Stromal-derived factor 1 and thrombopoietin regulate distinct aspects of human megakaryopoiesis

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The role of the chemokine binding stromal-derived factor 1 (SDF-1) in normal human megakaryopoiesis at the cellular and molecular levels and its comparison with that of thrombopoietin (TPO) have not been determined. In this study it was found that SDF-1, unlike TPO, does not stimulate αIIbβ3⁺ cell proliferation or differentiation or have an antiapoptotic effect. However, it does induce chemotaxis, trans-Matrix migration, and secretion of matrix metalloproteinase 9 (MMP-9) and vascular endothelial growth factor (VEGF) by these cells, and both SDF-1 and TPO increase the adhesion of αIIbβ3⁺ cells to fibrinogen and vitronectin. Investigating the intracellular signaling pathways induced by SDF-1 and TPO revealed some overlapping patterns of protein phosphorylation/activation (mitogen-activated protein kinase [MAPK] p42/44, MAPK p38, and AKT [protein kinase B]) and some that were distinct for TPO (eg, JAK-STAT) and for SDF-1 (eg, NF-κB). It was also found that though inhibition of phosphatidylinositol-3-kinase (PI-3K) by LY294002 in αIIbβ3⁺ cells induced apoptosis and inhibited chemotaxis adhesion and the secretion of MMP-9 and VEGF, the inhibition of MAPK p42/44 (by the MEK inhibitor U0126) had no effect on the survival, proliferation, and migration of these cells. Hence, it is suggested that the prolifeative effect of TPO is more related to activation of the JAK-STAT pathway (unique to TPO), and the PI-3K–AKT axis is differentially involved in TPO- and SDF-1-dependent signaling. Accordingly, PI-3K is involved in TPO-mediated inhibition of apoptosis, TPO- and SDF-1–regulated adhesion to fibrinogen and vitronectin, and SDF-1–mediated migration. This study expands the understanding of the role of SDF-1 and TPO in normal human megakaryopoiesis and indicates the molecular basis of the observed differences in cellular responses. (Blood. 2000;96:4142-4151)

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growth factor (VEGF). To ascertain the significance of our observations, we blocked phosphatidylinositol 3-kinase (PI-3K) activity and MAPK p42/44 pathway with the specific inhibitors LY294002 and U0126, respectively.

Our results suggest that SDF-1 and TPO activate human megakaryoblastic \( \alpha_{\text{IIb}}\beta_{\text{3}} \)-positive cells by overlapping (MAPKp42/44, p38, AKT), but distinct, intracellular signaling pathways (JAK-STAT for TPO and NF-κB for SDF-1). The result of these differences in intracellular signaling appears to be that SDF-1 increases the interaction of these cells with their microenvironment but has no effect on their proliferation, differentiation and survival, whereas TPO has the opposite role. Moreover, we demonstrate that the proliferative effect of TPO is more related to activation of the JAK-STAT pathway unique to TPO and that the PI-3K–AKT axis, which plays an important role in the biology of human megakaryoblasts, is differentially involved in TPO and SDF-1–dependent signaling.

Materials and methods

**Human CD34\(^+\)** cells, megakaryoblasts, and platelets

Light-density bone marrow mononuclear cells (BM MNCs) were obtained from consenting healthy donors, depleted of adherent cells and T lymphocytes (A–T– MNCs), and enriched for CD34\(^+\) cells by immunofluorescence selection with MiniMACS paramagnetic beads (Miltenyi Biotec, Auburn, CA) as described.\(^{10,18-20,30,31}\) The purity of isolated BM CD34\(^+\) cells was greater than 95%, as determined by fluorescence-activated cell sorter (FACS) analysis.

BM CD34\(^+\) cells were expanded in a serum-free liquid system, and growth of CFU-MK was stimulated with recombinant human (rh) TPO (50 ng/mL) and rhIL-3 (10 ng/mL) (both from R&D Systems, Minneapolis, MN) as described.\(^{19,21,30}\) After incubation for 11 days at 37°C, approximately 85% of the expanded cells were positive for the megakaryocytic-specific marker \( \alpha_{\text{IIb}}\beta_{\text{3}} \),\(^{22,30,31}\) and this population was further enriched to more than 95% purity (as determined by FACS analysis) by additional selection with immunomagnetic beads (Miltenyi Biotec), as previously described by us.\(^{20,31}\)

Gel-filtered platelets were prepared from 4 persons as previously described\(^{10,26}\) and used within 2 hours of preparation. Marrow aspiration from and blood donation by healthy volunteers was carried out with donors' informed consent obtained through the Institutional Review Board.

**Cell cycle analysis and detection of apoptosis by Annexin V binding assay, caspase-3 activation, and poly(ADP-ribose) polymerase cleavage**

DNA content as a measure of cell cycling was determined as previously described.\(^{22}\) Briefly, \( \alpha_{\text{IIb}}\beta_{\text{3}} \)-positive cells (2 \( \times 10^6 \)) were stimulated with appropriate ligands (IL-3, TPO, or SDF-1α or β) and after incubation were spun down; 250 to 500 μL RNase (50 μg/mL in phosphate-buffered saline (PBS)) was then added, and the cells were incubated at 37°C for 30 minutes and resuspended in 250 to 500 μL propidium iodine solution (50 μg/mL in sodium citrate; Sigma, St Louis, MO) before reincubation at room temperature for 30 minutes. Subsequently, the cells were analyzed using FACStar and the Cell Quest program.

Apoptosis was assessed by staining with FITC-Annexin V and flow cytometric analysis (FACScan; Becton Dickinson, Mountain View, CA) and by using the apoptosis detection kit (R&D Systems) according to the manufacturer’s protocol. Activation of caspase-3 and poly(ADP-ribose) polymerase (PARP) cleavage was determined by FACS and Western blot analysis, respectively, according to the manufacturers’ protocols (BD Pharmingen, San Diego, CA). Cellular extracts were assayed for telomerase activity using the PCR-based telomeric repeat amplification protocol assay as described.\(^{33}\)

**Chemotaxis, trans-Matrigel migration, Ca \( ^{2+} \) fluxes, and MMP and VEGF production**

Chemotaxis assays to SDF-1 (PeproTech, Rocky Hill, NJ; R&D Systems) or TPO (R&D) through an 8-μm pore filter were performed in Costar-Transwell 24-well plate (Costar Corning, Cambridge, MA) as described before.\(^{34}\) Results were calculated as a percentage of the input number of cells. All experiments were performed in triplicate.

Chemoattraction of megakaryoblasts across Matrigel was evaluated in a trans-Matrigel migration assay according to a method previously described by us.\(^{35,36}\) The lower chambers were filled with migration media containing 100 ng/mL SDF-1α or TPO, and percentage migration was calculated from the ratio of the number of cells recovered from the lower compartment to the total number of cells loaded. To examine the role of MMPs, specifically MMP-2 and MMP-9, in trans-Matrigel migration, megakaryoblasts were preincubated for 2 hours with 10 μg/mL MMP inhibitors rhTIMP-1 (a gift from Dr Dylan Edwards, University of East Anglia, United Kingdom) or rhTIMP-2 (a gift from Dr Rafael Fridman, Wayne State University, Detroit, MI) before they were loaded onto Boyden chambers, and the trans-Matrigel migration assay was conducted as before. To evaluate MMP secretion by megakaryoblastic cells, 2 \( \times 10^6 \) cells/mL were incubated for 3-24 hours at 37°C in 5% CO\(_2\) in the absence or presence of 100 ng/mL SDF-1 or TPO, and the cell-conditioned media were analyzed immediately by zymography as described previously.\(^{36,37}\) The intensity of the bands was quantitated using the National Institutes of Health ScionImage for Windows software (Scion, Frederick, MD).

Ca\(^{2+} \) flux studies on ex vivo–expanded megakaryoblasts were performed using a spectrophotofluorimeter, as previously described.\(^{10,34}\) Secretion of VEGF by normal human megakaryoblastic cells was evaluated by the Quantikine human VEGF immunoassay (R&D) according to the manufacturer’s protocol, as described.\(^{20}\)

**\( \alpha_{\text{IIb}}\beta_{\text{3}} \) Receptor activation and adherence assays**

Activation of \( \alpha_{\text{IIb}}\beta_{\text{3}} \) receptors was measured using the MoAb PAC-1 (Becton Dickinson) as previously described.\(^{38}\) The \( \alpha_{\text{IIb}}\beta_{\text{3}} \)-positive cells (1 \( \times 10^6 \)) were washed twice with PBS, resuspended in 50 μL PBS plus 2% fetal bovine serum, and treated with the appropriate ligands: thrombin (2 U/mL), SDF-1α (500 ng/mL), or TPO (100 ng/mL) for 5 minutes. Subsequently, 20 μL FITC-conjugated PAC-1 was added, and the cells were incubated for 15 to 20 minutes at room temperature in the dark; RGDG peptide\(^{39} \) was added to confirm specific binding of PAC-1 antibody. After staining, cells were analyzed by FACStar and the Cell Quest program.

Adherence assays of \( \alpha_{\text{IIb}}\beta_{\text{3}} \)-positive cells were performed as described.\(^{39} \) In brief, 96-well microtiter plates (Dynatech Labs) were covered with 4 μg/mL BSA, fibrinogen, vitronectin, fibronecin, or VCAM-1 (all from Sigma), and \( \alpha_{\text{IIb}}\beta_{\text{3}} \)-positive cells (1 \( \times 10^6 \)) were incubated for 30 minutes at 37°C in lymphocyte suspension buffer\(^{39} \) in the absence or presence of SDF-1 (500 ng/mL), TPO (100 ng/mL), SDF-1 + TPO (500 ng/mL + 100 ng/mL), 2 U/mL thrombin or IL-3 (100 ng/mL). Cell suspensions (100 μL) were applied to the wells and incubated for 1 hour at 37°C. The number of adherent cells was estimated by using the colorimetric phosphate assay as described.\(^{40}\)

**Phosphorylation of intracellular pathway proteins**

Western blot analysis was performed on extracts prepared from human \( \alpha_{\text{IIb}}\beta_{\text{3}} \)-positive cells (1 \( \times 10^7 \)), which were kept in RPMI medium containing low levels of BSA (0.5%) to render the cells quiescent. The cells were then divided and stimulated with optimal doses of SDF-1α or SDF-1β (500 ng/mL) or TPO (100 ng/mL) for 1 minute to 2 hours at 37°C, and cells were then lysed for 10 minutes on ice in M-Per lysing buffer (Pierce, Rockford, IL) containing protease and phosphatase inhibitors (Sigma). Subsequently, the extracted proteins were separated on either a 12% or 15% SDS-PAGE gel, and the fractionated proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH) as previously described.\(^{20,34} \) Phosphorylation of each of the intracellular kinases—MAPK p44/42, MAPK p38, JNK MAPK, p90
Table 1. CFU-MK colony formation by human CD34* cells in serum-free, semisolid methylcellulose medium

<table>
<thead>
<tr>
<th></th>
<th>SDF-1</th>
<th>TPO</th>
<th>SDF-1 + TPO</th>
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</thead>
<tbody>
<tr>
<td>100 ng/mL</td>
<td>0 ± 0</td>
<td>87 ± 26</td>
<td>89 ± 31</td>
</tr>
<tr>
<td>25 ng/mL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SDF-1 + TPO</td>
<td></td>
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</tbody>
</table>

Each data entry constitutes 3 independent clonogenic assays from 3 different donors. Data shown are mean ± 1 SD. CFU-MK indicates colony-forming units–megakaryocyte.

RSK, AKT, ELK-1 and STAT-1, -3, -5, and -6—was detected using commercial mouse phosphospecific monoclonal antibody (p44/42) or rabbit phosphospecific polyclonal antibodies for each of the remainders (all from New England Biolabs, Beverly, MA) with horseradish peroxidase–conjugated goat antirabbit IgG or goat antiantibody IgG as a secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA), as described.13,26 Equal loading in the lanes was evaluated by stripping the blots and reprobing them with the appropriate monoclonal antibodies: p42/44 anti-MAPK antibody clone 9102, anti p38 MAPK antibody clone 9212, an anti-JNK antibody clone 9252, an anti-AKT antibody clone 9272, an anti–ELK-1 antibody clone 9182, an anti–STAT-3 9112 (New England Biolabs), an anti–STAT-1 sc-464 and STAT-6 sc-1689 (Santa Cruz Biotechnology), an anti–STAT-5 89 or p90 RSK 78 (Transduction Laboratories, Lexington, KY). The membranes were developed with an ECL reagent (Amersham Life Sciences, Little Chalfont, UK) and subsequently dried and exposed to film (HyperFilm, Amersham Life Sciences). Densitometric analysis was performed using exposures that were within the linear range of the densitometer (Personal Densitometer SI; Molecular Dynamics, Sunnyvale, CA) and ImageQuant software (Molecular Dynamics).

Blocking of PI-3K and MEK

PI-3K and MAPK p42/244 activities were blocked by selective inhibitors. To assess the effects of the PI-3K inhibitor LY294002 (Sigma) or the MEK inhibitor U0126 (Calbiochem, La Jolla, CA), cells were preincubated with each of these compounds for 30 minutes before SDF-1α or TPO stimulation.

Electrophoretic mobility shift assay

To evaluate NF-κB pathways, nuclear extracts were prepared from normal human megakaryoblasts using a modified method of Pan et al,41 and electrophoretic mobility shift assay (EMSA) was performed using 2.5 μg nuclear extract as described previously.41 Oligonucleotides and their complementary strands for EMSA were obtained from Promega (Madison, WI) and Santa Cruz Biotechnology. The sequences were a consensus κB site (underlined), 5′-AGTTGAGGGAGCTTCCCCAGGC-3′ (NF-κB).41,42 gyr32 P/ATP (greater than 500 Ci/mmol) was from Amersham Pharmacia Biotech.

Statistical analysis

Arithmetic means and standard deviations of our FACS and chemotaxis data were calculated on a Macintosh computer PowerBase 180 (Apple, Cupertino, CA), using Instat 1.14 (GraphPad, San Diego, CA) software.

Table 2. Lack of SDF-1 effect on TPO + IL-3–dependent proliferation of human CD34* BM MNCs

<table>
<thead>
<tr>
<th></th>
<th>Suboptimal stimulation by TPO + IL-3</th>
<th>Suboptimal stimulation by TPO + IL-3 + SDF-1</th>
<th>Optimal stimulation by TPO + IL-3</th>
<th>Optimal stimulation by TPO + IL-3 + SDF-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 6</td>
<td>1.2 ± 0.7</td>
<td>1.2 ± 0.8</td>
<td>2.6 ± 0.5</td>
<td>2.7 ± 0.7</td>
</tr>
<tr>
<td>Day 11</td>
<td>3.6 ± 1.9</td>
<td>3.5 ± 2.2</td>
<td>6.8 ± 2.8</td>
<td>6.1 ± 3.5</td>
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Each data entry consists of data from 3 different donors. Data shown represent mean ± 1 SD.

SDF-1 does not affect proliferation of human CFU-MK and megakaryoblasts

It has been unknown whether SDF-1 stimulates human CFU-MK colony formation. In our previous studies, we used a plasma clot system19 that might have contained confounding growth factors. Hence, to remove the unwanted influence of growth factors and cytokines in a serum, we now evaluated the growth of CFU-MK or αmβ3* cells stimulated with SDF-1 or TPO in cultures supplemented with chemically defined artificial serum.18,20,30 SDF-1 was found not to have any effect, either on its own or acting as a costimulator with TPO, on the formation of human CFU-MK colonies from CD34* cells (Table 1). In addition, when human megakaryoblastic αmβ3* cells were expanded ex vivo from CD34* cells (in the presence of a suboptimal or an optimal dose of TPO + IL-3) and SDF-1 was added as a costimulator, we did not observe SDF-1 to affect proliferation or maturation of these cells at days 6 and 11 of culture (Table 2). Neither the number of αmβ3* cells in the cultures nor the intensity of expression of αmβ3* on the cell surface changed (data not shown). Because it has been recently suggested that SDF-1 may stimulate proliferation of CD34* cells if used at very low concentrations,43 we repeated these experiments using low doses of SDF-1 (10 pg to 1 ng/mL) and still did not observe any effect on cell proliferation (data not shown).

Cell cycle analysis of megakaryoblasts growing in the presence of SDF-1 or TPO was also undertaken. The αmβ3* cells expanded for 6 days (in the presence of TPO + IL-3) were transferred to cultures containing no growth factors or to cultures supplemented with TPO + IL-3, TPO, or SDF-1. Within 24 hours of withdrawal of TPO + IL-3, the number of αmβ3* cells in the S and G2/M phases of the cell cycle had decreased by 20% to 30% in comparison with the cultures supplemented with TPO + IL-3 or TPO alone (not shown). More important, the number of αmβ3* cells in the S and G2/M phases of the cell cycle also decreased (by approximately 20%-30%) in the cultures supplemented with SDF-1, as in the cultures depleted of TPO and IL-3 (not shown). Finally, stimulation of αmβ3* cells by SDF-1 did not increase telomerase activity tested by the telomeric repeat amplification protocol assay (data not shown), as it did in the TPO-stimulated cells. These observations together suggest that, unlike what occurs in murine cells,17 SDF-1 does not affect proliferation and maturation of human megakaryoblasts.

SDF-1 does not affect survival of human αmβ3* cells

TPO is a potent antiapoptotic factor for human CD34* cells,1 erythroid progenitors,44,45 and megakaryocytic cells,1 but whether SDF-1 influences the survival of normal human αmβ3* cells is unknown. To address this issue, human αmβ3* cells were cultured for 12 hours in the absence or presence of TPO or SDF-1. We found...
that cells that were withdrawn from TPO + IL-3 underwent apoptosis after 12 hours, as evidenced by an Annexin V binding assay (Figure 1Ai). The presence of TPO in the culture media prevented these cells from undergoing apoptosis (Figure 1Aii), but the presence of SDF-1 did not (Figure 1Aiii). Megakaryoblastic cells cultured in the presence of SDF-1 bound Annexin V to the same extent as cells cultured without TPO + IL-3 (Figure 1Aiii). These data clearly indicate that SDF-1 does not maintain or enhance megakaryopoiesis.

Because PI-3K (a potential target for TPO signaling) plays an important role in inhibiting apoptosis in human hematopoietic cells, normal human megakaryoblasts were exposed to the PI-3K inhibitor LY294002, and we found that the inhibition of PI-3K activity resulted in increases in Annexin V binding of cells (not shown), activation of caspase-3 (Figure 1B) and PARP cleavage (Figure 1C). Of note, inhibition of the MAPK p42/44 pathway by the MEK inhibitor (UO126) did not affect the survival of human megakaryoblasts (not shown).

**SDF-1 but not TPO induces Ca$^{2+}$ flux, chemotaxis, trans-Matrigel chemoaetraction, MMP-9, and VEGF production**

Next we extended our studies to define the roles of SDF-1 and TPO in the homing of human megakaryoblastic cells by examining their effects on chemotaxis, Ca$^{2+}$ fluxes, trans-Matrigel chemotraction, and production of MMP and VEGF.

We have reported that SDF-1 induces Ca$^{2+}$ flux in human megakaryoblasts, and TPO has been shown to enhance the platelet reactivity of other agonists. Because the chemotactic presence of TPO (lane 1), TPO (lane 3). A representative experiment of 3 is demonstrated. (C) Detection of PARP cleavage in normal human megakaryoblasts in the presence of TPO (lane 1), TPO + DMSO (lane 2), and TPO + LY294002 (30 μmol/L) (lane 3). A representative experiment of 3 is demonstrated.

We found that in comparison with SDF-1, which stimulated a measurable Ca$^{2+}$ flux in these cells, TPO added at various concentrations (physiological and high) did not (Figure 2A). Moreover, having previously shown that SDF-1 is a strong chemoattractant for human megakaryocytes, we examined whether TPO could attract these cells as well. We found that TPO, in contrast to SDF-1, did not attract human megakaryoblasts (Figure 2B). Of note, and in agreement with a recent report, SDF-1–induced chemotaxis was inhibited by LY294002 (Figure 2C) but not by
U0126 (not shown), suggesting the involvement of PI-3K in this process. Similarly, an SDF-1 gradient, unlike a TPO gradient, attracted αβ+ cells across the reconstituted basement membrane Matrigel (Figure 2D), and the MMP inhibitors (rhTIMP-1 and rhTIMP-2) reduced this trans-Matrigel chemotaxis by 68% and 52%, respectively (Figure 2D), suggesting that MMPs are involved in this process. We then determined whether αβ+ cells secrete MMPs, especially MMP-9 and MMP-2, and demonstrated for the first time that megakaryocytic cells constitutively secrete MMP-9 and that SDF-1 increases this secretion slightly (Figure 2E). SDF-1–induced trans-Matrigel migration and MMP-9 secretion were inhibited when cells were preincubated with LY 29002 (Figure 2F,G, respectively), suggesting the involvement of PI-3K in both processes.

Because normal human megakaryoblasts have been shown to secrete VEGF15,16 and because endogenously secreted VEGF plays an important role in the transendothelial migration of megakaryocytes,15 we next evaluated whether SDF-1 or TPO has any effect on the secretion of VEGF by normal human αβ+ cells. Table 3 shows that SDF-1, but not TPO, significantly stimulates the secretion of VEGF by these cells. These have been preincubated with LY29402, but not with U0126, did not respond to SDF-1 stimulation with increased VEGF secretion (not shown).

**SDF-1 and TPO activate αβ integrins and adhesion of human megakaryoblasts to fibrinogen and vitronectin**

Adhesion to extracellular ligands is an important step in megakaryocyte migration. Using the PAC-1 binding assay,38 we demonstrated that both SDF-1 and TPO activate αβ integrins on human megakaryoblasts (data not shown), which is consistent with previous findings by others that TPO enhances platelet reactivity51 and with our recent observations that SDF-1 also stimulates it.53 Consistent with these observations, we found that both SDF-1 and TPO increased the adherence of αβ cells to fibrinogen (Figure 3). Both cytokines, if added together, also induced adhesiveness to vitronectin (Figures 3, 4). Of note, adhesion of human megakaryoblasts was inhibited when the cells were pretreated with the PI-3K inhibitor, suggesting again the involvement of PI-3K in this process. Interestingly, the adhesion of human differentiating αβ cells to fibronectin and VCAM-1 was weak and not affected by SDF-1 or TPO (data not shown). Thus, it appears that both SDF-1 and TPO increase the adhesion of megakaryoblasts to their microenvironment.

**Phosphorylation of MAPK (p42/44 and p38) and AKT in normal human megakaryoblasts is induced by SDF-1 and TPO**

To explain the molecular basis of the different biologic effects of SDF-1 and TPO, we examined the intracellular signaling pathways induced by these cytokines in human megakaryocytic cells. It has been reported that the intracellular kinase MAPK p42/44 is phosphorylated in human cell lines, platelets, and murine megakaryoblasts after stimulation by both TPO or SDF-1.23-26 In this study, we examined the MAP kinases (p42/44, p38, and JNK) that have been reported to play an important role in regulating cell proliferation,34 including the intensity and kinetics of their activation in normal human megakaryoblasts. We found that both SDF-1 and TPO induced strong phosphorylation of MAPK p42/44 (Figure 5A); however, after stimulation with SDF-1, it was phosphorylated faster than with TPO (peak at 1 minute for SDF-1 vs 10 minutes for TPO) and more intensely (21-6 vs 11-3-fold increases, respectively) (Figure 5C). We correctly predicted that the activation of MAPK p42/44 should lead to phosphorylation of several MAPK substrates (p90 RSK and ELK-1), and this was confirmed for p90 RSK (Figure 5B) and ELK-1 (not shown). Again, though both SDF-1 and TPO stimulated strong phosphorylation of both substrates, SDF-1 induced an earlier and more intense response.

We next tested whether other members of the MAPK family (p38, JNK) are activated in αβ+ cells by either TPO or SDF-1. To address this question, αβ+ cells were made quiescent by BSA starvation and subsequently were stimulated with TPO or SDF-1; p38 was phosphorylated by both TPO and SDF-1, though the phosphorylation was found to be 3 times more intense after stimulation with SDF-1 (data not shown). In contrast, JNK showed no change in phosphorylation in αβ+ cells stimulated by either TPO or SDF-1 (not shown).

AKT is a serine-threonine kinase that plays an important role in the phosphorylation of several antiapoptotic proteins that may be key to normal hematopoiesis.47-50 It has been reported that integrin stimulation of human αβ+ cells results in the phosphorylation of

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**Table 3. Effects of SDF-1 and TPO on VEGF secretion by human megakaryoblasts (10⁶ cells/mL) cultured for 24 hours in serum-free medium**

<table>
<thead>
<tr>
<th>Condition</th>
<th>VEGF (pg/mL)</th>
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<tr>
<td>SDF-1 100 ng/mL</td>
<td>315 ± 69†</td>
</tr>
<tr>
<td>TPO 100 ng/mL</td>
<td>134 ± 37</td>
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Each data entry consists of 2 independent measurements from 3 different donors. Data shown are mean ± 1 SD. *Sensitivity of the ELISA assay for VEGF was > 5 pg/mL.

†P < .0001 compared to (--) and TPO.
AKT, but whether TPO stimulates AKT as part of its antiapoptotic effect and how this differs from SDF-1 have not been investigated. Examining the kinetics of AKT phosphorylation in megakaryoblastic cells (Figure 6), we observed that AKT is already phosphorylated 1 minute after stimulation by either TPO or SDF-1; however, SDF-1 phosphorylated AKT approximately 3 times more intensely than TPO (36-68- vs 10-64-fold increases, respectively). Moreover, LY294002 abolished SDF-1– or TPO-induced AKT phosphorylation, suggesting that AKT phosphorylation is PI-3K dependent (Figure 6C). Hence, it appears that though intracellular signaling through the c-mpl and the CXCR4 receptors overlaps and leads to phosphorylation of AKT with the same kinetics, one results in an antiapoptotic effect and the other does not. Whether the degree of phosphorylation by the 2 agonists is responsible for the observed difference or whether TPO and SDF-1 differently activate other proteins related to PI-3K–AKT axis requires further analysis.

AKT is also involved in the activation of NF-κB, a regulator of gene transcription. Because NF-κB regulates the expression of MMP-9 and VEGF, and, as we have shown above, the secretion of both these factors was up-regulated after stimulation by SDF-1 (Figure 2D, Table 3, respectively), we hypothesized that SDF-1 might influence NF-κB activation. As predicted, using EMSA, we detected biologically active NF-κB in nuclear extracts isolated from normal human megakaryoblastic cells stimulated by SDF-1 (Figure 7A). At the same time, we established that TPO did not influence NF-κB activation. We also observed that the activation of NF-κB by SDF-1 was PI-3K but not MEK dependent in human megakaryoblasts (Figure 7B).

SDF-1 induces phosphorylation of MAPK p42/44 and AKT in human CD34+ cells but not in platelets

We also investigated the responsiveness of CD34+ cells and circulating platelets to stimulation by SDF-1 or TPO. Examining CD34+ cells, we found that though both factors induced the phosphorylation of MAPK p42/44 and AKT (Figure 8A,B), only TPO stimulated the proliferation of these cells (Table 1) and, as reported previously, protected them from undergoing apoptosis. This observation again suggests that SDF-1 is not primarily directed toward maintaining or enhancing cell proliferation. In contrast to CD34+ and αβ3+ cells, the stimulation of human platelets by SDF-1 under similar conditions did not lead to phosphorylation of MAPK p42/44 and AKT (Figure 9A,B). This observation supports our hypothesis that the responsiveness of CXCR4 to stimulation by SDF-1 (phosphorylation of MAPK
p42/44, and AKT) decreases in the final stages of megakaryopoiesis–thrombocytopoiesis. In contrast, TPO stimulation of human platelets resulted in the phosphorylation of MAPK p42 and AKT (Figure 9).

SDF-1, in contrast to TPO, does not induce tyrosine phosphorylation of STAT family proteins in human megakaryoblasts

Despite the fact that the stimulation of human αβ3+ cells with SDF-1 led to the phosphorylation of MAPK p42/44, p38 and the nuclear protein ELK-1, SDF-1 (as shown above) had no effect on the proliferation or maturation of normal human megakaryoblasts. To understand the molecular basis of these findings, we looked at the activation of the JAK-STAT pathways in normal human αβ3+ cells. STAT proteins have been shown to play an important role in regulating cell proliferation and in signaling from the activated c-mpl receptor in various hematopoietic cell lines and normal human platelets. In particular, the stimulation of human platelets by TPO led to the phosphorylation of STAT-1, STAT-2, STAT-3, and STAT-5 proteins and of STAT-3 and STAT-5 in FDCP-2 cells genetically engineered to constitutively express human c-mpl. However, the effects of SDF-1 on the phosphorylation of STAT proteins in human megakaryocytic cells have not been studied.

We focused our studies on the phosphorylation of STAT-1 and STAT-3 at both Tyr705 and Ser727 and of STAT-3 and STAT-5 in FDCP-2 cells genetically engineered to constitutively express human c-mpl. However, the effects of SDF-1 on the phosphorylation of STAT proteins in human megakaryocytic cells have not been studied.

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SDF-1 enhances the effect of TPO on megakaryocyte formation. However, it does not support recent observations on a murine model suggesting that SDF-1 acts together with TPO to enhance the development of CFU-MK and that SDF-1 at low doses enhances the proliferation of peripheral blood CD34+ cells. We suggest that this difference in results may be due to the different culture systems used (serum-free medium vs serum-supplemented medium) or to the different target cells (human vs murine marrow cells).

We next evaluated the role of SDF-1 and TPO in the homing of human megakaryoblastic cells. We found that TPO, in contrast to SDF-1, does not induce Ca2+ flux and is not a chemoattractant for human αIIbβ3+ cells. We also demonstrated that human megakaryoblasts secrete MMP-9 constitutively and that SDF-1 slightly up-regulates it. Moreover, trans-Matrigel chemotaxis of megakaryoblasts to an SDF-1 gradient was decreased by the tissue inhibitors of MMPs rhTIMP-1 and rhTIMP-2. We also found that the stimulation of normal human megakaryoblasts by SDF-1, but not by TPO, increases endogenous secretion of VEGF by these cells. Because VEGF up-regulates the expression of E-selectin on human endothelium, we postulate that VEGF endogenously secreted by megakaryocytic cells may play an important role in the interaction of megakaryocytes with endothelium, their egress from the bone marrow, and proplatelet formation. Adhesion of human megakaryoblasts to fibrinogen and vitronectin was also increased by SDF-1, and both agonists were synergistic in activating binding of the latter. This supports the hypothesis that both SDF-1 and TPO may regulate the adhesiveness of megakaryoblasts in the hematopoietic microenvironment by activating αIIbβ3 and α5β1 integrins. Our data are also consistent with a recent report showing that SDF-1 activates integrins (αIIbβ3, and α5β1) on the surfaces of human CD34+ cells. Collectively, our data indicate that the homing of immature megakaryoblasts in bone marrow and egress of platelet-releasing megakaryocytes through the endothelial layer and subendothelial basement membranes may be regulated by changes in or responsiveness to an SDF-1 gradient.

To find an explanation at the molecular level for the differences in the biologic effects of SDF-1 and TPO, we investigated signal transduction pathways activated in normal human megakaryoblasts by these cytokines. First, we found that the stimulation of human αIIbβ3+ cells by both TPO and SDF-1 leads to the phosphorylation of the kinases MAPK p42/44 and p38 and their downstream targets.
activity by Ly294002 induced apoptosis in these cells. However, in a PI-3K–dependent manner and that the inhibition of PI-3K have now demonstrated that AKT is phosphorylated in normal human megakaryoblasts that are central to preventing stimulation of these cells. We found that only TPO and SDF-1 differentially activate other proteins related to the PI-3K–AKT axis downstream-regulated proteins, is activated after stimulation by SDF-1. The fact that NF-κB, which is one of the PI-3K–AKT axis downstream-regulated proteins, is activated after stimulation by SDF-1. The fact that NF-κB regulates the expression of MMP-9 and VEGF explains why the stimulation of normal human megakaryoblasts by SDF-1 leads to increased endogenous MMP-9 and VEGF secretion.

Because TPO has been shown to be crucial for the proliferation and differentiation of developing murine megakaryocytes through JAK-STAT pathways, we investigated whether similar pathways are activated after SDF-1 stimulation. Identification of these pathways could shed more light on the regulation of proliferation of normal human megakaryocytes and explain at a molecular level why TPO and not SDF-1, as demonstrated in this study, stimulated the proliferation of these cells. We found that only STAT-3 was phosphorylated at the serine residue (Ser727) after SDF-1 stimulation, in contrast to much of the STAT family of proteins, which are phosphorylated by TPO at tyrosine residues in megakaryocytes. Because it has been suggested that the phosphorylation of STAT-3 at Ser727 may play a role in the down-regulation of STAT-3 protein activation, the phosphorylation of STAT-3 at Ser727 by SDF-1 suggests that it may down-regulate STAT-3 in normal human megakaryoblasts. Hence, we suggest that the tyrosine phosphorylation of JAK-STAT proteins probably plays a crucial role in the proliferation of megakaryocytic cells after TPO stimulation but that activation of the MAPK p42/44 and p38 pathways is not an important intermediate step in the proliferation of these cells given that SDF-1 also activates them. Of note, we found that the inhibition of MEK by U0126 affected neither survival nor proliferation of human megakaryoblasts. These data are consistent with recent studies showing that the MAPK p42/44 pathway is not required for megakaryoblast formation, though it may regulate the transition from proliferation to maturation in this lineage.

In contrast, the phosphorylation of MAPK p42/44, p38, and AKT after stimulation with SDF-1 does not occur in human platelets, and we find this intriguing. We suggest that the differences between human megakaryoblasts and platelets in the composition of G and RGS proteins, coupled to the particular chemokine receptor, could explain these differences.

In summary, we demonstrated that though both TPO and SDF-1 are important in megakaryopoiesis and stimulate some of the same intracellular pathways, they have distinct biologic effects on human megakaryocytic cells. SDF-1, but not TPO, regulates some steps in the migration of these cells in the hematopoietic microenvironment (eg, chemotaxis and secretion of MMP-9 and VEGF). In contrast, TPO, but not SDF-1, permits growth of megakaryocyte precursors (eg, by enhancing proliferation and by inhibiting apoptosis); both factors regulate their adhesion. Hence, this study sheds light on the relation between 2 distinct cytokine axes critical in human megakaryopoiesis and the molecular basis of the observed differences in cellular responses.

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