Erythropoietin Induces Tyrosine Phosphorylation of Jak2, STAT5A, and STAT5B in Primary Cultured Human Erythroid Precursors

By Atsushi Oda, Kenichi Sawada, Brian J. Druker, Katsutoshi Ozaki, Hina Takano, Kazuki Koizumi, Yoshikazu Fukada, Makoto Handa, Takao Koike, and Yasuo Ikeda

We examined signaling by erythropoietin in highly purified human colony forming unit-erythroid cells, generated in vitro from CD34+ cells. We found that erythropoietin induces tyrosine phosphorylation of Jak2, STAT5A, and STAT5B. Tyrosine phosphorylation of Jak2 reaches a peak around 10 minutes after stimulation and is maximum at 5 U/mL of erythropoietin. Tyrosine phosphorylation of STAT5 is accompanied by the translocation of activated STAT5 to the nucleus as shown by electrophoretic mobility shift assay (EMSA) using 32Pi-labeled STAT5 binding site in the β-casein promoter. Tyrosine phosphorylation of STAT1 or STAT3 was not detected in human erythroid precursors after stimulation with erythropoietin. Crkl, an SH2/SH3 adapter protein, becomes commonly precipitated specifically with STAT5 from erythropoietin-stimulated erythroid cells; although it was shown to become associated with c-Cbl in the studies using cell lines. Thus, human erythroid precursors can be expanded in vitro in sufficient numbers and purity to allow its usage in signal transduction studies. This report sets a basis for further studies on signaling in primary cultured human erythroid precursors, which in turn contribute to our better understanding in the differentiation processes of erythrocytes and their precursors.

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ERYTHROPOIETIN is a glycoprotein hormone that is essential for normal erythropoiesis. In vitro, erythropoietin is absolutely required for the survival, proliferation, and differentiation of erythroid precursors, although a recent study showed that interleukin-6 (IL-6) and its soluble form of the receptor may also have a similar effect on the erythroid precursors in the presence of stem cell factor (SCF). The effect of erythropoietin is exerted through binding to the specific receptors on the surface of responding cells, leading to dimerization of the receptors and activation of Jak2 tyrosine kinase. Whether Jak2 is pre-associated with the erythropoietin receptor before the ligand binding or not is a controversial issue. A recent report suggests that the association is a multistep process, partially regulated through phosphorylation of the receptor on tyrosine and serine residues. It was reported by many that activation of Jak2 is accompanied by tyrosine phosphorylation of numerous proteins including Jak2 itself, STAT proteins, Shc, Vav, and the erythropoietin receptor, although tyrosine kinases other than Jak2 may also phosphorylate these proteins.

Colony-forming unit-erythroid (CFU-E) comprises only 0.5% or less of all cells in normal bone marrow. Colony-forming unit-erythroid cells in human peripheral blood in sufficient numbers to allow the examination of early signaling by erythropoietin.

MATERIALS AND METHODS

Reagents. HEPES, sodium dodecyl sulfate (SDS), 2-mercaptoethanol (2-ME), sodium orthovanadate, bovine serum albumin (BSA), DNase, chicken egg albumin, Iscove’s Modified Dulbecco’s Medium (IMDM), propidium iodide (PI), protein A-Sepharose, protein G-Sepharose, isopropyl β-D-thiogalactopyranoside (IPTG), Triton X-100, and Tris (hydroxymethyl) aminomethane (Tris) were purchased from Sigma (St Louis, MO). Polyvinylidene difluoride (PVDF) membranes (pore size, 0.45 μm) were from Millipore Corporation (Bedford, MA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) molecular standards and 32Pi were from Amersham (Arlington Heights, IL) or BioRad (Richmond, CA). Double stranded Poly (dl-dc) was from Pharmacia Biotech (Milwaukee, WI). Enhance chemiluminescence (ECL) reagents including secondary antibodies were purchased from Amersham. Insulin (porcine sodium, activity United States Pharmacopoeia [USP] U/mg) was purchased from Calbiochem and Behring Diagnostics, La Jolla, CA. Antiphosphotyrosine murine monoclonal antibody (4G10) was used as described. STAT5A and Jak2 antisera, and anti-STAT1 and glutathione S-transferase (GST) monoclonal antibodies were from Santa Cruz (Santa Cruz, CA). An anti-STAT5 monoclonal antibody was from Transduction Laboratories (Lexington, KY). Nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP) were from Gibco BRL (Gaithersburg, MD). For EMSA, an anti-STAT5 antiserum (a kind gift from Dr Hiroshi Wakao, University of Tokyo, Tokyo, Japan) described previously, was used. STAT5A and STAT5B antiseria were from R&D systems (Minneapolis, MN). Recombinant erythropoietin,

From the Division of Hematology, Department of Internal Medicine, Keio University, Tokyo, Japan; the Blood Center, Keio University, Tokyo, Japan; The Department of Internal Medicine II, Hokkaido University School of Medicine Sapporo, Hokkaido 060, Japan; and the Division of Hematology and Medical Oncology, Oregon Health Sciences University, Portland, OR.

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A.O. and K.S. contributed equally to this work.
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Address reprint requests to Atsushi Oda, MD, PhD, Department of Internal Medicine, School of Medicine, Keio University, 35 Shinnanomachi, Tokyo 160, Japan.

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IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), and SCF were kindly donated from Kirin Brewery Co Ltd (Tokyo, Japan). Erythropoietin was also kindly donated from Chugai Pharmaceutical Co Ltd (Tokyo, Japan). Vitamin B 12 and folic acid were from Sankyo Pharmaceutical Co (Tokyo, Japan) and Takeda Pharmaceutical Co (Osaka, Japan), respectively. Human IL-3 (104 chronic myelogenous leukemia U/mg) was from Amgen Biologicals (Thousand Oaks, CA). Dynabeads M 450 coated with goat antimouse IgG were from Dynal Inc (New York, NY). Fetal calf serum (FCS), penicillin, and streptomycin were from Flow Laboratories Inc (McLean, VA).

Ex vivo generation of erythroid progenitor cells. Recombinant human granulocyte colony-stimulating factor (G-CSF: Chugai Pharmaceutical Co and Kyohwa Hakkoh Pharmaceutical Co, Tokyo, Japan) was administered to healthy adult volunteers (who had previously signed consent forms approved by the Hokkaido University School of Medicine and the Hokkaido Red Cross Blood Center Committee for the Protection of Human Subjects), as described elsewhere.21 The mobilized peripheral blood (PB) CD34+ cells were isolated with immunomagnetic beads, as described in detail elsewhere.22,23 The cells were then cryopreserved and stored until use in a tank with liquid nitrogen. The frozen PB CD34+ cells were thawed, suspended in IMDM containing 30% heat inactivated and 100 U/mL DNase, and were centrifuged at 400g for 5 minutes at 4°C. The cells were washed two times with IMDM containing 20% FCS, and resuspended in IMDM containing 30% heat inactivated and 100 U/mL DNase, and were centrifuged at 10,000g for 5 minutes at 4°C. The supernatant was removed and the cells were washed two times with IMDM containing 20% FCS for 12 hours at room temperature. Two microliters of nuclear extracts (40 µg, containing 10 to 20 µg protein) were prepared from day-8 cells (2 × 107 cells) by lysis followed by high salt extraction as described previously.20 Two microliters of nuclear extracts were mixed with 20 µL of binding buffer [10 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1% NP-40, 5% glycerol, 1 mg/mL bovine serum albumin, and 2 mM mg/L poly(dI-dC). The mixture was incubated for 30 minutes at room temperature. Complexes were separated on 5% nondenaturing polyacrylamide gels in 0.25 × Tris borate EDTA buffer and detected by autoradiography.

 EMSA. Nuclear extracts (40 µg, containing 10 to 20 µg protein) were prepared from day-8 cells (2 × 107 cells) by lysis followed by high salt extraction as described previously.20 Two microliters of nuclear extracts were mixed with 20 µL of binding buffer [10 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1% NP-40, 5% glycerol, 1 mg/mL bovine serum albumin, and 2 mM mg/L poly(dI-dC). The mixture was incubated for 30 minutes at room temperature. Complexes were separated on 5% nondenaturing polyacrylamide gels in 0.25 × Tris borate EDTA buffer and detected by autoradiography.

 TF-1 cell. Human IL-3 and GM-CSF-dependent TF-1 cell line was kindly provided by Dr Hisamaru Hirai (University of Tokyo, Tokyo, Japan).29 Cells were maintained in IMDM containing 10% fetal calf serum (FCS) and 1 ng/mL human GM-CSF. Before stimulation with GM-CSF or IL-3, cells were incubated in IMDM containing 10% FCS without exogenous IL-3 or GM-CSF for 18 hours. After two washes, they were incubated in PBS (pH 7.4) at 37°C.

 GST binding assays. The GST-SH2 domain of Crkl fusion protein (GST-Crkl-SH2) was generated as previously described.30,31 The GST-fusion construct was transformed into Escherichia coli DH-5α and protein expression was induced by 0.5 mM/L IPTG to exponentially growing cells. The GST-Crkl-SH2 was isolated from sonicated bacterial lysates using glutathione sepharose beads. A total of 2.5 µg of GST-fusion proteins were incubated with 50 µL of glutathione sepharose beads in bacterial lysis buffer (150 mM/L NaCl, 16 mM/L NaHPO4, 4 mM/L NaH2PO4, [pH 7.3]) containing 10 µg of aprotinin, 1 mM/L orthovanadate, 1 mM/L PMSF, and 0.1% (β-mercaptoethanol). The beads were washed three times with PBS and were incubated
with cell lysates for 3 hours on ice. Proteins were separated by SDS-PAGE and transferred onto PVDF membranes for immunoblot analysis.

RESULTS

The purified fraction from mobilized PB contained 96.7% ± 1.6% (n = 3, mean ± SD) CD34+ cells. Cultivation of PB CD34+ cells for 8 days in serum-containing medium with erythropoietin, IL-3, and SCF resulted in a 120 ± 49-fold expansion of the total cell number (day-8 cells, n = 8) with a viability of 96% ± 3%. A total of 71% ± 13% cells expressed the specific erythroid marker Gly A, whereas CD34 and myelomonocytic marker CD13 were almost absent, as shown in Table 1. In morphologic analysis by May-Grunwald-Giemsa staining, day-8 cells predominantly consisted of immature erythroid cells with a purity of 92% ± 6%27 whereas most of the remaining cells showed a basophilic/eosinophilic-like feature. The maturation level of erythroid progenitor cells closely associates with colony stimulating factor (CSF) requirements for erythroid development.24 When CSF requirements of day-8 cells were analyzed in serum-free medium, the ECFC required erythropoietin alone but not IL-3 and SCF for erythroid development, which indicates that ECFC in day-8 cells predominantly consists of mature erythroid progenitor cells. The erythroid progenitor cells that gave rise to aggregates with 8 to 49 erythroblasts and that were equivalent to CFU-E, consisted 78.8% ± 2.5%, whereas the rest of ECFC gave rise to aggregates with 2 to 7 erythroblasts.

We then set out to examine some early signaling events in these human erythroid precursor cells. When Jak2 was immunoprecipitated from lyses of erythroid cells before and after stimulation with erythropoietin (10 U/mL), we saw the inducible tyrosine phosphorylation of Jak2 (Fig 1A, upper panel). Jak2 was equally immunoprecipitated before and after stimulation (Fig 1A, lower panel). Time course experiments of Jak2 tyrosine phosphorylation showed tyrosine phosphorylation within 1 minute that reached a peak within approximately 10 minutes after addition of erythropoietin and then decreased (10 U/mL; Fig 1B, upper panel). Erythropoietin induced protein tyrosine phosphorylation in erythroid cells in a dose-dependent manner (0.5 to 5 U/mL; Fig 1C, upper panel). Next, we examined whether STAT5A and B become tyrosine phosphorylated in erythroid cells stimulated with erythropoietin (10 U/mL, for 10 minutes). A STAT5 monoclonal antibody recognizes both STAT5A and STAT5B (Fig 2, lower panel). The latter moved faster than STAT5A in the gel. Both STAT5A and STAT5B became tyrosine phosphorylated (Fig 2, upper panel).

Table 1. Characterization of Expanded Cells

<table>
<thead>
<tr>
<th>Characterization of Expanded Cells</th>
<th>Value</th>
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<tbody>
<tr>
<td>Expansion rate</td>
<td>119.9-fold ± 48.6-fold</td>
</tr>
<tr>
<td>Viability</td>
<td>96.4% ± 2.7%</td>
</tr>
<tr>
<td>Purity of erythroid cells</td>
<td>91.4% ± 5.9%</td>
</tr>
<tr>
<td>Purity of ECFC</td>
<td>91.4% ± 5.9%</td>
</tr>
<tr>
<td>CSF combination</td>
<td>EP 40.5% ± 4.3%</td>
</tr>
<tr>
<td></td>
<td>EP + IL-3 41.1% ± 3.3%</td>
</tr>
<tr>
<td></td>
<td>EP + IL-3 + SCF 42.5% ± 4.0%</td>
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<tr>
<td>Surface Marker</td>
<td>Gly A 79.5% ± 6.1%</td>
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<td></td>
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<tr>
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<td>CD34 35.0% ± 15.6%</td>
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<tr>
<td></td>
<td>CD34 3.7% ± 1.1%</td>
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Values are mean ± standard deviation of eight independent experiments.

Abbreviations: ECFC, erythroid colony-forming cells; CSF, colony-stimulating factor; EP, erythropoietin; IL-3, interleukin-3; SCF, stem cell factor.
5D). Lysates from starved (−) erythroid cells or cells stimulated with 10 U/mL of erythropoietin for 10 minutes (+) were incubated with GST-Crk1-ST2. The SH2 domain showed only a trace amount of binding to STAT5 from starved cells with a significant increase using lysates from erythroid cells.

DISCUSSION

In this study, we examined erythropoietin-signaling in human erythroid precursors expanded in vitro. This corroborates the recent study of Dai et al. and indicates that these studies are feasible. Because these cells, unlike murine factor-dependent...
cell lines genetically engineered to express erythropoietin receptors or murine and human leukemic cell lines, regularly undergo full terminal differentiation and become reticulocytes, we believe that these studies are more likely to be reflective of in vivo situations, although the presence of the artifacts arising from in vitro culture still cannot be ruled out. When Drachman et al examined murine mature megakaryocytes and established cell lines for proximate signal transduction triggered by thrombopoietin, they found that there were significant differences in these cells, suggesting that the results of the studies using the established cell lines may not readily be extended to primary cells. We have previously reported that thrombopoietin induces tyrosine phosphorylation of STAT3 and STAT5 in thrombopoietin-stimulated human platelets. Drachman et al reported that the same set of STATs was tyrosine phosphorylated and activated in thrombopoietin-stimulated murine megakaryocytes. Thus, it is possible that the differences in activated STATs by erythropoietin and thrombopoietin may be important in the lineage-specific developments of both erythrocytic and megakaryocytic precursors.

The differences between the studies that used primary erythroids and those that used cell lines are not limited to STAT proteins. Barber et al have shown that Crkl becomes tyrosine phosphorylated and associated with c-Cbl on stimulation by erythropoietin. However, we found that in primary erythroids Crkl becomes associated with a 95-kD tyrosine phosphorylated protein, which has been identified as STAT5. This coimmunoprecipitation of Crkl and STAT5 is not likely caused by the cross reactivity of Crkl antisera with STAT5 because of the following three reasons (Fig 5): (1) Crkl antisera coimmunoprecipitated STAT5 mostly after stimulation of cells with erythropoietin, suggesting that, if this is a cross reactivity, the Crkl antisera erythropoietin-induced STAT1 tyrosine phosphorylation and its activation in murine erythroid cells. Further, in rat erythroid cells from fetal liver, STAT3 was tyrosine phosphorylated on stimulation with erythropoietin. Thus, these and our current data suggest the presence of species-specific differences in signaling by erythropoietin, although it is formally possible that different sensitivities used in detection of STAT5 activation may have contributed to the differences.

Further, our data suggest that erythropoietin and thrombopoietin may induce tyrosine phosphorylation and activation of different sets of STATs in human primary cells in spite of their similarity in amino acid sequence, although such differences may be obscured in the studies that used established cell lines. We have previously reported that thrombopoietin induces tyrosine phosphorylation of STAT3 and STAT5 in thrombopoietin-stimulated human platelets. Drachman et al reported that the same set of STATs was tyrosine phosphorylated and activated in thrombopoietin-stimulated murine megakaryocytes. Thus, it is possible that the differences in activated STATs by erythropoietin and thrombopoietin may be important in the lineage-specific developments of both erythrocytic and megakaryocytic precursors.

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must recognize only the activated form of STAT5. (2) Crkl immunoprecipitation and STAT5 coimmunoprecipitation were inhibited by Crkl immunizing peptides whose amino acid sequence is not found in STAT5. (3) Phenylphosphate inhibited coimmunoprecipitation of STAT5 without impairing the immunoprecipitation of Crkl, suggesting that Crkl-STAT5 interaction may be dependent on phosphotyrosine on STAT5, because we did not detect tyrosine phosphorylation of Crkl. The last supposition was supported by the results of in vitro binding of GST-Crkl-SH2 to STAT5 from stimulated erythroid cells (Fig 5D), suggesting that STAT5 could be binding to Crkl through its phosphotyrosine-dependent interaction with the SH2 domain of Crkl. However, STAT5 is thought to dimerize when tyrosine phosphorylated through its SH2 domain binding to the tyrosine phosphate of another STAT protein.38,39 Possible explanations for this finding include the fact that dimerization of STAT5 is required for binding to Crkl or that erythropoietin treatment results in an altered subcellular localization of Crkl or STAT5 that allows these proteins to interact. Alternatively, another protein could be involved in mediating the complex formation. Although more studies are necessary, our study indicates that activated STAT5 complex is not simply made of dimerized STAT5 in human erythroid cells and that the complex may include Crkl and possible other proteins as well. Taken together, our data suggest that the results of the studies that used the primary erythroids may be quite different from those obtained from the experiments that were solely dependent on cell lines, giving a rationale for further examinations of the signal transduction of primary human erythroid precursors (Fig 5). Further, the importance of examinations of proximal signal transduction was supported by the recent reports, suggesting that abrogation of tyrosine phosphorylation may be related to the increased erythropoiesis in polycythemic patients.33,40

![Fig 4](image_url)

(A, upper panel) Tyrosine phosphorylation of STAT3 was not tyrosine phosphorylated in the erythroid cells stimulated with erythropoietin for 10 minutes. The erythroid cells were lysed by the addition of an equal amount of a buffer containing 2% Triton X-100 before and after exposure to erythropoietin (10 U/mL). STAT3 was immunoprecipitated with specific anti-STAT3 antisera. As a positive control, TF-1 cells were stimulated with IL-6 (100 ng/mL) for 10 minutes. Immune complexes were resuspended in SDS sample buffer. Tyrosine phosphorylation of STAT3 was detected by 4G10 as described in Fig 1A. Bands were visualized by chemiluminescence. Lane 1, resting erythroid cells; lane 2, 10 minutes after exposure to erythropoietin (10 U/mL); lane 3, resting TF-1 cells; lane 4, 10 minutes after exposure to IL-6 (100 ng/mL). (lower panel) The same nylon membrane was stripped of the antibody and reprobed for STAT3. Bands were visualized by chemiluminescence. Lanes are the same as in upper panel. (B) Anti-STAT1 was used instead of STAT3 antisera.
Fig 5. (A and B) Crkl immunoprecipitates from erythropoietin-stimulated erythroid cells contain a tyrosine phosphorylated 95- to 100-kD protein, which is also recognized by a STAT5 monoclonal antibody. Erythroid cells were lysed by the addition of an equal amount of a buffer containing 2% Triton X-100 before and after exposure to erythropoietin (10 U/mL for 10 minutes). Crkl was immunoprecipitated with specific Crkl antisera. The Crkl immunoprecipitates were divided into two. Tyrosine phosphorylated proteins (A), STAT5 (B, upper panel), and Crkl (B, lower panel) in the Crkl immunoprecipitates were detected as described in Fig 1. (C) Crkl-STAT5 coimmunoprecipitation was inhibited by phenylphosphate (PP, 20 mmol/L) or Crkl immunizing peptide (20 μg/mL). Erythroid cells were divided equally to four samples. Three samples were incubated with erythropoietin (10 U/mL for 10 minutes). Crkl was immunoprecipitated with specific Crkl antisera. STAT5 (upper panel) and Crkl (lower panel) in the Crkl immunoprecipitates were detected as described in (A) and (B). (D) Bacterially expressed the SH2 domain of Crkl (GST-Crkl-SH2) binds to STAT5 from erythropoietin-stimulated erythroid cells. Lysates from starved (-) or erythropoietin-stimulated (10 U/mL for 10 minutes (+)) erythroid cells were analyzed for binding to GST-Crkl-SH2. Bound proteins were separated by SDS-PAGE, transferred to PVDF membranes and immunoblotted with STAT5 (upper panel) or GST (lower panel) monoclonal antibodies.
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