Plasma Endothelin-1, Cytokine, and Prostaglandin E2 Levels in Sickle Cell Disease and Acute Vaso-Occlusive Sickle Crisis

By Evangeline Graido-Gonzalez, James C. Doherty, Eric W. Bergreen, Gregory Organ, Margaret Telfer, and Marvin A. McMillen

The relative contributions of microvascular inflammation and vasomotor dysregulation to the development of acute vaso-occlusive crisis in sickle cell disease have been intensely studied. The present observational study was designed to examine the levels of circulating proinflammatory cytokines, anti-inflammatory cytokines, and vasoactive mediators during and after acute painful crisis. In symptomatic sickle cell patients, plasma levels of endothelin-1 and prostaglandin E2 were elevated during crises compared with healthy African-American controls. These levels had decreased, but not normalized, when patients were seen 1 to 3 weeks after discharge from hospital. Other mediators (tumor necrosis factor α [TNFα], interleukin-1β [IL-1β], IL-6, IL-8, and IL-10) were neither elevated in asymptomatic sickle cell disease nor in acute vaso-occlusive crisis. As a potent long-acting mediator of vasconstriction and inflammation, endothelin-1 may play a key role in the cycle of ischemia and inflammation that initiates and sustains pain of crisis. The downregulatory effects of prostaglandin E2 on immune cell function may contribute to the increased susceptibility to infection observed in patients with sickle cell disease.

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A CENTRAL CLINICAL ISSUE that determines quality of life in patients with sickle cell disease is the occurrence of painful crisis, an episodic event producing several days of excruciating pain. Reduced deformability of hemoglobin-S (Hgb-S)-containing red blood cells can dramatically increase the viscosity of blood and lead to mechanical blockage of the microcirculation, but the reversibility of the crisis and the lack of widespread tissue necrosis associated with the crisis lead to the question of whether vasospasm and inflammation might play important roles in the pathophysiology of pain crisis.

Significant recent advances have occurred in understanding the interactions of the irreversibly sickled cell with the microvasculature. These have coincided with better understanding of endothelial cell regulation of vascular smooth muscle and vasomotor tone. The endothelial cell is a key regulator of the contractile status of vascular smooth muscle via the constitutive and regulated expression of nitric oxide (NO) and by the induced expression of endothelins and eicosanoids. A major area of current study is whether the vascular occlusion and the ischemia of sickle cell disease may be caused by endothelial cell dysregulation mediated by abnormal erythrocytes. Several studies have examined inflammatory mechanisms that may mediate adhesion of sickle erythrocytes to the endothelium. There is evidence to suggest that sickle erythrocytes may have direct or indirect effects upon vascular tone, exclusive of adhesion events, and that local control of vascular tone is abnormal not only during sickle cell crisis, but also in the steady state course of the disease. Inflammatory events (cytokine production and adhesion molecular expression) may also play a role in the pathogenesis of sickle crisis.

Endothelin-1 (ET-1) is a potent vasoconstrictor and proinflammatory agonist that has been shown to be elevated in sickle cell disease. The present observational study was designed to investigate plasma levels of ET-1, proinflammatory cytokines (tumor necrosis factor α [TNFα], interleukin-1β [IL-1β], IL-6, IL-8), antiflammatory cytokines (IL-10), and counter-regulatory prostaglandin E2 (PGE2) in asymptomatic sickle cell disease and during pain crisis.

MATERIALS AND METHODS

Patients. Thirteen adult homozygous sickle cell patients had an extra tube of blood drawn at routine outpatient visits to confirm prior published results indicating high baseline levels of ET-1, giving a mean ET-1 level of 35 pg/mL with a range of 0 to 151 pg/mL (n = 13). In compliance with Michael Reese Hospital Human Investigations Committee guidelines, patients with confirmed homozygous sickle cell anemia were invited to participate in the study. Additional blood samples were obtained from a group of 11 comparably aged African-American consenting volunteers who were documented to be negative for sickle or C hemoglobin. These samples served as age- and race-matched healthy controls. For this study, blood was drawn from 13 consecutive consenting adult patients (20 to 50 years of age) with known homozygous sickle cell disease presenting in acute pain crisis without infection or other concurrent medical illness.

No patients were suspected to have narcotic dependency. Painful crisis was defined as a presentation to the emergency room with severe generalized trunk and extremity pain, defined by the patient as similar to previous episodes, and not accompanied by cough, pharyngitis, rhinitis, dyspnea, diarrhea, or physical evidence of pneumonia or urinary tract infection. Fever and leukocytosis to 20,000 per μL were allowed. These patients met predetermined criteria for admission and had not responded to fluids, Dilaudid, and intramuscular morphine in the emergency room over a 4-hour period.

In the hospital, they were placed on intravenous hydration with 5% dextrose and 0.45 N saline and received boluses of morphine until a Patient Control Access pump (PCA) could administer morphine. Patients required parenteral narcotic support for 3 to 7 days before oral acetaminophen with codeine was tolerable. Blood was collected for this study on admission and each morning for 3 days thereafter. At the first posthospitalization visit to the clinic, after a time ranging from 1 to 3 weeks, the postcrisis specimen from each patient was drawn.

Cytokine measurement. Blood was collected into standard 3-mL sodium-EDTA blood collection tubes that were centrifuged to isolate plasma. Plasma samples were evaluated for cytokine levels using a bead-based multiplex assay (Bio-Plex; Bio-Rad, Hercules, CA). This assay provides simultaneous quantification of IL-1α, IL-1β, IL-6, IL-8, TNFα, and IFNγ.

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plasma fractions. Plasma was immediately frozen at −80°C for assay at a later date.

Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used to determine plasma ET-1 and PGE2 (Amer sham Life Science, Buckinghamshire, UK) and TNFα, IL-1β, IL-6, IL-8, and IL-10 (Immunotech Inc, Westbrook, ME). Each kit uses a quantitative immunometric sandwich ELISA performed on a 96-well plate coated with monoclonal antibody against the specific peptide, cytokine, or prostanoid to be tested. During an incubation period ranging from 2 to 24 hours at 4°C to 27°C (depending on the specific assay), ET-1, TNFα, IL-1β, IL-6, IL-8, IL-10, and PGE2 bind to the specific affixed monoclonal antibody. A monospecific antibody conjugated to horseradish peroxidase is added. During an additional incubation, the conjugate antibody binds to the affixed antibody/peptide or antibody/cytokine complexes. Upon addition of appropriate substrate, a peroxidase-dependent color reaction occurs that is proportional to the amount of bound peptide, cytokine, or prostanoid. Plates are scanned using a Bio-Whittaker Microplate Reader 2001 (Bio-Whittaker, Inc, Walkersville, MD) set at the appropriate wavelength for the color-forming reaction. A standard curve is generated and specimen concentrations are determined by comparing sample optical density with the values on the standard curve. All samples in this study were performed in duplicate, and sample variation was estimated at approximately 5%.

Peak ET-1, cytokine, and PGE2 levels during crisis were recorded and were compared with postcrisis and control values. Results were compared by ANOVA and the Student’s unpaired two-tailed t-test. All data are presented as the mean ± standard error of mean.

RESULTS

Plasma ET-1 levels were significantly elevated relative to healthy controls (0.535 ± 0.508 pg/mL) in both patients in acute pain crisis (130.9 ± 23.1 pg/mL; *P < .0002) and those at postcrisis follow-up (23.69 ± 9.52 pg/mL; *P < .04) interpreted as their baseline symptom-free levels (Table 1). Postcrisis levels decreased significantly from levels drawn in crisis for all patients (*P < .0001).

Plasma PGE2 levels were also significantly elevated in crisis relative to healthy controls (316 ± 33.9 pg/mL; *P < .0001) and postcrisis patients (670.9 ± 61.0 pg/mL; *P < .0003; Table 1). Similar to the pattern observed with respect to ET-1 levels, PGE2 levels in crisis were elevated compared with healthy controls and decreased postcrisis (*P < .048). Neither the levels of ET-1 nor those of PGE2 varied significantly during the crisis sampling period (Fig 1).

Plasma levels of TNFα, IL-1β, IL-6, IL-8, and IL-10 were not different between healthy controls and sickle cell patients (*P < .05), although a trend was observed in which TNFα and IL-10 remained higher than controls during and after crisis. No difference was observed between the levels in crisis and those of the same patients at postcrisis follow-up (*P > .05; Table 2).

DISCUSSION

The endothelins are a family of 21 amino acid peptides first characterized by Yanagisawa in 1988.3 Four endothelin subtypes have been identified: ET-1, the most prevalent subtype, is the most potent vasoconstrictor yet described. ET-1 is not only a vasoconstrictor of large arteries and veins, but also constricts the resistance arterioles and postcapillary venules.10 ET-1 is rapidly internalized by its target cells, and infused pharmacologic doses are cleared from the circulation within minutes, principally by the lungs.11 The vasoconstriction lasts as long as 1 hour. The two specific ET-1 receptors, ETR-A and ETR-B, are G-protein–coupled membrane receptors on vascular smooth muscle cells, and the cell response (ie, smooth muscle contraction) results from inositol-triphosphate–mediated increases in intracellular calcium.12 These receptors differ in their specificities for the various endothelin subtypes and in their tissue distribution.13 The endothelins have been found to have elevated systemic levels in ischemic injury in acute respiratory distress syndrome, sepsis, and disseminated intravascular coagulation.14-16 Endothelin antagonists have been developed and are currently being evaluated.17

Endothelins are also proinflammatory agonists. Stimulation of cultured human peripheral blood monocytes with ET-1 causes monocyte production of inflammatory cytokines such as TNFα, IL-1β, IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), and substances that enhance neutrophil superoxide production.18-20 Endothelins cause neutrophil production of platelet-activating factor (PAF) and increase monocyte and neutrophil chemotaxis.21-23 Endothelins upregulate endothelial cell expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin, adhesion molecules that participate in the recruitment of leukocytes to sites of inflammation.24 Neutrophil proteases play a key role in cleaving bioactive ET-1 from its precursor molecule.25,26

Inflammation appears to play a significant role in the vaso-occlusive crisis in sickle cell disease. Systemic levels of TNFα and IL-1β increase in sickle cell disease, as do soluble forms of ICAM-1, VCAM-1, and E-selectin.27,28 TNFα increases adherence of sickled cells to vascular endothelium, and circulating reticulocytes in sickle cell disease express both α4β1 integrin and glycoprotein IV (CD36), adhesion molecules capable of binding to VCAM-1.29,30 Adherence of sickle erythrocytes in the microcirculation may initiate the impairment of microcirculatory blood flow.31 The inflammatory mediators (TNFα, IL-1β, IL-6, and IL-8) show small changes in our study of pain crisis that fail to reach significance, but may play a role at the tissue level, where autocrine and paracrine effects predominate.

PAIN CRISIS

We demonstrate elevation of plasma ET-1 levels during sickle cell pain crisis and a decrease in ET-1 levels to higher than normal in the same patients after symptomatic recovery. Several reports have demonstrated increased levels of ET-1 in patients with asymptomatic sickle cell disease.3-5 We used a

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**Table 1. Plasma ET-1 and PGE2 Levels in Healthy Controls, in Painful Crises, and During Recovery Phase**

<table>
<thead>
<tr>
<th>Condition</th>
<th>ET-1 (pg/mL)</th>
<th>PGE2 (pg/mL)</th>
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<tr>
<td>Healthy controls (n = 11)</td>
<td>0.525 ± 0.508</td>
<td>316.3 ± 46.9</td>
</tr>
<tr>
<td>Crisis sickle cell (n = 13)</td>
<td>130.9 ± 23.1†</td>
<td>825.9 ± 33.9†</td>
</tr>
<tr>
<td>Postcrisis sickle cell (n = 13)</td>
<td>23.9 ± 9.52*</td>
<td>670.9 ± 61.0*</td>
</tr>
</tbody>
</table>

*Statistically significant difference from controls (*P < .05).
†Statistically significant difference from postcrisis levels (*P < .05).
‡One to 3 weeks after discharge from hospital.
Fig 1. Plasma ET-1 and PGE\(_2\) concentrations during the first 72 hours of hospitalization for sickle cell crisis and at time of asymptomatic follow-up. Although plasma ET-1 (A) and PGE\(_2\) (B) levels appear to follow similar trends, neither varies significantly during the first 72 hours of hospitalization (P > .05). However, the mean peak crisis levels for each are elevated relative to those of the same patients when asymptomatic and are elevated relative to those of healthy age- and race-matched controls (Table 1). (ADM, admission time; 24H, 48H, and 72H indicate 24, 48, and 72 hours after admission, respectively; F/U, at time of postcrisis outpatient appointment). (●), Sickle cell; (■), control.

Table 2. Plasma Cytokine Levels in Healthy Controls, in Painful Crises, and During Recovery

<table>
<thead>
<tr>
<th></th>
<th>TNF(_\alpha) (pg/mL)</th>
<th>IL-1(\beta) (pg/mL)</th>
<th>IL-6 (pg/mL)</th>
<th>IL-8 (pg/mL)</th>
<th>IL-10 (pg/mL)</th>
</tr>
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<tbody>
<tr>
<td>Controls (n = 11)</td>
<td>44.41 ± 15.68</td>
<td>186.4 ± 39.7</td>
<td>39.67 ± 23.51</td>
<td>224.2 ± 62.0</td>
<td>4.142 ± 2.556</td>
</tr>
<tr>
<td>Crisis (n = 13)</td>
<td>61.21 ± 5.62</td>
<td>148.3 ± 31.0</td>
<td>41.74 ± 9.94</td>
<td>305.0 ± 34.2</td>
<td>20.53 ± 7.59</td>
</tr>
<tr>
<td>Postcrisis (n = 13)</td>
<td>66.61 ± 6.07</td>
<td>146.6 ± 12.9</td>
<td>14.62 ± 7.33</td>
<td>243.6 ± 37.3</td>
<td>17.14 ± 11.11</td>
</tr>
<tr>
<td>P values</td>
<td>&gt; .25</td>
<td>&gt; .25</td>
<td>&gt; .25</td>
<td>&gt; .25</td>
<td>&gt; .25</td>
</tr>
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sensitive ELISA technology to measure ET-1 levels, whereas the previous studies used radioimmunoassays.\(^6,7\)

The ET-1 levels observed may be derived from a variety of sources. Hypoxia is a potent stimulus to the production and release of ET-1 by vascular endothelial cells.\(^32\) There appears to be ET-1 production unique to sickle cell disease. Phelan et al\(^33\) demonstrated that cultured human umbilical vein endothelial cells experience a fourfold to eightfold greater transcriptional induction of the gene encoding ET-1 when exposed to sickled cells in vitro. Cultured bovine pulmonary artery endothelial cells transcribe increased amounts of ET-1 mRNA and release increased ET-1 peptide when exposed to plasma from sickle patients with acute chest syndrome and acute crisis.\(^7\) Plasma from the same patients at the time of symptom-free follow-up does not produce this effect.

ET-1 causes monocyte production of PGE\(_2\) in vitro.\(^34\) PGE\(_2\) downregulates inflammatory response by increasing intracellular cyclic AMP levels in immunohematologic cells.\(^35\) In monocytes and macrophages, PGE\(_2\) inhibits class II (Ia-DR) antigen expression, production of IL-1b, and antigen presentation.\(^36-38\)

In lymphocytes, PGE\(_2\) impairs IL-2 production by T-helper cells and decreases response to IL-2.\(^39\) PGE\(_2\) production by so-called suppressor macrophages may downregulate the inflammatory process, thus preventing harmful systemic inflammation.\(^40\) PGE\(_2\) levels increased significantly during crisis in the present study and remained elevated after symptoms had resolved.

Patients with sickle cell disease are at increased risk for serious bacterial infections. This has been attributed to functional asplenia, impaired opsonic function, and activation of the alternative complement pathway. Defects in cell-mediated immunity have also been described, and patients with severe variants of the disease demonstrate defective random neutrophil migration, chemotactic activity, and lymphocyte transformation index.\(^41,42\) These effects may be attributable to chronic exposure to downregulatory mediators such as PGE\(_2\) and IL-10.\(^43\)

The plasma levels of ET-1 and PGE\(_2\) in this study were elevated in 13 adult sickle cell patients in crisis compared with IL-6, and IL-8 were only variably increased, there were high plasma levels of ET-1 and 4 stimulate monocyte production of cytokines. Crit Care Med 25:958, 1977

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