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 vasoconstriction 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.

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 comparatively 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/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

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).

From the Department of Internal Medicine, the Department of Surgery, and the Division of Hematology, Michael Reese Hospital and University of Illinois College of Medicine at Chicago, Chicago, IL.
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Address reprint requests to Margaret Telfer, MD, Division of Hematology/Oncology, Michael Reese Hospital and Medical Center, 2929 S Ellis Ave Room RC-1200, Chicago, IL 60616.

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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 monoclonal 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 < 0.002) and those at postcrisis follow-up (23.69 ± 5.92 pg/mL; *P = 0.04) interpreted as their baseline symptom-free levels (Table 1). Postcrisis levels decreased significantly from levels drawn in crisis for all patients (*P = 0.001).

Plasma PGE2 levels were also significantly elevated in crisis relative to healthy controls (316 ± 33.9 pg/mL; *P < 0.001) and postcrisis patients (670.9 ± 61.0 pg/mL; *P = 0.003; 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 = 0.048). 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 < 0.05), 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 > 0.05; Table 2).

DISCUSSION

The endothelins are a family of 21 amino acid peptides first characterized by Yanagisawa in 1988.9–12 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 arteries 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 microrcirculatory 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.5,7 We used a

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

<table>
<thead>
<tr>
<th></th>
<th>ET-1 (pg/mL)</th>
<th>PGE2 (pg/mL)</th>
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<tbody>
<tr>
<td>Healthy controls (n = 11)</td>
<td>0.535 ± 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>23.6 ± 9.52**</td>
<td>670.9 ± 61.0**</td>
</tr>
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*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 PGE₂ 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 PGE₂ (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 |
|----------------------------------|-------------------------------|-----------------|-----------------|-----------------|-----------------|
|                                  | TNFα (pg/mL) | IL-1β (pg/mL) | IL-6 (pg/mL) | IL-8 (pg/mL) | IL-10 (pg/mL) |
| Controls (n = 11)                | $44.41 \pm 15.68$ | $186.4 \pm 39.7$ | $39.67 \pm 23.51$ | $224.2 \pm 62.0$ | $4.142 \pm 2.556$ |
| Crisis (n = 13)                  | $61.21 \pm 5.62$ | $148.3 \pm 31.0$ | $41.74 \pm 9.94$ | $305.0 \pm 34.2$ | $20.53 \pm 7.59$ |
| $P$ values                       | $> .25$         | $> .25$         | $> .25$         | $> .25$         | $> .07$         |
| Postcrisis (n = 13)              | $66.61 \pm 6.07$ | $146.6 \pm 12.9$ | $14.62 \pm 7.33$ | $243.6 \pm 37.3$ | $17.14 \pm 11.11$ |
| $P$ values                       | $> .25$         | $> .25$         | $> .25$         | $> .25$         | $> .25$         |
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.22 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 experience a fourfold to eightfold greater transcriptional induction of the gene encoding ET-1 when exposed to plasma from sickle cell 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 PGE2 in vitro.34 PGE2 downregulates inflammatory response by increasing intracellular cyclic AMP levels in immunohematologic cells.35 In monocytes and macrophages, PGE2 inhibits class II (Ia-DR) antigen expression, production of IL-1β, and antigen presentation.36-38 In lymphocytes, PGE2 impairs IL-2 production by T-helper cells and decreases response to IL-2.39 PGE2 production by so-called suppressor macrophages may downregulate the inflammatory process, thus preventing harmful systemic inflammation.40 PGE2 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.41-43 These effects may be attributable to chronic exposure to downregulatory mediators such as PGE2 and IL-10.43

The plasma levels of ET-1 and PGE2 in this study were elevated in 13 adult sickle cell patients in crisis compared with aged-matched African-American controls and with their own levels on asymptomatic follow-up. Whereas TNFα, IL-1β, IL-6, and IL-8 were only variably increased, there were high levels of counterregulatory PGE2 and IL-10. We conclude that endothelin could contribute to both the prolonged vasospasm and to inflammation in acute painful sickle cell crisis and that endothelin antagonist strategies might have utility in the treatment of this complex disorder.

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