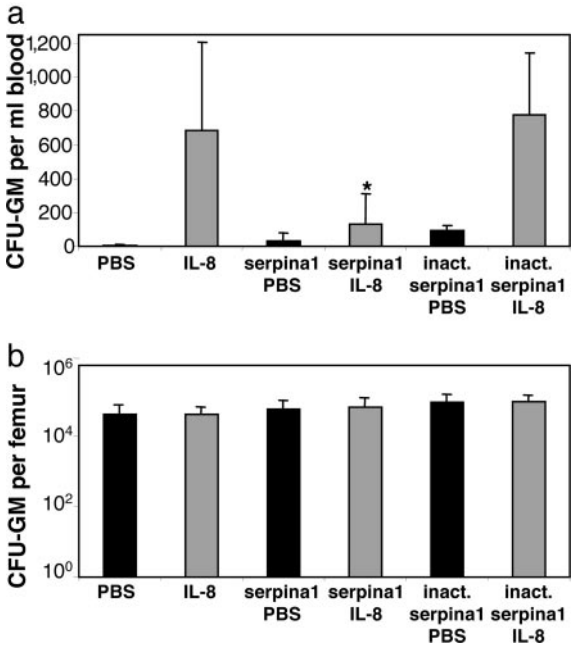

Fig. 4. Serpina1 inhibits IL-8-induced HSC/HPC mobilization. At 2 h and at 5 min before IL-8-induced HSC/HPC mobilization, human Serpina1 (300 μg) was administered to the mice by i.p. injection. Control mice received PBS or heat-inactivated Serpina1 ($n = 4$). Subsequently, the mice were mobilized with IL-8 (30 μg per mouse; $n = 14$) or received PBS as a control ($n = 9$) and the number of CFU-GM was evaluated in peripheral blood (*a*) and BM (*b*). Results from four independent experiments are shown as mean ± SD. *, $P < 0.05$ compared with IL-8-treated control mice.

showing that Serpina1 was responsible for the inhibitory activity in the BMEE obtained from low-dose-irradiated mice.

Serine proteases such as elastase are irreversibly inhibited by the serine protease inhibitor (serpin) Serpina1 and α_2-macroglobulin (α_2-MG) (28–30). Serpins belong to a single superfamily with highly conserved secondary structural elements and inhibit their target protease by covalent binding. This inhibition is irreversible and is required for effective control of the proteolytic cascade that may be initiated by the release of just a few protease molecules. In mammals, serpins have roles in a range of proteolytic processes, including blood clotting, inflammation, and turnover of extracellular matrix (31). α_2-MG lures a protease with a residue domain that it is allowed to cleave. This cleavage activates the α_2-MG molecule, leading to a conformational change by which it envelops the protease and prevents it attacking other substrates. However, molecules that consists of few amino acids may still enter the envelope and are subsequently degraded by the protease (30). Serpina1 functions by covalent binding and subsequent functional inhibition of target proteases. The interaction between Serpina1 and its target is directed by the so-called reactive center loop (RCL), a ≈20-residue domain that extends out from the body of the Serpina1 polypeptide and determines the specificity of the inhibitor. Serpina1, like a mousetrap, exists in a metastable state, and its energy is released by cleavage of the RCL. Elastase binds to and cleaves the RCL of Serpina1, which then transposes the protease to the other end of the molecule. By this process, the protease is crushed against the serpin, resulting in a loss of structural integrity that ensures its destruction (32).

Because elastase that is inhibited by α_2-MG still has the capacity to cleave the substrate that we use in our substrate-inhibition assay, we could not measure the inhibition of elastase by α_2-MG (30). Therefore, Serpina1 was considered as the primary candidate serine protease inhibitor to inhibit elastase

activity in our *in vitro* system. Quantitative RT-PCR of total BM cells confirmed that Serpina1 mRNA was increased relative to housekeeping genes after low-dose TBI. Also, Western blot analysis indicated that the relative Serpina1 protein concentrations were increased in BMEE derived from low-dose-irradiated mice. However, because of the lack of sufficient Ab for *in vivo* use, we were unable to study whether radiation-induced inhibition of HSC/HPC mobilization can be reversed by treatment with anti-Serpina1 Abs *in vivo*. Last, the importance of Serpina1 in HSC/HPC mobilization was shown by the absence of IL-8-induced HSC/HPC mobilization upon administration of Serpina1 *in vivo*. Heat-inactivated Serpina1 had no effect on IL-8-induced HSC/HPC mobilization, pointing toward a specific role of active Serpina1 in the inhibition of HSC/HPC mobilization rather than an artifact, due to nonspecific effects.

Proteases have been shown to be regulatory mediators of cytokine-induced HSC/HPC mobilization. Studies from our laboratory in rhesus monkeys indicated a critical role for the matrix metalloproteinase 9 (MMP-9; gelatinase B) that is stored in the azurophilic granules of neutrophils. IL-8-induced HSC/HPC mobilization coincided with induction of circulating MMP-9 as measured by zymography. Moreover, neutralizing Abs against MMP-9 prevented IL-8-induced HSC/HPC mobilization (21). Also, Levesque *et al.* (12, 13) have shown that neutrophil elastase and cathepsin G are involved in G-CSF- and cyclophosphamide-induced HPC mobilization. They found an increased concentration of VCAM-1 cleavage products in the plasma of G-CSF-mobilized patients. Concomitantly, VCAM-1 expression was decreased and neutrophil elastase and cathepsin G concentrations in the BM were increased (12, 13). Another substrate for neutrophil elastase and cathepsin G was independently identified by two groups who found that SDF-1 (CXCL12) was cleaved, leading to a reduced stem cell retention in the BM (10, 11). Also, neutrophil elastase, cathepsin G, proteinase 3, and MMP-9 are also capable of cleaving c-kit that is expressed on primitive hematopoietic cells (9). These results suggest that G-CSF-induced mobilization is mediated by interruption of the VCAM-1/VLA-4 pathway through cleavage by proteases that are present in the granules of neutrophils.

In this study, we show that not only serine proteases (including elastase and cathepsin G) but also serine protease inhibitors have an important role in the induction of HSC/HPC mobilization. Our finding that Serpina1 and possibly other serine protease inhibitors have an important role in HSC/HPC mobilization was strengthened by a finding of Winkler *et al.*, who showed a 70- to 1,000-fold reduction in Serpina1 and Serpina3 mRNA by using quantitative RT-PCR and immunoblotting during G-CSF-induced HSC/HPC mobilization in a murine model.** Also, a genetic linkage analysis of mobilization in AKXL recombinant inbred strains of mice revealed that a region on chromosome 12 (near 50 cM) close to where the Serpina1 gene is located, might be involved in stem cell mobilization (33). Recently, it has been shown (34) that Serpina1 and Serpina3 are involved in hematopoietic progenitor cell mobilization. These results explain why the induction of proteases, an event that occurs within hours after G-CSF administration, may initiate HSC/HPC mobilization only when the concentration of the natural protease inhibitors (i.e., Serpina1 is concomitantly decreased). The ratio between proteases and protease inhibitors will then increase dramatically in favor of the proteases and results in cleavage of adhesive interactions between stem cells and stromal elements in the BM microenvironment.

Based on these notions, we propose the following sequence of events during IL-8-induced HSC/HPC mobilization. Upon IL-8 injection, an instant neutropenia occurs because of entrapment of circulating neutrophils in the lungs and probably other organs, such as the BM. Underlying this entrapment is the IL-8-induced shedding of L-selectin and the up-regulation of the β_2 integrins, lymphocyte function-associated molecule 1 (LFA-1), and Mac-1 (CD11b), with subsequent firm adhesion to the endothelium. As soon as they are attached to the endothelium, neutrophils degranulate in response to activation by IL-8 and release MMP-9 and serine proteases (i.e., neutrophil elastase and cathepsin G). Besides serine proteases, also serine protease inhibitors reside in the BM. One of these serine protease inhibitors, Serpina1, is known to be a substrate for MMP-9 (35). Cleavage of Serpina1 by MMP-9 results in inactivation of this serine protease inhibitor. Also, the release of large quantities of chlorinated oxidants that bind to Serpina1 also reduces its inhibitory capacity (36). We hypothesize that during IL-8-induced HSC/HPC mobilization MMP-9 is released by neutrophils, an event leading to the inactivation of Serpina1 (Fig. 5).

Serpina1 would otherwise bind to neutrophil elastase to inhibit this serine protease irreversibly. However, its inactivation by MMP-9 increases the ratio between elastase and Serpina1 and results in a net increase in neutrophil elastase activity in the BM microenvironment. This increase leads to disruption of adhesive interactions and probably also to the degradation of the basement membrane and of the matrix molecules to which hematopoietic stem cells are attached in the microenvironment. In steady state, when the serine protease/serine protease inhibitor ratio favors the protease inhibitors, no HSC/HPC mobilization will occur.

Therefore, we propose that the delicate balance between serine proteases and their inhibitors is decisive in the initiation of cytokine-induced HSC/HPC mobilization.

Materials and Methods

Animals. We used 8- to 12-week-old male BALB/c mice (Charles River Nederland, Maastricht, The Netherlands) in all mobilization studies. The animals were maintained in the animal facilities of the Leiden University Medical Center under conventional conditions. All experimental protocols were approved by the institutional ethical committee on animal experiments.

Stem Cell Mobilization. Mobilization of HSC was induced by a single i.p. injection of 30 μg of IL-8 (Novartis Research Institute, Vienna) diluted in endotoxin-free PBS with 0.1% bovine serum. After 20 min, mice were killed by CO_2 asphyxiation and peripheral blood and BM cells were obtained.

G-CSF-induced mobilization was studied by i.p. administration of 10 μg of G-CSF per mouse per day (Filgrastim, Neupogen, a kind gift from Amgen, Breda, The Netherlands) for 3 consecutive days, starting at 24 h after TBI. In inhibition experiments, 300 μg of human Serpina1(α_1-antitrypsin; Serva, Heidelberg) was administered at 2 h and at 5 min before IL-8 administration. Serpina1 was heat-inactivated by boiling for 6 min at 96°C. Heat-inactivated Serpina1 did not inhibit elastase activity in the elastase substrate conversion assay. CFU-GM were cultured as described in ref. 24.

Irradiation Protocol. Mice were irradiated in Perspex chambers with a linear accelerator (Philips SL 75–5/6 mV; Philips Medical Systems, Best, The Netherlands) at a dose rate of 98 cGy/min. Total doses of 0 (sham-irradiated) or 0.5 Gy were administered.

Elastase Activity Assay. BMEE were obtained by flushing femurs with 250 μl of cold PBS. The cell suspension was centrifuged at 2,300 $\times$ *g* for 5 min, and the supernatant was stored at -20°C. Elastase activity was determined by using the chromogenic substrate *N*-Succinyl-L-Ala-Ala-Ala-*P*-nitroanilide (Sigma). To test for the presence of protease inhibitors, a standard amount of purified porcine pancreas elastase (Sigma) was added to the BMEE immediately before addition of the elastase substrate. Elastase activity

Winkler, I. G., Hendy, J., Horvath, A., Coughlin, P. & Levesque, J. P. *Exp. Hematol.* **31, 152 (abstr.).

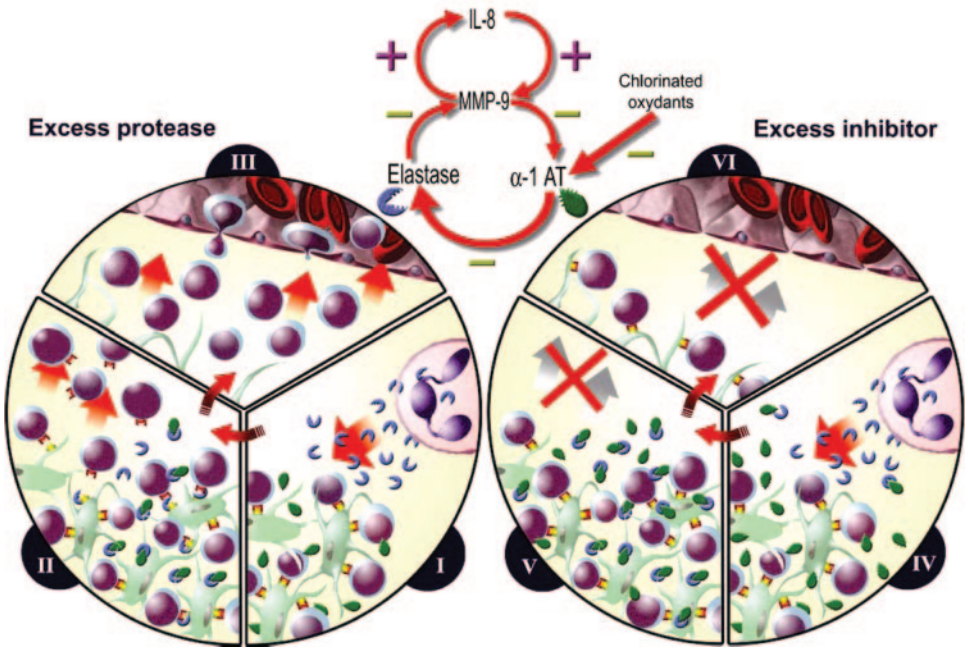

Fig. 5. Schematic representation of the role of neutrophils, proteases, and protease inhibitors during IL-8-induced HSC/HPC mobilization. Upon IL-8 injection, neutrophils up-regulate cell adhesion molecules, including lymphocyte function-associated molecule 1 (LFA-1) and Mac-1, with subsequent rolling and firm adhesion to the endothelium. (*I*) As soon as they are attached to the endothelium, neutrophils degranulate in response to activation by IL-8 and release MMP-9 and serine proteases, including neutrophil elastase. Serine protease inhibitors, including Serpina1, are present in high concentrations in plasma. (*II*) When the protease/protease inhibitor ratio favors the proteases (excess protease), the cytoadhesive interactions between HSC/HPC and stromal elements will be degraded. (*III*) Subsequently, HSC/HPC mobilize to the peripheral blood. (*IV* and *V*) Induction of protease inhibitors in the BM results in excess inhibitor (*VI*), and no HSC/HPC mobilization will occur. (*Upper*) Positive and negative regulation of protease activity. Cleavage of Serpina1 (α_1-antitrypsin, α_1 AT) by MMP-9 results in inactivation of this serine protease inhibitor. Serpina1 would normally bind to neutrophil elastase to inhibit this serine protease. However, its inactivation results in a net increase in neutrophil elastase activity in the BM microenvironment. Elastase, in turn, inactivates MMP-9, resulting in less inhibition of Serpina1. Besides inactivation of Serpina1, MMP-9 cleaves IL-8 into a molecule with a higher bioactivity. The presence of more bioactive IL-8 leads to higher levels of MMP-9.

was quantified by using an elastase standard during each individual test.

Preparation of Anti-Serpina1 Abs. Anti-Serpina1 Ab was prepared by purifying Serpina1 from pooled normal mouse serum (BALB/C, C57BL/10, and B10; Janssen). Serum was adjusted to pH 5.5 by adding one volume of 0.5 M sodium acetate (pH 5.5), and it was salted out by adding ammonium sulfate to 1.95 M. After 2 h at room temperature, the supernatant was recovered by centrifugation (4,000 $\times$ *g* for 20 min), and the precipitate was washed with 1.95 M ammonium sulfate. The combined supernatants were dialysed extensively against 1.75 M ammonium sulfate at 4°C and applied to a thiophilic adsorbent (Affi-T agarose; Kem En Tec, Copenhagen) equilibrated in 1.75 M ammonium sulfate (25, 26). By using a flow of ≈0.5 ml/min, the adsorbent was washed to its baseline with the same buffer and eluted with 1.5 M ammonium sulfate, followed by 0.1 M NaCl. After analyzing fractions by crossed immunoelectrophoresis with anti-human Serpina1 Abs (anti-α_1-antitrypsin; A012; DAKO), which cross-reacted slightly with mouse Serpina1, and SDS/PAGE, Serpina1 was found in high purity in the 1.5 M ammonium sulfate fraction as a single sharp band migrating at 53 kDa in reducing SDS/PAGE. Contrapsin and albumin constituted the main protein impurities. Albumin traces were removed by absorbing the preparation with Blue Sepharose (Bio-Rad).

This preparation was used for immunization of rabbits. Bleedings containing the desired Ab reactivity were pooled and Abs semi-purified by a conventional salting-out method (27). The resulting Ab was tested on a blot of mouse serum and reacted with a band of 53 kDa. Because the Serpina1 that is used for the immunizations is obtained from pooled normal mouse serum, the Ab is likely to recognize all five mouse Serpina1 proteins.

Western Blotting. We loaded 10 μg of protein on a 14% acryl/bisacrylamide gel and subsequently transferred onto an Immobilon-P transfer membrane filter (Millipore). Membranes were incubated with 1% blocking reagent (Roche Diagnostics) and then incubated with polyclonal rabbit anti-mouse Serpina1 Abs, followed by horseradish peroxidase polyclonal anti-rabbit IgG Abs (Promega), and revealed with the enhanced-chemiluminescence method. Densiometry was performed by using EAGLESIGHT software, and the irradiated extract vs. sham-irradiated extract ratio was calculated for each sample.

Real-Time PCR. For real-time PCR experiments, BM cells were collected, and RNA from 5 $\times$ 10^6 cells was isolated by using a Qiagen RNAeasy Mini kit (Westburg, Leusden, The Netherlands). Subsequently, cDNA was synthesized. The following primer pairs were used: Serpina1, CAACACCTCCTCCAAACC (forward) and CAGAAACTTCTCCACCAGC (reverse); housekeeping gene HPRT, GACTTGCTCGAGATGTCA (forward) and TGTAATCCAGCAGGTCAG (reverse); housekeeping gene β-actin AGACCTCTATGCCAACACAG (forward) and TAGGAGCCAGAGCAGTAATC (reverse); and housekeeping gene GAPDH, ATGGCCTTCCGTGTTCCTAC (forward) and CCTGCTTCACCACCTTCTT (reverse). Data were analyzed by using the software provided by the manufacturer and quantified by using the comparative C_T method ($2^{-\Delta\Delta C_T}$), which calculates changes in expression relative to the expression of housekeeping genes. All reactions were checked for aspecific products by analyzing the melting curve of the reaction.

Statistical Analysis. Differences were evaluated by using Student's *t* test. *P* values of <0.05 were considered statistically significant.

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