Placenta Growth Factor Activates Monocytes and Correlates with Sickle Cell Disease Severity.


Running Title: Placenta Growth Factor and Sickle Cell Disease Severity

Division of Hematology-Oncology, Childrens Hospital Los Angeles, Departments of Pediatrics and Pathology, and the Department of Biochemistry & Molecular Biology, Keck School of Medicine, University of Southern California.

Corresponding Author: Punam Malik, MD
Division of Hematology-Oncology, Mail Stop 54
Childrens Hospital Los Angeles,
4650 Sunset Boulevard, Los Angeles, CA 90027
Phone: 323-669-5438
Fax: 323-913-3087
Email: pmalik@chla.usc.edu

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ABSTRACT
Sickle cell disease (SCD) results in chronic hypoxia and secondarily increased erythropoietin concentrations. Leukocytosis and activated monocytes are also observed in SCD in absence of infection or vaso-occlusion (steady state), the reasons for which are unknown. We found that erythroid cells produced placenta growth factor (PIGF), an angiogenic growth factor belonging to the vascular endothelial growth factor (VEGF) family, and its expression was induced in bone marrow CD34+ progenitor cells in the presence of erythropoietin. Furthermore, the steady state circulating PIGF levels in subjects with severe SCD [≥ 3 vaso-occlusive crises (VOC)/year] were $18.5 \pm 1.2 \text{ pg/mL (n=9)}$ compared to $15.5 \pm 1.2 \text{ pg/mL (n=13)}$ in those with mild SCD (<3 VOC/year) and $11.3 \pm 0.7 \text{ (n=9)}$ in normal controls ($P<0.05$), showing a correlation between PIGF levels and SCD severity. In addition, PIGF significantly increased mRNA levels of the pro-inflammatory cytochemokines interleukin-1β, interleukin-8, monocyte chemoattractant protein-1 and VEGF in peripheral blood mononuclear cells (MNC) of normal subjects (n=4; $P<0.05$). Expression of these same cytochemokines was significantly increased in MNC from subjects with SCD at steady state (n=14), compared to normal controls. Of the leukocyte subfractions, PIGF stimulated monocyte chemotaxis ($P<0.05, n=3$). Taken together, these data show for the first time, that erythroid cells intrinsically release a factor, which can directly activate monocytes to increase inflammation. The baseline inflammation seen in SCD has always been attributed to sequelae secondary to the sickling phenomenon. We show that PIGF contributes to the inflammation observed in SCD and increases the incidence of vaso-occlusive events.
INTRODUCTION

Sickle cell disease (SCD) is characterized by sickling of RBCs due to polymerization of sickle hemoglobin upon de-oxygenation, resulting in vascular occlusion. Repeated sickling and oxygenation-deoxygenation damages RBC membranes and shortens their life span, causing hemolysis and anemia. Chronic hemolytic anemia, shortened lifespan of RBCs and vascular occlusions are the hallmarks of the disease. The bone marrow responds to hypoxia and anemia with an erythropoietic response, reflected by high circulating reticulocytes.

Another salient feature of SCD is leukocytosis that occurs despite absence of acute infection or inflammation. Activated monocytes, endothelial cells, neutrophils and the coagulation cascade observed in SCD at steady state, has been presumed to be secondary sequelae to the sickling phenomenon. While activated leukocytes have been shown to initiate and/or promote vaso-occlusion, the reasons for leukocytosis, activation of monocytes and neutrophils and increased adhesivity of endothelium at steady state, are largely unknown.

Activation of endothelial cells in SCD contributes to vascular occlusions. Vascular endothelial growth factor (VEGF), one of the most potent activators of endothelial cells that increases expression of intercellular adhesion molecule-1 (ICAM-1) on endothelial cells, is elevated in the plasma of patients with SCD. It and therefore, could increase adhesion of sickle RBCs to endothelial cells.

Placenta growth factor (PIGF) is another angiogenic growth factor belonging to the VEGF family. In contrast to wide spread distribution of VEGF, PIGF production was initially considered to be restricted to placental trophoblasts and umbilical vein endothelial cells. However, recent studies show that PIGF is expressed in non-placental tissues including erythroid cells, but not by other mature hematopoietic cells.

In addition, PIGF acts as a potent stimulant for VEGF secretion by monocytes and is co-expressed with VEGF in synovial fluid where high levels of PIGF homodimers and PIGF/VEGF heterodimers are found, suggesting that it plays a role in the inflammatory process. Since VEGF concentrations are elevated and erythropoiesis is expanded in individuals with SCD, we hypothesized that PIGF will be increased in the plasma of SCD patients and that PIGF may be
the intrinsic red cell factor that mediates the activation of leukocytes in SCD, resulting in vaso-occlusive events.

In this report, we show that PlGF production was increased in the bone marrow from subjects with SCD reflected by an increase in PlGF plasma levels. Furthermore, PlGF activated peripheral blood mononuclear cells, specifically monocytes, and increased the expression of VEGF, pro-inflammatory cytokines and chemokines by these cells. Finally, high PlGF concentrations correlated with increased incidence of vaso-occlusive events. Taken together, these data suggest that PlGF, an erythroid cell-derived factor, contributes to vascular occlusion and sickle cell disease severity.

MATERIALS AND METHODS

Subjects and samples. Heparinized blood was obtained from 22 subjects with SCD and nine normal controls according to a protocol approved by the Committee on Clinical Investigation [CCI, the Institutional Review Board (IRB)] at Childrens Hospital Los Angeles, University of Southern California. Plasma was separated within two hours of sample collection. All subjects studied were under baseline conditions or at steady state, defined as the absence of vaso-occlusive or infectious episodes within the three weeks before or three weeks after phlebotomy. Subjects with SCD were patients followed at the Childrens Hospital Los Angeles and were diagnosed by hemoglobin electrophoresis. Controls were healthy adult volunteers who did not have SCD. Disease severity classification was based upon the Pediatric Hydroxyurea Group Multicenter Trial28 with minor changes: Subjects were classified as having severe disease if they had three or more vaso-occlusive episodes per year requiring in-patient admission or emergency room visits. Subjects were considered having mild disease if they had two or less such vaso-occlusive crises per year.

The blood samples obtained were centrifuged at 0-4°C and 1000 x g for 15 minutes and plasma separated and stored at –80°C until it was assayed. Patients on chronic transfusion were excluded from the study. In 14 SCD subjects and 6 normal controls, EDTA anticoagulated blood samples were collected to separate leukocytes and leukocyte fractions. Normal bone marrow samples were obtained from voluntary donations. Bone marrow samples from SCD subjects were obtained from individuals who had voluntarily donated 5-10 mL of bone marrow when
under anesthesia for another clinically indicated procedure. All blood and marrow donations were voluntary, obtained after informed consent, using protocols approved by the institutional IRB.

**Separation of cell fractions from blood.** 1) Total leukocytes. Whole blood was subjected to hypotonic lysis by suspension in three volumes of sterile water for 30 sec followed by rapid addition of one-tenth volume 10X PBS to return the hemolysate isotonicity. The hemolysate was then centrifuged at 800 x g for 5 minutes to remove the red cell lysate. The pellet of white cells was washed once in PBS and re-suspended in serum free X-vivo 15 medium (Biowhittaker, Walkersville, MD) and used for studies on chemotaxis described below. 2) Peripheral blood mononuclear cells, composed mainly of lymphocytes and monocytes, were separated by layering freshly obtained peripheral blood on ficoll-hypaque (Pharmacia, Piscataway, NJ) and centrifugation at 1000 x g for 30 min at room temperature. The light density cells above the ficoll were removed, washed once in PBS and used for RNA analyses either directly or after resuspension in RPMI, with and without PI GF (250 ng/mL) for 1-4 four hours followed. 3) Bone marrow CD34+ cells were obtained as described previously. Briefly, fresh bone marrow samples were layered on ficoll-hypaque to obtain light density mononuclear cells (LD-MNCs), which were then enriched for CD34(+) cells via magnetic-associated cell sorting (MACS), with two cycles of positive selection using anti-CD34 antibody and immunomagnetic beads and the Midi-MACS columns (Miltenyi Biotech, Auburn CA). This typically results in a 90% to 95% pure population of CD34+ cells. CD34+ cells were placed in basal bone marrow medium, an IMDM based medium containing 20% fetal bovine serum, in different cytokine combinations as described below. 4) Erythroid (glycophorin A+) and non-erythroid (glycophorin A-) cells: Light density mononuclear cells, collected after density separation on Ficoll-Hypaque were washed once with PBS, counted and resuspended in Miltenyi buffer (1 x PBS, 0.5%BSA, 5mM EDTA; Miltenyi Biotech) at a concentration of 10^8 cells/300 µL. To prevent non-specific binding, human IgG (50 µg/100 µL; Baxter, Glendale, CA) was used as a blocking agent for 15 minutes at 6^o C. Thereafter, cells were labeled with anti-glycophorin-A FITC labeled antibody (Immunotech, Westbrook, ME), washed and labeled with anti-FITC micro-beads (Miltenyi Biotech) as per manufacturer’s instructions. Cells were then passed through Midi-MACS columns and the eluted population kept aside as glycophorin A- cells. The glycophorin A+ fraction was passed through a second column to improve purity. Typically, a double-column sort results in >90-95% purity of the isolated fraction.
**RNA Analyses:** RNA was extracted from bone marrow cells or from peripheral blood MNCs using the RNA-Stat (Tel-Test, Friendswood, TX) RNA isolation kit using the manufacturer's instructions. **1) RT PCR:** RNA was quantified and 1 µg of total RNA per sample was reverse-transcribed to cDNA in a 10 µL volume, using the Superscript RT-PCR kit (PE-Applied Biosystems, Foster City, CA). PCR was performed by amplifying 1 µL of cDNA at 94°C for 1 min, annealing x 1 min, 72°C x 1 min for 40 cycles on a Perkin Elmer 9600 thermocycler (PE Biosystems, Foster City, CA) using the following PlGF primer pairs: forward primer 5'ACAAGCTTCCTACGTGGAGCTGACGTTCT3' and reverse primer 5'AAATCTAGATCCTTCCGGCTTCATCTTCT3'. PCR conditions: 55°C x 5' and 95°C x 10' x 1 cycle followed by 60°C x 1', 95°C x 5 sec x 40 cycles. **2) Ribonuclease (RNase) Protection Assay:** RNase protection assays (RPA) were performed on total RNA extracted from cells using the Riboquant In Vitro Transcription Kit (Pharmingen, San Diego, CA) as per the manufacturer's protocol. Briefly, 32P labeled antisense RNA was synthesized in vitro using linearized templates for cytokines TNF-α and IL1-β and chemokines, MIP-1β, MCP-1, and IL-8, the angiogenic growth factor VEGF, and housekeeping genes GAPDH and L32. Excess in vitro synthesized radiolabeled RNA was hybridized overnight to 2 µg of total cellular RNA. The mixture was digested with RNase and protected fragments (double strands of cellular and in vitro synthesized RNA) were resolved on a 6.5% polyacrylamide gel and quantified by phosphoimager analysis (BioRad, Hercules, CA).

**Western Blot Analyses:** Cell pellets were lysed with 20 µl of lysis buffer (50 mM Tris HCl, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 25 mM NaF, 25 mM β-glycerophosphate, 0.3% NP-40, 100 mM PMSF, 1 µg/ml leupeptin and 0.5 µg/ml aprotinin) per two million cells. Whole cell lysates were left on ice for 30 minutes and centrifuged at 4°C at 10,000 g for 5 minutes. Lysate supernatants were then mixed with a 4x NuPage sample buffer (Invitrogen, Carlsbad, CA) and boiled for 5 minutes at 90°C. The proteins were separated on a 10% polyacrylamide gel, transferred to nitrocellulose membrane and blocked in 5% non-fat milk. The membranes were probed with monoclonal anti-human PIGF antibody (R & D Systems, Minneapolis, MN). The secondary antibody was a peroxidase-labeled anti-mouse antibody linked to an ECL-plus detection system (Amersham Biosciences, UK). The membranes were probed with the secondary antibody and subjected to ECL, as per manufacturer's protocol, then stripped and re-probed with antibody to human β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) as a loading control.
Enzyme linked immunosorbent assay (ELISA): Blood was centrifuged at 4°C at 1000 x g for 15 minutes and the resulting plasma stored at -80°C until it was assayed. Plasma samples were assayed in duplicate for PIGF using a Quantikine human PIGF ELISA kit (R & D Systems, Minneapolis, MN), according to the manufacturer’s directions. Optical density was measured at 450 nm and corrected for optical imperfections by subtracting the optical density at 550 nm in a Microplate Reader 3550-UV (Bio-Rad, Hercules, CA).

Electrophoretic Mobility Shift Assays (EMSA): Nuclear extracts were prepared from cells treated with erythropoietin-containing medium at the time intervals indicated and from untreated control cells. Cells were lysed in 50 mM KCl, 25 mM HEPES, pH 8.0, 100 mM DTT, 1% NP-40 and protease inhibitor cocktail (Sigma) for 5 min on ice. The nuclei were washed, pelleted and resuspended in extraction buffer (500 mM KCl, 25 mM HEPES, pH 8.0, 100 mM DTT, 10% glycerol and protease inhibitor cocktail) at 4°C for 30 min with constant shaking and the samples were spun at 13,000 x g for 5 min. Supernatant comprised of nuclear extracts (5 µg protein/sample) were incubated in 25 µL 10 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, 5 mM MgCl, 1 mM DTT, 10% glycerol, 40 mg/mL poly (dl-dC), and 50 mM KCl and 32P-labeled oligonucleotide probe carrying MTF-1 binding sites at room temperature for the indicated times. A 100-fold molar excess of unlabeled oligonucleotides was used as a competitor. The reaction mixtures were resolved on 4% acrylamide gel with 0.5% TBE buffer. The sequence of the MTF-1 oligonucleotide probe used for the EMSA was 5’-CACGCAGCTGCGGGCTCCGGCTGCGGGCTGGCCGGGGCGCTGCGGGCTGACCG-3’.

Leukocyte Chemotaxis Assay: Leukocytes were obtained from whole blood using hypotonic lysis, as described above. Leukocyte migration assay was performed with minor modification of a previously described technique33, in a modified Boyden chemotactic apparatus (Costar Transwell Migration Plate). Serum free X-vivo 15 medium (BioWhittaker), containing 0, 1 nM or 5 nM concentrations of PIGF was placed in the lower chamber and 50,000 leukocytes in 100 µL of X-vivo 15 were placed in the upper chamber. Filters were removed, fixed with methanol and stained with Diff-Quick (Baxter, Miami, FL). The number of cells that had transmigrated overnight were counted by an independent blinded observer and a differential count performed in 9 randomly chosen fields at 100x magnification and the counts were averaged. All experiments were performed in triplicate.
**Statistical methods:** For comparative analyses, P values were calculated using paired or unpaired T-tests using the Excel 5.0 software (Microsoft, Seattle, WA). P values less than 0.05 were considered significant.

**RESULTS**

**PIGF is produced by erythroid cells and is expressed in bone marrow of patients with SCD**

Normal bone marrow CD34+ progenitor cells were cultured in erythroid differentiation conditions for two weeks, as described previously\(^{32}\), in order to obtain RNA from a relatively pure population of erythroblasts. Control non-erythroid cell RNA was extracted from peripheral blood MNCs (composed of lymphocytes and monocytes) from two normal subjects. RT-PCR analyses were performed using PCR primers that spanned an intron to ensure that any contaminating genomic DNA would result in a different and larger sized band. PIGF mRNA expression was only observed in the erythroid cultures (Figure 1A).

The chronic anemia in SCD results in erythroid cell hyperplasia in the bone marrow. Since we found expression of PIGF in erythroid cells, it was therefore likely that PIGF production would be increased in SCD. We isolated RNA from normal and SCD bone marrow light density mononuclear cells. Bone marrow samples from subjects with β-thalassemia, another hemoglobinopathy with increased erythropoiesis, were also included. An RT-PCR analysis showed that PIGF transcripts were present in the thalassemia and SCD bone marrow light density mononuclear cells (LD-MNC), when visualized with ethidium bromide staining of the agarose gels, but not in normal bone marrow LD-MNC (Figure 1B). This finding was intriguing, since we detected PIGF transcripts in the erythroid cultures and a significant pool of erythroid cells is present in normal bone marrow. One possible explanation is that the level of PIGF expression in normal bone marrow was below the detection limit of ethidium bromide-visualized RT-PCR product but was detectable in SCD and thalassemia bone marrow due to the increased erythroid cell mass. Conversely, there could be factors in SCD and thalassemia subjects that could enhance production of PIGF.

Therefore, we improved the sensitivity of the assay and controlled for the number of erythroid cells by performing an RT PCR for glycophorin A: Light density mononuclear cells were isolated from 4 normal and 3 SCD bone marrow cells and a semi-quantitative RT-PCR analysis was performed for PIGF, glycophorin A and β-actin. The blot was transferred and probed with a
corresponding ³²P labeled cDNA probes. The main observations were: 1. There were less glycophorin A expressing cells in the light density fraction in SCD bone marrow, suggesting that sickle erythroid cells are denser than normal. 2. Short exposure of the blot allowed the detection of PIGF transcripts only in SCD light density mononuclear fraction (Figure 1C, PIGF⁵), while the prolonged exposure of the same blot (PIGF¹) showed small amounts of PIGF mRNA detectable in normal bone marrow as well. 3. Most importantly, the PIGF mRNA expression, when normalized for glycophorin A expression, was very high in SCD bone marrow. Taken together, these data suggest that erythroid cells in SCD express a disproportionately high concentration of PIGF.

It was intriguing, however, that despite high glycophorin A signal, there was very faint band for PIGF in normal bone marrow. We reasoned that 1) the RT-PCR was performed on relatively impure erythroid populations, since normal light density mononuclear cells contain few erythroblasts and are predominantly composed of lymphocytes and monocytes, and 2) the glycophorin A RT-PCR was only semi-quantitative. We therefore purified erythroid and non-erythroid cells from light density fraction of normal and SCD bone marrow cells, using human glycophorin-A-FITC labeled antibody followed by an immunomagnetic selection with anti-FITC micro-beads and performed Western Blot analyses on equal numbers of glycophorin A+ and glycophorin A- cells from each of the bone marrow samples (Figure 1D). Due to limited volume of SCD bone marrow, comparison of relative PIGF concentrations between normal and SCD glycophorin A+ cells were not possible. However, PIGF monomers and dimers were detected only in the glycophorin A+ erythroid cell populations. Of note, PIGF was predominantly present in monomeric form in hematopoietic cells, while the converse was seen in placental tissue. We are currently investigating this aspect. Fibroblast lysates were used as a negative control and show a faint PIGF band. Recently, PIGF expression has been reported to be inducible in fibroblasts²⁴.

A portion of the glycophorin A+ and glycophorin A- cells from the normal bone marrow were also subjected to RT-PCR analyses (Figure 1E). Due to limited cell numbers, cells from SCD bone marrow were only subjected to Western Blot analyses (Figure 1D). Figure 1E reaffirms that PIGF transcripts are predominant in the glycophorin A+ erythroid cells.

**PIGF expression is inducible by erythropoietin in bone marrow progenitor cells** The anemia in SCD (and β-thalassemia) causes tissue hypoxia that results in increased
CD34+ progenitor cells from a normal bone marrow were analyzed for PIGF mRNA by RT-PCR analyses soon after isolation (Figure 2, lane 1), or after a 12 hour exposure to 3U/mL of erythropoietin (Figure 2, lane 2). We also wanted to determine if PIGF production in erythroid cells was a result of erythropoietin or was generated by erythroid cells in an erythropoietin-independent manner. Erythropoietin is essential for erythroid differentiation. We therefore used an erythropoietin-independent erythroid differentiation protocol, previously shown to result in erythroid commitment and differentiation. CD34+ progenitor cells were cultured in an erythropoietin-independent erythroid medium (IL-6, sIL-6 receptor and SCF; lane 3) or in erythropoietin-independent erythroid medium with additional erythropoietin (3U/mL; lane 4). No PIGF expression was observed in erythropoietin-independent erythroid differentiation conditions (lane 3). However, with addition of erythropoietin to these conditions (lane 4), PIGF transcripts became evident, demonstrating that erythropoietin induced transcription of PIGF.

**Erythropoietin increases the nuclear DNA binding activity of metal response element-binding transcription factor-1** The VEGF promoter contains several hypoxia inducible factor (HIF-1α) response elements. In contrast, the PIGF promoter does not have HIF-1 response elements, but has nine consensus binding motifs for metal response element-binding transcription factor-1 (MTF-1). We therefore examined whether erythropoietin induced nuclear translocation and DNA binding of MTF-1. Due to limitations in the number of CD34+ progenitor cells that can be obtained from normal bone marrow, we utilized a Ewing’s sarcoma cell line (SK-N-MC cells). SK-N-MC cells express both PIGF and erythropoietin receptor and addition of erythropoietin to this cell line results in a dose-dependent increase in PIGF mRNA expression (manuscript submitted). SK-N-MC cells were treated with erythropoietin for different time intervals followed by isolation of nuclear extracts and hybridization to a radiolabeled oligonucleotide containing MTF-1 response elements (Figure 3). Nuclear DNA binding activity of MTF-1 was observed within 15 minutes of erythropoietin exposure, and was competed out by addition of excess non-radiolabeled probe. These studies indicate that erythropoietin induces nuclear translocation and DNA binding activity of MTF-1, suggesting that the increased transcription of PIGF by erythropoietin may involve MTF-1 binding to its response elements in the PIGF gene promoter.

**Plasma PIGF concentrations are elevated in subjects with SCD and are associated with increased incidence of vascular occlusive events** PIGF is a secreted angiogenic growth factor.
factor\textsuperscript{25} and we observed increased PIGF mRNA in bone marrow of subjects with SCD. We therefore determined whether this resulted in higher amounts of PIGF protein in peripheral blood, in subjects with SCD. PIGF concentrations in SCD plasma were 16.8 ± 0.9 pg/mL, (mean ± SEM), n=22, compared to 11.3 ± 0.7 pg/mL, n=9, in normal controls (P<0.001). Furthermore, PIGF concentrations increased with increasing disease severity (Figure 4): Of the 22 individuals with SCD, those with severe disease (n=9) had significantly higher circulating PIGF at steady state (18.5 ± 1.2 pg/mL) than those with mild disease (15.5 ± 1.2 pg/mL, n=13) compared to normal controls (11.3 ± 0.7, n=9). Each of these groups was significantly different from each other (P<0.05). Therefore, PIGF appears to modulate the clinical severity, probably by increasing the inflammatory tone in SCD.

**PIGF activates mononuclear cells and increases production of inflammatory cytokines and chemokines** Elevated levels of PIGF and PIGF/VEGF heterodimers have been observed in inflammatory joint disease\textsuperscript{26}. Activated monocytes and high concentrations of inflammatory cytokines have been reported in SCD in the absence of vascular occlusion or illness. We therefore, determined whether PIGF could mediate activation of leukocytes and increase production of inflammatory molecules.

Normal blood mononuclear cells (MNCs, n=4) were exposed to PIGF (250 ng/ml) and the mRNA expression profile of inflammatory cytokines TNF-\(\alpha\), IL1-\(\beta\); chemokines IL-8, MIP1-\(\beta\) and MCP-1; and VEGF was examined (Figure 5). There was increased mRNA expression of IL-1\(\beta\) (55 ± 11 fold, P<0.08), IL-8 (106 ± 44 fold, P<0.04), MCP-1 (7.4 ± 1.6 fold, P<0.01); and VEGF (14 ± 4.3 fold, P<0.02), as compared to untreated MNCs.

**The genes inducible by PIGF in normal MNCs were also expressed at high levels in MNCs from SCD subjects at steady state** We reasoned that if PIGF was activating normal MNCs, then expression of these cytokines and chemokines would also be elevated in SCD MNCs. RNA from peripheral blood MNCs from subjects with SCD at steady state was analyzed. The same pattern of increased mRNA expression of IL1-\(\beta\), IL-8, MCP-1 and VEGF was observed in MNCs from SCD-subjects, compared to controls, (Figure 6), with P values ranging from P<0.01 to P<0.001. These data suggest that the activation of monocytes previously reported in SCD\textsuperscript{5} may be secondary to increased release of PIGF from erythroid cells.
**PIGF increased chemotaxis of monocytes:** Of the mononuclear fractions, Flt-1, the receptor for PIGF, is expressed on monocyte-macrophages\(^{37}\). Monocytes have been shown to transmigrate towards a PIGF gradient\(^{38,39}\). In order to examine which of the leukocyte subfractions (monocytes or neutrophils) were responding to PIGF, we exposed leukocytes, obtained after hypotonic lysis of whole blood, to a PIGF gradient and measured their transmigration towards PIGF through a 3-micron pore membrane. Figure 7 shows the number of transmigrated leukocytes and their sub-populations (granulocytes and monocytes). PIGF increased the transmigration of leukocytes. However, it was the monocyte fraction that showed a statistically significant, dose-dependent increase in chemotaxis towards PIGF (n=3), substantiating that PIGF is affecting the monocyte fraction of the mononuclear cells.
DISCUSSION

Although the molecular defect in SCD has been known for years, the reasons for leukocytosis, monocyte and endothelial cell activation at steady state, in the absence of infection or vascular occlusion are largely unknown. Moreover, the disease phenotype is extremely variable and prolonged symptom-free episodes between VOC suggest factors other than sickling promote VOC. We show that erythroid cells expressed PIGF in response to erythropoietin. PIGF increased the expression of pro-inflammatory cytochemokines (IL1-β, IL-8 and MCP-1) and VEGF from normal mononuclear cells. This same pattern of increased mRNA expression of cytokines and chemokines was also seen in mononuclear cells from SCD subjects at steady state. We also found that plasma PIGF concentrations were elevated in individuals with SCD at steady state and correlated with the incidence of vaso-occlusive events.

Recently, both PIGF and VEGF-A have been shown to be secreted from erythroid cells in culture, indicating the specific role of erythroid cells in generation of angiogenic factors. Both VEGF and PIGF have been proposed to interact with either monocyte/macrophages or endothelial cells to promote transmigration of erythroid cells across endothelial barrier. The expanded erythropoiesis in SCD bone marrow may be responsible for increased PIGF observed in SCD bone marrow and in circulation. However, it was interesting that the light density fraction of bone marrow mononuclear cells from normal or SCD subjects appeared to have nearly comparable amounts erythroid cells, as determined by expression of glycophorin A, an erythroid-specific cytoskeletal protein. The light density cell separation technique most likely removed the relatively dense erythroid cells, observed in SCD secondary to the sickling phenomenon. The density profile of red cells is altered in SCD. Regardless, the levels of PIGF mRNA were disproportionately high in SCD bone marrow, when normalized to glycophorin mRNA, suggesting that the expression of PIGF in SCD erythroid cells is regulated by additional factors.

It was intriguing, however, that despite high glycophorin A signal, there was very faint band for PIGF in normal bone marrow. Tordjman et al. have shown that normal erythroid cells produce significant amounts of PIGF. When relatively purified erythroid and non-erythroid populations from the light density mononuclear fractions from normal and SCD bone marrow were analyzed, PIGF protein was present only in erythroid cells. These results are consistent with the previously published results.
We also found that PIGF transcripts were induced as early as 12 hours after erythropoietin exposure in CD34+ cells. However, Tordjman et al\(^2\) have previously reported the appearance of PIGF transcripts in CD34+ cells on the second day of culture. This discrepancy may be due to differences in methodology. In our study we used more cycles of cDNA amplification by PCR, increasing the sensitivity of the assay, and used different erythroid culture conditions.

Anemia causes secondarily increased erythropoietin levels and erythroid hyperplasia, resulting in increased erythropoietin concentrations\(^3\). We observed that PIGF expression was induced by erythropoietin. This response was associated with increased nuclear DNA binding activity of the MTF-1, suggesting that erythropoietin probably increases PIGF transcription via this mechanism\(^4\). PIGF induction has been shown to be MTF-1 mediated in embryonic fibroblasts\(^2\). However, its induction by erythropoietin has not been previously described. We observed more PIGF transcripts in bone marrow from patients with \(\beta\)-thalassemia major, another hemolytic anemia with increased erythropoietic stress. It would be of interest to determine if PIGF is increased in other hemolytic anemias or severe iron deficiency states which result in erythroid hyperplasia. Since erythropoietin increases release of an angiogenic growth factor, higher PIGF levels may be present in hemoglobin SC patients, which may contribute to the retinopathy seen commonly in hemoglobin SC disease. The increased production of angiogenic growth factors mediated by erythropoietin could have even wider implications: Erythropoietin, routinely given to alleviate cancer-therapy related anemia, could induce angiogenesis in tumors. In a separate study, we have found that pediatric tumor cells express erythropoietin receptor and addition of erythropoietin increases the release of angiogenic growth factors VEGF and PIGF from tumor cells (Malik and coworkers, unpublished results). Administration of erythropoietin to patients with cancer therefore, may promote tumor angiogenesis and survival, which need to be studied. PIGF does play a role in placental angiogenesis: PIGF concentrations have been shown to increase during gestation and lower PIGF concentrations are associated with pre-eclampsia of pregnancy\(^4\).\(^4\)

PIGF concentrations in SCD subjects correlated with incidence of vaso-occlusive events, suggesting its putative role in inflammation. One observes high levels of PIGF homodimers and PIGF/VEGF heterodimers in synovial fluid of patients with inflammatory joint disease\(^2\). The mechanism by which PIGF or PIGF/VEGF heterodimers modulate inflammation is unknown and whether this is due to signaling via different VEGF receptors has largely been undetermined.
Studies have shown that VEGF can bind two distinct kinase receptors: the fms-like tyrosine kinase, Flt-1 (VEGF receptor-1) and the kinase insert domain-containing receptor/fetal liver kinase, KDR/Flk-1 (VEGF receptor-2)\textsuperscript{39,45-47}, PIGF binds only to Flt-1 while VEGF can bind either Flt-1 or KDR/Flk-1 receptor on endothelial cells\textsuperscript{21,48-50}. Amongst the hematopoietic cells, Flt-1 is specifically present on monocytes\textsuperscript{26,38} or progenitor cells\textsuperscript{51}. It has also been shown that PIGF can induce VEGF release from monocytes\textsuperscript{26}. Its role in generating pro-inflammatory cytokines and chemokines from monocytes has not been described. Our studies show that PIGF causes a very significant increase in pro-inflammatory cytochemokine mRNA in monocytes. This effect was mediated via the Flt-1 receptor (as shown in the accompanying publication by Selvaraj et al., Blood, 2003).

These pro-inflammatory molecules could contribute to the activation of leukocytes and endothelial cells, a phenomenon observed in SCD at steady state\textsuperscript{7,8,27}. Studies in the literature show that these pro-inflammatory molecules (TNF-\(\alpha\), IL-1\(\beta\), IL-8) are increased in subjects with SCD\textsuperscript{52} and activate neutrophils in vitro\textsuperscript{53}. Although TNF-\(\alpha\) concentrations appeared unchanged under our experimental conditions, there was a transient increase in TNF-\(\alpha\) expression with PIGF in peripheral blood monocytes (Selvaraj et al., Blood, 2003). These pro-inflammatory molecules released by PIGF may be responsible, to some extent, for the increased incidence of vascular occlusions in SCD subjects. Recent studies show that leukocyte adhesion to endothelium may be the primary event in initiating vascular occlusion, secondarily causing RBCs from subjects with SCD to adhere to the leukocytes or to endothelium\textsuperscript{16,54}. Therefore, it is plausible that PIGF may increase the adhesion of leukocytes either by a) directly activating monocytes and increasing their adherence to endothelium, or, b) secondarily activating neutrophils through release of pro-inflammatory molecules from monocytes. And finally, PIGF increases VEGF production from monocytes. PIGF and VEGF have been shown to form heterodimers and may further modulate the inflammatory tone.

Hebbel and colleagues have elegantly shown that RBCs from subjects with SCD adhere abnormally to endothelial cells and their adhesiveness correlates with clinical severity\textsuperscript{17,55}. Increased expression of adhesion molecules both on sickle RBCs\textsuperscript{56,57} and endothelial cells\textsuperscript{9} have been shown to contribute to the increased adhesion. VEGF has been shown to increase the expression of cell adhesion molecules ICAM-1 and VCAM-1 on the endothelium, thereby increasing endothelial cell adhesivity\textsuperscript{9,18,58} PIGF shares significant homology with VEGF and
could induce a pro-adhesive endothelial state either through direct activation of endothelial cells or indirectly, by increasing monocyte release of VEGF or pro-inflammatory cytokines.

A perplexing feature of SCD is its highly variable clinical phenotype and intermittent episodes of vascular occlusions, with prolonged and varied symptom-free intervals, suggesting that phenomenon other than sickling may be involved. Several large clinical studies consistently show that the degree of anemia and leukocytosis at baseline conditions are two independent factors that predict the severity of SCD. In view of our findings, SCD subjects that are more anemic would have increased tissue hypoxia, higher erythropoietin concentrations, factors that would lead to higher PIGF expression. This would result in increased vascular occlusive events through its effect on monocytes. Croizat et al. have shown that the degree of anemia in SCD correlates directly with the erythropoietin concentrations in plasma and the clinical severity of disease. Our data suggest that these factors would increase PIGF levels, which would then secondarily activate monocytes, thus contributing to the clinical severity.

Moreover, very recently, Rafii and colleagues have shown that PIGF mobilizes hematopoietic progenitor cells by increasing MMP-9 expression in bone marrow. MMP-9 mediates cleavage of c-kit, allowing recruitment and egression of progenitor cells and leukocytes. A single injection of PIGF-adenoviral vector in mice resulted in leukocytosis for 2-3 weeks that was MMP-9 mediated. In comparison, the adeno-null vector had no effect. Therefore, PIGF may be also be contributing to the leukocytosis observed in SCD at steady state via MMP-9. Additionally, the increased cytokines, TNF-α and IL-8 released from PIGF stimulated monocytes could also promote leukocyte chemotaxis. Leukocytosis has been shown to correlate with the degree of anemia in several clinical studies. It will be interesting to correlate the degree of anemia and leukocytosis with PIGF levels and the degree of monocyte activation.

In conclusion, the molecular defect in SCD has been known for years, but the reasons for leukocytosis and activation of monocyte and endothelial cell at steady state, in the absence of infection or vascular occlusion are largely unknown. Activation of leukocytes and elevation of cytokines, chemokines and VEGF in SCD has always been attributed to events secondary to vascular occlusion and the resulting inflammation, as shown in the schema in Figure 8 (shown in blue). PIGF may provide the first link between these two phenomena (Figure 8, shown in red): we show that erythroid cells intrinsically release PIGF, a factor which directly
activates leukocytes, and specifically monocytes and may thus modulate the inflammatory tone and clinical severity of SCD.

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FIGURE LEGENDS

Figure 1. PIGF is produced by erythroid cells and is expressed in SCD bone marrow. A. RNA was extracted from normal peripheral blood mononuclear cells (composed mainly of monocytes and lymphocytes, lanes 1 and 2) and an erythroid culture derived from normal bone marrow (lane 4) and subjected to RT-PCR analyses. PIGF primers used spanned two exons, giving a larger intron-containing band for genomic DNA (lane 3). B. RT-PCR analysis on normal bone marrow cells (lanes 1 and 2), SCD bone marrow (SBM, lane 3), β-thalassemia major bone marrow (TBM, lane 4) and two normal umbilical cord blood mononuclear cells (UCB, lanes 5 and 6) using PIGF (top panel) and β-actin (bottom panel) primers. Lanes 7-10 are control lanes: H2O (lane 7) and the Molt T cell line (lane 8) as negative controls; 293 cell genomic DNA as a DNA control (lane 9) and endothelial cells (lane 10) as a positive control. C. RT-PCR analyses for PIGF, glycophorin A (an erythroid cell specific gene) and β-actin followed by transfer and probing, showing the proportion of PIGF transcription in normal versus SCD light density mononuclear cells. The short (PIGF_S) and long (PIGF_L) exposure of the PIGF blot is shown to depict the small amount of PIGF mRNA in normal bone marrow and relatively high amount in sickle bone marrow. D. Western blot analyses of glycophorin A+ (Gly+) and glycophorin A- (Gly-) light density mononuclear cells (LD-MNC). Lanes 1 and 2 represent Gly+ and Gly- cells from SCD LD-MNC (SBM), respectively; lanes 3 and 4 represent Gly+ and Gly- cells from normal bone marrow LD-MNC (NBM), respectively; lane 5 and 6 represent fibroblasts and placenta, as negative and positive controls, respectively. E. RT-PCR analysis for PIGF on the NBM Gly+ and Gly- cells, shown in panel D (lanes 1 and 2). Lanes 3 and 4 represent controls.

Figure 2. Erythropoietin induces PIGF expression in bone marrow progenitor cells. A. CD34+ progenitor cells from a normal bone marrow were analyzed for PIGF mRNA by RT-PCR analyses soon after isolation (lane 1), or after a 12 hour exposure either to 3U/mL of erythropoietin (lane 2), or to erythropoietin independent erythroid differentiation medium (IL-6, sIL-6 receptor and SCF; lane 3) or erythropoietin independent erythroid differentiation medium with additional erythropoietin (3U/mL; lane 4). The bottom panel shows an RT-PCR for β-actin for the corresponding samples. Lanes 5 and 6 consist of negative (H2O) and positive controls (human pulmonary microvascular endothelial cells), respectively.

Figure 3. Erythropoietin increases nuclear DNA binding activity of MTF-1 transcription factor. Electro mobility shift assay (EMSA) on nuclear extracts from SK-N-MC Ewing’s sarcoma cell line, which expresses both PIGF and a functional erythropoietin receptor, using a
radiolabeled oligonucleotide probe containing MTF-1 response elements. The blot shows increased nuclear DNA binding of MTF-1 to the probe within 15 minutes (lane 3), with a maximal binding at 30 min (lane 4). The response is abrogated with excess cold (non-radiolabelled) oligonucleotide MTF-1 probe (lane 5).

**Figure 4.** PIGF concentration is increased in plasma of subjects with SCD at steady state and correlates with incidence of VOC. PIGF concentration was determined on cell free heparinized plasma from SCD subjects and from normal controls, using ELISA (Y-axis). Subjects were classified as mild and severe disease based on the incidence of severe VOC/year (X-axis). Each point ( ) represents average values of duplicate samples from individual subjects. Mean values are represented as (.) with values adjacent to the symbol. Medians represented as (O). Error bars represent standard error of the mean.

**Figure 5.** PIGF activates normal mononuclear cells, increasing production of inflammatory cytokine IL-1β, chemokines MCP-1 and IL-8 and the angiogenic growth factor VEGF. A. Ribonuclease protection assay performed using RNA from normal peripheral blood MNCs from 4 subjects exposed to exogenous PIGF (250 ng/mL) for one hour (lanes 5-8) or left untreated (lanes 1-4). HeLa cell RNA was used as a control RNA (lane 9). B. Densitometry analyses was performed on appropriate exposures on the different bands and normalized for loading.

**Figure 6.** The cytokines and chemokines that are induced in normal MNCs with exogenous PIGF are also elevated in SCD MNCs at baseline. A. RNAse protection assay was performed using RNA from normal peripheral blood MNCs (A1-5, lanes 2-6) and those derived from subjects with SCD at steady state (S1-8, lanes 6-13). HeLa cell RNA was used as a control RNA.

**Figure 7.** PIGF increases monocyte chemotaxis. Total leukocytes from fresh peripheral blood obtained after hypotonic lysis of RBCs were placed in a Boyden chamber against no chemical gradient (white bars, control) or a PIGF gradient of 1 nM (striped bars) or 5 nM (black bars). Lipopolysaccharide (LPS, grey bars) gradient was the positive control, which promotes neutrophil transmigration. Cells that transmigrated across 3 micron pore filters were stained and the total leukocytes counted in 9 fields (panel A), and a differential count obtained on the transmigrating neutrophils (panel B) or monocytes (panel C). Each bar represents mean ± SD.
[n=3] and values that were statistically significant from controls are represented with an asterisk: $P<0.05 = ^*$ and $P<0.01 = ^{**}$. 

**Figure 8. A working model showing the link between increased erythropoiesis in SCD and inflammation.** In SCD, increased sickling of RBCs leads to vaso-occlusion, which has been suggested to activate leukocytes. Alternatively, we propose that increased PlGF, resulting from erythroid hyperplasia and increased erythropoietin levels in SCD activates leukocytes, specifically monocytes, and results in elevated pro-inflammatory cytokines and chemokines. These cytokines have been previously shown to upregulate expression of VCAM and ICAM-1 in cultured endothelial cells, and thereby increase the adherence of sickle RBCs and leukocytes through their counter-receptors. The increased adherence of these cells to endothelium has also been shown to cause vaso-occlusion in *in vivo* models. In this proposed schema of events in SCD, previously published associations are shown in blue, speculations are indicated with a dashed blue line and our data and its interpretations are depicted in red. We conclude that erythroid cells intrinsically release a factor, PlGF, which directly activates leukocytes, specifically monocytes, to modulate the inflammatory tone and clinical severity of SCD.

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Malik et al. Figure 1

A

B

C

NBM-LD MNC  SBM-LD MNC

PI GF

PL GF

Gly A

\( \beta \text{actin} \)
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A) Normal MNCs vs. SCD MNCs at steady state

B) Bar charts showing expression levels of MCP-1, VEGF, IL-1β, and IL-8 in C/H vs. BCD conditions.
Malik et al. Figure 7

[Graph showing data for Transmigrating Leukocytes, Neutrophils, and Monocytes under different conditions: Control, LPS, PIGF 1, PIGF 5.]

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Malik et al. Figure 8
Placenta growth factor activates monocytes and correlates with sickle cell disease severity

Natalya Perelman, Suresh K Selvaraj, Sandeep Batra, Lori R Luck, Anat Erdreich-Epstein, Thomas D Coates, Vijay K Kalra and Punam Malik