The Effect of CXCL12 Processing on CD34+ Cell Migration in Myeloproliferative Neoplasms

Sool Yeon Cho1, Mingjiang Xu1,2, John Roboz1, Min Lu1, John Mascarenhas3, and Ronald Hoffman1,2

Abstract

Primary myelofibrosis (PMF) and polycythemia vera (PV) are chronic myeloproliferative neoplasms. PMF and, to a lesser degree, PV are characterized by constitutive mobilization of hematopoietic stem cells (HSC) and progenitor cells (HPC) into the peripheral blood (PB). The interaction between the chemokine CXCL12 and its receptor CXCR4 plays a pivotal role in determining the trafficking of CD34+ cells between the bone marrow (BM) and the PB. PMF, but not PV, is associated with downregulation of CXCL12 by CD34+ cells due to epigenetic events. Both PV and PMF patients have elevated levels of immunoreactive forms of CXCL12 in the BM and PB. Using electrospray mass spectrometry, the PB and BM plasma of PV and PMF patients was shown to contain reduced amounts of intact CXCL12 but significant amounts of several truncated forms of CXCL12, which are lacking in normal PB and BM plasma. These truncated forms of CXCL12 are the product of the action of several serine proteases, including dipeptidyl peptidase-IV, neutrophil elastase, matrix metalloproteinase-2 (MMP-2), MMP-9, and cathepsin G. Unlike CXCL12, these truncates either lack the ability to act as a chemoattractant for CD34+ cells and/or act as an antagonist to the action of CXCL12. These data suggest that proteolytic degradation of CXCL12 is characteristic of both PV and PMF and that the resulting truncated forms of CXCL12, in addition to the reduced expression of CXCR4 by CD34+ cells, lead to a profound mobilization of HSC/HPC in PMF. Cancer Res 70(8): 3402–10. ©2010 AACR.

Introduction

Primary myelofibrosis (PMF) is a chronic myeloproliferative neoplasm (MPN), which is characterized by constitutive mobilization of hematopoietic stem cells (HSC) and progenitor cells (HPC) into the peripheral blood (PB). The abnormal trafficking of the HSC and HPC likely leads to the establishment of extramedullary sites of hematopoiesis, which determine in part the clinical course of patients with this disorder (1, 2). Over 60% of cases of PMF are characterized by the acquisition of a mutation of the tyrosine kinase Janus-activated kinase 2 (JAK2V617F) and/or the thrombopoietin receptor (MPLW515L/K; ref. 3).

In adults, the majority of HSC are normally found in the bone marrow (BM), but HSCs/HPCs are also constitutively present in limited levels in the circulation (4). The localization of HSC/HPC within the marrow is dependent on the dynamic interaction between hematopoietic cells and the microenvironment. The interaction between BM environment cells and HSC/HPC has led to the development of the concept of stem cell niches and stem cell mobilization. Extensive evidence has stressed the importance of two pathways, one dependent on the α4/β1 integrin and the other on CXCR4/CXCL12 [stromal cell–derived factor-1 (SDF-1)] signaling in retaining HSC/HPC within the marrow. There is evidence that three events are operational following granulocyte colony-stimulating factor (G-CSF) administration leading to HSC/HPC mobilization: activation of proteases, attenuation of adhesion molecule function, and disruption of CXCR4/CXCL12 signaling (4). Several laboratories have suggested that one of the central defects in PMF is dysregulation of the marrow stem cell niche, which leads to persistent and profound constitutive mobilization of HSC/HPC (5–9). This underlying defect in the stem cell niche has been attributed to profound alterations in the CXCR4/CXCL12 axis, which can occur as a consequence of downregulation of CXCR4 expression by PMF CD34+ cells due to hypermethylation of the CXCR4 promoter and the elaboration of a variety of serine proteases, including matrix metalloproteinase-9 (MMP-9) and neutrophil elastase (NE), each of which are capable of degrading a number of marrow matrix proteins, including vascular adhesion molecule-1 and CXCL12 (5–9). Passamonti and coworkers have suggested that JAK2V617F might activate granulocytes, thereby contributing to the mobilization of CD34+ cells in MPN patients (10).
CXCL12 is a major chemoattractant for human HSC/HPC (5). Increased CXCL12 production has been observed in the marrow following DNA-induced damage and has been suggested to improve BM recovery and facilitate stem cell engraftment posttransplantation (4). In addition, increased plasma CXCL12 levels have been observed following HSC/HPC mobilization due to the infusion of sulfated glucans (11). Our laboratory has previously reported that the levels of immunoreactive forms of plasma CXCL12 in PMF patients were significantly higher than the levels observed in normal adults and that immunostaining of marrow biopsy showed increased CXCL12 deposition within marrow spaces (6). The presence of increased CXCL12 in both the marrow and plasma of PMF patients has led to efforts to better define the role of CXCL12 in stem cell trafficking in PMF. In this report, however, we show that cleavage of CXCL12 by several proteases, which are overexpressed in PMF patients, likely eliminates the marrow homeostatic function of CXCL12 and likely contributes to the constitutive HSC/HPC mobilization characteristic of this MPN.

**Materials and Methods**

**Chemicals and reagents.** Full-length human recombinant CXCL12 [1–67 amino acid (aa) sequence, carrier free) was purchased from the Millipore Co. Synthetic truncates of CXCL12 were kindly provided by Dr. L. Pelus of the Indiana University School of Medicine. Mouse recombinant epidermal growth factor (EGF) was purchased from R&D Systems, Inc. and was used as an internal standard (IS). Ultrafiltration membrane filters (30-kDa cutoff) were purchased from Millipore Co. The following lyophilized proteolytic enzymes were purchased from Sigma Co.: dipeptidyl peptidase-IV (CD26), NE, cathepsin G (CG), MMP-2, and MMP-9. All high-performance liquid chromatography (HPLC) grade solvents were purchased from Fisher Scientific, Inc.

**Patients and healthy control subjects.** Human PB samples were obtained from normal volunteers or patients with PMF or polycythemia vera (PV) after informed consent was provided according to the guidelines of the Institutional Review Board of the Mount Sinai School of Medicine. All patients with PMF or PV met the WHO diagnostic criteria for PMF or PV (12). None of the patients were receiving cytotoxic agents at the time of the study, and none had evidence of transformation to acute leukemia. Fresh normal marrow was purchased from AllCells LLC. Cord blood (CB) collections were provided by Cord Blood of New York Blood Center.

**Preparation of PB and BM plasma and purification of CD34+ cells.** PB and marrow samples were collected in tubes containing sodium heparin. These tubes were first centrifuged at 1,800 rpm (740 g) at room temperature for 30 min, and the platelet-free plasma was removed, divided into 1-mL vials aliquots, and immediately frozen at −80°C. Plasma samples were selected randomly from a bank of samples collected from PB (n = 21) or PMF (n = 15) patients. Normal plasma samples were also collected from healthy volunteers (n = 10). One hundred microliters of each plasma specimen were ultrafiltrated using a 30-kDa cutoff membrane filter, followed by centrifugation for 30 min at 9,000 × g. BM plasma samples were prepared from normal or PV BM by similar methods. It was not possible to prepare PMF BM plasma because the marrow is characteristically in-aspirable in such patients. The plasma levels of NE and MMP-9 were measured by ELISAs as described previously (5).

PB or CB collections were layered onto Ficoll-Hypaque (1.077 g/mL; Amersham Biosciences), and the mononuclear cells (MNCs) were separated after density centrifugation. The percentage of CD34+ cells within the PB MNCs of PMN patients was determined using a FACS.Canto Flow Cytometer (Becton Dickinson) as described previously (13). A CD34+ cell population was isolated from the CB MNCs using a CD34+ isolation kit (Miltenyi Biotec). The purity of the CD34+ cell population was then analyzed, and only cell fractions showing a CD34+ cell purity of at least 85% were used for subsequent experiments.

**Mass spectrometry instrumentation.** Truncated forms of CXCL12, which are the products of action of proteases, have been previously detected using mass spectrometry (14–20). A combined liquid chromatograph–mass spectrometer (LC-MS) system consisting of a binary HPLC pump (Model 1525, Waters Co.) connected to a triple quadruple mass spectrometer equipped with an electro spray ionization (ESI) source (Model QuattroLC, Waters Co.) was used in these studies. Samples were either introduced directly into the mass spectrometer (no HPLC column) or onto a HPLC column connected to the mass spectrometer using an autosampler (Model 717, Waters Co.). Two types of HPLC columns were used, one for samples incubated with purified proteases and the other for PB and BM plasma samples.

**Obtaining mass spectra of CXCL12 and synthetic truncated standards.** Aliquots of each standard (10 μg/mL) were directly injected into the mass spectrometer. The ESI source was operated in the positive ion mode under the following conditions: capillary voltage, 3 kV; cone voltage, 55 V; source temperature, 80°C; desolvation temperature, 250°C; nitrogen nebulizer gas flow, 80 L/h; desolvation gas flow, 800 L/h. During data acquisition, the mass spectral scanning time was 1 s to cover a 500-Da to 2,000-Da mass range; 180 scans were made during the acquisition of data. Molecular masses of each analyte were obtained from multiple-charged ion profiles using transformation software (Waters MassLynx 4.0).

**Incubation of CXCL12 with proteases.** Aliquots of CXCL12 (25 μg/mL of water) were added to individual vials of CD26 (500 μg/mL), NE (500 μg/mL), or CG (42 μg/mL), respectively. Aliquots of CXCL12 (50 μg/mL of water) were also incubated with MMP-2 or MMP-9 (20 μg/mL). The mixtures were incubated for 30 min at 37°C. Each incubation sample was ultrafiltrated using 30-kDa cutoff membrane filter and then centrifuged for 30 min at 9,000 × g.

**Mass spectra of the truncated products in incubates.** Aliquots of each ultrafiltrate (10 μL) were injected into the HPLC column using an autosampler. The HPLC column (a size exclusion type Biopac AV-1) was 1-cm long and 4.0 mm in diameter (GL Sciences, Inc.). The eluent was a mixture of water-acetonitrile, containing 0.1% formic acid,
used in a linear gradient elution mode; starting with 20% acetonitrile, the concentration of acetonitrile was increased to 100% in 50 min. The HPLC eluent flow rate was 0.2 mL/min.

The ESI source was operated using conditions described above. During data acquisition, mass spectral scanning time was 2.5 s to cover a 500-Da to 2,000-Da mass range (600 mass units/s) and 1,200 scans were made during acquisition. Molecular masses of the truncated products of CXCL12 were generated from the multiple-charged ion profiles as described above and confirmed by comparison with the mass spectra of the synthetic standard of truncated products. Diagnostic ions to be used for analyses in patient or normal samples were selected from multiple-charged ion profiles.

**Mass spectrometric analysis of individual truncates in PB and BM plasma from MPN patients and normal subjects.** Twenty-five microliters of mouse EGF (10 μg/mL) were added to 50 μL of each ultrafiltrate of PB or BM plasma. Fifteen microliters of aliquots were injected into the LC-MS system, which included a reverse-phase column (Model ODS-100 V, 3-cm long, 1 mm in diameter, Tosoh Biosciences, Inc.). Operating conditions for gradient elution and the ESI source were the same as described for the products of the incubation steps. Truncated forms of CXCL12, resulting from the action of individual proteolytic enzymes, were quantified by selected ion recording (SIR) of the following masses (m/z): 980 for intact CXCL12, 952 for the truncated product due to CD26 (2 aa removed), 940 for the truncated product due to NE (3 aa removed), 929 for the truncated products due to MMP-2 and MMP-9 (4 aa removed in both cases), 915 for the truncated product due to CG (5 aa removed), and 1,029 for IS (EGF). Quantification of intact CXCL12 and individual truncates was performed using conventional calibration curves established by analyzing sets of normal plasma samples to which increasing quantities (range, 1–160 ng/mL in five steps) of CXCL12 standard, the synthetic standard of the –2 aa truncation product, and constant quantities (10 μg/mL) of IS were added. It is noted that there was no need to establish calibration curves for each individual truncation products of CXCL12, because the mass spectral intensity responses of the individual truncation products were the same (within experimental errors) as that of intact CXCL12 and also the –2 aa truncation product. The same methodology (with reduced sample volumes when indicated) was used for the analysis of normal and patient BM plasma samples.

**HPC assays.** CB CD34+ cells were assayed for HPC in semi-solid media as described previously in the presence or absence of CXCL12 or its individual truncates (13). Briefly, 5 × 10^5 cells were plated per dish in duplicate cultures containing 1 mL Iscove’s modified Dulbecco’s medium (IMDM) with 1% methylcellulose, 30% fetal bovine serum, 5 × 10^{-5} mol/L 2-mercaptoethanol (Stem Cell Technologies), to which 100 ng/mL SCF, 100 ng/mL interleukin-3 (IL-3), 100 ng/mL IL-6, and 100 ng/mL granulocyte macrophage colony-stimulating factor in the presence or absence of CXCL12 or its truncates (100 ng/mL) were added. Colonies were enumerated after 14 d of incubation.

**Migration assay.** The in vitro migratory behavior of CB CD34+ cells was performed as previously described using 6.5-mm diameter, 5-μm pore transwell plates (Corning; ref. 21). Briefly, transwell filters were coated overnight at 4°C with 10 ng/cm^2 of fibronectin (Sigma) at a concentration of 20 μg/mL in PBS. The coating solution was aspirated and replaced by a 1% bovine serum albumin (BSA) solution in PBS at 37°C for 30 min to block nonspecific binding sites. Before cells were added to the upper compartment, the coated transwell filters were washed twice with buffer (IMDM with 0.5% BSA). Then, 1 to 2 × 10^5 CD34+ cells suspended in 100 μL of buffer containing 125 ng/mL CXCL12 or its various truncated forms were added to the lower compartment. After incubation at 37°C for 4 h, nonmigrating and migrating cells were harvested from the upper and lower compartments, respectively. Nonmigrating cells were recovered following two washes, each consisting of a 5-min treatment with an enzyme-free cell dissociation buffer (Life Technologies) at 37°C, followed by vigorous pipetting. The harvested cells in the two fractions were enumerated using a hemocytometer. The percentage of migrating cells was calculated by determining the ratio of the number of cells recovered from the lower compartment to the total number of cells loaded in the upper compartment.

**Flow cytometric analysis of CB CD34+ cell apoptosis.** CB CD34+ cells were incubated in the presence of 125 ng/mL of CXCL12 or its truncated forms for 48 h. The cells were then stained with antihuman CD34 monoclonal antibody conjugated to APC in combination with antihuman Annexin V conjugated to FITC both before and after this incubation step. Immediately before analysis, 1 μg of propidium iodide (Sigma) was added to exclude nonviable cells. At least 10,000 viable cells were examined with a FACSCanto flow cytometer and analyzed using cell Quest software (Becton Dickinson).

**Statistical analysis.** The results are expressed as the mean ± SD of data obtained from varying numbers of separate experiments. Differences between groups were compared by using either a Student’s t test or ANOVA. Statistical significance was assumed for P < 0.05. Correlations between the clinical features of the MPN patients and the plasma levels of intact or total truncated CXCL12 were performed by linear regression analysis using the least-squares method.

**Results.**

**Mass spectral analysis of CXCL12 and synthetic standards of CXCL12 truncation products.** When full-length CXCL12 (1–68 aa sequence) is exposed to human serum, it undergoes processing initially at the COOH terminus (loss of lysine) to produce a 1 to 67 aa sequence (18, 19). Accordingly, we have used the commercially available CXCL12 (1–67 aa sequence) form for both the incubation experiments with proteolytic enzymes and also for the quantification of CXCL12 in normal and patient plasma. The molecular mass of CXCL12 was confirmed based on the multiple-charged ion pattern obtained in ESI. Conventional transformation (deconvolution software) of the +6,
+7, +8, and +9 multiple-charged ions yielded the molecular mass to be 7,832 Da compared with the expected mass of 7,831 Da (Supplementary Fig. S1A). The +8 multiple-charged peak (m/z 980) was selected for the detection and quantification of CXCL12 in patient plasma samples using the SIR technique. The multiple-charged ion profiles in the ESI mass spectra of the synthetic standards of the truncation products were as expected, and the observed molecular masses were in agreement with the calculated values, usually within 1 or 2 mass units. The molecular mass of IS of EGF was determined to be 6,171 Da (Supplementary Fig. S1B). The +6 charged ion (m/z 1,030 Da) was selected for the efforts to quantify CXCL12 and its truncated forms.

Detection of CXCL12 and identification of its truncates. CXCL12 (1–67 aa sequence) was incubated, in separate experiments, with individual proteolytic enzymes. The truncation products were identified from the typical +6, +7, and +8 multiple-charged ion patterns (Supplementary Fig. S2). The identities of the truncation products were confirmed using synthetic standards. The sequences generated were 3 to 67 by CD26, 4 to 67 by NE, 5 to 67 by MMP-9, and 6 to 67 by CG. The number and type of amino acids removed from intact CXCL12 by incubation with individual enzymes are listed in Table 1. A comparison of the masses of the multiple-charged ions calculated from the sequences of CXCL12 and its truncated products, measured using synthetic standards, and determined in the incubated samples is shown in Supplementary Table S1. It is noted that the calculated as well the measured molecular masses shown in Supplementary Table S1 are in agreement with those based on the number of amino acids removed from CXCL12 shown in Table 1.

Quantification of intact CXCL12 in normal and MPN plasma. Intact CXCL12 was quantified in normal and patient plasma using SIR of the diagnostic +8 charged ions of the analyte and the +6 charged ions of the IS (Supplementary Fig. S3). The calibration curves for quantification of CXCL12 were linear, e.g., y = 0.0013x + 0.027 with R² = 0.99. Using the monitoring of CXCL12 (m/z 980) and IS (m/z 1030) by SIR, the concentration of intact CXCL12 in normal human plasma was determined as 16.6 ± 9.4 ng/mL (n = 10). The concentrations of CXCL12 was 7.9 ± 3.6 ng/mL in PV (n = 21) plasma and 5.8 ± 4.0 ng/mL in PMF plasma (n = 15; Table 2). The concentration of intact CXCL12 was significantly decreased in both PV (P < 0.01) and PMF (P < 0.005) plasma compared with normal plasma.

Detection and quantification of truncated products in normal and patient PB plasma. In normal human plasma, no detectable amount (<1.0 ng/mL) of any truncated form of CXCL12 was found. However, all truncated products were detected in both PMF and PV plasma, confirming the presence of these proteases in the patient plasma (Fig. 1). The characteristic peak of +8 multiple-charged ions was selected for the quantification of individual truncation products. Concentrations of individual truncates were calculated via calibration curves obtained by adding known quantities of increasing CXCL12 and/or -2 aa standards to normal plasma. IS was added to each calibration and patient plasma sample as described (Supplementary Fig. S3). The concentration of each of the truncated products in normal and patient plasma is summarized in Table 2. The total quantity of truncated products was 4-fold and 5-fold greater than that of intact CXCL12 in the PV and PMF patient samples, respectively (Fig. 2).

Detection of CXCL12 and its truncated products in BM plasma from normal subjects and patients with PV. Intact CXCL12 (1–67 aa sequence) was detected in BM plasma both from normal and patients with PV. Normal BM contained significantly more intact CXCL12 than PV BM. Whereas truncation products were below the level of detection in normal BM (<1 ng/mL), all four truncation products detected in the PB plasma of PV patients were also found in the BM plasma of PV patients, suggesting that a similar proteolytic environment exists both in the marrow and PB. A comparison of the CXCL12 truncation products and intact CXCL12 in BM plasma from normal subjects and patients with PV is shown in Fig. 3. No truncated products were detected in the normal BM, and all known truncated products were found in the patient BM.

Correlation of MPN patient parameters and levels of intact or truncated CXCL12. We intended to correlate the patient JAK2V617F allele burden, WBC count, and the percentage of PB CD34+ cells with both the concentration of intact and total truncated forms of CXCL12 in MPN patient plasma. There was no correlation between the granulocyte JAK2V617F allele burden and the concentration of either intact or total truncated forms of CXCL12 (data not shown). Also, the total number of WBCs did not correlate with the levels of either intact CXCL12 or total truncated forms of CXCL12 (r = 0.50, data not shown) in MPN patients. The absolute number of PB CD34+ cells, however, seemed to be correlated with the levels of total truncated forms of CXCL12 (r = 0.676) but not with the levels of intact CXCL12 (data not shown).

Effect of CXCL12 and its truncates on CD34+ cell migration. The effects of CXCL12 and its truncates on the migration of CB CD34+ cells were assessed. As shown in Fig. 4, full-length CXCL12 dramatically increased the migratory behavior of CB CD34+ cells. This chemotractant capacity was
not documented with each of CXCL12 truncates. In fact, CXCL12 (5–67) diminished the ability of intact CXCL12 to attract CB CD34+ cells.

**Effect of CXCL12 and its truncates on CD34+ cell apoptosis and hematopoietic colony formation.** The addition of CXCL12 or any of its truncates forms to suspension cultures did not increase the percentage of CB CD34+ cells undergoing apoptosis as measured using Annexin V staining (data not shown). Furthermore, the ability of CB CD34+ cells to generate hematopoietic colonies in semisolid media was also not influenced by the addition of CXCL12 or any of its truncated forms (data not shown).

**Discussion**

A growing body of evidence suggests that the interaction of CXCL12 with CXCR4 plays an important role in regulating HSC/HPC trafficking (4, 22). In the BM, CXCL12 is expressed

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**Table 2. Concentration of CXCL12α and its individual truncation products in plasma from normal subjects and patients with PMF and PV (mean ± SD, ng/mL)**

<table>
<thead>
<tr>
<th>Form of CXCL12α (aa sequence)</th>
<th>Normal (n = 10)</th>
<th>PMF (n = 15)</th>
<th>PV (n = 21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact (1–67)</td>
<td>16.6 ± 9.4</td>
<td>5.8 ± 4.0</td>
<td>7.9 ± 3.6</td>
</tr>
<tr>
<td>2aa (3–67)</td>
<td>ND</td>
<td>7.8 ± 6.9</td>
<td>9.4 ± 3.4</td>
</tr>
<tr>
<td>3aa (4–67)</td>
<td>ND</td>
<td>8.4 ± 7.5</td>
<td>7.4 ± 5.2</td>
</tr>
<tr>
<td>4aa (5–67)</td>
<td>ND</td>
<td>8.2 ± 6.2</td>
<td>8.8 ± 4.9</td>
</tr>
<tr>
<td>5aa (6–67)</td>
<td>ND</td>
<td>4.3 ± 4.2</td>
<td>5.5 ± 4.3</td>
</tr>
<tr>
<td>Total truncation products</td>
<td>ND</td>
<td>28.7 ± 19.9</td>
<td>31.1 ± 7.8</td>
</tr>
</tbody>
</table>

NOTE: Comparison of CXCL12α concentrations between normal and patient plasma yielded P < 0.005 for PMF and P < 0.01 for PV. Abbreviations: aa, number of amino acids removed; ND, not detected; concentration < 1.0 ng/mL.

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**Figure 1.** Transformed (computer reconstructed) electrospray mass spectra showing the presence of truncated products of CXCL12. A, plasma from normal healthy subjects. No detectable amount of any of the truncated products of CXCL12 were found; detection limit, <1 ng/mL. B, each of the truncated products was detected in the plasma of patients with PV studied, indicating the presence of these specific proteases in the plasma of these patients. C, each of the truncated products was detected in plasma of each of patients with PMF, indicating the proteases present in the plasma of these patients. The peak intensities of IS were too large to be shown as part of the mass spectra. For quantification data, see Table 2.
by osteoblasts, endothelial cells, stromal cells, and spindle-shaped reticular stromal cells distributed throughout the BM (5, 22). Although CXCR7 has been described as an alternate receptor for CXCL12, CXCR4 is the only functional chemokine receptor present on HSC/HPC accounting for the prominent role of this receptor in the homing and maintenance of HSC within the marrow niches (23). G-CSF mobilization of HSC/HPC is associated with a decrease in CXCL12 protein in the BM and decreased expression of CXCR4 on HSC/HPC (5). The reduced expression of CXCR4 following G-CSF administration has been attributed to the accumulation of active neutrophil proteases, including NE and CG, which can cleave between the GHS epitope and the first transmembrane domain of CXCR4, a cleavage known to inactivate the chemotactic properties of CXCR4 (5). The reduction of marrow CXCL12 following G-CSF administration is a consequence of the accumulation of several proteases, which are able to directly cleave and inactivate CXCL12, and/or the suppression of CXCL12 production by BM osteoblasts secondary to a loss of osteoblast lineage cells (4, 11).

The constitutive mobilization of HSC/HPC, which characterizes the MPN, seems to share many features associated

![Figure 2](image_url)

**Figure 2.** Comparison of total concentration of each of the truncated forms of CXCL12 with that of intact CXCL12 in plasma from normal volunteers and patients with PMF and PV. The total concentrations of truncated products in the patients were 3-fold to 4-fold greater than that of intact (full-length) CXCL12. Also note that a detectable amount of truncated CXCL12 was not present in normal plasma (<1.0 ng/mL).

![Figure 3](image_url)

**Figure 3.** Electrospray mass spectra of the BM plasma from a normal subject and a patient with PV. Comparison of transformed mass spectra of normal BM plasma (A) and BM plasma from a patient with PV (B). Truncated products were not observed in the normal BM plasma, and each of the truncated products was found in the patient BM plasma. All peaks are normalized with respect to the truncated product representing the removal of two amino acids from the end terminal of CXCL12. The intensity of CXCL12 is significantly larger in normal BM than that in patient BM.
with G-CSF HSC/HPC mobilization but also has several distinguishing characteristics (2, 5, 6). CD34+ mobilization associated with PMF is sustained and more profound than that associated with mobilization following the administration of G-CSF (2). In addition, serum G-CSF levels have been reported to be extremely low in patients with PMF (24), making it unlikely that the abnormal CD34+ cell trafficking in PMF can be attributed to a chronic elevation of G-CSF. In addition, MPN-associated CD34+ cell mobilization is associated with an increase in the immunoreactive forms of CXCL12 in both the plasma and marrow of patients with PV and PMF, whereas G-CSF mobilization is accompanied by a reduction in marrow CXCL12 (4, 6). There is, in both normal volunteers receiving G-CSF and MPN patients, a proteolytic environment characterized by increased plasma levels of NE and MMP-9 (4, 5). These proteases, as well as CD26 and CG, are capable of degrading CXCL12. We have previously reported that the cellular progeny of the MPN HSCs/HPCs, including Mks, mononuclear cells, and neutrophils, are the major sources of such proteases in PMF patients (5, 25). The possible mechanism underlying increased protease activity in MPN could be due to increased numbers of protease-producing cells and/or increased ability of MPN cells to produce these proteases. This explanation seems plausible, because most patients with MPN have been shown by Passamonti and coworkers to have granulocyte activation patterns similar to those induced by G-CSF (10).

Chemokine truncation results in a variety of effects, including inactivation of the chemokine, changing the chemokine into an effective receptor antagonist, enhancing activity of the chemokine, or in some cases no effect at all (22, 26–28). CXCL12 has been reported to undergo shortening to different forms based upon the action of various enzymes (27, 28). Such CXCL12 truncation products have recently been shown to play a role in the development of human disease (28–30). Proteolytic cleavage of constitutively expressed CXCL12 in the brains of patients with HIV-associated dementia has been shown to produce neuronal membrane perturbation with ensuing neurotoxicity and apoptosis leading to neurodegeneration (29, 30). In this report, we provide evidence that CXCL12 truncates also play a role in the constitutive mobilization of CD34+ cells associated with MPN. We show that, although immunoreactive forms of CXCL12 are increased in the plasma and marrow of MPN patients, the overwhelming amount of this material really represents truncated forms of CXCL12. In fact, PMF and PV plasmas contain 35% and 41%, respectively, of the amount of intact CXCL12 present in normal plasma. Only the full-length CXCL12 is fully competent as a chemoattractant for CB CD34+ cells. Although normal PB and BM plasma contains few truncated forms of CXCL12, the corresponding plasma isolated from MPN patients contains truncates, which are the consequence of degradation by CD26, NE, MMP-2, MMP-9, and CG. In both PV and PMF, over 50% of the immunoreactive forms of CXCL12 are actually a truncated form of CXCL12, which lacks the ability to chemoattract CB CD34+ cells. Furthermore, the degradation product of CXCL12 produced by MMP-2 and MMP-9 (CXCL12; 5–67 aa sequence) was shown to be capable of reducing the chemoattractant capacity of full CXCL12, suggesting that this truncate might act as a receptor antagonist. The increased presence of PMF CD34+ cells at extramedullary sites, such as spleen and liver, could be due to the presence of intact CXCL12 at those sites (31, 32). This possible explanation requires further investigation. Alternatively, the localization of CD34+ cells to spleen and liver in PMF might be due to a distinct pathway other than CXCL12/CXCR4. This hypothesis is raised because osteoblasts, which are a major source of CXCL12 in the marrow, are not present at these extramedullary sites (33).

The pattern of CXCL12 truncates present in the plasma of PV patients was similar to that observed in the marrow,
showing that the degradation pattern of CXCL12 observed in plasma was a reflection of the protease activity in the marrow. Because the marrow in PMF cannot be routinely aspirated due to marrow fibrosis, a similar comparison between PMF plasma and marrow was not possible. Based upon the toxic effects of CXCL12 truncates on neural cells observed in patients with HIV-1 and dementia (28–30), we explored if these truncates might account for the marrow failure associated with PMF. These truncates did not, however, affect the degree of CD34+ cell apoptosis or ability of CD34+ cells to generate hematopoietic colonies in vitro.

Because PV and PMF plasmas, but not normal plasma, were observed to contain similar large amounts of CXCL12 truncates, one would assume that the degree of constitutive stem cell mobilization would be similar in the two disorders. PMF is, however, associated with a greater degree of CD34+ cell mobilization than that observed in the other Philadelphia chromosome-negative MPN (2). The constitutive mobilization of CD34+ cells in PV patients seems to be a consequence of the reduced amount of intact marrow CXCL12 and the predominance of CXCL12 truncates within the marrow that lack chemotactic capabilities and those that act as receptor antagonists. Furthermore, the degree of CD34+ cell mobilization was shown to be related to the amount of truncated CXCL12. The greater degree of mobilization of CD34+ cells observed in PMF is likely due to the combination of the truncation of CXCL12 by serine proteases and down-regulation of CXCR4 cell expression by CD34+ cells, which is a consequence of epigenetic silencing of the promoter for CXCR4 (9). These two events provide potential targets for the development of novel therapeutic agents for the treatment of patients with PMF. We and others have shown that chromatin-modifying agents can lead to increased expression of CXCR4 by PMF CD34+ cells (21, 34). In addition, several protease inhibitors that might alter the degradation of CXCR12 are currently being evaluated in clinical trials (35). The evaluation of these classes of drugs in MPN patients requires additional studies.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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