Serine protease inhibitors serpina1 and serpina3 are down-regulated in bone marrow during hematopoietic progenitor mobilization

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Mobilization of hematopoietic progenitor cells into the blood involves a massive release of neutrophil serine proteases in the bone marrow. We hypothesize that the activity of these neutrophil serine proteases is regulated by the expression of naturally occurring inhibitors (serpina1 and serpina3) produced locally within the bone marrow. We found that serpina1 and serpina3 were transcribed in the bone marrow by many different hematopoietic cell populations and that a strong reduction in expression occurred both at the protein and mRNA levels during mobilization induced by granulocyte colony-stimulating factor or chemotherapy. This decreased expression was restricted to the bone marrow as serpina1 expression was maintained in the liver, leading to no change in plasma concentrations during mobilization. The down-regulation of serpina1 and serpina3 during mobilization may contribute to a shift in the balance between serine proteases and their inhibitors, and an accumulation of active neutrophil serine proteases in bone marrow extravascular fluids that cleave and inactivate molecules essential to the retention of hematopoietic progenitor cells within the bone marrow. These data suggest an unexpected role for serpina1 and serpina3 in regulating the bone marrow hematopoietic microenvironment as well as influencing the migratory behavior of hematopoietic precursors.

Hemopoietic progenitor cells (HPCs) are responsible for the renewal of all mature blood cells. In adult mammals, the majority of HPCs reside in the BM. Transient increases in the number of HPCs circulating in the peripheral blood (mobilization) occur in response to a wide variety of stimuli including strenuous physical exercise, myelosuppressive chemotherapy, polyanions, chemokines, and hematopoietic growth factors (1). Mobilized HPCs are now the favored source of transplantable cells to reconstitute hematopoiesis after high-dose chemotherapy. Currently, the agent most commonly used to elicit HPCs mobilization is G-CSF used alone or in combination with myelosuppressive chemotherapy (2, 3). The administration of G-CSF induces a 10- to 100-fold increase in the level of circulating HPCs in both humans and mice. G-CSF–induced mobilization is time and dose dependent, involving a rapid neutrophilia (evident within hours) and a gradual increase in HPC numbers in the blood peaking between 4 and 7 d of G-CSF administration. Mobilization with chemotherapeutic agents such as cyclophosphamide (CY) occurs during the recovery phase after the chemotherapy-induced neutropenia, that is, days 6–8 in mice, and days 10–14 in humans. Although mobilized HPCs collected from the peripheral blood are extensively used to rescue hematopoiesis in patients undergoing high-dose myeloablative chemotherapy, the exact molecular mechanisms responsible for the mobilization of HPCs from the BM into the peripheral blood remain unclear.

Essential for the retention of HPCs in the BM are adhesive and chemotactic interactions. Particularly important are (a) the adhesive interaction between the vascular cell adhesion molecule VCAM-1 (CD106) expressed by the BM stroma with its counter receptor integrin α5β1 (VLA-4) expressed by HPCs, and (b) HPC chemotaxis due to binding of the chemokine CXCL12 (SDF-1) produced by the BM stroma, to its cognate receptor CXCR4 (CD184) expressed at the surface of HPCs.
Blocking either of these interactions; the VCAM-1–α4β1 adhesion interaction (4–6) or the CXCL12–CXCR4 chemotactic interaction (7, 8), by means of antibodies, antagonists, or tissue-specific gene-targeted deletion has been shown to result in mobilization of HPCs in vivo.

Our group has shown that HPC mobilization induced by G-CSF or CY coincides with the accumulation of high concentrations of active neutrophil serine proteases within the BM (9, 10). The predominant proteases were identified as neutrophil elastase (NE) and cathepsin G (CG), both released by neutrophils upon activation (9, 10). Once released into the BM extracellular fluid, these proteases can disrupt locally the two important mechanisms by which HPCs home to and remain within the BM: (a) adhesion to the BM stroma by cleaving the extracellular domain of VCAM-1 (9, 10), and (b) chemotaxis of HPCs by degrading and inactivating the chemokine CXCL12 (11, 12) and cleaving the 1st extracellular domain of CXCR4 expressed by human HPCs (12). As previous studies have shown, inactivation of either is sufficient to induce mobilization. However, a mechanism explaining how high concentrations of active neutrophil serine proteases accumulate in the BM during mobilization remains unclear.

Neutral serine protease activity is controlled in vivo by naturally occurring serine protease inhibitors or serpins (13, 14). Serpins function like mousetraps with a protruding reactive center loop. Cleavage of this reactive center loop by a specific protease triggers a spring-like mechanism, which entraps the protease within the serpin structure destroying the protease catalytic architecture (15–17). Once formed, the inactive serpin–protease complex is rapidly cleared from the body. Although serpin structure is very conserved, the reactive center loop sequence is highly variable between different serpins allowing the targeting of specific proteases. Two groups of serpins located on chromosome 14 in humans and chromosome 12 in mice (referred to as “clade A” serpins; 14, 18) can inactivate neutrophil serine proteases in blood and tissues. These are SERPINA1 (also known as α1-proteinase inhibitor or α1-antitrypsin: AAT) and SERPINA3 (α1-antichymotrypsin: ACT; 19, 20). Although humans express a single copy of each SERPINA1 and SERPINA3 gene, in the house mouse Mus musculus the serpin1 gene has replicated five times (serpin1a–c; 21–23) whereas the serpin3 gene has replicated 14 times (serpin3a–n; 23, 24).

To explain how high concentrations of active neutrophil serine proteases accumulate in the bone marrow during HPC mobilization, we hypothesize that (a) serine protease inhibitors, in particular serpins, are present in steady-state BM preventing the action of serine proteases released by BM neutrophils in steady-state conditions, and (b) that the balance between serine proteases and their inhibitors shifts in mobilized BM leading to the accumulation of active NE and CG.

Herein we report that, serpina1 and serpina3 are both transcribed in steady-state BM and are present at sufficient concentrations to inhibit local serine proteases. During mobilization induced by either G-CSF or CY, both protein and mRNA concentrations of serpina1 and serpina3 in the BM dramatically drop associated with an accumulation of active neutrophil serine proteases and concomitant cleavage and inactivation of molecules essential for the retention of HPCs within the BM.

RESULTS
Inhibitors of serine proteases are present in steady-state BM extracellular fluids
We have reported previously a dramatic increase in active serine protease levels in the BM at the peak of mobilization induced either by G-CSF or CY (9, 10, 12). This could be due to (a) a corresponding increase in protease production/release, and/or (b) a drop in the level of inhibitors that control the activity of these proteases. To determine whether naturally occurring protease inhibitors are involved in the regulation of protease activity in the BM, recombinant VCAM-1 was incubated with pooled BM fluids from three saline-injected mice (Fig. 1, lane 1), three G-CSF day 4 mice (lane 3), or a mixture of the two (lane 2). As expected from our previous work, no cleavage of VCAM-1 was observed when incubated with BM fluids from saline-injected nonmobilized mice, whereas cleavage of VCAM-1 did occur when incubated with BM fluids from G-CSF day 4 mice (9, 10). Interestingly, when both BM fluids were mixed together (lane 2) cleavage was significantly reduced. This result demonstrates that naturally occurring protease inhibitors are present in the BM fluids from steady-state (saline-injected) mice in sufficient quantities to significantly inhibit the proteolytic activity of BM fluids from mice mobilized with G-CSF.

When these same pooled BM fluids from G-CSF day 4 mice were incubated with purified human SERPINA1 (α1-antitrypsin), complete inhibition of VCAM-1 cleavage was
observed, suggesting that murine steady-state BM may contain similar serine protease inhibitors (Fig. 1, compare lanes 1, 3, and 4). Inhibitors of matrix metalloproteinases such as BB-94 (Fig. 1, lane 5) or O-phenanthroline (lane 6) did not prevent VCAM-1 cleavage. Together these results indicate that the proteases in G-CSF–mobilized murine BM fluids able to cleave VCAM-1 are predominately serine proteases (inhibited by SERPINA1) and not matrix metalloproteinases. Consequently, the inhibition of protease activity by steady-state BM fluids is not due to tissue inhibitors of metalloproteinases but rather to endogenous serpins or nonserpin inhibitors of serine proteases.

Levels of serpina1 protein drop in the BM during mobilization

Serpina1 protein, the main inhibitor of NE in tissues, was analyzed in BM extracellular fluids by immunoblotting. Whereas serpina1 (53 kD) was present in BM fluids before and after mobilization, levels were significantly decreased during HPC mobilization on days 2–6 after G-CSF administration (Fig. 2 A). Similarly, levels of serpina1 were significantly decreased in the BM fluids from CY-treated mice between days 6–10 again corresponding to the peak of mobilization in these mice (Fig. 2 B). This decrease in serpina1 was restricted to the BM as no corresponding

Figure 2. Concentration of serpina1 protein in BM extracellular fluids drops during mobilization induced by G-CSF or CY. Aliquots of BM extracellular fluids or blood plasma were taken at indicated time-points of mobilization induced by G-CSF (A) or by CY (B) and analyzed by immunoblotting with a rabbit anti–human SERPINA1 cross-reacting with mouse serpina1. The 53-kD intact, 50-kD cleaved, and 76-kD complexed forms of serpina1 are indicated by arrows as determined in Fig. 3 A. Panels C and D show the concentration of serpina1 (○), active NE (□), and CG (△) in BM extracellular fluids from G-CSF– (C) and CY-mobilized (D) mice. Results are expressed in picomoles per femur. Values are mean ± SD of three mice per time-point. E shows a calibration curve for the quantification of serpina1 protein. Serial dilutions of mouse plasma were immunoblotted with serpina1 antibody and fluorescent intensity of the 53-kD serpina1 band measured using the Odyssey Infrared Imaging System. F shows the same CY-mobilized BM extracellular fluids and plasma as B, but immunoblotted with rabbit anti–human α2-macroglobulin.
change in serpina1 was observed in the matching plasma from these same mice (Fig. 2, A and B, lower panels).

Interestingly, a large increase in serpina1 was observed specifically on day 3 after CY injection. As the BM is very damaged at this time-point, we hypothesized that this peak in serpina1 in the BM could be due to blood infiltration across the endothelium into the BM. This was confirmed by immunoblotting the same BM fluids as above for α2-macroglobulin, a major plasma protein. Because of its large size (four 160-kD subunits linked by disulfide bridges), α2-macroglobulin cannot normally diffuse through vascular walls from the blood into tissues. Although α2-macroglobulin was readily detected in all blood samples, it was only detected in the BM extravascular fluids of mice at day 3 after CY administration, but absent from all other BM fluids (Fig. 2 F).

In another series of experiments, serpina1 concentration in BM fluids from individual mice were directly measured from immunoblots using a secondary antibody conjugated to the infrared fluorescent dye Alexa Fluor 680 and the Odyssey Infrared Imaging System. The advantage of this system is that the fluorescent intensity of a band is directly proportional to serpina1 concentration (Fig. 2 E, see standard curve). Large amounts of serpina1 (54.3 ± 1.3 picomole [2.9 µg/femur]) were measured in steady-state BM fluids (Fig. 2 C), dropping to 11.0 ± 1.2 picomole [0.5 µg/femur] on day 6 of G-CSF mobilization and rebounding (to 185 ± 49 picomole [9.8 µg/femur]) during the recovery from mobilization. A similar drop in serpina1 concentration (down to 5.1–7.6 picomole/femur) was observed in the BM of CY-mobilized mice at days 6–10 during mobilization, with serpina1 levels again rebounding to 61–66 picomole/femur on days 12–14 (Fig. 2 D).

As no specific antibodies for murine NE and CG are available, it was not possible to directly measure changes in protease concentration during mobilization. Instead we measured proteolytic activity using specific chromogenic substrates and then calculated the molar concentration of serpina1 (53 kD) in steady-state BM fluids is sufficient to inhibit all NE and CG present in a steady-state femur. How- ever, the 53-kD “intact” serpina1 band disappeared when incubated with increasing concentrations of NE for 30 min at 37°C (Fig. 3 A). Indeed the 53-kD “intact” serpina1 band disappeared when incubated with increasing concentrations of NE, with a concurrent 3.5-fold increase in the 50-kD band (cleaved serpina1) and sixfold increase in the 76-kD band (complexed serpina1). The concentration of 50-kD cleaved and 76-kD complexed serpina1 bands were then quantified in BM extracellular fluids from G-CSF– and CY-mobilized control mice were used to calculate the 100% baseline.

We first confirmed the identity of the 50- and 76-kD bands detected on previous blots (Fig. 2, A and B), by incubating mouse plasma with increasing concentrations of NE and then immunoblotted with rabbit anti-SERPINA1 to reveal the displacement of intact 53-kD serpina1 and appearance of cleaved 50-kD and complexed 76-kD serpina1 band in the presence of excess serine protease. The relative intensities of 50-, 53-, and 76-kD bands were measured using the Odyssey Infrared Imaging System (Fig. 3 B). BM extracellular fluids were taken from mice mobilized with G-CSF or CY at indicated time-points and then immunoblotted and the relative intensities of the 50, 53, and 76-kD serpina1 bands measured as described above. Values from non-mobilized control mice were used to calculate the 100% baseline.

Figure 3. Relative variations in intact serpina1, cleaved serpina1, and serpina1–protease complexes during mobilization. [A] Mouse plasma was incubated with increasing concentrations of purified human NE and then immunoblotted with rabbit anti-SERPINA1 to reveal the disappearance of intact 53-kD serpina1 and appearance of cleaved 50-kD and complexed 76-kD serpina1 band in the presence of excess serine protease. The relative intensities of 50-, 53-, and 76-kD bands were measured using the Odyssey Infrared Imaging System. (B) BM extracellular fluids were taken from mice mobilized with G-CSF or CY at indicated time-points and then immunoblotted and the relative intensities of the 50, 53, and 76-kD serpina1 bands measured as described above. Values from non-mobilized control mice were used to calculate the 100% baseline.

production of serpina1 by BM cells during mobilization would also result in the observed decrease in intact serpina1 levels within the BM fluid but without an associated increase in cleaved or complexed serpina1 forms.

We first confirmed the identity of the 50- and 76-kD bands detected on previous blots (Fig. 2, A and B), by incubating mouse plasma with increasing concentrations of NE and then immunoblotted with rabbit anti-SERPINA1 to reveal the displacement of intact 53-kD serpina1 and appearance of cleaved 50-kD and complexed 76-kD serpina1 band in the presence of excess serine protease. The relative intensities of 50-, 53-, and 76-kD bands were measured using the Odyssey Infrared Imaging System. (B) BM extracellular fluids were taken from mice mobilized with G-CSF or CY at indicated time-points and then immunoblotted and the relative intensities of the 50, 53, and 76-kD serpina1 bands measured as described above. Values from non-mobilized control mice were used to calculate the 100% baseline.
be the predominant contributor to the large amounts of serpina1 observed within BM.

Reduction of serpina1a–e mRNA levels in the BM during mobilization

To further investigate endogenous BM production of serpina1, total RNA was extracted from BM cells of six individual mice injected with either saline for 4 d, G-CSF for 4 d, or 8 d after a single injection of CY, and quantitative real-time RT-PCR performed. Results were standardized using primers specific for murine vimentin, a cytoskeleton protein ubiquitously expressed.

Using primers that amplify transcripts from the five murine serpina1 genes (serpina1a–e), we observed a dramatic decrease in serpina1a–e mRNA levels in the BM of mice mobilized with G-CSF (8,000-fold decrease) or CY (790-fold decrease; Fig. 4 A). When primers to β2-microglobulin (β2m), another ubiquitously expressed gene, were substituted for the pan-serpina1 primers, there was no difference between saline-, G-CSF–, or CY-injected mice. These results confirm that serpina1 genes are transcribed in the BM, and that the concentration of transcripts decreases during mobilization. Furthermore, the decrease in concentration of mouse serpina1a–e transcripts is specific (no changes are found with other RNAs such as β2m) when compared with the vimentin.

To confirm real-time RT-PCR results, a repeat PCR was stopped at 30 cycles for serpina1 (mid-log phase of reaction) or 25 cycles for β2m, run on 8% PAGE and bands visualized with ethidium bromide. After 30 cycles, serpina1 product (140 bp) was detectable in RNA from the BM of three individual saline-injected mice but not in the three G-CSF–mobilized mice (Fig. 4 B).

Real-time RT-PCR was performed from BM at different time-points of G-CSF–induced mobilization. The decrease in serpina1 mRNA on days 2, 4, and 6 (Fig. 4 C) corresponds to the timing of decreased serpina1 protein in BM fluids (Fig. 2) as well as the rise in colony-forming cells mobilized into the peripheral blood (Fig. 4 E).

Similar decreases in serpina1a–e mRNA levels were observed in the BM at the peak of CY-induced mobilization (days 6–8). Together these data indicate that a significant two to three log decrease in serpina1a–e mRNA concentration occurs in the BM at the peak of mobilization even when two completely different mobilization protocols (either G-CSF, a cytokine, or CY, a cytotoxic agent) are used.

Transcription of the neutrophil proteases NE, CG, as well as VCAM-1 and CXCR4 in the BM remains unchanged during mobilization

In sharp contrast to serpina1, no significant change in NE or CG transcripts was observed during mobilization induced by G-CSF or CY (Fig. 4 D and Fig. 5). However this is not surprising as these two proteases are transcribed in myeloid progenitors (see Fig. 6 E and reference 25) and stored in...
azurophil granules where they are released upon granulocyte activation (26) or in response to mobilizing agents (27).

No significant changes in the transcription of other molecules reported to be important for the retention of HPCs in the BM such as VCAM-1 or CXCR4 were observed (Fig. 5). Taken together, these results support the notion that a significant drop in serpin production combined with neutrophil activation and degranulation (27), both contribute to the increased active serine protease levels observed in mouse BM at the peak of G-CSF– and CY-induced mobilization.

Serpina3 mRNA decreases in the BM at the peak of mobilization

Using the same approach, we followed the mRNA levels of six murine serpina3 genes that encode serpins with an NH₂-terminal secretion sequence and thus are potentially secreted by cells (serpina3b, serpina3c, serpina3d, serpina3k, serpina3m, and serpina3n). A similar decrease in transcripts (between 8- and 1,400-fold) was found in the BM at the peak of mobilization induced by either G-CSF or CY (Fig. 5, bottom) with the exception of serpina3b, which was not transcribed in the BM of BALB/c mice. We also analyzed by real-time RT-PCR, transcripts of another five murine serpina3 genes that have no secretion sequences and are thus presumed to remain cytoplasmic (serpina3e, serpina3f, serpina3g, serpina3h, and serpina3i), the majority of which were detected in the BM of steady-state BALB/c mice (Table I). Similar to the secreted serpina3, the mRNA levels of these other serpina3 genes were significantly decreased at the peak of mobilization (fold reduction in BM transcription at day 4 of G-CSF was 85 with P < 0.001 for serpina3e and serpina3i, 621 with P < 0.01 for serpina3g, and 1,592 with P < 0.01 for serpina3h). Serpina3f was not expressed in steady-state or mobilized BALB/c BM.

Decrease in serpina1 and serpina3 transcripts within sorted BM cell populations during mobilization

The exact identity of cell types expressing serpina1 and serpina3 in mouse BM has not been thoroughly investigated. We sorted BM cells from saline- and G-CSF–treated mice at day 4 based on lineage markers to determine which cell types were expressing serpina1 and serpina3 and whether serpin transcription in these cells was down-regulated during mobilization.

We first examined myeloid cells as they are the predominant cell type accumulating in the BM at the peak of mobilization (Fig. 6 A). Bone marrow myeloid cells were sorted on the basis of positive CD11b (Mac-1) expression and relative maturity on the basis of Gr-1 expression (Fig. 6, B and C) with primitive-mono/myeloid cells being Gr-1dim (sort gate R3), immature myeloid intermediate for Gr-1 staining (sort gate R4), and mature BM neutrophils Gr-1bright (sort gate R2). With G-CSF–induced mobilization a twofold increase in both the proportion and total cell number of primitive Mac-1⁺ Gr-1dim/myeloid cells (R3 plus R4 regions) was found in the BM.

By real-time RT-PCR, serpina1a-e mRNA transcripts were greatest in BM neutrophils (Mac-1⁺ Gr-1bright) with one or two log less mRNA in more primitive Gr-1intermediate and Gr-1dim cells, respectively (Fig. 6 D). Importantly, at the peak of G-CSF mobilization, a dramatic and significant transcriptional down-regulation of serpina1a-e genes was observed when compared with saline-injected BM in all myeloid subsets. This transcriptional down-regulation was not just specific to serpina1a-e genes, but was also found with the secreted α₁-antichymotrypsin serpina3c (mRNA concentration was 5-fold lower in BM neutrophils and 10-fold lower in more primitive myeloid cells) during mobilization. However, no change was found for other mRNAs transcribed by these cells during mobilization. For example, although transcription of the protease CG is higher in more primitive myeloid cells (consistent with packaging in primary azurophil granules), administration of G-CSF was not found to alter CG transcript levels in any of the three myeloid cell populations sorted from the BM (Fig. 6 E).

A similar 100–1,000-fold decrease in serpina1a-e mRNA levels was also observed during mobilization for other sorted BM cell populations such as B220⁺ B lymphocytes, CD4⁺...
and CD8+ T lymphocytes, and total CD45+ hematopoietic cells (unpublished results).

**Decreases in serpina1 transcripts is restricted to the BM of mobilized mice**

During mobilization, a large number of the mobilized HPCs home to the spleen. We therefore queried whether the drop in serpina1 and serpina3 transcripts during mobilization was a general phenomenon or restricted to the BM. For this purpose, we analyzed serpina1 mRNA levels in liver, BM, and spleen by real-time RT-PCR and confirmed these results at the protein level by immunohistochemistry of tissue sections.

SERPINA1 and SERPINA3 proteins are present in the blood at high concentrations at 1.5 and 0.5 mg/ml, respectively, in humans (28). They are mainly produced by the liver and released into the blood. The regulation of expression has not been exhaustively studied in the mouse. As expected, we found high levels of serpina1a–e mRNAs (Fig. 7A, left) and serpina1 protein expression (Fig. 7B, left) in liver cells, with no change in mRNA or protein expression during G-CSF–induced mobilization. Conversely, serpina1a–e transcripts dropped dramatically (1,000-fold) in mouse BM during G-CSF–induced mobilization (Fig. 7A) with a corresponding sharp drop in protein levels (Fig. 7B, middle).

Interestingly, although serpina1a–e transcription in the spleen of mice was minimal, it increased during mobilization (Fig. 7A). This finding was confirmed at the protein level by immunohistochemistry showing accumulation of cell clusters producing serpina1 proteins within the myeloid areas of the

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**Table I. Murine serpin genes and primer sequences**

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<tr>
<th>Old nomenclature</th>
<th>Sequence of reactive loop, P,P1’</th>
<th>NH2-terminal secretion sequence</th>
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aMSa is the canonical human α1-antitrypsin–reactive center loop P1P1'; bLSb is the canonical human α1-antichymotrypsin–reactive center loop P1P1'. cDenotes that PCR was performed with a lower annealing temperature of 55°C. dOligonucleotides for serpina3e and serpina3i do not distinguish between these two transcripts.
spleen from mobilized mice but barely detectable in the spleens from nonmobilized mice. However, no increase in serpina1a–e mRNA was found in Gr-1<sup>+</sup> cells sorted from spleens of mobilized versus nonmobilized mice (unpublished results) suggesting that the increase in serpina1 protein and mRNA levels in the spleen after the administration of G-CSF is most likely due to the migration of serpin-producing cells (such as myeloid cells) from the BM into the spleen. This is supported by the findings that the spleen of saline-injected mice contained 6 ± 2% Gr-1<sup>+</sup> neutrophils increasing to 25 ± 5% Gr-1<sup>+</sup> neutrophils after 4 d of G-CSF. When absolute numbers are considered, this corresponds to 5 ± 2 × 10<sup>6</sup> neutrophils/spleen in nonmobilized animals compared with 38 ± 7 × 10<sup>6</sup> neutrophils/spleen with mobilization.

RT-PCRs using primers for serpina3c revealed a similar phenomenon, that is a drop in mRNA levels in the BM and increased mRNA concentrations in spleen during mobilization (Fig. 7 A, right). In the liver, mobilization also resulted in a 10-fold decrease in serpina3c transcripts.

**DISCUSSION**

In this paper we report that steady-state BM contains a molar excess of serpina1 compared with the serine proteases NE and CG. As serpins irreversibly block serine proteases with 1:1 stoichiometry (19, 20), this concentration of serpina1 is sufficient to inhibit neutrophil serine proteases released in steady-state BM. Furthermore, we report that mobilization is associated with a sharp drop in the concentration of both serpina1 protein and mRNA levels in the BM, which may contribute to the accumulation of active NE and CG within this tissue. The decrease in intact 53-kD serpina1 protein in mobilized BM was not accompanied by a rise in serpina1–protease complexes nor in cleaved serpina1 levels, which normally occurs when serpina1 is cleaved by serine proteases. Therefore our data suggests that the decrease in transcription of endogenous serpina1 in mobilized BM was not compensated by liver-derived serpina1 diffusion from the blood. Taken together, these data support the hypothesis that the rise in NE and CG proteolytic activity in the BM during mobilization is likely to be due to the dual effects of both an increase in neutrophil activation as well as a decrease in the replenishment of serine protease inhibitors such as serpina1 and serpina3. Furthermore after stopping G-CSF treatment, a rebound in serpina1 and serpina3 mRNA and protein levels in the BM is observed possibly contributing to the cessation of mobilization. Of note, we have also found similar results in other inbred mouse strains such as C57BL/6J, C3H/HeJ, and 129SvJ confirming that this phenomenon is not restricted to a particular mouse strain (unpublished results).

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**Figure 6.** Serpina1 transcripts are decreased in all BM mono/myeloid populations during HPC mobilization. (A) Number of nucleated cells and Mac-1<sup>+</sup> (CD11b<sup>+</sup>) mono/myeloid cells in the femoral BM during the course of G-CSF administration. Data represent mean ± SD from six to nine mice per group. (B) Phenotypic expression and sorting of primitive mono/myeloid Mac-1<sup>+</sup> Gr-1<sup>dim</sup> cells (gate R3), immature myeloid Mac-1<sup>+</sup> Gr-1<sup>int</sup> cells (gate R4), and mature Mac-1<sup>+</sup> Gr-1<sup>bright</sup> neutrophils (gate R2) from mouse BM after 4 d of saline (left) or G-CSF (right) injection. Percentages in sorting gates indicate the proportion of these cells among total BM-nucleated cells. (C) Giemsa staining of the R3, R4, and R2 populations sorted as above. Top row corresponds to cells sorted from BM of saline-injected mice whereas the bottom row corresponds to cells sorted from the BM of G-CSF-injected mice. (D) Serpina1 mRNA expression levels in R3, R4, and R2 populations sorted from the BM of mice injected for 4 d with saline (gray columns) or G-CSF (white columns). Results are expressed as mRNA concentration relative to vimentin mRNA on a log scale. (E) Cathepsin G (CG) mRNA expression levels in R3, R4, and R2 populations sorted from the BM of mice injected for 4 d with saline (gray columns) or G-CSF (white columns). Results are expressed as mRNA concentration relative to vimentin mRNA on a log scale.
Interestingly, hepatic transcription of \textit{serpina1} was not altered during HPC mobilization, resulting in constant blood plasma levels. This would explain in part why there is no active NE or CG in the blood and no cleavage of VCAM-1 on the lumen of BM sinuses that are in direct contact with blood (9). VCAM-1 expression in the spleen does not change during mobilization (9). In this study using immunohistochemistry as well as real-time RT-PCR, we show that cells producing \textit{serpina1} proteins accumulate in the spleen during mobilization, possibly protecting VCAM-1 from proteolytic cleavage by neutrophil serine proteases in this tissue.

The human genome has only one copy of the \textit{SERPINA1} and \textit{SERPINA3} genes and their target protease specificity is well established (29). Both human \textit{SERPINA1} and \textit{SERPINA3} proteins can inhibit NE and CG in vitro, however human \textit{SERPINA1} preferentially targets NE (with the reactive center loop $P_1P_1^\alpha$ amino acids “MS”; 19) whereas \textit{SERPINA3} preferentially targets CG (with reactive center loop $P_1P_1^\alpha$ amino acids “LS”; 20). The reactive center loop of many of the murine \textit{serpina1} and \textit{serpina3} proteins have diverged (30), although several still exhibit the canonical $\alpha_1$-antitrypsin reactive center loop (murine \textit{serpina1a}, \textit{serpina1b}, \textit{serpina3b}, and \textit{serpina3n}) whereas others have the canonical $\alpha_1$-antichymotrypsin–reactive center loop (murine \textit{serpina1e}, \textit{serpina3c}, \textit{serpina3d}, and \textit{serpina3j}; Table I). Therefore members of both the murine \textit{serpina1} and \textit{serpina3} families are likely to be involved in the inhibition of serine protease activity in steady-state BM as shown in Fig. 1.

An interesting finding of this study is that although the level of \textit{serpina1a}–e transcripts dropped dramatically in the BM of mice during mobilization, hepatic mRNA levels did not alter resulting in no changes in plasma \textit{serpina1} concentrations. Earlier studies on human \textit{SERPINA1} transcription/expression reveal five alternative transcription starts can be used depending on the tissue in which expression occurs (with three transcription start sites in leukocytes, one in cornea and one in hepatocytes; 31). In this study we found the reduction in \textit{serpina1} transcripts was specific to BM leukocytes during...
mobilization whereas hepatic transcription remained unchanged. Although we cannot exclude mechanisms involving mRNA destabilization, this suggests that different tissue-specific serpina1 transcriptional start sites are used by murine leukocytes and/or hepatic cells. In contrast to SERPINA1, the human SERPINA3 gene appears to use a single promoter regardless of tissue-type in which it is expressed (31).

Both human SERPINA1 and SERPINA3 genes are reported to be “acute phase proteins” as expression increases two- to fivefold in the liver during inflammation leading to increased levels of SERPINA1 and SERPINA3 proteins in plasma (32, 33). Whether a similar increase in the transcription of hepatic murine serpina1 occurs during inflammation remains controversial (34, 35). A few studies have reported that several cytokines (such as IL-6, IL-1ß, or TNF-α) increase SERPINA1 transcription in human monocytes (36, 37). However, to our knowledge, no down-regulation of human or mouse SERPINA1 or SERPINA3 in response to cytokines/growth factors has been reported previously in leukocytes or even hepatocytes.

An intriguing finding of this study is that the same murine BM Gr-1+ myeloid cells contain large amounts of serine proteases as well as large amounts of serine protease inhibitors, which would suggest that the serpins and proteases, although present in the same cells, are stored in different compartments. This is likely to be the case as serpina1 transcription is greatest in BM neutrophils, whereas serine protease (NE and CG) transcription was highest in primitive mono/myeloid cells (Fig. 6). It is known that in granulocyte precursors, the timing of transcription determines in which granules the protein products are stored (25, 38). For instance, the serine proteases synthesized by primitive myeloid cells are packaged predominantly in primary azurophil granules, whereas granule proteins synthesized by more mature BM neutrophils are more likely packaged in secondary or tertiary granules (39). The release of these distinct granules by a neutrophil occurs at different stages during the inflammatory process (26), so although contained within the one cell, both the serine proteases and their specific inhibitors may be released separately. Alternatively, serpins may be constitutively secreted with the proteases remaining sequestered in granules.

In conclusion, our study is the first to suggest a novel role of serpina1 and serpina3 proteins in regulating the homeostasis of the hematopoietic microenvironment, which ultimately determines the fate of hematopoietic stem cells. This conclusion is supported by other studies using genetic linkage analysis showing that the Aat locus containing the serpina1 genes, is significantly linked to BM cellularity in inbred mouse strains (40). As reported previously, many proteoglycans (41), extracellular matrix proteins (42-44), cytokines, and chemokines (45, 46) expressed in the BM hematopoietic microenvironment and playing a major role in determining the fate of hematopoietic stem cells, are substrates of the neutrophil serine proteases that these serpins inhibit. Similarly, an earlier report has shown that the addition of either recombinant human SERPINA1 or antileukoproteinase to serum-free culture media boosts in vitro proliferation and survival of HPCs, probably by protecting cytokines from the proteases released by differentiated leukocytes (47). Therefore we propose that (a) serpina1 and serpina3 proteins play a major role in maintaining the molecular and functional integrity of the BM hematopoietic microenvironment, (b) the stoichiometric balance between these serpins and neutrophil serine proteases is inverted locally in the BM during systemic administration of G-CSF or chemotherapy, and (c) the removal of the serpin-protease balance allows proteases to evade their naturally occurring inhibitors, accumulate in active form and to contribute to the mobilization of HPCs.

MATERIALS AND METHODS
Mobilization of mice, tissue collection, and clonogenic assays.
All procedures on animals have been approved by the Animal Experimentation Ethics Committee of the Peter MacCallum Cancer Centre. Male 10–12-wk-old BALB/c mice were injected with 125 μg/kg recombinant human G-CSF (Neupogen Filgastrim; Amgen) twice daily (days 0–5) or an equal volume of saline (control mice). Mice were harvested at days 2, 4, and 6 after G-CSF injection or allowed to rest and harvested at days 8 and 10. Cyclophosphamide-treated mice received a single intraperitoneal injection of 200 mg/kg CY (Amershams Biosciences) on day 0.
Peripheral blood was collected in EDTA. Plasma was separated and red cells lysed with 10 mM NaHCO3, pH 7.4, and 150 mM NH4Cl. Nucleated cells were plated in double-layer nutrient agar clonogenic cultures (1,500 cells/dish) in the presence human IL-1α, mouse IL-3, mouse CSF-1, and rat KIT ligand as described previously (9). Colonies were scored after a 2-wk culture at 37°C in the presence of 5% O2, 10% CO2, and 85% N2.

The BM content of one femur from each mouse was flushed into 1 ml PBS on ice. After centrifugation for 5 min at 400 g, the supernatant fraction (BM extracellular fluid) was removed and used directly in Western blots or for the digestion of recombinant human VCAM-1. The BM cell pellet, as well as the liver and spleen were used for RNA extraction, immunostaining and cell sorting, or to generate frozen sections.

Flow cytometry and cell sorting.
Bone marrow and spleen cells were stained using 1 μg/ml of directly conjugated mAb (BD Biosciences) for myeloid cells (Gr-1+FITC and CD11b-FITC), for B lymphocytes (B220-PE), for T lymphocytes (mix of CD3-FITC, CD4-FITC, CD5-FITC, and CD8α-FITC), and for total hematopoietic cells (CD45-FITC), and for their digestion of recombinant human VCAM-1. The BM cell pellet, as well as the liver and spleen were used for RNA extraction, immunostaining and cell sorting, or to generate frozen sections.

RNA extraction and quantitative real-time RT-PCR.
Total RNA was extracted using Trizol (Invitrogen). After DNase treatment and reverse transcription using random hexamers, quantitative real-time PCR with SYBR green (ABI Systems) was performed after manufacturer’s instructions using the oligonucleotide combinations shown in Table I. RNA levels were standardized by parallel RT-PCRs using primers to two different house-keeping genes, vimentin (a cytoskeletal protein, forward primer 5’-CACCCTGCA-GTATCCAGACA-3’; and reverse 5’-GATTCAACTTCTCCTGCAA-GGT-3’) and β2-microglobulin (forward primer 5’-TTCACCCCAC-TGAGACTGAT and reverse 5’-GCTTCGGTCCGCTGCATA-3’). Additional RT-PCRs for the serine proteases NE (forward primer 5’-ACCCTCAT-TGCCAGAAGACTTC-3’ and reverse 5’-CCTGCACGACTGACCCGAAA-TTT-3’), CG (forward primer 5’-AGCCAGGGGAAGATCATTGGA-3’ and reverse 5’-TGGATCAGAAAGATGCGCTG-3’) as well as VCAM-1 (forward primer 5’-CTGGGAGCGTGAGGGTCCCGAAGT-3’ and reverse 5’-GCCACTGAAATTCCTGCTG-3’) and CXCR4 (forward primer 5’-GGCTGACTGTTACTTTGGGAAA-3’ and reverse 5’-CCGGTCAC-GGCTATGAA-3’) were also performed. A PCR from each sample before reverse transcription was also performed to confirm the absence of contaminating genomic DNA.
VCAM-1 cleavage and immunoblotting. BM extracellular fluids collected from three mice at day 4 of G-CSF–induced mobilization were pooled and mixed with an equal volume (10 µl) of either pooled BM fluids from three nonmobilized mice or with PBS. Where indicated, protease inhibitors were added at the following concentrations: 1 mg/ml human α1-antitrypsin (Sigma-Aldrich), 1 µM BB-94/Batinastat (British Biotech Pharmaceuticals), 1 mM O-phentholamine. After a 20-min incubation at room temperature, 10 µg recombinant human VCAM-1 (98-kD glycosylated extracellular domain; R&D Systems) was added and the mixture reincubated at 37°C for 20 min. An equal volume of loading buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS) was then added and samples boiled for 3 min, before separation by electrophoresis on 10% SDS-PAGE and transfer onto nitrocellulose membrane. Membranes were immunoblotted using a goat anti–human VCAM-1 antibody as described previously (9, 10).

Immunoblotting for serpin A and α1-macroglobulin, and measurements of serpin A concentration. 10 µl aliquots of pooled BM extracellular fluids at indicated time-points of G-CSF or saline administration were mixed with an equal volume of loading buffer with 10 mM dithiothreitol, boiled for 3 min, and separated by electrophoresis on 10% SDS-PAGE. After transfer onto nitrocellulose membranes and blocking in Odyssey blocking buffer, membranes were incubated for 1 h with an anti–human α1-antitrypsin (SERPINA1) Ab cross-reacting with mouse serpin A diluted 1/3,000 in Odyssey blocking buffer with 0.5% Tween 20. After extensive washes with PBST, membranes were incubated with a 1/3,000 dilution of Alexa Fluor 680–conjugated goat anti–rabbit IgG (Molecular Probes). Visualization and quantification were performed on the Odyssey Infrared Imaging System (LI-COR Biosciences) equipped with two solid phase lasers at 680 and 800 nm with a resolution of 169 µm. The integrated intensity of the serpin A band in the samples was compared with that of mouse plasma serpin A and the moles per femur calculated, assuming the concentration of murine serpin A in blood to be the same as in humans, 1.5 mg/ml (28), with a molecular weight of 53,000. Some experiments were performed with BM extracellular fluids from three individual mice from each time-point group and average concentration ± SD were calculated.

Immunoblotting for α1-macroglobulin were performed by incubating the membranes with a 1/3,000 dilution of a rabbit anti-human α1-macroglobulin (Sigma-Aldrich) followed by an incubation with a 1/10,000 dilution of a horseradish peroxidase–conjugated donkey F(ab)2 anti–rabbit IgG (Jackson ImmunoResearch Laboratories). Blots were revealed by enhanced chemoluminescence.

Measurement of protease catalytic activity. The concentrations of active NE and CG in BM extracellular fluids were measured using the chromogenic substrates methyl-O-succinyl (Metsuc)–Ala-Ala-Pro-Val-paranitroanilide (pNA) and Suc-Ala-Ala-Pro-Phe-pNA (Calbiochem-Nova-bioschem) as described previously (9, 10). Measurements were calibrated using serial dilutions of purified human NE and CG (Elastin Products) and molar concentrations were calculated using molecular weight of 30,000 and 29,000 for murine NE and CG, respectively.

Immunohistochemistry. Frozen sections of mouse liver and spleen were air dried and then fixed for 30 min in methanol containing 0.3% H2O2 to destroy endogenous peroxidases. Slides were then blocked in 4% SSC containing 5% BSA, 5% skim milk powder, and 0.05% Triton X-100 for 2 h before incubation with either 25 µg/ml purified rabbit anti–human α1-antitrypsin or nonimmune-purified rabbit IgG in PBST with 5% skim milk and 5% BSA overnight at 4°C. After extensive washes, slides were incubated with a 1/500 dilution of horseradish peroxidase–conjugated donkey F(ab)2 anti–rabbit IgG with minimal cross-reactivity to mouse proteins for 2 h at room temperature, and then washed. Staining was revealed using 0.5 mg/ml 3,3′-diaminobenzidine in 50 mM Tris-HCl, pH 7.4, 0.3% H2O2 before counterstaining with hematoxylin and mounting with Aquamount (BDH).

For BM wax-embedded sections, femoral BM was fixed by perfusing 0.05% glutaraldehyde, 2% paraformaldehyde into the descending femoral aorta and femurs processed exactly as described by Nilsson et al. (48). Longitudinal 3.5-µm sections were cut, dewaxed in xylene, and then rehydrated using progressive baths from ethanol to water. After antigen retrieval (12 min boiling in 10 mM sodium citrate, pH 6.0), endogenous peroxidase elimination and antigen labeling were performed as described above.

Statistics. Levels of significance were calculated using the nonparametric Mann–Whitney test.

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