The only curative therapy for sickle cell disease (SCD) is allogeneic hematopoietic stem cell (HSC) transplantation. Gene therapy approaches for autologous HSC transplantation are being developed. Although earlier engraftment is seen when cells from GCSF-mobilized blood are transplanted than when bone marrow is transplanted, administration of GCSF to patients with SCD can cause significant morbidity. We tested whether primitive hematopoietic progenitors are spontaneously mobilized in the blood of patients with SCD during acute crisis (AC-SCD patients). The frequency of myeloid-lymphoid–initiating cells (ML-ICs) and SCID-repopulating cells (SRCs) was significantly higher in blood from AC-SCD patients than in blood from patients with steady-state SCD or from normal donors. The presence of SRCs in peripheral blood was not associated with detection of long-term culture–initiating cells, consistent with the notion that SRCs are more primitive than long-term culture–initiating cells. As ML-ICs and SRCs were both detected in blood of AC-SCD patients only, these assays may both measure primitive progenitors. The frequency of ML-ICs also correlated with increases in stem cell factor, GCSF, and IL-8 levels in AC-SCD compared with steady-state SCD and normal-donor sera. Because significant numbers of ML-ICs and SRCs are mobilized in the blood without exogenous cytokine treatment during acute crisis of SCD, collection of peripheral blood progenitors during crisis may yield a source of autologous HSCs suitable for ex-vivo correction by […]
Spontaneous circulation of myeloid-lymphoid–initiating cells and SCID-repopulating cells in sickle cell crisis

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The only curative therapy for sickle cell disease (SCD) is allogeneic hematopoietic stem cell (HSC) transplantation. Gene therapy approaches for autologous HSC transplantation are being developed. Although earlier engraftment is seen when cells from GCSF-mobilized blood are transplanted than when bone marrow is transplanted, administration of GCSF to patients with SCD can cause significant morbidity. We tested whether primitive hematopoietic progenitors are spontaneously mobilized in the blood of patients with SCD during acute crisis (AC-SCD patients). The frequency of myeloid-lymphoid–initiating cells (ML-ICs) and SCID-repopulating cells (SRCs) was significantly higher in blood from AC-SCD patients than in blood from patients with steady-state SCD or from normal donors. The presence of SRCs in peripheral blood was not associated with detection of long-term culture–initiating cells, consistent with the notion that SRCs are more primitive than long-term culture–initiating cells. As ML-ICs and SRCs were both detected in blood of AC-SCD patients only, these assays may both measure primitive progenitors. The frequency of ML-ICs also correlated with increases in stem cell factor, GCSF, and IL-8 levels in AC-SCD compared with steady-state SCD and normal-donor sera. Because significant numbers of ML-ICs and SRCs are mobilized in the blood without exogenous cytokine treatment during acute crisis of SCD, collection of peripheral blood progenitors during crisis may yield a source of autologous HSCs suitable for ex-vivo correction by gene therapy approaches and subsequent transplantation.


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Nonstandard abbreviations used: sickle cell disease (SCD); homozygous mutation for sickle hemoglobin (HbSS); peripheral blood (PB); natural killer initiating cell (NK-IC); alopophycocyanin (APC); sickle cell disease in steady state (SS-SCD); normal (NL); sickle cell disease in acute crisis (AC-SCD); hematopoietic stem cell (HSC); stem cell factor (SCF); long-term culture–initiating cell (LTC-IC); myeloid-lymphoid–initiating cell (ML-IC); SCID-repopulating cell (SRC).

patients and is often referred to as the hallmark of the disease. Crises are more common during infancy and in the third and fourth decades of life. The mortality rate is considerably increased in those adults with more frequent painful crises.

The median life expectancy of patients with SCD in the USA is 42 years for men and 48 years for women (1). The only curative therapy is hematopoietic cell transplantation. The first allogeneic hematopoietic cell transplantation for SCD was carried out in 1984 (2). More recently, nonmyeloablative conditioning regimes have been used, as dramatic clinical improvements can be observed with low rates of hematopoietic chimerism in SCD patients (3, 4). Because of the lack of allogeneic HLA-matched donors and toxicity associated with allogeneic hematopoietic cell transplantation, various approaches to the genetic modification of autologous hematopoietic stem cells (HSCs) are currently being investigated (5, 6).

Use of cytokines, particularly GCSF, to mobilize HSCs and progenitors in the blood has revolutionized autologous hematopoietic cell transplantation (7). Other cytokines enhance mobilization of stem and progenitor cells into the peripheral blood (PB), including stem cell factor (SCF), IL-3, and thrombopoietin (8–10). Combinations of these factors, particularly SCF and GCSF, increase the number and quality of progenitors mobilized (11). However, the
use of cytokines in SCD may have a detrimental effect in patients in acute crisis, as shown by recent reports of fatalities following administration of GCSF (12, 13).

Several studies have noted that increased numbers of CD34+ cells circulate in the PB of SCD patients. The number of erythroid blast-forming units is elevated in the blood of patients with homozygous mutation for sickle hemoglobin (HbSS) and HbS β-thalassemia (14), suggesting increased erythropoiesis in response to anemia and increased sensitivity of progenitors to erythropoietin. Other studies have shown that CFCs (15) and long-term culture-initiating cells (LTC-ICs) (16) are increased in the blood of SCD patients. The mechanism for this is not clear. Levels of IL-8, a chemokine known to mobilize stem and progenitor cells in animal models (17), are increased in SCD patients in acute chest crisis, possibly as a result of infections (18). Increased levels of GCSF have been found in the bronchoalveolar fluid in SCD patients in acute chest crisis (19). Levels of IL-3 are consistently elevated in severe SCD patients, and high levels of SCF, another cytokine implicated in hematopoietic stem cell (HSC) mobilization (20), have also been shown in SCD patients in acute chest crisis (21). Finally, GM-CSF levels are raised in SCD and can be directly correlated to the increased hematopoiesis seen in moderate to severe SCD (22).

HSCs are able to self-renew and to give rise to all hematopoietic cell lineages. In mouse models, both the phenotype and the function of HSCs have been characterized using competitive in vivo repopulation assays (23). To measure human HSCs, alternative in vivo or in vitro assays are needed, as competitive repopulation assays cannot be performed. Several in vitro assays have been developed that assess primitive human progenitors. A stroma-based system, termed myeloid-lymphoid-initiating cell (ML-IC) assay, has been developed in our laboratory that measures human engraftment. They were sublethally irradiated with either 5 µg/kg GCSF (Filgastrim; Amgen Inc., Thousand Oaks, California, USA) for 6 days (n = 4) or 5 µg/kg GCSF and 20 µg/kg SCF (Amgen Inc.) for 6 days (n = 4).

**Cytokine measurement.** All serum samples were frozen at –70°C and thawed once at the time of the assay. All samples were assayed in duplicate. GCSF, SCF, GM-CSF, and IL-8 levels were measured using an ELISA kit (Quantikine Kit; R&D Systems Inc., Minneapolis, Minnesota, USA). The assay sensitivities were 0.5 pg/ml for GCSF, 0.8 pg/ml for SCF, 0.1 pg/ml for GM-CSF, and 0.5 pg/ml for IL-8. One-hundred-microliter samples were dispensed in 96-well microtiter plates of the Quantikine Kit and incubated at room temperature for 2 hours. The plates were then rinsed four times with wash buffer and incubated for 2 hours with anti-cytokine HRP conjugate that corresponded to each of the cytokines tested. The bound enzyme was then detected by incubation with tetramethylbenzidine and hydrogen peroxide as a substrate and then quantified using a microplate reader (Bio-Rad, Melville, New York, USA).

**NOD/SCID transplantation.** NOD/SCID mice were kept in a pathogen-free mouse facility with sterilized food and bedding in accordance with guidelines from the University of Minnesota’s Institutional Animal Committee. Mice aged 6–8 weeks were used for studies of human engraftment. They were sublethally irradiated with 275 cGy (dose rate 50 cGy/min) in a...
cesium irradiator. Twenty-four hours after irradiation, CD34+ cells from SCD patients or normal donors were injected via the tail vein. Mice received all CD34+ cells selected from 50 ml PB (between \(2.0 \times 10^5\) and \(3.7 \times 10^5\) CD34+ cells) with \(2.6 \times 10^6\) to \(2.8 \times 10^6\) irradiated normal carrier cells, or \(0.5 \times 10^6\) to \(3.0 \times 10^6\) CD34− cells from SCD patients in acute crisis or steady state. As control, mice were injected with all CD34+ cells selected from 200 ml PB from normal volunteer donors (\(2.0 \times 10^4\) to \(5.0 \times 10^4\) CD34+ cells with \(3 \times 10^6\) irradiated normal carrier cells). After 6 weeks, mice were sacrificed by cervical dislocation following carbon dioxide asphyxiation. Bone marrow was collected by flushing of both femurs and both tibiae with RPMI (GIBCO BRL; Life Technologies Inc., Grand Island, New York, USA) and 20% FCS. Erythrocytes were lysed using a hypertonic lysis buffer, and bone marrow cells were incubated for 30 minutes in PBS containing 10% mouse and 10% rat serum (Caltag Laboratories Inc., Burlingame, California, USA). Samples were then stained with anti-human CD45–phycoerythrin, anti-mouse CD45–FITC, and anti-human CD14, –human CD19, –human CD33, and –human CD34 APC (Becton Dickinson Immunocytometry Systems, San Jose, California, USA) to detect multilineage engraftment of human cells using appropriate isotype-control antibodies. Bone marrow harvested from both femurs and both tibiae of mice with about 2% human CD45 cells was transplanted 1:1 into secondary irradiated NOD/SCID recipients, which were again sacrificed and analyzed 6 weeks after transplantation.

**PCR analysis of samples.** Using the DNeasy Tissue Kit (QIAGEN Inc., Valencia, California, USA) according to the manufacturer’s instructions, we isolated genomic DNA from aliquots of blood, bone marrow, and spleen samples collected from animals transplanted with human SCD CD34+ cells frozen at \(-20^\circ\text{C}\) in RNAlater (Ambion Inc., Austin, Texas, USA). PCR amplification of a 345-bp fragment of the \(\beta\)-globin gene was accomplished using primers BG02 (TCCTAAGCCAGTGCCAGAAGAG) and BG05 (CTATTGGTCTCCTTAAACCT) (IDT, Coralville, Iowa, USA) and Pfu Turbo Hotstart DNA polymerase (QIAGEN Inc.) in 50 \(\mu\)l as follows: 95°C for 3 minutes, followed by 35 or 40 cycles of 95°C for 10 seconds, 55°C for 30 seconds, and 72°C for 1 minute.

<table>
<thead>
<tr>
<th>Age</th>
<th>Crisis etiology or steady state (SS-SCD)</th>
<th>WBCs (\times 10^9) per ml PB on day of sample collection</th>
<th>Hemoglobin (g/dl) on day of sample collection</th>
<th>Hemoglobin F (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>31</td>
<td>Infection</td>
<td>28.9</td>
<td>6.7</td>
<td>6.1</td>
</tr>
<tr>
<td>35</td>
<td>Acute pain</td>
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<td>12.4</td>
<td>8.1</td>
</tr>
<tr>
<td>22</td>
<td>Infection</td>
<td>12.9</td>
<td>7.8</td>
<td>7.5</td>
</tr>
<tr>
<td>26</td>
<td>Sequestration</td>
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<td>30</td>
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<td>21.6</td>
<td>8.8</td>
<td>8.8</td>
</tr>
<tr>
<td>32</td>
<td>Acute painful crisis</td>
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<td>8.9</td>
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<td>10.0</td>
</tr>
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<td>10.3</td>
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</tr>
<tr>
<td>25</td>
<td>Chest crisis</td>
<td>19.1</td>
<td>6.9</td>
<td>6.4</td>
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<tr>
<td>19</td>
<td>Infection</td>
<td>33.6</td>
<td>9.2</td>
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<td>28</td>
<td>Infection</td>
<td>30.1</td>
<td>11.1</td>
<td>9.2</td>
</tr>
<tr>
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<td>Chest crisis</td>
<td>38.8</td>
<td>10.6</td>
<td>7.9</td>
</tr>
<tr>
<td>31</td>
<td>Infection</td>
<td>23.1</td>
<td>8.9</td>
<td>5.5</td>
</tr>
<tr>
<td>19</td>
<td>Steady state</td>
<td>6.9</td>
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<tr>
<td>28</td>
<td>Steady state</td>
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<td>12.0</td>
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<tr>
<td>35</td>
<td>Steady state</td>
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<td>12.5</td>
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<td>27</td>
<td>Steady state</td>
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<td>Steady state</td>
<td>2.6</td>
<td>7.7</td>
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</tr>
</tbody>
</table>

WBC, white blood cells.

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**Figure 1**

FACS analysis for CD34+CD38-Lin− cells in PB of NL donors and SS-SCD and AC-SCD patients. CD34+ cells were selected as described in Methods, stained with CD34 APC, CD38 FITC, and lineage phycoerythrin (Lin-PE) antibodies, and selected by FACS. Live cells were gated in R1, and then CD34+ cells were gated against lineage cells. R2 represents the CD34−Lin− gate. Cells in R2 were gated as CD34 versus CD38. The R3 gate, CD34−CD38− (not shown here), was used to sort for single-cell deposition for assays.
30 seconds, 50–60°C for 30 seconds, and 72°C for 1 minute, followed by 72°C for 10 minutes. PCR products were digested with DdeI restriction endonuclease and analyzed by electrophoresis through 2% agarose gels. Gels were stained with ethidium bromide (Sigma-Aldrich, St. Louis, Missouri, USA) and digitally photographed with a Kodak EDAS 290 gel documentation system (Eastman Kodak Co. Scientific Imaging Systems, Rochester, New York, USA).

Quantitative PCR for human cell engraftment. Real-time PCR was performed using an ABI PRISM 7700 sequence detector with the accompanying software (version 1.6; Applied Biosystems, Foster City, California, USA). Reaction conditions for amplification of human microsatellite DNA were as follows: 40 cycles of two-step PCR (95°C for 15 seconds, 60°C for 60 seconds) after initial denaturation (95°C for 10 minutes) with 1 µl of DNA as template, 50 µM of each primer, and 1x SYBR-green PCR Master Mix (Applied Biosystems). The PCR primers were designed using Primer Express software (version 1.0; Applied Biosystems) as follows: hSat(f), ATTCACGTCACAAACTGAACATTC; hSat(r), CGTTTGAAATGTCCGTTTGTAGAT. The result was normalized to 18S rRNA, forward primer 5′-GTAACCCGTTGAACCCCATT-3′ and reverse primer 5′-CCATCCAATCGGTAGTAGCG-3′ DNA, and calculated in reference to a mixed sample of human K562 and murine AFT024 cells.

Results

Increased numbers of primitive progenitors are present in the PB during acute crisis of SCD. We initially evaluated the number of CD34+CD38–Lin– cells in PB of SCD patients and normal (NL) donors (Figures 1 and 2). PB from NL donors contained 6.7 (range 1–11) CD34+CD38–Lin– cells per 10^5 PBMCs, and PB from AC-SCD patients compared with NL donors and SS-SCD patients. Mononuclear cells were selected from blood from normal donors, SS-SCD patients, and AC-SCD patients. Following enrichment for CD34+ cells and Lin– cells, CD34+CD38–Lin– cells were selected by FACS. Significantly more CD34+CD38–Lin– cells were detected among mononuclear cells from AC-SCD blood compared with SS-SCD and NL-donor blood. Differences between the groups were evaluated by Student’s t test.

Figure 3

Significantly more LTC-ICs (a), NK-ICs (b), and ML-ICs (c) are present in CD34+CD38–Lin– cells from AC-SCD patients than in those from NL donors or SS-SCD patients. Single CD34+CD38–Lin– cells, selected by FACS from normal donors (44–66 wells plated), SS-SCD patients (66–88 wells plated), and AC-SCD patients (66–132 wells plated), were plated in ML-IC assays as previously described (24). After 2 weeks, single CD34+CD38–Lin– cell progeny were replated in four individual wells, two of which were maintained under LTC-IC and two under NK-IC conditions. For LTC-IC cultures, wells were overlaid after 5 weeks with clonogenic medium, and the presence of CFCs was determined 2 weeks later. NK-IC cells were harvested after 5 weeks and evaluated by FACS for the presence of CD56+ NK cells or CD19+ B cells. An LTC-IC was determined as a well in LTC-IC cultures where CFCs were present without NK and/or B cells being present in the companion NK-IC cultures. An NK-IC was determined as a well in NK-IC cultures where NK and/or B cells were present without CFCs being present in the companion NK-IC cultures. An ML-IC was identified when progeny of the initial CD34+CD38–Lin– cell gave rise to at least one LTC-IC and at least one NK-IC. Significantly more LTC-ICs, NK-ICs, and ML-ICs were present among CD34+CD38–Lin– cells in AC-SCD blood compared with blood from SS-SCD patients and NL donors. Values shown as 0 represent frequencies below the detection level of the assays. As we plated between 44 and 132 wells, this indicates that fewer than one ML-IC was present among 44–132 CD34+CD38–Lin– cells. Differences between the groups were evaluated by t test.
patients with SCD in steady state contained 13.7 (range 1–23) CD34+CD38–Lin– cells per 10^5 PBMCs. PB from patients with SCD in acute crisis (AC-SCD patients) had an average of 250 (range 38–880) CD34+CD38–Lin– cells per 10^5 PBMCs, significantly more than in SS-SCD patients or normal controls (P < 0.01).

We next examined the proportion of CD34+CD38–Lin– cells, from the three donor groups, that were ML-ICs, LTC-ICs, and NK-ICs. For these studies, CD34+CD38–Lin– cells were selected from 50–200 ml PB. Because the number of CD34+CD38–Lin– cells was significantly lower in blood of NL donors and SS-SCD patients, fewer CD34+CD38–Lin– cells were available for evaluation from these individuals (11–66 from NL donors and 28–88 from SS-SCD patients) than from AC-SCD patients (66–132 cells). We could not detect ML-ICs among PB CD34+CD38–Lin– cells from normal donors or SS-SCD patients. CD34+CD38–Lin– cells in PB from normal donors included no cells that gave rise to NK-ICs, and 0.29% (range 0–0.5%) of cells that gave rise to LTC-ICs only upon replating (Figure 3, a and b). However, because 4.35% of CD34+CD38–Lin– cells gave rise to at least one LTC-IC and one NK-IC, they were considered ML-ICs (Figure 3c). No CD34+CD38–Lin– cells from SS-SCD patients or from NL donors gave rise to LTC-ICs and NK-ICs; therefore, no ML-ICs could be detected (P < 0.05 for LTC-IC frequencies, P < 0.01 for NK-IC frequencies, and P < 0.001 for ML-IC frequencies vs. NL or SS-SCD). A strong correlation was seen between the presence of LTC-ICs (r^2 = 0.74) or NK-ICs (r^2 = 0.96) and the presence of ML-ICs in the blood of AC-SCD patients.

We next tested whether PB CD34+ cells from SCD patients included SRCs. Fifty milliliters of blood was obtained from patients with SCD in acute crisis or steady state, and 200 milliliters was obtained from normal donors. CD34+ cells were selected, and all CD34+ cells were injected in sublethally irradiated NOD-SCID mice. Because of the differences in the number of CD34+ cells obtained from a given volume of PB among AC-SCD patients, SS-SCD patients, and NL donors, the total number of CD34+ cells injected varied significantly between the groups: 2.68 × 10^5 ± 815

Table 2
Engraftment of CD34+ cells from PB of normal donors, SS-SCD patients, and AC-SCD patients

<table>
<thead>
<tr>
<th>Cell type</th>
<th>CD34+ dose</th>
<th>% Human engraftment in NOD/SCID bone marrow</th>
<th>FACS</th>
<th>Quantitative PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control (n = 5)</td>
<td>2.0 × 10^5 to 5.0 × 10^5</td>
<td>0%</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>SS-SCD (n = 5)</td>
<td>2.1 × 10^5 to 3.6 × 10^5</td>
<td>0%</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>AC-SCD</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. 1</td>
<td>2.0 × 10^5</td>
<td>1.9</td>
<td>1.75</td>
<td></td>
</tr>
<tr>
<td>No. 2</td>
<td>3.1 × 10^5</td>
<td>0.6</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>No. 3</td>
<td>2.6 × 10^5</td>
<td>1.4</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>No. 4</td>
<td>2.5 × 10^5</td>
<td>0.6</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>No. 5</td>
<td>3.7 × 10^5</td>
<td>0.6</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>No. 6</td>
<td>2.2 × 10^5</td>
<td>0.6</td>
<td>0.6</td>
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</tr>
<tr>
<td>No. 7</td>
<td>3.3 × 10^5</td>
<td>2.1</td>
<td>1.9</td>
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</tr>
<tr>
<td>No. 8</td>
<td>3.0 × 10^5</td>
<td>2.0</td>
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<td></td>
</tr>
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</table>

CD34+ cells selected by immunomagnetic beads from 200 ml PB of five NL donors, 50 ml PB of five SS-SCD patients, and 50 ml PB of eight AC-SCD patients were infused with irradiated carrier cells via the tail vein in NOD/SCID mice irradiated with 275 cGy. After 6 weeks, animals were sacrificed and marrow, blood, and spleen cells collected. The presence of human cells was determined by FACS (staining for human and mouse CD45+ cells) and by quantitative PCR as described in Methods.

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mal mouse bone marrow was used as a control. Lanes 1–3, spleens from mice nos. 1–3. Lane 13, K562 cells. Lane 14, cord blood CD34+ cells. Lanes 4–9, bone marrow from mice nos. 1–6. Lanes 10–12, PB from mice nos. 1–3. Lane 13, K562 cells. Lane 14, cord blood CD34+ cells. Normal mouse bone marrow was used as a control.

Figure 5
PCR confirmation of human hematopoietic cell engraftment in NOD/SCID mice transplanted with AC-SCD CD34+ cells. PCR for human DNA is shown. Lanes 1–3, spleens from mice nos. 1–3. Lanes 4–9, bone marrow from mice nos. 1–6. Lanes 10–12, PB from mice nos. 1–3. Lane 13, K562 cells. Lane 14, cord blood CD34+ cells. Normal mouse bone marrow was used as a control.

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1,820–2,180 pg/ml) (Figure 6b), GM-CSF (Figure 6c), and IL-8 (Figure 6d) were measured in serum of NL donors and SCD patients. SCF was undetectable in the serum of normal donors. Levels of SCF in serum of AC-SCD patients were 1,049 pg/ml (range 431–1,914 pg/ml) (P < 0.01 for AC-SCD patients vs. SS-SCD patients and NL donors). Levels of IL-8 were also significantly higher in AC-SCD patients (4,820 pg/ml; range 466–7,015 pg/ml) than in SS-SCD patients (86 pg/ml; range 41–465 pg/ml) and NL donors (58 pg/ml; range 46–98 pg/ml) (P < 0.01 for AC-SCD vs. SS-SCD patients). ML-IC frequency in blood of AC-SCD patients correlated with SCF levels at $r^2 = 0.78$, GCSF levels at $r^2 = 0.50$, IL-8 levels at $r^2 = 0.72$, and GM-CSF levels at $r^2 = 0.58$.

Addition of SCF to GCSF results in increased mobilization of primitive progenitors in the PB of lymphoma patients. To further determine whether the presence of high levels of multiple cytokines in serum of AC-SCD patients might be responsible for the mobilization of ML-ICs in the blood, we evaluated the number of ML-ICs, LTC-ICs, and NK-ICs in the PB of patients with Hodgkin disease or non-Hodgkin lymphoma who were treated with either GCSF alone or GCSF plus SCF to mobilize PB progenitors for transplantation. Increased numbers of ML-ICs were present in lymphoma patients whose blood was mobilized with GCSF plus SCF (6.25% ML-ICs [range 3.03–8.33%] per CD34+CD38–Lin– cell) compared with patients whose blood was mobilized with GCSF alone (1.7% ML-ICs [range 1.41–2.27%] per CD34+CD38–Lin– cell). As we observed for AC-SCD and SS-SCD patients, the number of LTC-ICs among PB CD34+CD38–Lin– cells was only marginally different (GCSF plus SCF, 18.22%, range 15.9–31.82%; GCSF alone, 13.45%, range 7.58–18.94%), whereas fewer NK-ICs were detected among PB CD34+CD38–Lin– cells from lymphoma patients treated with GCSF alone (1.52%, range 0–3.13%) compared with patients treated with GCSF plus SCF (8.91%, range 4.55–15.17%) (Figure 7, a–c).

Figure 6
Elevated serum levels of SCF (a), GCSF (b), GM-CSF (c), and IL-8 (d) in serum of AC-SCD patients compared with NL donors and SS-SCD patients. Serum was collected from ten normal donors, seven SS-SCD patients, and 13 AC-SCD patients, and then frozen at −80°C. Levels of SCF, GCSF, GM-CSF, and IL-8 were measured using ELISA. Differences between groups were analyzed by unpaired t test.
Discussion

SCD is the most common hematopoietic disease that results from a single gene defect. It would be treated successfully if efficient gene transfer into HSCs could be accomplished. Because transplantation of HSCs collected from mobilized blood brings about earlier hematopoietic recovery, it has replaced bone marrow HSC transplantation (28). However, toxicity caused by administration of cytokines to mobilize HSCs into the PB of patients with SCD has been observed (29). Several studies have shown that committed erythroid blast-forming unit progenitors as well as LTC-ICs are present in the blood of SCD patients (15). We show here, for the first time to our knowledge, that primitive progenitors, such as ML-ICs with both lymphoid and myeloid potential, as well as SRCs are spontaneously mobilized in AC-SCD patients. The presence of both ML-ICs and SRCs in the PB of AC-SCD patients is associated with significantly higher levels of SCF and GCSF in serum of these patients.

The LTC-IC assay measures primitive progenitors that, after 5 weeks, generate progenitors with myeloid but not lymphoid differentiation potential. However, a number of studies have shown that LTC-ICs do not correlate with engraftment of human hematopoietic progenitors (30–33). We have used the ML-IC assay to measure primitive progenitors in vitro. Although LTC-ICs and, to a lesser extent, NK-ICs could be detected in the PB of SS-SCD patients, ML-ICs were only found in the PB of AC-SCD patients. The ML-IC assay measures single progenitors that can give rise to secondary progenitors that reinitiate not only LTC-IC cultures, but also long-term lymphoid cultures in which NK cells and B cells are produced (24). The multilineage differentiation of ML-ICs is thus one of the main characteristics of HSCs. Because the ML-IC assay measures progenitors in an in vitro system, it does not address another property of HSCs, namely the ability to reconstitute hematopoiesis in vivo. To demonstrate reconstitution of hematopoiesis, transplantation is required, such as in NOD/SCID murine recipients.

In addition to ML-ICs, SRCs were also present in the blood of AC-SCD patients but not SS-SCD patients or NL donors. Our observations therefore confirm the notion that the presence of LTC-ICs in human samples at week 5 does not correlate with the presence of SRCs (34). However, we demonstrate here that the presence of ML-ICs, seen only in AC-SCD patients, correlates with the presence of SRCs, found only in PB of AC-SCD patients. Infusion of CD34+ cells from as little as 50 ml AC-SCD blood gave rise to 0.6–1.9% human CD45+ cells after 6 weeks. Moreover, cells from two AC-SCD patients that engrafted in primary NOD/SCID recipients could be transferred to secondary recipients. Although we could not detect any ML-ICs or SRCs among CD34+CD38−Lin− cells from 200 ml blood of NL donors and 50 ml blood of SS-SCD patients, there is evidence that nonmobilized PB of NL donors contains hematopoietic reconstituting cells (35, 36). Therefore, we believe that if larger volumes of blood were to be evaluated, ML-ICs and SRCs would be detectable in blood of SS-SCD patients and NL donors. Nevertheless, our studies indicate that a much higher number of ML-ICs and SRCs is detectable per ml of blood in AC-SCD patients than in SS-SCD patients and NL donors. SCF levels are five- to 20-fold higher in AC-SCD patients than in SS-SCD patients, and they correlate strongly with the presence of ML-ICs. SCF is an early-acting...
cytokine that activates the c-Kit receptor, a transmembrane tyrosine kinase receptor (37) expressed on some but not all HSCs (38, 39) and most hematopoietic progenitors. Administration of SCF to mice for 7 days results in depletion of candidate bone marrow stem cells and a corresponding reduction in radioprotective activity (40). A concomitant increase in both these hematopoietic parameters, as well as multilineage long-term reconstituting activity, is observed in spleen and PB (41). We also found that mobilization of ML-ICs in the blood of patients with lymphoma is enhanced when SCF is added to GCSF. Thus, increased levels of SCF in the blood of AC-SCD patients may be an important factor contributing to the spontaneous mobilization of primitive progenitors in the blood during sickle cell crisis.

Levels of IL-8 also correlated highly with the presence of ML-ICs. Fibbe et al. showed that mobilization of HSCs in mice and nonhuman primates by IL-8 (42) depends on the metalloproteinase gelatinase B. Whether these mechanisms also operate in human AC-SCD patients needs to be determined. Our data also confirm the findings of increased IL-8 and GM-CSF in SCD patients. We also found a correlation between GCSF levels and GM-CSF levels with the presence of ML-ICs, although the correlation was less strong than with SCF or IL-8. Expression of VCAM-1 is downregulated significantly when HSCs are mobilized using GCSF, apparently as a result of proteolysis by neutrophil-derived elastase and cathepsin G (43). Mechanisms underlying mobilization of HSCs by GM-CSF (44) have not yet been elucidated.

In some patients, acute crisis was associated with a bacterial or viral infection (Table 1). Thus, elevated levels of GCSF, GM-CSF, SCF, and IL-8 may be related, in this group of patients, to an anti-infectious response. However, in more than 50% of patients, no infection was diagnosed. The mechanism underlying the elevated levels of cytokines in this group of patients, therefore, cannot likely be attributed to an anti-infectious response but may be caused by tissue ischemia.

In conclusion, we demonstrate here, for the first time to our knowledge, that in AC-SCD patients primitive hematopoietic cells with ML-IC and SRC characteristics are spontaneously mobilized in the PB. This mobilization is correlated significantly with high serum levels of HSCs, including SCF, IL-8, and, to a lesser extent, GM-CSF and GCSF during acute crisis. The presence of large numbers of CD34+ cells, CD34+CD38−Lin− cells, and functionally defined primitive progenitors such as ML-ICs and SRCs in the PB of AC-SCD patients even in the absence of mobilizing cytokines or chemotherapy indicates that HSCs could be collected by apheresis at the time of exchange transfusion during crisis (two collections would yield more than 2.1 × 10^6 CD34+ cells per kg for a 70-kg patient). This would remove the need for bone marrow harvests or potentially dangerous cytokine-induced mobilizations.

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