Chemokine Regulation of Human Megakaryocyte Development

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We have previously shown that platelet factor 4 (PF4), a platelet-specific CXC chemokine, can directly and specifically inhibit human megakaryocyte colony formation. Herein we present additional studies characterizing the inhibitory effect of CXC chemokines on human megakaryocyte development. We first corroborated our initial studies by showing that recombinant human (rH) PF4, like the native protein, inhibited megakaryocytopoiesis. We then examined the inhibitory properties of other CXC family members. Neutrophil activating peptide-2 (NAP-2), a naturally occurring N-terminally cleaved βTG peptide, was found to inhibit megakaryocytopoiesis with two to three orders of magnitude greater potency than PF4. Structure function studies showed that an N-terminal mutation, which eliminated NAP-2’s neutrophil activating properties (NAP-2ΔN), also abrogated its ability to inhibit megakaryocyte development. Further investigations of this type demonstrated that a chimeric PF4 protein (AELR/PF4) in which PF4’s N-terminus was replaced with the first four amino acids of NAP-2 was also a potent inhibitor of megakaryocytopoiesis. Interleukin (IL)-8, another CXC chemokine, and three CC chemokines (macrophage inhibitory protein-1α [MIP-1α], MIP-1β, and C10) also specifically inhibited megakaryocyte colony formation at NAP-2 equivalent doses. CXC and CC chemokine inhibition was additive suggesting that the effects might be mediated through a common pathway. The inhibitory effects of NAP-2 and MIP-1α could not be overcome by adding physiologically relevant amounts of recombinant human megakaryocyte growth and development factor (MGDR) (50 ng/mL) to the cultures. Using Northern blot and reverse transcriptase-polymerase chain reaction (RT-PCR) based analyses, we documented mRNA expression of IL-8 receptor isoforms α and β in total platelet RNA and in normal human megakaryocytes, respectively. Based on these results, we hypothesize that chemokines play a physiologic role in regulating megakaryocytopoiesis. Because chemokines are elaborated by ancillary marrow cells, both autocrine and paracrine growth control is suggested, the effects of which might be exerted, in part, through α and β IL-8 receptors.

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2 (NAP-2), is a potent neutrophil activator. Of note, two different IL-8 receptors (IL-8R) are expressed by neutrophils. One is designated α or type 1 IL-8R and binds IL-8 efficiently, but NAP-2 poorly. A second β or type 2 IL-8R has also been described, which binds IL-8 well, and NAP-2 with moderate affinity. The CC or β chemokines, in which the first two cysteine residues are immediately adjacent to each other, represent the other branch of this family. CC chemokines such as macrophage inhibitory protein (MIP)-1α appear to be important monocyte stimulating agents and may also selectively suppress human megakaryocyte development. A CC chemokine receptor has been described. The PF4 and βTG studies described above suggested that neutrophil activating potency and megakaryocyte colony inhibitory activity paralleled each other. To test this hypothesis, we examined the effect of the neutrophil activator NAP-2 on human megakaryocyte development in vitro. We also sought to determine the megakaryocyte colony inhibitory activity of other members of this chemokine family. Herein, we present data showing that a number of CXC and CC chemokines are potent inhibitors of human megakaryocytopoiesis and that megakaryocytes express a number of chemokine receptors. These data suggest that chemokines, perhaps elaborated by ancillary cells in the marrow microenvironment, may play a physiologically significant role in regulating human megakaryocyte development by both autocrine and paracrine mechanisms.

**MATERIALS AND METHODS**

**Cells**

Light-density bone marrow mononuclear cells (MNC) were obtained from normal consenting volunteers. Hematopoietic colonies were enumerated using a Coomassie Protein Assay Reagent Kit with bovine serum albumin as a standard (Pierce, Rockford, IL), which were allowed to grow to an optical density in 10 mmol/L isopropl-thiogalactopyranoside that induced recombinant protein expression. The recombinant proteins were processed as previously described. Recombinant proteins were purified from the supernatants of bac-

**Hematopoietic Colony Assays**

All assays were performed with either peripheral blood or bone marrow obtained from normal consenting volunteers. Hematopoietic colonies were grown and identified as previously described. In brief, colony assays were performed with either unseparated light-density marrow nuclear cells (MNC) or with MNC depleted of monocyte-macrophages and T-lymphocytes as previously described. Final plated cell concentrations were 2 x 10^5/mL. Cultures were supplemented with 30% vol/vol of aphaenic anemia serum, except when supplemented with recombinant human MGD5 (generously provided by Dr Pamela Hunt, Amgen Corp, Thousand Oaks, CA). Megakaryocyte and granulocyte colonies were supplemented with rH IL-3 (29 U/mL) and rH granulocyte-macrophage colony-stimulating factor (GM-CSF) (5 ng/mL) (Genetics Institute, Cambridge, MA), except for the megakaryocyte studies that tested MGD5 effect. Erythroid colonies were supplemented with recombinant erythropoietin (5 U/mL) (Amgen Corp). Recombinant human chemokines were added just before plating.

Megakaryocyte colonies were enumerated after 12 days in culture by indirect immunofluorescence using either a highly-specific rabbit antihuman platelet membrane glycoprotein antiserum R41 or a monoclonal anti-GPIb/IIa complex antibody A249. Binding of the probe antibody was detected with a species appropriate fluoroscent-conjugated secondary antibody (Meloy, Springfield, VA). A cluster of three or more intensely fluorescent cells were defined as one colony. Plates were read by two separate individuals, including one blinded to plate designation. Results were in agreement between both readers within 5% to 10%. Results are reported as the mean ± standard error (SE) of colonies enumerated.
**IL-8Ra and IL-8Rβ Platelet Northern Blot**

The IL-8Ra and IL-8Rβ cDNAs were kindly provided by Dr Ingrid U. Schraufstatter (Scripps Institute, La Jolla, CA) in the expression vector pSFFV.neo. The ~1.2-kb coding regions for both the IL-8Ra and IL-8Rβ cDNAs, the 3.3-kb platelet glycoprotein Ib (GPIIb), and the 0.4-kb βTG cDNA inserts26–28 were released from their respective vectors by EcoRI digestion. All of the above cDNA inserts were purified from an agarose gel using GENECLEAN (Biolol, Vista, CA). These inserts were then labeled using α-32P-dCTP, random primers, and Klenow to ~106 CPM/μg DNA.

Total platelet RNA was prepared from platelet rich plasma (PRP) obtained from 100 mL of sodium citrate anticoagulated blood. Only the top two-thirds of the PRP was used. After spinning down the platelets, platelet RNA was prepared using the guanidinium thiocyanate/acid phenol single-step RNA isolation technique. The amount of RNA applied per lane was equivalent to ~25% of the total yield. The RNA was fractionated on gels containing formaldehyde followed by transfer to GeneScreen Plus membrane (DuPont Co, Wilmington, DE) and hybridization to the labeled probe as previously described.

**Reverse Transcriptase-Polymerase Chain Reaction of Purified Megakaryocyte mRNA**

Human megakaryocytes were isolated from the marrow of remunerated, normal adult volunteers. The isolation of greater than 99% pure megakaryocytes was accomplished as previously described using counterflow centrifugal elutriation (Beckman J2-21M; Standard elutriation motor; Beckman Instruments; Mountainview, CA) to obtain an initial megakaryocyte enrichment, followed by isolation of morphologically recognizable mature megakaryocytes with a micro-manipulator. RNA was isolated from ~50 megakaryocytes in less than 3 hours’ time.

mRNA was isolated from these cells using the Quick-Pre mRNA Purification Kit (Pharmacia, Piscataway, NJ). The final mRNA pellet was washed with 75% ethanol and resuspended in 10 μL of triple distilled autoclaved water. Reverse transcription of the megakaryocyte mRNA was performed using 4 μL of the original sample heated to 65°C for 10 minutes and then cooled on ice for 3 minutes. A total of 100 U of Moloney murine leukemia virus reverse transcriptase (RT) (GIBCO BRL, Gaithersburg, MD), 50 ng of random primers (Boehringer Mannheim, Indianapolis, IN), 40 U of RNasin (Promega, Madison, WI), and dNTPs (50 μM each) were added to the tube and incubated for 1 hour at 37°C. Specific oligo primer pairs were used for the polymerase chain reaction (PCR) amplification with the megakaryocyte random cDNA were as follows: IL-8Ra: 5'-AGTGCTC-AATATTCAGATCCC-3' (sense oligonucleotide; 1 to 20 base, beginning at the transcriptional start site) and 5'-AGATTCATAGAC-AATATTACAGATCC-3' (antisense oligonucleotide; 500 to 481 base); and IL-8Rβ: 5'-GAGGACCCAGGTGATCCAGG-3' (sense, 816 to 835 base) and 5'-GAGGACCCAGGTGATCCAGG-3' (antisense, 500 to 481 base) respectively. Ten microliters of the reverse transcription reaction or 100 ng of the appropriate IL-8R cDNA or water was used in a 100-μL PCR reaction using 30 ng of both sense and antisense primers for each receptor and 2.5 U Taq polymerase (Promega) using manufacturer’s supplied buffer and 2.5 μM/mL MgCl2. PCR conditions were melting at 95°C for 1 minute, annealing at 60°C, and extension at 72°C for 30 rounds. A total of 10 μL of each final PCR product was size-fractionated on a 1.2% agarose gel.

**RESULTS**

**Hematopoietic Effects of Recombinant PF4**

We first determined whether rH PF4 made in a prokaryotic system would manifest effects on megakaryocytopoiesis similar to those we previously reported with highly purified, serum-derived material. Mature rH PF4 protein was synthesized in Escherichia coli as described in Materials and Methods. Its amino acid sequence was identical to the native protein, except that the recombinant material retained an initiating methionine residue. Biologic integrity of the engineered protein was demonstrated in chemotactic studies where the rH PF4 was as effective as serum-derived PF4. In a plasma clot assay, both recombinant and native material inhibited megakaryocyte colony formation in an identical manner throughout the dose range tested (Fig 1A). In agreement with our previously reported results, inhibition was dose-dependent and most significant at concentrations ≥10 μg/mL. Also in agreement with our previous results, the numbers of cells/colony (Fig 1B) and the size of cells comprising the colonies (Fig 1C) were also diminished in the presence of exogenous PF4. Finally, inhibition was megakaryocyte lineage specific (data not shown).

**NAP-2, AELR/PF4 and Mutation Proteins**

We have previously shown that βTG does not inhibit in vitro megakaryocytopoiesis. However, it is now known that a neutrophil cathepsin G N-terminal cleavage product of βTG, a 70-amino acid protein termed NAP-2, is a biologically active form of this protein. NAP-2 is a potent activator of neutrophils, binding to the IL-8Rβ on neutrophils. To extend our structure/function studies and to begin to understand the mechanism by which chemokines inhibit megakaryocyte development, we tested whether NAP-2, as opposed to βTG, would inhibit megakaryocytopoiesis. As shown in Fig 2A, megakaryocyte colony formation was much more sensitive to the inhibitory effects of NAP-2 than PF4. Where PF4 inhibited in the μg/mL range, half maximal inhibitory concentrations of NAP-2 were in the 10 to 100 ng/mL range. These concentrations were comparable to those needed for NAP-2 to activate neutrophils. As was the case with PF4, NAP-2’s inhibitory effect was lineage-specific. It has previously been reported that at PF4 concentrations ≥10 μg/mL inhibition of CFU-E and CFU-GM might be observed. However, we did not observe such effects in our cultures and no definitive changes in CFU-E or CFU-GM colony formation were observed with increasing doses of NAP-2.

We also sought to determine if NAP-2 inhibited megakaryocyte development directly, or indirectly via a secondary effect on accessory marrow cells. To address this question, marrow mononuclear cells were depleted of monocyte-macrophages and T-lymphocytes and then enriched for CD34+ cells using immunomagnetic beads before exposure to NAP-2. Purity of the CD34+ cells in this population consistently exceeded 85%. At 50 μg/mL, PF4 decreased the number of megakaryocyte colonies to 8% of the control, and at 750 ng/mL, NAP-2 decreased expression to 10% of the control. These results support the hypothesis that the chemokines tested exerted a direct inhibitory effect on megakaryocyte progenitor cells.

Because NAP-2 is thought to activate neutrophils through IL-8Rβ, we tested other NAP-2 and PF4 mutant constructs whose interactions with the neutrophil IL-8 receptors have been previously defined. For example, alanine substitutions
Inhibitory Effects of Other Chemokines

We tested additional chemokines to see whether they too could inhibit megakaryocytopenia. IL-8 was almost as effective as NAP-2 in inhibiting megakaryocytopenia in the 5 to 500 ng/mL concentration range (Fig 3A). Surprisingly, the CC chemokine MIP-1α was equally effective (Fig 3A). Inhibition with both of these chemokines was again noted to be lineage specific (data not shown). Two additional CC chemokines were tested and also inhibited megakaryocytopenia. MIP-1β decreased megakaryocyte colony formation to 52% of the untreated control value at 500 ng/mL, and a culture supernatant containing C-10, another member of the CC chemokine family, decreased megakaryocyte colonies to 35% of control values at a concentration of 2% (vol/vol).

We then sought to determine whether the inhibitory effects of the CXC and CC chemokines might be additive or synergistic. Progenitor cells were therefore cultured with the individual chemokines at varying doses, or with the two added together. As shown in Fig 3B, the combination of NAP-2 and MIP-1α had greater inhibitory effect than either chemokine individually. There does not appear to be a synergistic effect.
Rather, the combination of NAP-2 and MIP-1α better approximates an additive effect. If the inhibition observed is additive, CXC and CC chemokines may inhibit megakaryocyte development by signaling through a common pathway. It should also be noted that even at the highest combined chemokine dose, megakaryocyte colony formation was not completely extinguished. When compared with an untreated control group, ~30% of maximal colony formation was still observed even at the highest combined dose level. The nature of the difference(s) between this resistant subpopulation and those progenitor cells that were sensitive to the chemokine inhibitory effects remains to be determined.

Finally, we also determined whether the chemokine inhibitory effect could be abrogated by MGDF stimulation. We first established that, in our culture system, megakaryocyte colony growth was maximal at MGDF doses ~20 ng/mL. At such doses, the number of colonies observed in the culture dishes was comparable to the number observed when optimal concentrations of aplastic anemia serum plus IL-3 and IL-6 were used as stimulators. We then cultured progenitor cells in the presence of 50 ng/mL of MGDF and varying doses of either NAP-2 or MIP-1α. As shown in Fig 4, in comparison to the numbers of colonies obtained in the presence of MGDF alone, NAP-2 at >5 ng/mL and MIP-1α at >50 ng/mL significantly inhibited colony formation. Therefore, even in the presence of a significant physiologic stimulator, used at maximally effective concentrations, these chemokines appeared capable of blunting megakaryocyte colony growth.

Demonstration of the Expression of IL-8Ra and IL-8Rβ by Megakaryocytes

The above studies suggested that chemokines exert a direct effect on megakaryocyte development. If our interpretation of the experiments was correct, it follows that megakaryocytes should express the receptors that bind these ligands. To provide these important data, we pursued two independent, but complementary approaches. First, we isolated total platelet RNA and looked for IL-8Ra and IL-8Rβ. The presence of these messages in the total RNA pool would provide evidence that these receptors might, in fact, be expressed by megakaryocytes. We also performed RT-PCR for these same messages on an essentially pure population of normal human megakaryocytes.

Platelet RNA was extracted from the top two-thirds of platelet-rich plasma of low-speed centrifuged blood. White cell contamination was approximately 1 cell per 5,000 platelets. Duplicate lanes of total RNA were hybridized to cDNAs for the L-8 receptors and platelet GPIIb and PG as platelet-specific positive controls. As seen in Fig 5A, there were single detectable bands of the expected size of ~3 kb for both of the IL-8 receptors and 3.3 kb for GPIIb. An intense signal was detected with βTG at 0.8 kb with additional bands seen at ~1.2 and 1.8 kb. Total peripheral blood neutrophils detect two major IL-8R bands of 2.4 and 3.0 kb.

<table>
<thead>
<tr>
<th>Name</th>
<th>Amino Acid Sequence</th>
</tr>
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<tbody>
<tr>
<td>NAP-2 (wild type)</td>
<td>AEELRRCMC ... THCN ... DGRKICLDQAPRIKKIVQKLAGDESAD</td>
</tr>
<tr>
<td>NAP-2&lt;sup&gt;2E3-A&lt;/sup&gt;</td>
<td>AQLRRCMC ... THCN ... DGRKICLDQAPRIKKIVQKLAGDESAD</td>
</tr>
<tr>
<td>PF4 (wild type)</td>
<td>EAREDQDQCLC ... PHCP ... NGRKICLDQQPLYKIKKIKKILES</td>
</tr>
<tr>
<td>AEELR/PF4</td>
<td>AEELRCMC ... PHCP ... NGRKICLDQQPLYKIKKIKKILES</td>
</tr>
</tbody>
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Amino acid differences from wild type are underlined.
while HL60 cell lines only express the 3.0 kb band, similar to the single band we detected in platelet RNA.42

Even though the blots depicted in Fig 5 were hybridized under high stringency conditions, it is possible that we were only detecting a single IL-8R species because of the ~70% homology in the coding regions of the IL-8R cDNAs. Therefore, to provide additional proof that we were, in fact, detecting both IL-8Ra and IL-8Rβ, we performed RT-PCR reactions on mRNA isolated from a small number of 100% pure normal human megakaryocytes.43 Primers chosen were unique for the two IL-8 receptors, as shown by the fact that the primers do not yield cross-amplify using IL-8 receptor cDNAs (data not shown). These RT-PCR studies demonstrate that both IL-8 receptors are expressed in megakaryocytes (Fig 5B).

**DISCUSSION**

We have previously shown that PF4, a platelet-specific CXC chemokine, can directly inhibit in vitro megakaryocytopoiesis. We now show that other CXC chemokines, NAP-2, IL-8, and even the more distantly related CC chemokines, MIP-la and β, and C10, have a similar direct inhibitory effect on in vitro megakaryocyte development as manifested by the appearance of fewer colonies composed of smaller numbers of less mature cells. Inhibition is observed when chemokines are added to progenitor cell cultures at concentrations equivalent to those at which they activate neutrophils and monocytes,44 suggesting that the inhibitory signals they generate may be of physiologic significance. Studies performed with mutated NAP-2 and PF4 proteins also support this hypothesis as the ability to activate neutrophils and to inhibit megakaryocytopoiesis closely parallel each other. Another highly suggestive finding is that megakaryocytes express both α and β isoforms of the IL-8 receptor. Nevertheless, although the presence of these receptors provides a mechanism for the observed inhibitory effects of some of the CXC chemokines, further studies will be needed to define the full repertoire of megakaryocyte chemokine receptors and, in particular, whether CC chemokine receptors also exist on these cells.
Although many stimulatory cytokines have been described, relatively few inhibitory proteins have been elucidated. The best-studied examples of these include the interferons (INFs), tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), and MIP-1α.44-47 The physiologic role of these cytokines in the regulation of human hematopoiesis remains speculative.44 This is, in part, because the biologic effects of these cytokines are context dependent, a situation that has tended to generate apparently contradictory reports on their activity. For example, MIP-1α appears to specifically inhibit the growth of cycling hematopoietic cells,46 but when admixed with IL-3 or GM-CSF appears to promote colony growth in a synergistic manner.49 In addition, TNF-α and the INFs appear to be general suppressors of hematopoietic cell growth, while the activity of TGF-β is similar to MIP-1α in that it appears to block proliferation of growth factor stimulated progenitor cells, but alone may stimulate CFU-GM and CFU-E. These observations have prompted speculation that some of these inhibitors, in particular TGF-β and MIP-1α, may play a role in maintaining stem cells in a G0 state when hematopoiesis is otherwise stimulated.44 In support of this hypothesis, it has been reported that inhibition of TGF-β expression with antisense DNA increases the apparent number of assayable multilineage progenitor cells in cord blood.48 These apparently discordant results may also be explained by the observation that the inhibitory effects of MIP-1α and TGF-β depend on the maturation of the progenitor that has been exposed to this chemokine.49,46,49,50 Many investigators have reported that MIP-1α either has no effect on, or actually stimulates the growth of more mature progenitors, while it suppresses the growth of more primitive cells.49,50 The fact that the chemokines we have investigated have no apparent effect on CFU-GM derived colony formation is in accord with these results. Why the apparently mature CFU-Meg are inhibited by these chemokines when CFU-GM are not, remains unknown.

Whether the findings we report are of physiologic significance remains unclear. The fact that members of this family can inhibit megakaryocyte development at concentrations three orders of magnitude lower than PF4 is suggestive of physiologic relevance because concentrations in this range are likely achievable in vivo. Further, the ability to have significant inhibition despite maximal levels of TPO also supports a potential in vivo role. Nevertheless, establishing whether there is a correlation between megakaryocyte/platelet mass and chemokine concentrations, either in plasma or in the marrow, will have to be investigated. This will be particularly important in patients with inflammatory states who may be expected to have relatively high circulating chemokine levels, and perhaps paradoxically, reactive thrombocytosis. Measurement of MGDF levels will be of equal importance then, as we have already speculated that stimulatory growth factors may overcome the effects of apparently weaker negative regulators.46 Indeed, we have also hypothesized that negative regulatory loops, whether autocrine or paracrine in nature, are more likely to play a role in regulating basal, or nonstimulated, megakaryocyte production.46 If shown to be of physiologic significance, these studies may represent a significant advance in the development of new pharmacologic strategies to regulate disordered or inappropriate thrombopoiesis.
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