Mechanism Of Monocyte Activation And Expression Of Proinflammatory Cytochemokines By Placenta Growth Factor


Running Title: Mechanism of PlGF induced monocyte activation in SCD

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Abstract
Monocytes from patients with sickle cell disease (SCD) are in an activated state. However, the mechanism of activation of monocytes in SCD is not known. Our studies showed that placenta growth factor (PlGF) activated monocytes and increased mRNA levels of cytokines (TNF-α and IL-1β) and chemokines (MCP-1, IL-8 and MIP-1β) in both normal monocytes and in the THP-1 monocytic cell line. This increase in mRNA expression of cytochemokines was also reflected in monocytes derived from subjects with SCD. We studied the PlGF-mediated downstream cellular signaling events that caused increased transcription of inflammatory cytochemokines and chemotaxis of THP-1 monocytes. PlGF-mediated cytochemokine mRNA and protein expression was inhibited by PD98059 and Wortmannin, inhibitors of MEK kinase and PI3 kinase, respectively, but not by SB203580, a p38 kinase inhibitor. PlGF caused a time dependent transient increase in phosphorylation of ERK-1/2, which was completely inhibited by Wortmannin, indicating that activation of PI3 kinase preceded MEK activation. PlGF also induced transient phosphorylation of AKT. MEK and PI3 kinase inhibitors, and antibody to flt-1 abrogated PlGF-induced chemotaxis of THP-1 monocytes. Over expression of a dominant-negative AKT or a dominant-negative PI3 kinase p85 subunit in THP-1 monocytes attenuated the PlGF-mediated phosphorylation of ERK-1/2, cytochemokine secretion and chemotaxis. Taken together, these data show that activation of monocytes by PlGF occurs via activation of flt-1, which results in activation of PI3 kinase/AKT and ERK-1/2 pathways. Therefore, we propose that increased levels of PlGF in circulation play an important role in the inflammation observed in SCD via its effects on monocytes.

Introduction
The clinical manifestations of sickle cell disease (SCD) include chronic hemolytic anemia, frequent infections and recurrent episodes of painful crises1-7. Vascular
occlusion leading to episodes of painful crises and damage to various end organs is the major cause of morbidity and mortality in SCD\textsuperscript{1,3,6,8}. Hebbel and coworkers have shown that the extent of adherence of SS RBC to cultured endothelial cells appears to parallel the clinical severity of vaso-occlusive events in SCD\textsuperscript{9}. However, SCD patients with an identical defect in their $\beta$-globin genes show wide variability in the frequency and severity of vasoocclusive crises, and can remain asymptomatic for prolonged periods\textsuperscript{2}. This could be due to the epistatic effects of co-inherited genes such as $\alpha$-globin and $\gamma$-globin\textsuperscript{10-13}, both of which have been shown to alter disease severity\textsuperscript{13,14}. However, the effects of $\alpha$ and $\gamma$ globin gene expression are not always predictable, suggesting that there must be factors other than those directly related to the RBC in the pathophysiology of vasoocclusion in SCD.

Clinically, it has been noted that leukocytosis is associated with increased frequency of vaso-occlusive crisis (VOC)\textsuperscript{15,16}. Moreover, a chronically elevated white cell count is a clear indicator of mortality\textsuperscript{17}, frequency of acute chest syndrome\textsuperscript{8,18} and development of stroke\textsuperscript{19,20} in SCD. Since polymorphonuclear neutrophils (PMN) and monocytes are activated during infection and inflammation\textsuperscript{15,21}, we hypothesize they may play a role in the initiation and/or potentiation of vaso-occlusive episodes. Treatment of individuals with SCD with hydroxyurea causes a decrease in white blood cell count and a decrease in myeloperoxidase activity, indicating that reduced leukocytosis and leukocyte activation results in improvement in the incidence of vasoocclusive crises\textsuperscript{22-24}. A recent study\textsuperscript{25} shows that monocytes in SCD patients are in an activated state as they express more IL-1$\beta$ and TNF-$\alpha$ compared to normal monocytes. Furthermore, mononuclear cells from SCD patients can activate cultured endothelial cells, as judged by the increased expression of cell adhesion molecules and tissue factor in endothelial cells\textsuperscript{25}. However, the mechanism by which monocytes in SCD patients become activated is relatively not understood.

In the preceding paper, we show that plasma levels of PIGF are higher in SCD patients compared to normal controls and they correlate with disease severity. The data also suggest that the higher levels of PIGF present in patients with SCD may be due to
hypoxia, increased erythropoiesis and erythropoietin concentrations due to the anemia.
We have also shown that PIGF activates mononuclear cells (MNCs) resulting in increased gene expression of cytokines (TNF-α, IL-1β) and chemokines (MCP-1, IL-8 and MIP-1β).

The present study shows that PIGF activated the monocyte fraction of the MNCs. We have examined the cellular signaling mechanism by which PIGF interaction with peripheral blood monocytes (PBM) and THP-1 monocytes results in increased expression of proinflammatory cytokine chemokines. We show that interaction of PIGF with Flt-1 receptor on monocytes caused downstream activation of PI3 kinase/AKT and ERK-1/2 pathways, which increased expression of aforementioned inflammatory cytokines and chemokines. We also show that PIGF induced activation of Src kinase, PI3 kinase/AKT and MAP kinase in monocytes promotes chemotaxis of monocytes.

**Materials and Methods**

**Materials.** Media and other cell culture reagents were obtained from Invitrogen Corporation (Grand Island, NY). Genistein, PP2 [4-amino-5- (4-chlorophenyl)-7-(t-butyl) pyrazolo [3,4-d] pyrimidine], Wortmannin, LY294002 [2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one], PD98059 (2’-Amino-3’-methoxyflavone) and Actinomycin D were obtained from Calbiochem (La Jolla, CA). SB203580 and U73122 were purchased from Biomol (Plymouth Meeting, PA). ^32^P-UTP was obtained from ICN Biomedical Inc. (Irvine, CA). Antibodies to AKT, phospho AKT and phospho ERK1/2 were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Antibodies to ERK1/2 and all other secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). DuoSet ELISA development system for quantitation of chemokines, anti-human VEGF-R1 (Flt-1) and recombinant human placental growth factor were obtained from R&D Systems Inc. (Minneapolis, MN). All other reagents not listed otherwise were obtained from Sigma (St. Louis, MO).

*Isolation of human peripheral blood monocytes.* Blood was obtained from normal healthy volunteers and sickle cell anemia patients (HbSS) after obtaining informed consent.
consent according to a protocol approved by the institution review committee at the USC Comprehensive Sickle Cell Center, LAC-USC Hospital. Human peripheral blood monocytes were isolated from blood collected in EDTA as anticoagulant\textsuperscript{26}. Briefly, ten volumes of blood sample (30 mL) were mixed with one volume (3 mL) of a solution composed of 6\% Dextran 500 in 0.9\% NaCl. The tube was allowed to stand at room temperature for 45 minutes, which resulted in the sedimentation of erythrocytes. The leukocyte-rich plasma was harvested, layered over Nyco-prep media, density 1.068 g/mL (Accurate Chemical and Scientific Corporation, Westbury, NY) at a ratio of 2:1, and centrifuged at 600 g for 15 minutes. The monocyte fraction was further purified according to manufacturer’s instructions. Finally, these cells were allowed to adhere for 1-2 hr in a 60 mm petri dish in the tissue culture incubator. By this procedure non-adherent lymphocytes were removed. Monocytes isolated by this procedure had purity in the range of \textasciitilde90\% as assessed by labeling with MO2-FITC antibody (Coulter Diagnostics, Hialeah, FL) followed by FACscan analysis as previously described\textsuperscript{26} with a yield of 55-75\%.

\textbf{Cell culture.} THP-1, a promonocytic cell line was obtained from the American Type Culture Association (ATCC; Manassas, VA). Cells were cultured in RPMI-1640 medium containing 10\% heat inactivated fetal bovine serum.

\textbf{RNase protection assay.} THP 1 monocytes were treated with PIGF for various time periods and total RNA was isolated from cultured cells with TriZOL reagent (Life Technologies). Assays were performed with a custom made Riboquant Multiprobe RNase protection assay system (Pharmingen). In brief, the isolated RNA (10 µg) was hybridized with $^{32}$P labeled probes overnight at 56°C followed by RNase digestion according to manufacturer’s instructions. GAPDH was used as an internal control. After digestion, the protected fragments were resolved on a 5\% denaturing polyacrylamide gel, transferred to Whatman filter paper 3, which was dried and later exposed to X-ray film. The intensity of bands corresponding to TNF-\textalpha, MIP-1\textbeta, IL-1\textbeta, MCP-1, IL-8 and GAPDH mRNA were analyzed using a gel documentation system. Values are
expressed as relative expression of mRNA normalized to house keeping GAPDH mRNA.

**Quantification of chemokines.** THP-1 cells (1 x 10^6 cells/mL) were incubated in serum free RPMI-1640 in the presence or absence of PIGF (250 ng/mL). At the end of the indicated periods, ranging from 1 to 24 hours, the medium was collected, and cell debris removed by low speed centrifugation at 1500 rpm for 10 minutes. Supernatant was stored at -80°C until further use. Levels of chemokines (MIP-1β, MCP-1 and IL-8) in the clarified supernatants were assayed using specific DuoSet, ELISA development systems (R&D Systems Inc, Minneapolis, MN) according to the manufacturers instructions.

**Western Blot analysis of MAPK and AKT.** For western blot analysis, THP-1 cells were incubated in RPMI-1640 medium containing 2% fetal bovine serum (FBS) for 12-18 hr. Medium was aspirated and replaced with fresh medium prior to treatment with PIGF. Where indicated cells (5 x 10^6 cells) were incubated with pharmacological inhibitors for 30 minutes prior to PIGF treatment. At the end of the treatment, medium was aspirated and cells were lysed in 100 µL of 1X SDS sample buffer. The lysate was centrifuged at 14,000 g for 20 minutes at 4°C and the supernatant heated at 95-100°C for 5 minutes. An aliquot (10 µg) of supernatant was subjected to electrophoresis on a 10% SDS-PAGE gel followed by transfer to a nitrocellulose membrane (BioRad). Activation of AKT, and ERK1/2 was assessed using 1:1000 dilutions of antiphospho specific antibodies (Cell Signaling Technology, Inc. Beverly, MA). Horseraddish peroxidase conjugated secondary antibodies were used to develop the membrane. The protein bands were detected with Supersignal chemiluminescence substrate (Pierce Biotechnology, Inc. Rockford, IL). The intensity of bands was quantified by scanning utilizing an Alpha Imager 2000 gel documentation system (Alpha Innotech Corporation, San Leandro, CA). Blots were stripped and reprobed using a 1:1000 dilution of antibodies against the nonphosphorylated forms of AKT and ERK1/2 to monitor protein loading.
**Chemotaxis assay.** Chemotaxis was assayed in 96-well plates (Neuro Probe Inc., Gaithersburg, MD) having Transwell inserts of 5 µm pore size. Briefly, THP-1 monocytes were washed twice and resuspended in RPMI-1640 medium containing 2% serum, and then loaded onto inserts at 5 x 10^3 cells/50 µL for each well. 30 µL of RPMI-1640 medium containing the chemoattractants (PIGF/MCP-1) at the indicated concentrations were placed in the bottom compartment. After 2 hours of incubation at 37°C with 5% CO₂, cells were scrapped from the upper chamber and washed with PBS (100 µL) to remove non-migrated cells. This was followed by addition of PBS containing 2 mM EDTA. After 15 minutes at 4°C, the plate was centrifuged at 200 g in a Jouan Centrifuge CT-422 with a plate carrier. The filter was removed and cells in the well were counted by the trypan blue exclusion method. To investigate the effect of inhibitors involved in the signal transduction, THP-1 cells were preincubated for 30 minutes with the indicated inhibitors before loading on to Transwell inserts. Each sample was tested in triplicate.

**Transfection of monocytes.** THP-1 cells were transfected with a plasmid expressing a dominant-negative (DN) mutant of AKT and a dominant negative mutant PI3 kinase (DNM-Δp85), generously provided by David Ann, USC School of Pharmacy, Los Angeles. 10 µg of plasmid was incubated with 10 µL of lipofectamine (Invitrogen) in 100 µL of serum free DMEM for 15 minutes. The liposome-DNA complexes were added to the THP-1 cell suspension (2 x 10^6 cells/mL) and incubated at 37°C for 24 hours in tissue culture incubator as described²⁷. These cells were then treated with PIGF to determine the activation of ERK-1/2.
Results

Expression of cytokines and chemokines in monocytes in normal and SS patients.

Previous studies have shown that both polymorphonuclear neutrophils (PMN) and monocytes are in activated state in SCD, which may contribute to increased adherence of PMN/monocyte to endothelium. Moreover, it has been shown that intracellular levels of TNF-α and IL-1β are higher in monocytes of SCD patients compared to African American and Caucasians controls. Thus we examined the mRNA expression of a panel of inflammatory cytokines and chemokines by RPA analysis in RNA isolated from peripheral blood monocytes (PBM) from subjects with SCD and from normal controls. As shown in Figure 1, expression of TNF-α (Panel A) and IL-1β (Panel B) were several fold higher in monocytes of SCD relative to normal subjects. Expression of the chemokines MCP-1 (Panel D) and IL-8 (Panel E) were also several fold higher in monocytes from SCD compared to normal control. However, MIP-1β expression (Panel C) was only modestly increased by 1.2-1.4 fold.

Role of placenta growth factor in activation of monocytes

These studies indicated that monocytes in SCD were in activated state in vivo, generating cytokines and chemokines. We have also shown that PlGF, a factor produced by erythroid cells was responsible for activation of the mononuclear cell fraction, composed of monocytes and lymphocytes. Furthermore, we show that plasma PlGF concentration was directly related to the incidence of vaso-occlusive events. Thus we hypothesized that interaction of PlGF with the circulating monocytes may induce expression of pro-inflammatory cytokemokines. As shown in Figure 2, treatment of PBM from normal individual with PlGF caused an increase in the mRNA expression of cytokines (TNF-α and IL-1β) and chemokines (MCP-1, IL-8 and MIP-1β) as determined using RPA analysis. Similarly, treatment of THP-1, a promonocytic cell line with PlGF caused a time-dependent increase in the expression of the same cytokines and
chemokines (Figure 3). Since both primary monocytes and the THP-1 monocyctic cells exhibited a similar profile of cytochemokine expression in response to PIGF, we used THP-1 cells for subsequent studies.

**Effect of PIGF treatment on the expression of cytokines and chemokines by THP 1 cells**

As shown in Figure 4A, there was a dose dependent effect of PIGF (25-250 ng/mL) on the mRNA expression of cytokine and chemokines in THP-1 monocytes, with optimal effect at 250 ng/mL. This dose of PIGF was used in all experiments, unless otherwise indicated. We then measured if increased mRNA expression of cytokines and chemokines in THP-1 monocytes was due to increased stability of mRNA. Extremely low basal levels of IL-1β, MCP-1, IL-8 and MIP-1β mRNA in THP-1 monocytes precluded determination of mRNA half-life of these pro-inflammatory cytochemokines in response to PIGF. We could measure TNF-α mRNA stability due to a significant basal level of expression. To measure the mRNA half-life (t½) of TNF-α, the transcriptional inhibitor actinomycin D was added to the culture medium 2 hours post PIGF treatment, and mRNA was analyzed at different time points. As shown in Figure 4 B the t½ of TNF-α mRNA in untreated THP-1 monocytes was 45 minutes which did not change significantly upon PIGF treatment (t½ = ~50 minutes) in agreement with the previous results.

**Effect of cell signaling inhibitors on PIGF induced cytokine and chemokine gene expression**

Since PIGF treatment of THP-1 monocytes caused increased gene expression of both cytokines (TNF-α and IL-1β) and chemokines (MCP-1, IL-8 and MIP-1β), we determined the effect of pharmacological inhibitors, which have been previously shown to be specific for specific kinases in the signaling pathway. As shown in Figure 5A,
pretreatment of THP-1 cells with genistein (25 µg/mL), a protein tyrosine kinase inhibitor\textsuperscript{30} inhibited PIGF induced mRNA expression of TNF-α (25%), IL-1β (15%), MCP-1 (35%), and MIP-1β (25%), indicating that PIGF induced activation of protein tyrosine kinase is involved in induction of the pro-inflammatory cytochemokines.

As shown in Figure 5A, pretreatment of THP-1 monocytes with PD 98059, a specific inhibitor of mitogen-activated protein kinase kinase (MAPKK / MEK)\textsuperscript{31}, attenuated the mRNA expression of cytokines (TNF-α and IL-1β) and chemokines (MCP-1, IL-8 and MIP-1β) by ~50%, indicating that activation of MAP kinase was required. It is pertinent to note that SB 203580, a selective p38 MAP kinase inhibitor\textsuperscript{32}, did not significantly affect the PIGF induced expression of these cytochemokines (data not shown). Similar results were obtained with these inhibitors when peripheral blood monocytes were used (Figure 5B). These results indicate that PIGF mediated cytochemokine induction does not involve p38 MAP kinase activation but involves MEK activation.

**Role of PI3 kinase and Src kinase in PIGF induced mRNA expression of cytochemokines**

To delineate the cellular components upstream of the MAP kinases, we used LY 294002 and Wortmannin, PI3 kinase inhibitors\textsuperscript{32}, and PP2, a Src kinase inhibitor\textsuperscript{33}. As shown in Figure 5A, Wortmannin (100-200 nm) reduced mRNA expression of these cytochemokines by 50-75%, in a dose dependent manner. However, LY 294002, a weak PI3 kinase inhibitor\textsuperscript{32} inhibited expression somewhat moderately (~40%). These results indicate that PIGF induced activation of PI3 kinase is required for increased expression of these cytochemokines.

PIGF induced expression of TNF-α was inhibited by 50% in response to PP2, while expression of MIP-1β and MCP-1 were mildly affected (15-20%). These results indicate that activation of Src kinase family of tyrosine kinase is required for downstream activation of mRNA of TNF-α. In contrast, the expression of IL-1β and IL-8 increased by
~ 50% in the presence of PP2 indicating a negative regulatory role of Src kinase on IL-1β and IL-8 inducing pathways.

**Time and dose dependent effect of PIGF induced secretion of chemokines by THP-1 monocytes**

We then determined if the increased transcription of cytochemokines translated to increased protein expression. The release of chemokines from THP-1 monocytes treated with PIGF (250 ng/mL) was determined over a period of 24 hours. As shown in Figure 6, PIGF treatment resulted in a time dependent (1, 2, 4, 6, 8 and 24 hr) increase in the secretion of MCP-1, IL-8 and MIP-1β, with optimal secretion occurring at 4-6 hr. There was a 3-fold increase in MIP-1β secretion (481 ± 12.5 pg/mL in PIGF treated vs, 150 ± 16 pg/mL in untreated THP-1 monocytes), a two fold increase in IL-8 secretion (56 ± 3.0 pg/mL in PIGF treated vs. 27 ± 3 pg/mL in untreated) and a 2.5-fold increase in secretion of MCP-1 (16 ± 0.25 pg/mL in PIGF treated vs. 6.5 ± 0.15 pg/mL in untreated) in response to treatment of THP-1 monocytes with PIGF (250 ng/mL). As shown in Figure 7, PIGF exhibited increase in the secretion of chemokines (MCP-1, IL-8 and MIP-1β) in a dose dependent manner (25-250 pg/mL).

**Effect of kinase inhibitors on PIGF induced secretion of chemokines by THP-1 cells**

Since PIGF induced chemokine (MCP-1, IL-8 and MIP-1β) mRNA expression was attenuated by PI3 kinase inhibitor (Wortmannin) and MAP kinase inhibitor (PD98059), we examined the effect of these inhibitors on PIGF-induced secretion of these chemokines. As shown in Figure 8 both Wortmannin and PD 98059 reduced release of MIP-1β and MCP-1 by ~ 50%. IL-8 secretion was inhibited 25% by Wortmannin and 30% by PD 98059. The extent of inhibition of the release of these chemokines by PI3 kinase inhibitor (Wortmannin) and MAP kinase inhibitor (PD 98059) was similar to the
effect on transcription of these genes (Figure 5). Genistein reduced the secretion of MIP-1β and MCP-1 by 40% and 50%, respectively. In contrast, genistein had little effect on PIGF-induced secretion of IL-8. PP2, a Src kinase inhibitor, inhibited PIGF-induced secretion of MIP-1β and MCP-1 by ~ 25%. However, PP2 inhibitor did not significantly effect PIGF induced secretion of IL-8 (Figure 8).

**PIGF induced activation of AKT in THP-1 cells**

Since PI3 kinase inhibitor attenuated PIGF-induced gene expression of cytokines and chemokines, we examined the role of the serine kinase AKT, a downstream target of PI3-K kinase. The full activation of AKT by growth factors requires phosphorylation on Serine 473, which correlates with AKT kinase activity34. THP-1 monocytes were stimulated with PIGF (250 ng/mL) for 5, 15, 30, 60 seconds up to 30 minutes, followed by a western blot analysis using an antibody to AKT phosphorylated at Ser473. AKT phosphorylation increased from 5-30 seconds (Figure 9) and gradually declined thereafter. By 30 minutes the phosphorylation of AKT decreased below the basal level, indicating that AKT phosphorylation by PIGF is transient. Increased phosphorylation of AKT occurred without changes in its protein levels (Figure 9).

**PIGF induced activation of ERK-1/2 in THP-1 cells**

Previous studies have shown that monocytes express Flt-1 (VEGFR-1) but not Flk-1 (VEGFR-2) receptor35. We examined whether PIGF phosphorylated ERKs and required signaling thru Flt-1. RT-PCR analysis of THP-1 monocytes revealed the presence of Flt-1 mRNA but not flk-1 mRNA (data not shown). As shown in Figure 10A, treatment of THP-1 monocytes with PIGF (250 ng/mL) increased the phosphorylation of both ERK-1 (p44) and ERK-2 (p42). The peak phosphorylation of ERK-1/ -2 was observed at 3 minutes and declined thereafter. Moreover, antibody to Flt-1 inhibited PIGF-induced phosphorylation of ERK-1 and ERK-2 by 100% and 75%, respectively (Figure 10A).
THP-1 monocytes were then treated with specific inhibitors prior to the addition of PIGF. In the presence of Wortmannin, a PI3K inhibitor, ERK phosphorylation was completely inhibited in PIGF-stimulated monocytes. Moreover, U 73122 (1 µM), a phospholipase C inhibitor\textsuperscript{36}, completely abrogated PIGF-induced ERK-1 phosphorylation and reduced the phosphorylation of ERK-2 by more than 80%. As a positive control, PD 98059, an inhibitor of MEK, completely abolished ERK phosphorylation in THP-1 monocytes treated with PIGF.

These above results were then confirmed in peripheral blood monocytes. As shown in Figure 10B, PIGF-induced phosphorylation of ERK at 5 minutes (optimal phosphorylation) was attenuated by both PI3 kinase and MEK inhibitors. Taken together, these findings indicate that activation of Flt-1 receptor, phospholipase C, and PI3 kinase/AKT by PIGF is required for activation of ERK in monocytes.

Effect of over expression of dominant negative isoforms of PI3 kinase and AKT on PIGF induced phosphorylation of ERK.

To specifically address the role of PI3 kinase/AKT in PIGF-induced modulation of ERK phosphorylation, we over expressed a dominant negative AKT (DNAKT) and a dominant negative PI3 kinase p85 subunit (DNM-Δp85). As shown in Figure 11, over expression of DNAKT in THP-1 monocytes significantly attenuated the ability of PIGF to promote phosphorylation of ERK-12 by 0.5-3 minutes. It is pertinent to note that PIGF caused optimal phosphorylation of ERK at 3 minutes (Figure 10). Similarly transfection of THP-1 monocytes with DNM-Δp85 resulted in reduced phosphorylation of ERK1/2 in response to PIGF. These results are consistent with the data obtained with pharmacological inhibitors and confirm that PIGF induced phosphorylation of ERK involves activation of PI3 kinase/AKT.
Effect of expression of Dominant negative PI3 Kinase and AKT on PIGF induced secretion of chemokines by THP-1 monocytes

Our studies showed that Wortmannin reduced PIGF induced secretion of chemokines (Figure 8). To unequivocally establish that the effect of pharmacological inhibitor of PI3 kinase was specific, we expressed dominant negative PI3 Kinase and AKT in THP-1 cells and studied PIGF induced secretion of chemokines (MIP-1β, MCP-1 and IL-8). As shown in Figure 8, expression of DnPI3K in THP-1 reduced PIGF induced secretion of IL-8 by 45%, of MCP-1 by 30% and of MIP-1b by 30%. Similar results were obtained when THP-1 cells were transfected with DnAKT. These data are in accord with the results obtained with the use of Wortmannin, a PI3 kinase inhibitor. These studies and data obtained with wortmannin, a PI3 kinase inhibitor, show that PIGF induced signaling resulting in the secretion of chemokines involves activation of PI3 kinase.

Chemotactic response of THP-1 monocytes to PIGF

We determined whether PIGF increased monocyte chemotaxis. As shown in Figure 12A, PIGF caused a dose-dependent increase in the migration of monocytes. To determine whether the migration of THP-1 monocytes required a PIGF gradient we performed a checkerboard analysis. As shown in Table 1, the maximal increase in the migration of monocyte occurred in response to a positive concentration gradient between the two compartments of the Boyden chamber, which decreased as the difference in the concentration of PIGF between lower and upper compartments declined. These results indicate that the response of THP-1 monocytes to PIGF was a result of chemotaxis and not of chemokinesis, as has been observed with VEGF induced chemotaxis of monocytes. It is pertinent to note that MCP-1 (100 ng/mL), a positive control in these experiments, induced chemotaxis of THP-1 monocytes to a level slightly lower than PIGF alone (Figure 12 B).
**Effect of pharmacological inhibitors on PlGF induced THP-1 monocyte chemotaxis**

We determined whether pharmacological inhibitors of PlGF signaling also resulted in attenuation of monocyte chemotaxis. As shown in Figure 12B, Genistein (tyrosine kinase inhibitor), PP2 (Src kinase inhibitor), U73122 (phospholipase C inhibitor), Wortmannin (PI3 kinase inhibitor) and PD 98059 (MAP kinase inhibitor), reduced THP-1 chemotaxis in the range of 90-100%. Moreover, expression of dominant negative AKT (DNAKT) and a dominant negative PI3 kinase p85 subunit (DNM-Δp85) in THP-1 monocytes resulted in reduced (~50%) chemotaxis in response to PlGF (Figure 12B). However, MCP-1 induced chemotaxis of THP-1 monocytes was not inhibited by Wortmannin (Figure 12B). Similar results with MCP-1 induced chemotaxis have been observed in peripheral blood monocytes\(^{38}\). Furthermore, we observed that antibody to flt-1 completely abrogated PlGF induced chemotaxis of THP-1 cells (Figure 12 B). These results indicate that PlGF induced cellular signaling plays a role in chemotaxis of monocytes.
Discussion
Patients with sickle cell disease have abnormally high base-line leukocyte count, which is also observed in sickle transgenic mice\textsuperscript{39,40}. Moreover, it has been observed that the base line count of leukocytes is a very strong independent risk factor for severity of disease\textsuperscript{40}. The abnormally high levels of leukocytes above the base line reflects ongoing chronic inflammation at steady state. Recent studies show that monocytes isolated from SCD blood are in the activated state\textsuperscript{25}. Furthermore increased levels of IL-1\textsuperscript{β} and TNF-\textsuperscript{α}\textsuperscript{41,42}, and IL-8\textsuperscript{43} have been reported in sera from patients with SCD. However, the nature of stimuli, which cause monocyte activation in SCD, is not known.

A recent study shows that erythroid cells, but not other hematopoietic cells express both vascular endothelial growth factor-A (VEGF-A) and placenta growth factor (PIGF)\textsuperscript{44}. We have observed (Malik and coworkers, unpublished results), that PIGF is increased in SCD plasma and correlates with disease severity. Moreover, PIGF was also shown to activate the mononuclear cell fraction. In the present study, we show that the monocyte fraction that expresses the Flt-1 receptor\textsuperscript{45} in the mononuclear cells, is the population affected by PIGF.

We show that monocytes isolated from SCD patients, are in a highly activated state as demonstrated by increased gene expression of cytokines (TNF-\textsuperscript{α} and IL-1\textsuperscript{β}) as well as chemokines (MCP-1, IL-8 and MIP-1\textsuperscript{β}) compared to monocytes from normal individuals. These data are consistent with studies of Belcher et al\textsuperscript{25}, wherein they observed increased protein expression of both TNF-\textsuperscript{α} and IL-1\textsuperscript{β} in monocytes isolated from patients with SCD. Our data shows increased gene expression of cytokines (TNF-\textsuperscript{α} and IL-1\textsuperscript{β}) and chemokines (MCP-1, IL-8 and MIP-1\textsuperscript{β}) in peripheral blood monocytes (PBM) treated with PIGF. We also utilized a monocyte cell line THP-1, which showed an identical pattern of gene expression in response to PIGF. Thus we utilized the THP-1 monocytes as a model system for elucidating the signaling mechanism in response to PIGF.
Our studies show that PlGF treatment of monocyte causes dose dependent increase in the expression of proinflammatory cytochemokines. The increase in gene expression of these cytokines (TNF-α and IL-1β) and chemokines (MCP-1, IL-8 and MIP-1β) could have occurred as a result of increased mRNA stability. Since basal levels of IL-1β, MCP-1, IL-8 and MIP-1β mRNA in THP-1 monocytes were not detectable, one could not determine mRNA half-life of these inflammatory cytokines in response to PlGF. However, there was a basal level of expression of TNF-α mRNA, which increased several fold in response to PlGF. Therefore, half-life of TNF-α was determined. The half-life for TNF-α mRNA did not change in PlGF treated and in untreated THP-1 monocytes. Our studies show that PlGF treatment of THP-1 monocytes causes 3-fold, 2-fold and 1.5 folds increase in the secretion of chemokines MIP-1β, IL-8 and MCP-1, respectively, indicating concomitant increase in the translation of the corresponding mRNA.

We evaluated the signaling pathways involved in PlGF mediated induction of cytochemokines by using various low molecular weight pharmacological inhibitors known to block specific signaling events. Specific inhibition of MEK-1/2 kinase (ERK-1/2 phosphorylating kinase) reduced mRNA expression of these cytokines in THP-1 monocytes and PBM. In both PBM and THP-1 monocytes, PlGF induced expression of these cytochemokines was not inhibited by a selective p38 kinase inhibitor. These results are in sharp contrast to the effect of PlGF in trophoblast cells wherein PlGF causes activation of p38 kinase but not ERK-1/2 activity. However, PlGF has been shown to cause activation of ERK-1/2 activity in human umbilical vein endothelial cells (HUVEC). We also observed that PlGF caused time dependent increase in the phosphorylation of ERK-1/2 in THP-1 monocytes. These studies thus show that PlGF induced signaling in monocytes involves activation of MEK kinase leading to phosphorylation of ERK-1/2.

Moreover PlGF induced activation of ERK-1/2 phosphorylation was inhibited by antibody to Flt-1, indicating that PlGF signaling in monocytes involves interaction with its receptor Flt-1. We next delineated the intermediate signaling events that occur
between Flt-1 binding to PIGF and its downstream MEK activation. We show that inhibitors of PI3 kinase and phospholipase C completely inhibited PIGF induced phosphorylation of ERKs. To confirm that PI3 kinase/AKT is upstream of ERK, we studied the effect of PIGF on the phosphorylation of AKT. These studies revealed that PIGF causes transient activation of AKT (phosphorylation of AKT at Serine 473) at a very early time point (0.5 minute) followed by decrease at 1 minute. Furthermore, transfection of THP-1 cells with either dominant negative AKT plasmid (DNAKT) or dominant negative PI3 kinase p85 subunit resulted in decrease in the phosphorylation of ERK-1/2 by PIGF. Taken together these studies show that PIGF mediated activation of ERK-1/2 involves Flt-1, activation of phospholipase C and PI3 kinase/AKT as illustrated in the schema in Figure 13. Since PIGF induced cytochemokine gene expression was inhibited ~50% by Wortmannin and slightly less ~40% by LY 294002 (a weak PI3 kinase inhibitor), these results suggest that PIGF induced signaling leading to the expression of cytokines and chemokines is PI3-kinase dependent and independent. The PI3-kinase dependent pathway involves activation of ERK-1/2 (schema, Fig. 13), while PI3-kinase independent pathway may involve activation of AP-1. Further studies are required to delineate, which of the transcription factors (NF-κB, AP-1, Egr-1, CREB-1) may play a role in the regulation of PIGF induced cytochemokine gene expression.

Recent studies have shown that VEGF induced signaling in endothelial cells involves activation of Src and PLCγ1. Our studies showing that PP2, a Src kinase inhibitor, blocks PIGF induced expression of TNF-α indicates that Src activation is involved in downstream activation of TNF-α. In contrast, PIGF induced expression of IL-1β and IL-8 was 50% increased in the presence of Src kinase inhibitor PP2, indicating a negative regulatory role of Src kinase on IL-1β and IL-8 expression. Similar negative regulatory roles have been observed with VEGF, where tissue factor expression is augmented by inhibition of PI3 kinase. We suggest that inhibition of Src kinase augments other pathways (PI3 kinase/AKT and ERK-1/2) leading to increased expression of IL-1β and IL-8. Based on these studies one can develop potential anti-inflammatory therapeutic targets to ameliorate monocyte activation in sickle cell disease and thus prevent vaso-occlusive crises. Hebbel and colleagues have shown that inhibition of the transcription
factor NF-κB by sulfasalazine, an anti-inflammatory agent, showed beneficial effects in a few SCD patients.

Since monocytes are activated in response to PIGF, we determined whether binding of PIGF to Flt-1 in monocyte leads to functional response. Our studies show that PIGF causes chemotaxis of THP-1 monocytes in response to PIGF gradient, in agreement with a previous report\textsuperscript{37}. Our studies demonstrate that PIGF induced chemotaxis of THP-1 monocytes required activation of Src kinase, PI3 kinase and MEK kinase (ERK-1/2) indicating the importance of Src-PI3k/AKT-MAP kinase pathway in regulating the monocyte chemotaxis in response to PIGF (Figure 13). Moreover, we show that chemotaxis of monocytes mediated by PIGF gradient was completely abrogated by antibody to flt-1 indicating the importance of PIGF receptor flt 1 in chemotaxis.

Our studies show that PIGF causes activation of monocytes resulting in the expression of cytochemokines suggests that PIGF may play a role in inflammation in sickle cell disease. This is in accordance with the recent studies of Luttun et al\textsuperscript{49} wherein they showed that anti-flt-1, but not anti-flk-1, reduced the mobilization of bone marrow-derived myeloid progenitors into the circulation as well as inhibited the migration of flt-1 expressing monocytes to the sites of inflammation in the arterial wall, thereby reducing progression of atherosclerotic plaques. Moreover, these studies\textsuperscript{49} show the importance of PIGF, but not VEGF, in inflammation and atherosclerotic plaque formation. The role of PIGF in inflammation is further supported by the finding\textsuperscript{50} that targeted overexpression of PIGF-2 in the skin of mice resulted in exaggerated inflammation and edema, whereas PIGF deficient mice show greatly reduced inflammation in the skin.

Sickle cell disease has phenotypic characteristics of an inflammatory disease\textsuperscript{40} is indicated by the observation that one finds higher than normal leukocyte counts, elevated levels of serum cytokines, and increase in soluble intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). What triggers inflammation in endothelium and surrounding environment of circulating SS RBC is subject of investigation? It has been shown that interaction of sickle red blood cells,
not normal RBC, causes activation of endothelium\(^{51}\) indicating that sickle RBC can provoke inflammatory response. Recent studies of Kaul and Hebbel\(^{52}\) show that hypoxia/reoxygenation can trigger inflammatory response in transgenic sickle mice, but not in normal mice, indicating the role of inflammation in sickle cell disease\(^{40}\). We suggest that due to increased erythropoiesis in SCD, one finds increased levels of PIGF, which may act as a trigger on monocytes to produce cytochemokines, resulting in inflammatory response.

In conclusion, we have presented data showing that placenta growth factor causes activation of monocytes resulting in the generation of proinflammatory cytokines and chemokines. The PIGF induced signaling involves interaction with Flt-1 receptor followed by activation of PI3 kinase/AKT and MEK Kinase (ERK-1/2). The possibility of PIGF activation of other pathways, such as NF-\(\kappa\)B, is not ruled out, as inhibitors of PI3 kinase partially (50-75%) inhibit cytochemokine expression. The cytochemokines released from the monocytes can activate neutrophils and endothelial cells, augmenting cell adhesion molecule expression and vascular occlusion. Our results imply that PIGF is one of the factors in the plasma responsible for the activation of monocytes in sickle cell disease. Therapeutic strategies aimed at reducing monocyte activation may be beneficial in ameliorating vaso-occlusive crises in sickle cell disease. We suggest that both PIGF and flt-1 are potential candidates for therapeutic modulation of inflammation in sickle cell disease.

**Acknowledgements:**

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References


34. Kohn AD, Kovacina KS, Roth RA. Insulin stimulates the kinase activity of RAC-PK, a pleckstrin homology domain containing ser/thr kinase. EMBO J. 1995;14:4288-4295.


LEGENDS:

Figure 1: RPA analysis of mRNA expression of cytokines and chemokines in monocytes derived from normal and SCD patients. Peripheral blood monocytes were isolated from the whole blood of SCD patients and normal individuals. RNA was isolated using TriZOL reagent and 2 µg of RNA from each sample was analyzed by RNase protection assay using a Riboquant Multi-probe RNase protection assay system (Pharmingen, San Diego, CA). RPA was performed with riboprobes for A) TNF-α, B) IL-1β, C) MIP-1β, D) MCP-1, E) IL-8 and F) GAPDH.

Figure 2: Effect of PIGF on mRNA expression of cytokines and chemokines in normal peripheral blood monocytes. Peripheral blood monocytes isolated from whole blood of normal healthy individuals were treated with PIGF (250 ng/mL) for a time period of 2 hours. RPA was performed with riboprobes as indicated in Figure 1. A representative data is shown from three sets of experiments.

Figure 3: Upregulation of cytokine and chemokine mRNA expression in THP-1 monocytes by PIGF. THP-1 cells were treated with PIGF for time periods (1-24 hours) and RNA was isolated. 10 µg RNA samples were hybridized with 32P labeled antisense mRNA probes, and digested with RNase and T1 nuclease. The protected hybridized probe fragments were resolved on 5% polyacrylamide gel. The intensity of radioactive bands in the autoradiogram were quantitated by Alpha Imager documentation system. The relative mRNA levels were determined by normalizing band intensities of TNF-α, IL-1β, MIP-1β, MCP-1 and IL-8 with that of GAPDH housekeeping gene. The data presented are representative of one of three replicate experiments.

Figure 4: A) Dose dependent increase in the expression of cytochemokines in PIGF treated THP-1 cells: THP-1 cells were treated with different concentrations of PIGF ranging from 25-250 ng/mL for 2 hours. RNA was isolated by TriZoI reagent and 10 µg of RNA was used for RPA analysis. B: Effect of PIGF on the stability of TNF-α mRNA. After the treatment of THP-1 cells with PIGF (250 ng/mL) for 2 hours, actinomycin D (10µg/mL) was added. At the indicated time periods RNA was isolated. TNF-α mRNA expression was determined by RPA as described in Figure 2. The relative mRNA levels were determined by normalizing band intensities of TNF-α with that of GAPDH housekeeping gene. A representative data is shown from two experiments.
Figure 5: Effect of signaling inhibitors on PIGF induced cytokine and chemokine gene expression. THP-1 cells (A) and PBM (B) were preincubated with pharmacological inhibitors for 30 minutes, followed by treatment with PIGF (250 ng/mL) for 2 hours. RNA was isolated and 10 µg was used for RPA analysis. The data is representative of three sets of experiments.

Figure 6: Time course of PIGF mediated secretion of chemokines from THP-1 cells. THP-1 cells (1 x 10^6 cells/mL) were treated with PIGF (250 ng/mL) for time periods (1 to 24 hours). Cell free supernatant was collected and 100 µl from the total one mL supernatant was used for determining levels of (A) MIP-1β, (B) MCP-1 and (C) IL-8 using the DuoSet elisa from R&D systems. Data are means ± SD of n=3, each experiment run in duplicate.

Figure 7: Effect of PIGF dose on the secretion of chemokines from THP-1 cells. THP-1 cells (1 x 10^6 cells/mL) were treated with PIGF (25-250 ng/mL) for time periods 1 and 4 hours. The supernatant was collected and (A) MIP-1β, (B) MCP-1 and (C) IL-8 levels quantified as indicated in Figure 6. Data are expressed as means ± S.D of n=3.

Figure 8: Effect of signaling inhibitors on the PIGF induced release of chemokines in THP-1 monocytes. THP-1 monocytes (1 x 10^6 cells/mL) were treated with various pharmacological inhibitors for 30 minutes. THP-1 cells were transfected with a plasmid expressing a dominant-negative (DN) mutant of AKT (DNAKT) and a dominant negative mutant PI3 kinase (DNM-Δp85) as described in the methods. The cells were then treated with PIGF for 4 hours and the cell free supernatant was collected and analyzed for the release of A) MIP-1β, B) MCP-1 and C) IL-8.

Figure 9: PIGF induced AKT phosphorylation in THP-1 cells. THP-1 cells were grown overnight in RPMI medium containing 2% FBS. Cells were then starved for 2 hours in serum free media and treated with PIGF for the indicated time periods. Cell lysates were analyzed for phosho-AKT by western blot using anti-phospho AKT (pSer473) antibody. Equal loading of the gels was confirmed by probing with antibody specific for unphosphorylated AKT (lower panel, AKT).

Figure 10: A) PIGF induced ERK-1/2 activity in THP-1 monocytes. THP-1 cells were incubated with RPMI containing 2% serum overnight. The cells were then incubated with fresh
serum free media for 2 hours and then treated with PIGF (250 ng/mL) for the indicated time periods. Where indicated THP-1 cells were preincubated with U73122 (1 µM), Wortmannin (200 nM), PD98059 (10 µM) and Ab-Flt-1 (5 µg/mL) for 30 minutes before treatment with PIGF. Cell lysates were analyzed for ERK-1 and -2 activities by western blot using anti-phospho MAPK (Thr202/Tyr204) antibodies (upper panel, pERK-1/2). Blots were stripped and reprobed with antibodies specific for unphosphorylated ERK-1/2 (lower panel, ERK-1/2). The experiment shown is representative of three experiments. B) PIGF induced ERK-1/2 activity in peripheral blood monocytes. Peripheral blood monocytes (1 x 10^6 cells/mL) were incubated in RPMI-medium containing 1% FBS for 1 hour followed by treatment with PIGF (250 ng/mL) for 3 minutes. Prior to treatment, the cells were either incubated with Wortmannin (200 nM) or PD98059 (10 µM) for 30 minutes. Cell lysates were prepared and 10 µg of the protein was subjected to 10% SDS-PAGE. The blot was probed with phospho ERK-1/2 antibody (upper panel), stripped and reprobed with total ERK-1 antibody (lower panel) to show equal loading.

Figure 11. Effect of transfection of dominant negative AKT and DNMP-885 PI3 kinase on PIGF mediated ERK phosphorylation in THP-1 monocytes. THP-1 cells were transfected with either DNAKT or DNMP-885 PI3 kinase as described in Materials and Methods. Transfected cells were treated with PIGF for 3 minutes. Cell lysates were probed with antibody to ERKs as described in Figure 10. The data is representative of three experiments.

Figure 12: Dose dependent effect of PIGF on the chemotaxis of THP-1 monocytes. (A) THP-1 cells (5 x 10^3 cells) were added to the upper compartment of a Neuroprobe chemotaxis chamber. The indicated concentrations of either PIGF/MCP-1 were added to the lower compartments, the chambers were incubated for 2 hours at 37°C, and then the migrated cells were stained and counted as described in the text. Results show mean ± S.D of 4 independent experiments. (B) For inhibitor studies, THP-1 monocytes were pretreated with the respective pharmacological inhibitors or antibody for 30 minutes. THP-1 cells transfected with the dominant negative mutants of AKT and PI3 kinase were also used for the chemotaxis assay.

Figure 13: Working model of PIGF induced intracellular signal transduction cascade in monocytes. On interaction of PIGF with Flt-1 on monocytes, protein tyrosine kinase, Src kinase, PI3 kinase/AKT and MAP kinase pathways are activated. A parallel activation of Phospholipase C occurs, which activates MAP kinase.
Figure 1:

A: TNF-α
B: IL-1β
C: MIP-1β
D: MCP-1
E: IL-8
F: GAPDH
Kabra et al., Figure 2

- [Image of a figure showing the effects of PI GF on various proteins such as TNF-α, IL-1β, MIP-1β, MCP-1, IL-8, and GAPDH.]

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Kaira et al., Figure 3

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- **A**: THF-α
- **B**: IL-1β
- **C**: MIP-1β
- **D**: MCP-1
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Kaira et al., Figure 4A

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- TNF-α
- IL-1β
- MIP-1β
- MCP-1
- IL-8
- GAPDH
Kabra et al., Figure 48
Kalra et al, Figure 5A
Kaira et al., Figure 5B

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A: TNF-α
B: IL-1β
C: MIP-1β
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**Kaira et al., Figure 10A**

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**Kaira et al., Figure 10B**

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**Graph:**

- **p ERK-1/2**
- **ERK-1/2**
Kalra et al., Figure 12

[A]

Number of THP-1 cells migrated

PIGF [nM]

[B]

Migrated THP-1 cells [%]

Condition: PIGF, MIF-1
Kaipa et al., Figure 13
Table 1: PIGF induces THP-1 monocyte chemotaxis

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Different concentrations of PIGF were added to the upper and/or lower compartments of the neuroprobe chamber. The number of migrated cells were counted for each sample after 2 hours. Data from a representative experiment are shown.
Mechanism of monocyte activation and expression of proinflammatory cytochemokines by placenta growth factor

Suresh K Selvaraj, Ranjit K Giri, Natalya Perelman, Cage Johnson, Punam Malik and Vijay K Kalra