The Critical Role of Src Homology Domain 2–Containing Tyrosine Phosphatase-1 in Recombinant Human Erythropoietin Hyporesponsive Anemia in Chronic Hemodialysis Patients

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Abstract. The molecular mechanism of anemia that is hyporesponsive to recombinant human erythropoietin (rHuEPO) in hemodialysis patients without underlying causative factors has not been investigated fully in hematopoietic stem cell system. Circulating CD34+ cells (1 × 10^6) were isolated from rHuEPO hyporesponsive hemodialysis patients (EPO-H; n = 9), patients who were responsive to rHuEPO (EPO-R; n = 9), and healthy control subjects (n = 9). The patients with known causes of EPO hyporesponsiveness were eliminated from the current study. The cells were cultured in STEM PRO 34 liquid medium, supplemented with rHuEPO, IL-3, stem cell factor, and granulocyte-macrophage colony stimulating factor for 7 d and then transferred to a semi-solid methylcellulose culture medium for performing burst forming unit-erythroid (BFU-E) colony assay. Expression of src homology domain 2 (SH2)-containing tyrosine phosphatase-1 (SHP-1), phosphorylated Janus kinase 2 (p-JAK2), and phosphorylated signal transducer and activator of transcription 5 (p-STAT5) was assessed with Western blot analysis. In EPO-H patients, SHP-1 antisense or scrambled S-oligos were included in the culture medium, and its effects were evaluated. The number of circulating CD34+ cells was not statistically different among the three groups, and their proliferation rates were similar for 7 d in culture. However, BFU-E colonies were significantly decreased in EPO-H patients compared with EPO-R and control groups. The mRNA and protein expression of SHP-1 and p-SHP-1 was significantly increased, whereas that of p-STAT5 was reduced in EPO-H patients. The inclusion of SHP-1 antisense S-oligo in culture suppressed SHP-1 protein expression associated with p-STAT5 upregulation, increase in p-STAT5–regulated genes, and partial recovery of BFU-E colonies. In EPO-H hemodialysis patients, the EPO signaling pathway is attenuated as a result of dephosphorylation of STAT5 via upregulation of SHP-1 phosphatase activity, and SHP-1 may be a novel target molecule to sensitize EPO action in these patients.

Since 1990, administration of recombinant human erythropoietin (rHuEPO) has dramatically improved the status of anemia in chronic hemodialysis (CHD) patients. Also, it has reduced complications related to heart disease and contributed to a better outcome in mortality and morbidity (1). However, it is still difficult to overcome rHuEPO hyporesponsiveness, in which CHD patients have persistent severe anemia despite high-dosage rHuEPO administration and correction of underlying factors, such as depleted iron storage (2), inadequate dialysis (3), severe hyperparathyroidism (4), and deficiency of water-soluble vitamins (5). Previously, we isolated circulatory CD34+ hematopoietic stem cells in hHuEPO hyporesponsive CHD (EPO-H) patients, and they were subjected to colony assay using semisolid methylcellulose culture medium. A reduced number of burst-forming units-erythroid (BFU-E) was noted in EPO-H patients. Using cDNA array technique, we also observed downregulation of various cytokines and growth factors, including IL-6, IL-9, vascular endothelial growth factor, granulocyte-macrophage colony-stimulating factor (GM-CSF), and leukemia inhibitory factor, and of receptors for thrombopoietin, IL-9, and colony-stimulating factor 1 (6). These results suggest that specific impairment of autocrine/paracrine regulatory loops in BFU-E cells contributes to the development of rHuEPO hyporesponsiveness.

To gain further insights into the molecular mechanism of rHuEPO hyporesponsiveness, we used this assay system and surveyed the gene expression profile of EPO signal transduction molecules that belong to the Janus-associated kinase 2 (JAK2) signal transducer and activators of transcription 5 (STAT5) pathway in BFU-E–derived cells using cDNA array technique. Although several signaling pathways have been

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described in the literature (7–9), JAK2/STAT5 seems to be the major downstream signaling molecules of the EPO receptor in hematopoietic progenitor cells (HPC). Upon EPO binding to its receptor, EPO receptors dimerize, and this allows adjacent JAK2, a tyrosine kinase associated with the intracellular domain of EPO receptor, to approximate with each other with resultant transphosphorylation of active sites of JAK2 (10). After the activation of JAK2, it subsequently phosphorylates tyrosine residues of the intracellular domains of EPO receptor. After the binding of STAT5 to phosphorylated EPO receptors, STAT5 is phosphorylated by JAK2, and it then dissociates from EPO receptors. STAT5 then forms dimer and translocates into the nucleus to activate the transcription of specific genes, including mouse oncostatin M protein and hemoglobin (11). The src homology domain 2 (SH2)-containing tyrosine phosphatase-1 (SHP-1) is another partner in JAK2-STAT5 signaling pathway in erythroid progenitors. SHP-1, also known as hematopoietic cell phosphatase, is a protein tyrosine phosphatase located in the cytoplasm of hematopoietic cells originally discovered in human breast carcinoma cDNA library (12). According to previous reports, SHP-1 binds to the negative regulatory domain of EPO receptor via its src-homology 2 (SH2) domains and causes dephosphorylation of JAK2, thus functioning as a negative regulator of intracellular signal transduction (13). Patients with polycythemia vera have diminished expression of SHP-1 mRNA in their colony-forming unit-erythroid (CFU-E) cells, which indicate a pivotal role for SHP-1 in the regulation of human erythroid progenitors (14). By surveying gene expression of these signaling molecules in BFU-E cells derived from EPO-H patients, we found that SHP-1 mRNA was significantly upregulated compared with rHuEPO-responsive CHD (EPO-R) patients. We hypothesized that upregulated SHP-1 suppresses the intracellular signaling events after EPO binding to its specific receptor, and it contributes to the persistent rHuEPO hyporesponsiveness in CHD patients. In the present investigation, we isolated circulating CD34+ cells from EPO-R and EPO-H patients, and they were cultured in the liquid medium, STEM PRO 34, which supports the growth and expansion of hematopoietic stem cells. These culture systems enabled us to investigate the protein expression and tyrosine phosphorylation status of JAK2-STAT5 signaling molecules, including SHP-1. After investigating the upregulated protein levels and phosphorylation of SHP-1 in EPO-H patients, we further elucidated the relevance of this signaling pathway in EPO-H patients using antisense oligodeoxynucleotide technology.

Materials and Methods

EPO-H patients (n = 9) and EPO-R patients (n = 9) were enrolled in this study. All CHD patients underwent hemodialysis therapy three times a week for at least 1 yr by using polysulfon dialysis membrane and bicarbonate dialyse (Kindaly-AF2P, Fusco, Osaka, Japan). Age- and body mass index–matched healthy control subjects (CON; n = 9) without renal dysfunction and anemia were also recruited. EPO-H patients had hematocrit (Ht) levels <25% during past 24 wk despite intravenous administration of rHuEPO, 9000 IU/wk, the maximum administration dosage regulated by the Federation of National Health Insurance in Japan. EPO-R patients maintained their Ht levels >30% with <1500 IU/wk intravenous rHuEPO administration for 24 wk. All CHD patients met the following criteria: (1) on HD therapy for >1 yr; (2) no evidence of malignancy, chronic infection, chronic inflammation, renal cysts, or diabetes; (3) no history of angiotensin-converting enzyme inhibitors or angiotensin II receptor antagonists within the past 6 mo; (4) no hematologic diseases that cause anemia, such as iron deficiency, pure red cell aplasia, and α- and β-thalassemia; (5) no gastrointestinal bleeding; (6) malnutrition; and (7) underdialysis or secondary hyperparathyroidism. Written informed consent was obtained from all patients before the investigation.

Selection and Culture of CD34+ Cells

Blood samples were withdrawn into heparinized tubes from arteriovenous fistula at the initiation of HD. Isolation of circulating CD34+ cells was performed as described previously (6). First, peripheral blood mononuclear cells were isolated by Ficoll-Paque PLUS (Pharmacia Biotech, Piscataway, NJ). Then, early hematopoietic progenitors (CD34+ cells) were isolated using Dynal CD34 progenitor cell selection system (Dynal AS, Oslo, Norway) as follows. In brief, peripheral blood mononuclear cells were incubated with Dynabeads M-450 CD34 and rosetted CD34+ cells, washed with isolation buffer (PBS without Ca2+ and Mg2+, 2% BSA, 0.6% citrate, and 100 IU/ml penicillin-streptomycin solution) and then released from Dynabeads using DETACHaBEAD CD34. At this point, 1 × 10^6 CD34+ cells were subjected to colony assay described in the next section. The purified 1 × 10^4 CD34+ cells were transferred into STEM-PRO 34 SFM liquid medium (Invitrogen, Carlsbad, CA) supplemented with 3 IU/ml rHuEPO, 50 ng/ml recombinant human stem cell factor (rHuSCF), 10 ng/ml recombinant human GM-CSF (rHuGM-CSF), and 10 ng/ml recombinant human IL-3 (rHuIL-3) (15). Cells were then seeded at a density of 1 × 10^4 cells/ml in 24-well plates and incubated at 37°C in a humidified atmosphere flushed with 5% CO2/5% O2/90% N2, and culture was maintained for 7 d.

Colony Assay of HPC

For assessing differentiation potential of erythroid progenitors, HPC that were cultured in liquid medium for 7 d were transferred into 1 ml of semisolid methylcellulose medium (Methocult H4434; Stem Cell Technologies, Vancouver, BC, Canada). Cells were seeded at the density of 1 × 10^3/ml in 35-mm Petri dishes. The methylcellulose medium contained 30% FCS, 1% BSA, 2 mM l-glutamine and 10^-4 M 2-mercaptoethanol, 3 IU/ml rHuEPO, 50 ng/ml rHuSCF, 10 ng/ml rHuGM-CSF, and 10 ng/ml rHuIL-3. The dishes were incubated at 37°C in a humidified atmosphere with 5% CO2/5% O2/90% N2 for 14 d, and the number of BFU-E colonies was counted using an inverted microscope.

Western Blot Analysis of SHP-1, Phospho-JAK2, and Phospho-STAT5

The rabbit polyclonal antibodies against SHP-1, phospho-JAK2 (p-JAK2), and phospho-STAT5 (p-STAT5) were obtained from Upstate Biotechnology (Lake Placid, NY). Anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Sigma Aldrich, St. Louis, MO) was used to verify the equal loading of proteins. The cultured CD34+ cells in liquid medium (1 × 10^6 cells/ml) were washed twice at 4°C in STEM-PRO 34 in 1.5-ml microtubes and centrifuged. The pellets were lysed in 400 μl of RIPA buffer that contained 150 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1% Triton-X, 0.5% SDS, 0.5% sodium deoxycholic acid (DOC), 0.5 μg/ml leupeptin, 2.0 μg/ml aprotinin, and 1 μM PMSF. The extracts were centrifuged for
30 min at 14,000 rpm, and the supernatants were collected for immuno blot analysis. The samples (10 μg of protein) were heated in Laemmli buffer at 100°C for 5 min, subjected to 10% SDS-PAGE, and blotted on polyvinylidene difluoride membranes. The blots were immersed in PBS-Tween (pH 7.4; 137 mM NaCl, 8.1 mM Na₂HPO₄, 12H₂O, 2.7 mM KCl, 1.5 mM KH₂PO₄, and 0.1% Tween 20) that contained 5% nonfat dry milk for 1 h, then were incubated with primary antibodies against SHP-1, p-JAK2, and p-STAT5 overnight at 4°C. After washing three times for 5 min in PBS-Tween, the blots were incubated with secondary anti-rabbit antibody conjugated to horseradish peroxidase (Upstate Biotechnology) for 1 h at room temperature and washed again three times. Autoradiograms were developed using ECL-Plus system (Amersham Life Science, Arlington Heights, IL). The optical density ratio of each band to that of GAPDH was determined using NIH image program version 1.61 (National Institutes of Health, Bethesda, MD) (16).

**Assay of SHP-1 Tyrosine Phosphorylation**

In assay of SHP-1 phosphorylation, 400 μg of CD34+ cell lysates was preadsorbed using 40 μl of protein A Sepharose CL4B (Amersham) in the presence of normal rabbit serum. Supernatants were incubated with 4 μg of anti–SHP-1 antibody, and then immune complexes were adsorbed with 80 μl of protein A Sepharose CL4B overnight at 4°C. The samples were centrifuged, and immunoprecipitates were washed four times in RIPA buffer. The proteins were eluted with 30 μl of Laemmli buffer and subjected to SDS-PAGE. The blots were incubated with mouse monoclonal anti-phosphotyrosine antibody (Cell Signaling, Beverly, MA) and visualized by ECL Western blotting system.

**Quantitative Real-Time Reverse Transcription–PCR**

To quantify the mRNA expression of SHP-1, bcl-xl, oncostatin M, and hemoglobin αl in the HPC, we performed real-time reverse transcription–PCR (RT-PCR). First-stranded cDNA were synthesized from 1.0 μg of total RNA using ribonuclease H-MMLV (moloney murine leukemia virus) reverse transcriptase and were added to the Lightcycler-Mastmix (0.5 μM specific primers, 4 mM MgCl₂, and 2 μl of Master SYBR Green; Roche Diagnostics, Mannheim, Germany). PCR was optimized and performed in a real-time PCR cycler (Lightcycler; Roche Diagnostics) (17). For SHP-1, the denaturation at 95°C for 10 min was followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 62°C for 5 s, and extension at 72°C for 10 s. For bcl-xl, oncostatin M, hemoglobin αl, and GAPDH, the denaturation at 95°C for 10 min was followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 15 s, and extension at 72°C for 12 s. The copy number of SHP-1, bcl-xl, oncostatin M, and hemoglobin αl cDNA was normalized with a housekeeping gene (GAPDH), and the relative expression ratios were calculated. Gene-specific primers for human SHP-1 (Genbank accession no. NM_002831), bcl-xl (Z23115), oncostatin M (NM_020530), hemoglobin αl (NM_0002831), and GAPDH (M33197) were as follows: SHP-1 forward (5'–GCTGGACGTGGACATTGC–3'), SHP-1 reverse (5'–ATGCCTGGATCTCGACT–3'), bcl-xl forward (5'–TTCTGAGGGGGTACT–3'), bcl-xl reverse (5'–ATGGTTGAGGAGCAGA–3'), oncostatin M forward (5'–AGACCTTCAATGCCACAC–3'), oncostatin M reverse (5'–TCTCCACA GTCTCGATGGTCA–3'), hemoglobin αl forward (5'–CGGTA CACTCCGCTCTAA–3'), hemoglobin αl reverse (5'–CCACACGGGCAAGAAGCAT–3'), GAPDH forward (5'–TGAGGAGGTA CACTGG–3'), and GAPDH reverse (5'–TCCACCACCTGGTTCTGA–3').

**SHP-1 Antisense Experiment Using CD34+ Cells Derived from EPO-H Patients**

Forty micrograms of phosphorothioated SHP-1 antisense oligode oxynucleotide (ODN), 5'-GAGGTCTCGTGAAACCACCTCACCATCCT-3' (18), or phosphorothioated scrambled ODN, 5'-GGCAGGTTCTCGGACACCGATACTT-3', was incorporated into 0.1 AU of hemagglutination virus of Japan (HVJ)-envelope vector (GenomeONE-Neo; Ishihara Sanyyo, Osaka, Japan). CD34+ cells were isolated from EPO-H and CON groups, and 1 × 10⁶ cells were suspended in 1 ml of STEM-PRO 34 liquid medium. HVJ-E vector carrying SHP-1 antisense ODN was added into culture media and centrifuged at 2000 rpm for 1 h at 37°C. The cells were further cultured in STEM-PRO 34 SFM Complete Medium supplemented with rHuEPO, rHuSCF, rHuGM-CSF, and rHuIL-3 for 7 d. They were then subjected to Western blot analysis, quantitative real-time RT-PCR, and BFU-E colony assay as described above. The transfection efficiency into CD34+ cells was evaluated by using pEGFPLuc (BD Biosciences, Palo Alto, CA), pcDNA3.1/V5-His-TOPO/lacZ (Invitrogen), and FITC-labeled SHP-1 antisense ODN. Two days after the DNA transfection into CD34+ cells using HVJ-E vector, luciferase activity was evaluated by Luciferase Reporter Assay Kit (BD Biosciences), and α-galactosidase activity was visualized by α-Gal staining set (Roche).

**Statistical Analyses**

Data are presented as mean ± SD. Statistical analysis was performed by Wilcoxon nonparametric test. P < 0.05 was considered significant.

**Results**

**Clinical Characteristics of CHD Patients**

The EPO-H group revealed significantly lower Ht (22.3 ± 1.50%), even after the intravenous administration of a high dosage of rHuEPO, whereas the EPO-R group had a higher Ht (32.9 ± 1.2%) with low dosage of rHuEPO (Table 1). It has been reported that hemoglobin concentration improves in 90 to 95% of patients treated (4), and we also found that ~5% of CHD patients met criteria of the EPO-H group in our hospitals (Table 2). Serum erythropoietin, ferritin, and percentage of transferrin saturation revealed that the possibility of iron deficiency was excluded in both EPO-H and EPO-R groups. The nutritional status (body mass index and serum albumin levels), intact parathyroid hormone levels, the efficacy of HD (Kt/V urea values), and C-reactive protein did not show any significant differences between the two groups, and the patients in EPO-H group lacked clinical evidence of malnutrition, inadequately controlled hyperparathyroidism, underdialysis, and inflammation. The number of isolated CD34+ cells before culture was 1.16 ± 0.42 × 10⁴/ml in the EPO-H group and 1.39 ± 0.39 × 10⁴/ml in the EPO-R group, and there was no statistical difference (Table 1).

**Colony Assays for BFU-E and GM-CFU Using Circulating CD34+ Cells**

An equal number of purified CD34+ cells (1 × 10⁴ cells) was subjected to semisolid methylcellulose cultures and maintained for 14 d. The number of BFU-E was significantly less in the EPO-H group compared with the CON and
Table 1. Clinical characteristics of CHD patients

<table>
<thead>
<tr>
<th>No. of the patients</th>
<th>EPO-R</th>
<th>EPO-H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>6/3</td>
<td>5/4</td>
</tr>
<tr>
<td>Body mass index</td>
<td>23.2±1.0</td>
<td>23.4±1.3</td>
</tr>
<tr>
<td>Months after the initiation of CHD therapy</td>
<td>94.9±22.7</td>
<td>86.0±38.1</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>167</td>
<td>71</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>3.6</td>
<td>3.2</td>
</tr>
<tr>
<td>High sensitivity C-reactive protein (mg/ml)</td>
<td>0.29±0.20</td>
<td>0.20±0.21</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>10.7±0.5</td>
<td>7.2±0.6b</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>32.9±1.2</td>
<td>22.3±1.5b</td>
</tr>
<tr>
<td>White blood cell count (×10³/µl)</td>
<td>5529±199</td>
<td>5333±1183</td>
</tr>
<tr>
<td>Ferritin (ng/ml)</td>
<td>60.3±17.0</td>
<td>55.5±26.0</td>
</tr>
<tr>
<td>Total iron-binding capacity (µg/dl)</td>
<td>249±44</td>
<td>247±39</td>
</tr>
<tr>
<td>Unsaturated iron-binding capacity (µg/dl)</td>
<td>189±39</td>
<td>191±35</td>
</tr>
<tr>
<td>Percentage of transferin saturation (%)</td>
<td>23.3±7.7</td>
<td>24.5±6.1</td>
</tr>
<tr>
<td>Ferritin (ng/ml)</td>
<td>147±80</td>
<td>173±92</td>
</tr>
<tr>
<td>Erythropoietin (mU/ml)</td>
<td>20.5±9.4</td>
<td>23.1±7.2</td>
</tr>
<tr>
<td>Intact parathyroid hormone (pg/ml)</td>
<td>167±73</td>
<td>123±71</td>
</tr>
<tr>
<td>Highly sensitive C-reactive protein (mg/ml)</td>
<td>0.29±0.20</td>
<td>0.20±0.21</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>3.6±0.2</td>
<td>3.7±0.2</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>167±16</td>
<td>151±17</td>
</tr>
<tr>
<td>Kt/V urea</td>
<td>1.42±0.13</td>
<td>1.46±0.11</td>
</tr>
</tbody>
</table>

* Data are mean ± SD.

CHD, chronic hemodialysis; EPO-H, rHuEPO hypersensitive CHD patients; EPO-R, rHuEPO responsive CHD patients.

Table 2. Known causes of EPO hypersensitivity in CHD patients (N = 256)

<table>
<thead>
<tr>
<th>Causes of EPO Hyporesponsiveness</th>
<th>Patient Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron deficiency</td>
<td>6</td>
</tr>
<tr>
<td>Chronic inflammation by autoimmune diseases or other causes</td>
<td>4</td>
</tr>
<tr>
<td>Chronic infection</td>
<td>3</td>
</tr>
<tr>
<td>Malignancies</td>
<td>3</td>
</tr>
<tr>
<td>Malnutrition</td>
<td>3</td>
</tr>
<tr>
<td>Gastrointestinal bleeding</td>
<td>1</td>
</tr>
<tr>
<td>Severe hyperparathyroidism</td>
<td>1</td>
</tr>
<tr>
<td>Underdialysis</td>
<td>0</td>
</tr>
<tr>
<td>Angiotensin-converting enzyme inhibitors or angiotensin II receptor antagonists</td>
<td>0</td>
</tr>
<tr>
<td>Pure red cell aplasia</td>
<td>0</td>
</tr>
<tr>
<td>Thalassemias</td>
<td>0</td>
</tr>
<tr>
<td>Unknown causes</td>
<td>13</td>
</tr>
</tbody>
</table>

EPO-R groups (Figure 1A). Furthermore, colony size was significantly smaller in the EPO-H group compared with the CON and EPO-R groups (Figure 1B). The number of GM-CFU was not significantly different among the three groups (Figure 1C).

**Liquid Culture of HPC and Colony Assays for BFU-E**

The purified CD34+ cells (1 × 10⁴ cells) were cultured in liquid medium for 7 d, then the cultured cells (1 × 10⁴ cells) were transferred into 1 ml of semisolid methylcellulose medium and maintained for 14 d to access BFU-E colony formation. The number of proliferated HPC at day 7 in liquid medium showed no statistical difference among the CON, EPO-H, and EPO-R groups (Figure 2A). However, the number of BFU-E was significantly less in the EPO-H group compared with the CON and EPO-R group (P < 0.01; Figure 2, C through F). There was no statistical difference in the number of GM-CFU among the CON, EPO-H, and EPO-R groups (Figures 1A and 2F). Thus, we investigated CD34+ cells after 7 d of liquid culture to elucidate molecular mechanism of reduced BFU-E in EPO-H patients.

**Western Blot Analysis of SHP-1, p-SHP-1, p-JAK2, and p-STAT5**

The relative optical density of SHP-1 to GAPDH (Figure 3E) in the EPO-H group (1.62 ± 0.21) was significantly higher compared with the EPO-R group (0.90 ± 0.15; P < 0.01; Figure 3, A and F) and the CON group (0.69 ± 0.14; P < 0.001; Figure 3, A and F). Furthermore, tyrosine phosphorylation of SHP-1 was significantly enhanced in the EPO-H group compared with both CON and EPO-R groups (P < 0.001; Figure 3, B and G). In contrast, the p-STAT5/GAPDH ratio was significantly higher in the EPO-R group (1.26 ± 0.20) and the CON group (1.43 ± 0.14) than in the EPO-H group (0.78 ± 0.13; P < 0.01; Figure 3, C and H). Because there were no significant differences in p-JAK2/GAPDH among the three groups (Figure 3, D and I), it suggested that the upregulated p-SHP-1 mainly dephosphorylated p-STAT5 in cultured HPC derived from EPO-H patients.

**Quantitative Real-Time RT-PCR**

mRNA was isolated from the HPC that were cultured in liquid medium for 7 d, and they were subjected to quantitative real-time RT-PCR. SHP-1 mRNA expression, i.e., the copy number ratio (hematopoietic cell phosphatase/GAPDH), significantly upregulated in the EPO-H group (0.67 ± 0.09 × 10⁻²) compared with the EPO-R group (0.095 ± 0.04 × 10⁻²) and the CON group (0.10 ± 0.03 × 10⁻²; P < 0.001; Figure 4).

**Gene Transfection Efficiency into CD34+ Cells by HVJ-E Vector**

CD34+ cells (1 × 10⁴) were suspended in 1 ml of STEM-PRO 34 liquid medium, and they were centrifuged in the presence of HVJ-E vector only or HVJ-E vector carrying
pEGFPLuc. After 2 d in culture, CD34+ cells were lysed and subjected to luciferase assay. Luciferase activity was significantly increased in pEGFPLuc transfected CD34+ cells (2207 ± 105 relative light units) compared with control (138 ± 26 RLU; Figure 5A). By transfecting pcDNA3.1/V5-His-TOPO/lacZ by HVJ-E vector, blue-stained cells were observed by α-Gal staining, and transfection efficiency was ~30%. Transfection efficiency of FITC-labeled ODN was also ~30% of CD34+ cells.

**Figure 1.** Number and area of burst forming unit-erythroid (BFU-E)-derived colonies in methylcellulose cultures. Circulating CD34+ cells (1 × 10^4 cells) were cultured in methylcellulose with 10 ng/ml IL-3, 3 U/ml erythropoietin (EPO), 50 ng/ml stem cell factor, and 10 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF). The number of BFU-E is significantly less in the erythropoietin-hyperresponsive (EPO-H) group compared with control (CON) and erythropoietin-responsive (EPO-R) groups (A). Colony size is significantly smaller in the EPO-H group compared with the CON and EPO-R groups (B). Numbers of GM-CFU are not significantly different among the three groups (C). *P < 0.01.

**SHP-1 Antisense Experiment Using CD34+ Cells Derived from EPO-H Patients**

CD34+ cells that were derived from the EPO-H and CON groups were treated with HVJ-E vector carrying SHP-1 antisense ODN or HVJ-E vector carrying scrambled ODN. They were further cultured in liquid media and subjected to BFU-E colony assay and Western blot analysis. After 14 d of culture on methylcellulose plates, BFU-E colonies significantly recovered by the treatment of SHP-1 antisense ODN compared with scrambled ODN-treated CD34+ cells derived from EPO-H patients (P < 0.05; Figure 5, B through D). In contrast, SHP-1 antisense ODN did not alter the number of BFU-E compared with scrambled ODN-treated CD34+ cells in CON. Thus, we further checked the status of SHP-1 and p-STAT5 by Western blot analysis and quantified mRNA in p-STAT5-regulated genes by real-time RT-PCR. By Western blot analysis, SHP-1/GAPDH ratios were significantly decreased in the antisense ODN group (0.93 ± 0.14) compared with the scrambled ODN-treated CD34+ cells (1.72 ± 0.18; P < 0.01; Figure 5E).
turn, p-STAT5/GAPDH ratios significantly increased by SHP-1 antisense ODN (1.78 ± 0.30) compared with scrambled ODN-treated CD34+/H11001 cells (1.26 ± 0.28; *P < 0.05; Figure 5F).

To confirm further the functional activation of p-STAT5 in the SHP-1 antisense ODN-treated cells, we evaluated mRNA expression of p-STAT5–regulated genes, such as bcl-xL, oncostatin M, and hemoglobin 1. Copy number ratios of bcl-xL/GAPDH (0.08 ± 0.001) and oncostatin M/GAPDH (0.225 ± 0.054) in the antisense ODN-treated group were significantly higher compared with scrambled ODN–treated CD34+/H11001 cells, bcl-xL/GAPDH (0.05 ± 0.001), and oncostatin M/GAPDH (0.153 ± 0.013; *P < 0.01; Figure 5, G and H). We found that hemoglobin 1 mRNA abundantly expressed, and there were no statistical differences between antisense (hemoglobin 1/GAPDH ratio, 4.88 ± 6.62) and scramble ODN-treated (4.80 ± 8.33) CD34+/H11001 cells (Figure 5I).

Discussion

It has not been fully investigated how CHD patients develop chronic renal failure (CRF)-associated anemia or why these patients invariably reveal the hyporesponsiveness to exogenous administration of rHuEPO. There are numerous causes for EPO hyporesponsiveness, such as iron deficiency, chronic inflammation, malignancies, malnutrition, underdialysis, secondary hyperparathyroidism, and gastrointestinal bleeding. Even after the elimination of these causes, 5 to 10% of CHD patients still developed hyporesponsiveness to rHuEPO (4). Previously, we reported that the number of circulating CD34+/H11001 hematopoietic progenitors in such EPO-R patients is comparable to EPO-R patients and normal subjects (6). However, the number of peripheral blood BFU-E, examined by a methylcellulose culture, is significantly reduced in EPO-R patients despite administration of rHuEPO. We analyzed and compared the gene expression profile by extracting mRNA from BFU-E.
colonies and demonstrated the impairment of the autocrine/paracrine regulatory loop of various hematopoietic cytokines in EPO-H patients (6). Because we collected only a minute amount of cells from BFU-E colonies, we were not able to examine the activation of various signaling molecules. To address this issue, we isolated circulating CD34+ cells and cultured them in STEM PRO 34 liquid culture media, supplemented with prescribed cytokines, which enabled us to examine the activation or phosphorylation of signaling molecules downstream of the cytokine receptors. Furthermore, BFU-E colony formation of CD34+ cells, isolated from peripheral blood in EPO-H and EPO-R patients, was not altered by the expansion in STEM PRO 34 liquid culture. Thus, this proliferation assay system is appropriate and suitable for evaluation of BFU-E formation and signaling events of various cytokine receptors. By using the proliferation assay system, we demonstrated that mRNA and protein expression and tyrosine phosphorylation of SHP-1 significantly increased in CD34+ cells derived from EPO-H patients compared with the EPO-R group.

Western blot analysis revealed that p-STAT5 was significantly lower in the EPO-H group compared with the EPO-R group, whereas there was no difference in p-JAK2. Therefore, we consider that upregulated phosphorylated SHP-1 mainly dephosphorylates p-STAT5 and thus negatively regulates EPO signaling in HPC and contributes to the rHuEPO hyporesponsiveness in EPO-H patients (Figure 6). It is surprising that EPO-H patients without known causes of EPO hyporesponsiveness have similar abnormalities in the EPO signaling pathway, SHP-1 and STAT5. In human CD34+ cells and erythroblasts, erythropoietic factors activate three signaling pathways, JAK-STAT, mitogen-activated protein kinase p42/44, and phosphatidylinositol 3-kinase/Akt axes, and these processes are regulated by orchestrated activation of multiple signaling cascades. In preliminary data, we did not recognize apparent differences in phosphorylation of AKT and mitogen-activated protein kinase p42/44 in CD34+ cells in EPO-H patients; thus, the changes in phosphorylation of SHP-1 and STAT5 seems to be specific events in EPO-H patients.

In addition to EPO signaling, SHP-1 negatively regulates the IL-3 signaling, i.e., SHP-1 associates with the α-chain of IL-3 receptor via the amino-terminal SH2 domain of SHP-1 after the binding of ligand to the receptor, which then leads to dephosphorylation of the phosphorylated tyrosine residues. Paling et al. (19) demonstrated that SHP-1 negatively regulates IL-3 signaling in BaF/3 cells, potentially via regulation of tyrosine phosphorylation of STAT5. In the process of hematopoietic cell differentiation, IL-3 mainly regulates the proliferation of immature hematopoietic progenitors (GM-CFU and early BFU-E), whereas EPO promotes both proliferation and differentiation of later stages of erythroid progenitors, including late BFU-E. One can speculate that SHP-1 negatively regulates early BFU-E via dephosphorylation of IL-3 receptor and STAT5 and plays a role in rHuEPO hyporesponsiveness. Matsuzaki et al. (20) reported that BFU-E formation in EPO-H CHD patients was suppressed under increasing concentration of IL-3 (0.01 to 1 ng/ml), whereas normal response in GM-CFU formation similar to the control subjects was observed in the presence of low-concentration IL-3 (0.01 ng/ml). Lower sensitivity to IL-3 for BFU-E formation indicated that SHP-1-mediated inhibition of IL-3 signaling is critical in early BFU-E colonies and not in GM-CFU formation. However, it remains unclear why SHP-1 selectively inhibits BFU-E colony formation in EPO-H patients because IL-3 signaling is also critical for differentiation of myeloid progenitor series of cells, and SHP-1 as a tumor suppressor in lymphoma or leukemia patients has been suggested (21,22).

The human SHP-1 gene consists of 17 exons that span 17 kb, including two alternative 5’ exon 1, which are driven by two different promoters (21,23). The hematopoietic form of the SHP-1 transcript is initiated at a downstream promoter, promoter 2, separated by 7 kb from the upstream promoter 1. The downstream promoter 2 is active exclusively in cells of the hematopoietic lineage, whereas promoter 1 is active in various cells of nonhematopoietic origin. Promoter 1 has two characteristic motifs for expression of SHP-1: E-box, located 190 bp upstream of the transcription starting site (CAP site), and NF-kB binding site, located 105 bp upstream of the E-box. In contrast, promoter 2 contains an inverted GATA box, a CCAAT box, and a TATA box region. Thus, the molecular genetic approaches do not provide enough data to explain promoter 2–driven hematopoietic-specific expression of SHP-1 and its transcriptional regulation. For instance, diminished or abolished expression of SHP-1 protein was reported in various hematologic cell lines: B cell lymphoma, T cell lymphoma, natural killer cell lymphoma, chronic myelogenous leukemia, B cell acute lymphoblastic leukemia, and polycythemia vera. In these leukemic cell lines, DNA deletion or single-base mutations in SHP-1 gene were not detected, and the absence of expression of SHP-1 mRNA could be due to the gene silencing resulting from the methylation of the cysteine residue in the CpG islands located in the region encompassing promoter 2 and the first exon of hematopoietic isoform.

Why does SHP-1 significantly increase in CD34+ cells derived from EPO-H patients? First, we can raise the possibility that multiple causative factors, such as uremic toxins, inflammation, and iron deficiency, may be involved in upregulated expression of SHP-1. However, after the exclusion of CHD patients with such known causes for rHuEPO hyporesponsiveness, EPO-H patients revealed profound EPO hyporesponsiveness. As described above, there are no characteristic transcription factor binding sites in the promoter region of SHP-1; thus, we cannot negate the possibility that there are unknown conditions in the uremic state that provoke rHuEPO hyporesponsiveness. Because upregulated expression of SHP-1 gene persisted even after the in vitro culture in STEM PRO 34 liquid media or methylcellulose supplemented with various growth factors, the other plausible reason for upregulated expression of SHP-1 gene in EPO-H patients may be attributed to single nucleotide gene polymorphism in promoter regions or to the modification of the degree of methylation of the SHP-1 gene. As mentioned above, absence of expression of SHP-1 mRNA in leukemic cells could be due to the gene silencing resulting from increased methylation of the cysteine residue in the CpG islands located in the region encompassing promoter
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Figure 5. Schematic drawing of signal pathways in response to EPO. EPO receptor dimerization after EPO binding is schematically shown. Dimerized EPO receptors allow two JAK2 to approximate with each other and transphosphorylate active sites of JAK2. JAK2 subsequently phosphorylates tyrosine residues in the intracellular domains of EPO receptor, and then STAT5 binds to phosphorylated EPO receptors. STAT5 is phosphorylated by JAK2, dissociates from EPO receptors, and then forms dimer and translocates into the nucleus to activate the transcription of specific genes, including mouse oncostatin M protein and hemoglobin. SHP-1 binds to the negative regulatory domain of EPO receptor via its src-homology 2 (SH2) domain and causes dephosphorylation of JAK2 and STAT5; thus, it functions as a negative regulator of EPO signal transduction.

Figure 6. Schematic drawing of signal pathways in response to EPO. EPO receptor dimerization after EPO binding is schematically shown. Dimerized EPO receptors allow two JAK2 to approximate with each other and transphosphorylate active sites of JAK2. JAK2 subsequently phosphorylates tyrosine residues in the intracellular domains of EPO receptor, and then STAT5 binds to phosphorylated EPO receptors. STAT5 is phosphorylated by JAK2, dissociates from EPO receptors, and then forms dimer and translocates into the nucleus to activate the transcription of specific genes, including mouse oncostatin M protein and hemoglobin. SHP-1 binds to the negative regulatory domain of EPO receptor via its src-homology 2 (SH2) domain and causes dephosphorylation of JAK2 and STAT5; thus, it functions as a negative regulator of EPO signal transduction.

2 and the first exon of hematopoietic isoform. Thus, we may further investigate whether frequency of methylation of CpG islands decreases in CD34+ cells in EPO-H patients.

Because upregulated expression of SHP-1 was noted in EPO-H patients, antisense technology was used to confirm the functional role of SHP-1 in the pathogenesis of rHuEPO hyporesponsiveness. There have been some reports regarding gene transfection into hematopoietic stem cells using adenovirus (24,25), electroporation (26,27), and recombinant HVJ (28). Recently, HVJ-E vector has come into use to minimize the damage to transfected cells (29), and this is a novel tool that can be used easily to transfer expression plasmids, ODN, and proteins (30). In our initial studies, transfection efficiency of FITC-labeled SHP-1 antisense ODN was ~30% without significant cell toxicity. Such transfected CD34+ cells could be maintained in culture media and evaluated for the BFU-E colony formation and expression of signal transduction molecules SHP-1 and STAT5. After culture in methylcellulose media, the treatment of SHP-1 antisense ODN decreased SHP-1 protein expression and upregulated p-STAT5, and it resulted in the partial recovery of BFU-E colony formation. The current data indicate that SHP-1 is a novel therapeutic target in the treatment of EPO-H patients who undergo CHD therapy. In addition, the inhibition of SHP-1 may be beneficial to improve the responsiveness to rHuEPO in CRF patients while reducing the dosage of rHuEPO administration.

In conclusion, we demonstrated that intracellular signal transduction of EPO receptor is attenuated as a result of dephosphorylation of STAT5 via SHP-1 phosphatase activity, thus contributing to rHuEPO hyporesponsiveness in hematopoietic progenitors in EPO-H CHD patients. By suppression of SHP-1 protein using antisense ODN, we confirmed that SHP-1 is relevant in the pathogenesis of rHuEPO hyporesponsiveness in CHD patients. Thus, the future efforts should be directed for screening of specific inhibitors of SHP-1 phosphatase activity, and these agents may improve the quality of life in CRF patients and also reduce the financial burden in CHD patients.

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* indicates statistical significance compared to control group.

** indicates statistical significance compared to control group.

*** indicates statistical significance compared to control group.

P values are shown in the figure.
References

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