Protein Phosphatase 2A Plays an Important Role in Stromal Cell-Derived Factor-1/CXC Chemokine Ligand 12-Mediated Migration and Adhesion of CD34<sup>+</sup> Cells

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Protein Phosphatase 2A Plays an Important Role in Stromal Cell-Derived Factor-1/CXC Chemokine Ligand 12-Mediated Migration and Adhesion of CD34+ Cells

Sunanda Basu,$Nicole T. Ray, Simon J. Atkinson, and Hal E. Broxmeyer

Migration of hemopoietic stem and progenitor cells (HSPC) is required for homing to bone marrow following transplantation. Therefore, it is critical to understand signals underlying directional movement of HSPC. Stromal cell-derived factor-1 (SDF-1)/CXCL12 is a potent chemoattractant for HSPC. In this study, we demonstrate that the serine-threonine protein phosphatase (PP)2A plays an important role in regulation of optimal level and duration of Akt/protein kinase B activation (a molecule important for efficient chemotaxis), in response to SDF-1. Inhibition of PP2A, using various pharmacological inhibitors of PP2A including okadaic acid (OA) as well as using genetic approaches including dominant-negative PP2A-catalytic subunit (PP2A-C) or PP2A-C small interfering RNA, in primary CD34+ cord blood (CB) cells led to reduced chemotaxis. This was associated with impairment in polarization and slower speed of movement in response to SDF-1. Concomitantly, SDF-1-induced Akt phosphorylation was robust and prolonged. Following SDF-1 stimulation, Akt and PP2A-C translocate to plasma membrane with enhanced association of PP2A-C with Akt observed at the plasma membrane. Inhibition of PI3K by low-dose LY294002 partially recovered chemotactic activity of cells pretreated with OA. In addition to chemotaxis, adhesion of CD34+ cells to fibronectin was impaired by OA pretreatment. Our study demonstrates PP2A plays an important role in chemotaxis and adhesion of CD34+ CB cells in response to SDF-1. CD34+ CB cells pretreated with OA showed impaired ability to repopulate NOD-SCID mice in vivo, suggesting physiological relevance of these observations. The Journal of Immunology, 2007, 179: 3075–3085.

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THOUGH CORD BLOOD (CB)1 contains significant numbers of hemopoietic stem and progenitor cells (HSPC), its applicability in HSPC transplantation remains largely for use in children (1). Enhanced homing of CB HSPC has the potential to increase applicability of CB transplantation in adults (2). Stromal cell-derived factor-1 (SDF-1)/CXCL12 is an important player in HSPC homing and engraftment (3–5). SDF-1 binds to CXCR4, stimulating a series of intracellular events downstream of this G protein-coupled receptor (GPCR) (6–8). Major processes regulated by the SDF-1/CXCR4 axis in vivo are highlighted in Fig. 1 (9–11). The importance of SDF-1 in homing and engraftment of HSPC is demonstrated by immunomaging experiments that suggest that specific microvascular domains in bone marrow express high levels of E-selectin and SDF-1; in vivo colocalization studies confirmed that HSPC were restricted to these vessel beds (12). Because chemotaxis is indispensable to homing, understanding molecular mechanisms underlying SDF-1-directed chemotaxis of HSPC is crucial for designing more efficient HSPC transplantation protocols.

Initiation of chemotaxis requires binding of a chemoattractant to its receptor, which results in activation of the PI3K pathway. Activation of PI3K leads to generation of phosphatidylinositol (3,4) biphosphate and phosphatidylinositol (3–5) trisphosphate that recruit various pleckstrin homology (PH) domain-containing proteins, including Akt (13–15). Recruitment of PH domain-containing proteins, which bind phosphoinositides on the membrane, and their regulation, is a key event, downstream of G protein activation, leading to pseudopodia production (16). Although activation of Akt is critical for efficient cell movement, it is transient and highly regulated (17).

A balance of kinases and phosphatases is necessary for cells to maintain appropriate transducing signal levels (17). In Dictyostelium, in addition to PI3K, phosphatase and tensin homologue (PTEN) determines level and duration of phosphatidylinositol (3–5) trisphosphate production (18), which in turn regulates duration of association of PH domains with the membrane. PTEN, therefore, indirectly regulates activity of various PH domain-containing proteins, including Akt, whose activity is tightly regulated during chemotaxis (14). Although PI3K and its downstream target, Akt, have been shown to play an important role in chemotaxis of mammalian leukocytes (19–21), the complete regulation of this pathway is not yet clearly understood. For example, there are conflicting reports on the role of PTEN in chemotaxis of mammalian leukocytes (22, 23). In addition to PTEN, Akt activation is also regulated by protein phosphatase (PP)2A (24). PP2A is a multimeric serine-threonine phosphatase that is highly conserved in eukaryotes (25). It is a holoenzyme consisting of a heterotrimer of 65-kDa structural subunit A, 55- to 130-kDa regulatory subunits.

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In this study, we investigated a role for PP2A in SDF-1-mediated responses of primary human CD34+ CB cells, a population enriched for HSPC. Because limited numbers of cells are obtained from each CB collection and CD34+ cells are present at low frequency in CB, it is difficult to get enough of these rare cells to do in-depth molecular analysis of intracellular signaling with them. Most such studies have been done with established cell lines, casting doubt as to the relevance of such information in terms of primary cell function. Thus, it is extremely important that these studies be done in primary cells. For these reasons, we chose to evaluate an intracellular signaling role for PP2A in SDF-1-mediated chemotaxis and adhesion in primary CD34+ CB cells. To compensate for low numbers of primary CD34+ cells, we expanded these cells ex vivo, with maintenance of cellular function, and used them for intracellular biochemical analysis. Our study elucidates a role for a previously unrecognized intracellular signaling molecule that mediates SDF-1-dependent cell migration and adhesion in primary CD34+ CB cells. Inhibition of PP2A in primary CD34+ cells isolated from umbilical CB led to prolonged SDF-1-induced Akt phosphorylation; this was associated with impaired chemotaxis and SDF-1-stimulated adhesion of CD34+ cells to fibronectin, suggesting PP2A plays an important role in SDF-1-mediated functional responses of CD34+ CB cells.

Materials and Methods

Reagents

The following Abs were used for cell staining: FITC-conjugated CD34 (Miltenyi Biotec), PE-conjugated CXCR4, and allophycocyanin-conjugated CD38 (BD Pharmingen). For confocal microscopy, anti-PP2A-catalytic subunit (PP2A-C) (Upstate Biotechnology) and anti-CXCR4 Ab (Santa Cruz Biotechnology) were used. Phalloidin rhodamine was purchased from Invitrogen Life Technologies. Abs used for Western blot were anti-phospho-ERK1/2 mouse mAb, ERK1/2 rabbit polyclonal Ab, anti-phospho-Akt (Ser473 and Thr308) rabbit Ab and corresponding vector alone (pcDNA) (a gift from Dr. H. Nakshatri, Indiana University School of Medicine, Indianapolis, IN) were cotransfected with plasmid-encoding GFP (pmaxGFP, Amaxa) in pooled freshly isolated CD34+ cells using Amaxa Nucleofection kit. A total of 2–3 µg of each plasmid (pcDNA and pcDNAACAkt) and 0.75 µg of GFP encoding plasmid was used per transfection. Following transfection, cells were cultured for 20 h in medium containing above mixture of cytokines. After 20 h, cells were sorted on the basis of GFP expression; some of the sorted cells from each transfection were used for chemotaxis assay, and the rest of the cells were lysed and analyzed by Western blot.

Migration assay

Chemotaxis assays were performed using 24-well chemotaxis chambers, pore size 5.0 µM (Corning Costar). CD34+ cells in 100 µl of chemotaxis medium (IMDM plus 1% BSA) were added to the membrane. Chemotaxis medium alone (600 µl) or containing 200 ng/ml SDF-1 was added to bottom well. After 4 h at 37°C in 5% CO2, cells migrated to the lower chamber and input cells were counted for 30 s using FACScan under identical flow conditions. In a few experiments, SDF-1 was added to both the upper and lower well, and the movement of cells was assessed, as described above.

Time-lapse video microscopy and motility analysis using Dunn chamber

Motility of CD34+ cells was recorded using Dunn chamber (30). Glass coverslips were coated with Retronectin (human fibronectin fragment CH296; Takara Shuzo) and blocked using 2% BSA. CD34+ cells were allowed to adhere for 15 min at 37°C. Coverslips were mounted on the Dunn chamber with a gradient of 0–200 ng/ml SDF-1 between the inner and outer wells of the chamber. Cell positions were tracked using particle-tracking capabilities in Metamorph 6.1 software (Universal Imaging). Length and breadth of cells were measured using Photoshop (Adobe Systems). Ratio of length to breadth expresses extent of polarization of cells in response to SDF-1.

Progenitor cell colony assay

Methycellulose progenitor assay was performed, as described (31).

Adhesion assays

Adhesion assay was performed, as described (28).

Confocal microscopy

Effect of SDF-1 stimulation on subcellular localization of Akt/Pkt (Thr308) was studied using Zeiss LSM510 confocal microscope using ×63 objective lens and 5×–10× digital zoom. After 20 h of cytokine-expanded CD34+ cells were stimulated with SDF-1 (200 ng/ml) for various lengths of time, permeabilized with formalin, and fixed using methanol. The cells were then stained with either Akt or pAkt (Thr308) Ab (Cell Signaling Technology), washed, and stained with anti-rabbit Alexa 488 Ab ( Molecular Probes). Then cells were stained with PP2A-C Ab (Upstate Biotechnology), washed, and stained with anti-rabbit Alexa 647 Ab. Finally, cells were stained with 4′,6-diamidino-2-phenylindole.

Western blot analysis and coimmunoprecipitation

Whole cell protein lysates were prepared, and Western blot was done, as described (28). For coimmunoprecipitation, cells were treated, washed with PBS, and lysed in radioimmunoprecipitation assay buffer (50 mM Tris-HCl

Expansion and pretreatment of CD34+ CB cells

Enriched CD34+ cells were expanded using 100 ng/ml recombinant human thrombopoietin, 100 ng/ml human Flt-3 ligand, and 50 ng/ml recombinant human stem cell factor, as described previously (28). Freshly isolated or cytokine-expanded CD34+ cells were pretreated with OA or medium alone (IMDM plus 2% BSA) for 40 min, washed, and used. In experiments evaluating the effect of P38K inhibitor LY294002 on inhibition of chemotaxis by OA, cells were pretreated with the inhibitor(s) and then used directly in chemotaxis assay.

PP2A activity

PP2A activity of OA-pretreated or untreated CD34+ cell lysates was measured using specific phosphopeptide and buffering conditions, as per manufacturer’s instructions (Promega).

Transfection of CD34+ cells with small interfering RNA (siRNA) and plasmid

We used siRNA SMARTpool PP2A-C, α isoform from Upstate Biotechnology/Chemicon International for silencing α PP2A-C subunit in freshly isolated CD34+ CB cells. Transfection was done by electroporation using the Nucleofection System (Amaxa), according to the manufacturer’s instructions. Cells were transfected with 2 µg of oligonucleotides with control siRNA (Qiagen) or PP2A-C siRNA under identical conditions and cultured for 40 h. Control siRNA was labeled with fluorescein and used to monitor transfection efficiency. In cells transfected with control siRNA, >90% of cells were found to be fluorescein positive by flow cytometer, 4 h posttransfection. For the first 20 h, cells were kept in mixture of cytokines described above. For the next 20 h, cytokine concentrations were halved to slow cell proliferation. After 40 h, cells were analyzed for chemotaxis. Protein expression was studied using Western blot. Plasmins encoding wild-type (WT) PP2A-C or dominant-negative mutant of PP2A-C (DNPP2A-C; provided by Dr. B. A. Hemmings, Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland) or constitutively active Akt (CA-Akt) (29) and corresponding vector alone (pcDNA) (a gift from Dr. H. Nakshatri, Indiana University School of Medicine, Indianapolis, IN) were cotransfected with plasmid-encoding GFP (pmaxGFP, Amaxa) in pooled freshly isolated CD34+ cells using Amaxa Nucleofection kit. A total of 2–3 µg of each plasmid (pcDNA and pcDNAACAkt) and 0.75 µg of GFP encoding plasmid was used per transfection. Following transfection, cells were cultured for 20 h in medium containing above mixture of cytokines. After 20 h, cells were sorted on the basis of GFP expression; some of the sorted cells from each transfection were used for chemotaxis assay, and the rest of the cells were lysed and analyzed by Western blot.

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isolated and cytokine-expanded CD34 cells. Results from a representative experiment in which PP2A-C enzymatic activity in CD34 cell suspensions pooled from three CB collections were determined, as described in Materials and Methods. B, Viability of freshly isolated CD34 cells pretreated with OA (i) or with medium (ii) 1 μM OA alone cultured in IMDM containing 2% BSA alone (☐) or along with 200 ng/ml SDF-1 (■). Viability cell counts were enumerated using trypan blue. Independent wells were used for each day cell count. Data are from one of two independent experiments.

(pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, and 2 mM EDTA) containing protease inhibitor mixture (Roche), sodium fluoride, and sodium vanadate. Immunoprecipitation was done using anti-PP2A-C or anti-Akt Abs using catch and release kit (Chemicon International). Proteins in the immunocomplex were resolved on 8% SDS-PAGE.

Mice
NOD-SCID mice were bred and maintained in ventilated sterile microisolation cages. Experiments were approved by Animal Ethics Committee of Indiana University. Eight-week-old mice were given a sublethal dose of 300 cGy from a cobalt source 4 h before transplantation. Freshly isolated NOD-SCID CB cells were preincubated with OA or medium alone for 40 min and washed twice with PBS. Cells were then injected i.v. Mice were sacrificed after 30–35 days (32). Bone marrow cells were flushed from femurs, and percentages of human cells were determined by staining cells with anti-human CD45-allophycocyanin Ab (BD Pharmingen). Various blood cell lineages were determined with anti-human CD34-FITC, CD38-FITC, CD33-PE, and CD19-FITC Abs (BD Pharmingen).

Statistical analysis
Data are represented as mean ± SD, unless indicated otherwise. Student’s two-tailed t test was used for statistical analysis. Level of significance is indicated by p value.

Results
Due to limited numbers of CD34+ cells present in CB collections for intracellular biochemical analysis of signal transduction events, we expanded these cells ex vivo using thrombopoietin, Flt-3 ligand, and stem cell factor. After 4 days of expansion, cells still remain essentially CD34+ (28). To verify intact cellular responses, effects on chemotaxis and adhesion were performed with freshly isolated and cytokine-expanded CD34+ cells.

Effects of OA on CD34+ CB cells
OA is a cell-permeable molecule that inhibits PP2A in vitro at 100-fold lower concentrations than PP1 (33). Binding of OA to PP2A-C is tight and withstands a lysis process (34). OA has a 50% inhibitory concentration of 1 nM in cell-free systems; however, much higher concentrations are required to observe its effects using intact cells. This is due to relatively inefficient transport of this molecule into the cell and higher concentrations of PP2A in some cells (26, 33, 35). Concentrations of OA up to 1 μM preferentially inhibit PP2A enzymatic activity in cells, without detectable intracellular inhibitory effects on PP1, PP2B, or PP2C (26). As seen in Fig. 1A, PP2A activity in the soluble fraction of lysates from CD34+ cells pretreated with various concentrations of OA was reduced by 60–70% with 100 nM OA, with further reductions at 1 μM. Viability of CD34+ cells was unaffected by OA pretreatment when cells were cultured in medium containing 20% FCS without or with SDF-1 (52.0 ± 3.2% and 73.6 ± 2.0% for medium vs 51 ± 3% and 61.5 ± 2% for OA, without and with SDF-1, respectively). Similarly, after 3 days in culture in 2% BSA, no significant difference in survival of CD34+ cells, cultured in the absence or presence of SDF-1, was observed between OA-pretreated and control CD34+ cells (Fig. 1B, i and ii).

Effect of PP2A inhibition on chemotaxis of CD34+ cells toward SDF-1
Pretreatment with OA inhibited chemotactic response of both freshly isolated (Fig. 2Ai) and cytokine-expanded (Fig. 2Aii) CD34+ cells to SDF-1. The effect was dose dependent; chemotactic activity was reduced by 50% with 100 nM OA, and reduced up to 90% with 1 μM OA (Fig. 2B). OA pretreatment affected SDF-1-directed chemotaxis without effects on chemokinesis of the CD34+ cells. In the absence of a net SDF-1 gradient, motility of the cells pretreated with OA was comparable to background chemotaxis. As additional controls for effects of PP2A, we evaluated fostriecin and inhibitor-2, other serine-threonine phosphatase inhibitors, on chemotaxis of CD34+ cells. Fostriecin, an antitumor
antibiotic, is a highly selective inhibitor of PP2A and inhibits PP2A at 10,000–40,000 times lower concentrations than that required for inhibition of PP1 in vitro (36, 37). Fostriecin, when used at 5 μM, inhibited chemotaxis of CD34+ cells toward SDF-1 to a level comparable to that observed with 100 nM OA. In contrast, inhibitor-2, a selective inhibitor against PP1 in vitro and in vivo (38, 39), did not affect chemotaxis of CD34+ cells at concentrations up to 2 μM (Fig. 2B). These findings suggested that PP2A plays a role in SDF-1-directed chemotaxis of CD34+ CB cells.

To extend our above noted findings that used pharmacological inhibitors to more specific inhibition of PP2A, we transfected...
Freshly isolated CD34⁺ CB cells with WT PP2A-C or a DNPP2A-C (H118N) demonstrated 70% decrease in migration to SDF-1 compared with cells transfected with WT PP2A-C (Fig. 2C). We also evaluated CD34⁺ CB cells transfected with control siRNA or PP2A-catalytic subunit (PP2A-C) siRNA. Forty hours after transfection, expression of PP2A-C was reduced by 40% (Fig. 2D). This was accompanied by significantly reduced (35 ± 0.02%) chemotaxis of CD34⁺ CB cells toward SDF-1 (Fig. 2Di). PP1 was unaffected by PP2A-C siRNA transfection (Fig. 2Dii), demonstrating the specificity of PP2A-C siRNA. These findings confirm our observations using pharmacological inhibitors, and clearly establish that specific inhibition of PP2A impairs migration of CD34⁺ CB cells to SDF-1.

CD34⁺ cells are enriched, but not purified for HSPC. We, therefore, also assessed whether OA had any effects on SDF-1-induced migration of progenitor cells in CD34⁺ populations, as determined by colony assays for erythroid, multipotent, and granulocyte-macrophage progenitor cells. SDF-1-induced chemotaxis of CD34⁺ cells was significantly decreased (Fig. 2B) by OA pretreatment, but its inhibitory effects were similar for the different progenitor cells because it did not change the ratio of various progenitors in the migrated cell populations (Fig. 2Ei). As seen in Fig. 2Eii, OA did not affect the numbers or ratio of the input progenitor cell populations. Similarly, the proportion of phenotypically defined immature CD34⁺/CD38⁻/low and mature CD34⁺/CD38⁺ progenitors that migrated in response to SDF-1 was unaffected by OA pretreatment (medium, 88.97 and 7.89% vs OA, 88.92 and 8.59%). Thus, the inhibitory effect of OA on SDF-1-induced chemotaxis is similar for primitive and more mature progenitors, by both phenotypic and functional analysis.

**Effect of OA on adhesion of CD34⁺ cells to fibronectin**

Homing requires integrin-mediated adhesion interactions of HSPC with endothelium. In addition, adhesion to stroma is required for proper engraftment and retention of HSPC in bone marrow (41). SDF-1-mediated adhesion of freshly isolated (Fig. 3i) and cytokine-expanded (Fig. 3ii) CD34⁺ cells to fibronectin was significantly reduced by pretreatment of cells with OA.

**OA impairs ability of CD34⁺ cells to polarize**

Chemoattractant regulation of actin polymerization is a major event in remodelling of cytokskeleton and is the driving force behind directed cell movement (42). SDF-1-induced F-actin polymerization in CD34⁺ cells was unaffected by OA pretreatment of fresh and expanded cells (data not shown). To understand why OA-pretreated CD34⁺ cells do not migrate efficiently toward SDF-1 despite being able to undergo SDF-1-induced F-actin polymerization as efficiently as untreated CD34⁺ cells, we evaluated the ability of cells to polarize in response to SDF-1. Motility experiments using Dunn chamber demonstrated that OA pretreatment impaired the ability of the cells to polarize in response to SDF-1 (Fig. 4, A and B). The speed of movement was also reduced when cells were pretreated with OA (Fig. 4C). To further confirm that inhibition of PP2A was responsible for reduced speed of movement of CD34⁺ cells toward SDF-1, CD34⁺ cells transfected with either WT
PP2A-C or DNPP2A-C were evaluated for their chemotactic activity using Dunn chamber. Similar to OA-pretreated cells, CD34\(^+/\)H11001 cells transfected with DNPP2A-C also exhibited reduced speed of movement compared with cells transfected with WT PP2A-C (Fig. 4D).

OA results in prolonged SDF-1-induced Akt phosphorylation

Because pretreatment of CD34\(^+/\) cells with OA results in reduced chemotactic activity without influencing expression of CXCR4, we evaluated intracellular signaling events. SDF-1 induces Akt and ERK phosphorylation (7, 28). Both pathways have been implicated in chemotaxis (20, 28). Stimulation of CD34\(^+/\) cells with SDF-1 enhanced phosphorylation of Akt at Ser473 and Thr308 (Fig. 5Ai).

To measure the net change in phosphorylation of Akt in response to SDF-1 stimulation, we calculated the ratio of phosphorylated Akt to total Akt for each time point and then determined the fold change in phosphorylation following SDF-1 stimulation in relation to baseline values (Fig. 5Aii, iii and iv). Although SDF-1-induced phosphorylation of Akt at Ser473 appeared to be elevated more in OA-pretreated compared with control cells (Fig. 5A, i and ii), due to large intersample variability, the difference (both the fold change and kinetics) did not attain statistical significance. Akt phosphorylation at Thr308 was significantly higher and more prolonged in cells pretreated with OA (Fig. 5A, i and iii). At 10 min post-SDF-1 stimulation, pAkt (Thr308) returned to near basal level in control cells, whereas the level of pAkt (Thr308) was significantly elevated and
nearly 1.5-fold higher than basal level in OA-pretreated cells. SDF-1 stimulation of OA-pretreated and untreated CD34⁺ cells led to enhanced ERK1/2 phosphorylation that peaked at 3 min post-SDF-1 stimulation and thereafter declined (Fig. 5Bi). OA pretreatment did not significantly alter the time-related enhancement of SDF-1-induced ERK1/2 phosphorylation (Fig. 5Bii), although ERK1/2 phosphorylation at baseline was more robust in OA-pretreated cells.

Interaction of Akt with PP2A and β-arrestin-2 following SDF-1 stimulation

The importance of transient Akt activation for effective chemotaxis has been demonstrated in Dictyostelium (14). Because OA pretreatment of CD34⁺ cells resulted in impaired chemotaxis and prolonged Akt phosphorylation, we explored the effect of sustained activation of Akt on chemotaxis of CD34⁺ cells toward SDF-1. CD34⁺ cells were cotransfected with plasmids expressing CAAkt (or control vector), and GFP-CD34⁺ cells expressing CAAkt were identified by their GFP expression (Fig. 6A). Approximately 35–40% of cells were transfected. Twenty hours after transfection, GFP⁺ cells from both cultures (CD34⁺ cells transfected with control vector and CAAkt) were sorted and analyzed for chemotaxis. Overexpression of CAAkt in CD34⁺ cells (Fig. 6Aii) resulted in 49% inhibition of chemotaxis of CD34⁺ cells toward SDF-1 (Fig. 6Aiii). CAAkt expression did not affect chemokinesis or background movement of CD34⁺ cells (results not shown).

Serine-Threonine protein phosphatases negatively regulate Akt activity in various cellular systems (27, 43). Because OA pretreatment resulted in significantly greater and more prolonged Akt phosphorylation at Thr308 in response to SDF-1 stimulation, we considered it likely that inhibition of serine-threonine phosphatase by OA affected Akt dephosphorylation. To better understand regulation of Akt by PP2A, we performed coimmunoprecipitation experiments on cytokine-expanded CD34⁺ cells, because small numbers of CD34⁺ cells in a CB collection make it almost impossible to perform coimmunoprecipitation experiments using freshly isolated CD34⁺ cells. Coimmunoprecipitation experiments demonstrated that Akt and PP2A-C exist in a complex in CD34⁺ cells, and SDF-1 stimulation increases the amount of PP2A-C associated with Akt (Fig. 6, B and C). PP1 was not associated with Akt, in either unstimulated cells or in cells stimulated with SDF-1 (data not shown). In agreement with other reports (14, 15), Akt (Fig. 6D, a–e) and phosphorylated Akt (Thr308) (Fig. 6Df) translocated to the plasma membrane of CD34⁺ cells following SDF-1 stimulation. PP2A-C also translocated to the plasma membrane upon SDF-1 stimulation. These latter findings extend our coimmunoprecipitation observations and suggest that SDF-1-enhanced association of Akt with PP2A-C occurs primarily at the plasma membrane.

In addition to a classical role in desensitization of GPCR signaling, β-arrestin-2 also acts as a scaffolding protein, linking GPCR to a second wave of cell signaling via different signaling pathways (44, 45). β-arrestin-2 was found in the Akt-PP2A complex after, but not before, SDF-1 stimulation (Fig. 6B).

Inhibition of PI3K leads to partial recovery of chemotaxis in OA-pretreated CD34⁺ cells

Because enhanced and prolonged Akt phosphorylation is at least in part responsible for inhibition of chemotaxis of CD34⁺ cells, we reasoned that inhibition of Akt phosphorylation in OA-pretreated cells may partially recover their chemotactic activity. Akt phosphorylation is positively regulated by PI3K and is negatively regulated by PP2A (24). To test whether reduction of Akt phosphorylation in OA-pretreated cells indeed recovers their chemotactic activity toward SDF-1, we evaluated effects of PI3K inhibition on SDF-1-directed chemotaxis. Pretreatment of CD34⁺ cells with LY294002, a specific inhibitor of PI3K (46), resulted in reduced chemotactic response of CD34⁺ CB cells toward SDF-1. However, it indeed led to partial recovery of chemotactic activity in OA-pretreated CD34⁺ CB cells (Fig. 7A). This effect was observed only when low-dose (2 μM) LY294002 was used. Pretreatment of CD34⁺ cells with low-dose LY294002 along with OA cells also led to a reduction in Akt phosphorylation at both Thr308 and Ser473 compared with cells pretreated with OA alone following SDF-1 stimulation (Fig. 7B). These findings demonstrate that PI3K is stimulated in these cells following SDF-1 stimulation and PP2A activity is required to regulate optimal levels and duration of Akt phosphorylation.
Inhibition of GSK3 leads to reduced SDF-1-directed chemotactic activity

To determine how prolonged Akt phosphorylation might result in reduced chemotactic activity, we assessed the activation status of its substrates GSK3α and GSK3β. In addition to its important role in metabolism and proliferation (47), GSK3 plays an important role in cell migration (48). GSK3 activity is negatively regulated by phosphorylation at serine residues (49). PP2A has been reported to directly dephosphorylate GSK3β purified from rabbit skeletal muscle (50). Consistent with this report (50), we found that inhibition of PP2A by OA pretreatment led to increased level of GSK3 phosphorylation, particularly at 1 μM OA, and this was further enhanced upon SDF-1 stimulation (Fig. 7C). Because PP2A directly regulates GSK3 activity (51) and also dephosphorylates Akt (24, 50), we evaluated whether activated Akt in SDF-1-stimulated OA-pretreated cells played a role in GSK3 phosphorylation. Pretreatment of cells with 2 μM LY294002, even in the presence of 1 μM OA (Fig. 7B), resulted in decreased Akt phosphorylation with concomitant reduction in GSK3 phosphorylation at Ser21/9 sites in response to SDF-1. This demonstrates that inhibition of Akt phosphorylation leads to activation of GSK3 following SDF-1 stimulation, and is consistent with our observation that GSK3 associates with Akt in CD34+ cells upon SDF-1 stimulation (Fig. 7B). Moreover, in CD34+ cells transfected with CAAkt, although the direct interaction of CAAkt with GSK3 could not be evaluated due to limited numbers of CD34+ cells, GSK3 phosphorylation was enhanced in cells transfected with CAAkt. This further suggested that activated Akt associates with GSK3 and phosphorylates it (Fig. 6Aii). Unlike the interaction of GSK3 with Akt, which was only observed upon SDF-1 stimulation, interaction of GSK3 with PP2A-C was detected in both SDF-1-unstimulated and -stimulated CD34+ cells (Fig. 6C). Pretreatment of CD34+ CB cells with the specific GSK3 inhibitor, GSK3 inhibitor IX, significantly decreased SDF-1-directed chemotaxis (Fig. 7D) without affecting cell viability (data not shown), confirming that GSK3 plays a role in SDF-1-directed chemotaxis. Thus, Akt interacts with GSK3 in CD34+ cells following SDF-1 stimulation, and inhibition of GSK3 activity impairs chemotaxis of CD34+ cells.

OA pretreatment reduces engraftment of CD34+ cells in NOD-SCID mice

SDF-1 plays an important role in migration (homing) and retention of HSPC in bone marrow (3, 4, 12). NOD-SCID mice are a useful model to evaluate the engrafting properties and stem cell characteristics of human CD34+ cells (52). Consistent with our in vitro findings, inhibition of PP2A by OA significantly reduced engraftment of CD34+ cells in NOD-SCID mice (Fig. 8, i and ii), without impairing the ability of transplanted cells to give rise to different cell lineages (Fig. 8B). This suggests an involvement of PP2A in the in vivo engraftment of human CD34+ CB cells in NOD-SCID mice.

Discussion

SDF-1 is the most potent known chemoattractant for both human and mouse HSPC. Homing and engraftment of mouse and human HSPC are SDF-1 dependent (2–5, 12). However, much remains to be determined regarding the intracellular signaling molecules responsible for directional movement of HSPC toward SDF-1. Our report not only directly implicates PP2A in this effect through both pharmacological inhibitors and more specific PP2A inhibition using genetic approaches, but also elucidates the intracellular molecules involved in this regulation. Most importantly, the cellular and intracellular studies were done with primary cells highly enriched for HSPC. Such intracellular signaling molecule interactions have rarely, if ever, been previously studied with these primary cells because of their rareness and limiting numbers. We have now shown that PP2A is an important player in SDF-1-mediated chemotaxis and adhesion of human HSPC in vitro. Inhibition of PP2A impairs ability of CD34+ cells to polarize in response to SDF-1. PP2A activity following SDF-1 stimulation is required for maintaining appropriate levels and duration of Akt phosphorylation for effective chemotaxis.
Although activation of Akt/PKB by chemoattractants plays an important role in controlling aspects of cell polarity and cell movement, inhibition of PI3K, using low-dose LY294002, leads to partial recovery of chemotactic response in cells in which PP2A activity has been inhibited. Concentrations of OA up to 1 μM can be used to selectively inhibit PP2A without detectable intracellular inhibitory effect on PP1, PP2B, or PP2C. In addition to OA, by using fostriecin, a more specific PP2A inhibitor, and DNPP2A-C as well as PP2A-C siRNA, we confirmed that inhibition of PP2A impairs chemotaxis of CD34+ cells toward SDF-1, and that this was specific for PP2A. Neither PP2A-C siRNA nor DNPP2A-C affected either CXCR4 or integrin molecule expression on CD34+ cells (data not shown).

Inhibition of SDF-1-mediated chemotaxis and adhesion by OA pretreatment was not due to differential changes in CXCR4 expression upon SDF-1 stimulation (data not shown). However, Akt phosphorylation at Thr308 was more robust and prolonged in OA-pretreated cells stimulated with SDF-1 and most likely a reason for impaired SDF-1-mediated chemotaxis upon OA pretreatment. Expression of CAAkt impairs chemotaxis in Dictyostelium (14); the same occurred when we expressed CAAkt (an Akt variant lacking its PH domain) in CD34+ cells, Akt phosphorylation is modulated following SDF-1 stimulation, and PP2A plays an important role in regulating both the levels and duration of Akt phosphorylation. Immunoprecipitation experiments demonstrated that there is some basal association of Akt with PP2A in CD34+ cells, Akt phosphorylation is modulated following SDF-1 stimulation, and PP2A interacts with Akt, leading to a decrease in Akt activity. Interaction of PP2A with Akt was not affected by OA pretreatment; however, interaction of GSK3 with Akt seemed to be reduced in OA-pretreated cells. The reason for this is unclear. Increased translocation of Akt and PP2A to the plasma membrane was apparent after SDF-1 stimulation. The kinetics of Akt phosphorylation in CD34+ cells following stimulation with SDF-1 was slightly different from what has been described for other cells. This difference in kinetics of Akt translocation is most likely cell-type dependent. Optimal Akt phosphorylation in response to SDF-1 for effective chemotaxis is achieved by a balance of kinase, and phosphatase signal is illustrated by the fact that inhibition of PI3K, using low-dose LY294002, leads to partial recovery of chemotactic response in cells in which PP2A activity has been inhibited.
been inhibited by OA, although by itself LY294002 inhibited chemotaxis of the cells. Thus, Akt phosphorylation following SDF-1 stimulation appears to be regulated by an optimal balance of PI3K and PP2A signals in CD34⁺ cells.

Interestingly, β-arrestin-2 was detected by communoprecipitation in the Akt-PP2A complex following SDF-1 stimulation. In addition to its classical role in desensitization of GPCR signaling, β-arrestins can also act as a scaffolding protein (44, 45). A recent study suggested β-arrestin-2 is a positive mediator of dopaminergic synaptic transmission, and attributed this to its role as a signaling intermediate through a kinase/phosphatase scaffold (60). β-arrestin-2-deficient lymphocytes are defective in chemotaxis toward SDF-1 (61). It is therefore, possible that following SDF-1 stimulation, β-arrestin-2 acts as a scaffold to bring Akt and PP2A together. This interaction may be critical for effective SDF-1-directed chemotaxis of CD34⁺ CB cells.

Recently, GSK3 has been found to play a pivotal role in establishment of cell polarity by influencing microtubule stability. This was important for axon development and astrocyte migration (62, 63). However, global inhibition of GSK3 impairs polarity of cells (62). Cell migration is both positively and negatively affected by GSK3 (64–66). Cell migration may involve cyclic transient activation and inactivation of GSK3 as well as modulation of cellular localization of GSK3 (66). Phosphorylation of GSK3 leads to its inactivation (49). We found that following SDF-1 stimulation, GSK3 communoprecipitated with Akt in CD34⁺ cells, and inhibition of GSK3 impaired chemotaxis. Additionally, expression of CAkt impaired chemotaxis, and CAkt-expressing CD34⁺ cells had higher levels of pGSK3. This suggests that PP2A activity is primarily required for optimal level and duration of activation of Akt, which in turn regulates activation of GSK3 by regulating its phosphorylation status. SDF-1 stimulation of OA-pretreated cells leads to robust and prolonged Akt activation, which in turn leads to prolonged phosphorylation and inactivation of GSK3, thereby impairing chemotaxis. In addition to its association with Akt following SDF-1 stimulation, GSK3 was also found to be constitutively associated with PP2A-C in CD34⁺ cells and phosphorylated in CD34⁺ cells in the absence of SDF-1 stimulation when the cells were pretreated with 1 μM OA. Therefore, the profound decrease in chemotaxis when cells are pretreated with higher concentration of OA (e.g., 1 μM) is most likely due to strong inhibition of GSK3 that is mediated by two pathways. One pathway may be due to the direct effect of PP2A inhibition on GSK3 phosphorylation, and the other due to phosphorylation of GSK by activated Akt in response to SDF-1 stimulation. GSK3 inhibition impaired the chemotaxis response, but not as profoundly as 1 μM OA. Effects of inhibitors in whole cells are limited by various factors, including permeability, and it is possible that inhibition of GSK3 activity by GSK3 inhibitor IX in CD34⁺ cells at the dose used is not as strong as when cells are pretreated with 1 μM OA. Based on our current findings, we propose the following model to describe the intracellular role of PP2A for effective chemotaxis of CD34⁺ cells toward SDF-1 (Fig. 9).

Homing to bone marrow and engraftment in a proper microenvironment is required for a transplanted HSPC to be able to even establish the hematopoietic environment, and derailed cerebellar neuron migration in C. elegans. SDF-1 is also required for the interaction and retention of HSPC in the niche (67). We have found that repopulation of NOD-SCID mice transplanted with CD34⁺ cells pretreated with OA was significantly reduced on average greater than 60% less than mice transplanted with control CD34⁺ cells. The reduced SDF-1-directed chemotaxis and adhesion of HSPC to fibronectin in response to OA pretreatment are likely reasons for this phenomenon. Of possible relevance, it has been reported that chronic myeloid leukemia blast cells have reduced chemotactic activity and adhesion to fibronectin in response to SDF-1 (68). A more recent study showed that PP2A activity in chronic myeloid leukemia blast progenitors is significantly reduced (69). Taking these studies together with our findings suggests that PP2A may play an important role in SDF-1-mediated responses of both normal and malignant hematopoietic cells. Thus, further studies to understand the role of PP2A in normal and diseased hemopoiesis are warranted.

Our data, which demonstrate a role for PP2A in efficient chemotaxis of CD34⁺ CB cells primarily via modulation of Akt activation, may have potential therapeutic implications in disease states and in use for enhancing the homing and engraftment of HSPC.

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Disclosures

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References


