Expression and Extracellular Release of Transferrin Receptors During Peripheral Erythroid Progenitor Cell Differentiation in Liquid Culture

By Naoaki Shintani, Yutaka Kohgo, Junji Kato, Hitoshi Kondo, Kohshi Fujikawa, Etsu Miyazaki, and Yoshiro Niitsu

The expression and extracellular release of transferrin receptor (TR) was investigated in vitro model system of erythroid differentiation. Human peripheral blood mononuclear cells were cultured with interleukin-3 (IL-3) for 7 days, and with erythropoietin (EPO) for an additional 8 days. After EPO stimulation, IL-3-stimulated blastic cells were serially differentiated into mature erythrocytes. [3H]-thymidine incorporation of cultured cells increased linearly from day 0 to 5, followed by a decrease. Flow cytometric analysis showed an increase of TR expression from day 0 to 5, followed by a slight decrease. By metabolic labeling with [125I]methionine and immunoprecipitation, the cell lysate exhibited a 95-kD band corresponding to the intact TR on sodium dodecyl sulfate-polyacrylamide gel electrophoresis/autoradiography at day 5, when polychromat erythroblasts had their peak. The culture supernatant solubilized by tween-20 exhibited a 95-kD and an 85-kD band on days 5 and 8, which corresponded to the intact and the truncated forms of TR, respectively. The 95-kD band was more intense at day 5 than at day 8. The reverse transcriptase-polymerase chain reaction assay showed that the receptor mRNA expression was parallel to receptor synthesis. Thus, the synthesis and expression of TR on erythrocytes is associated mainly with cell proliferation in the early phase, and with both cell proliferation and hemoglobin production in the middle to late phases of maturation. Concomitantly, the extracellular release of TR from erythrocytes occurs in the middle to late phases of maturation. These data suggest that polychromat erythroblasts release soluble TR as both intact and truncated forms and may be an important source of serum TR implicated as an index for erythropoietic activity in the marrow.

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From the Department of Internal Medicine, Section 4, Sapporo Medical University School of Medicine, Sapporo; and the Gastroenterology Unit, Division of Endoscopy, National Cancer Center Hospital, Tokyo, Japan.

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Address reprint requests to Yutaka Kohgo, MD, PhD, Department of Internal Medicine, Section 4, Sapporo Medical University School of Medicine, South-1, West-16, Chuo-ku, Sapporo 060, Japan.

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(Katayama Chemical, Osaka, Japan), 5% phytohemagglutinin-stimulated leukocyte conditioned medium (PHA-LCM), and 2 U of recombinant EPO was incubated at 37°C in a humidified atmosphere containing 5% O₂ and 5% CO₂. The culture plates were evaluated under an inverted microscope on day 7 for colony forming units-erythroid (CFU-E), and on day 14 for burst forming units-erythroid (BFU-E). All determinations were made in triplicate.

[³H]Thymidine uptake assay. Cells were harvested in quadruplicate samples in flat-bottom, 96-well microtiter plates (Falcon, Oxnard, CA) at a density of 1 X 10⁶ cells/200 μL/well in IMDM containing 10% FCS and 7.4 X 10⁻⁴ Mq/mL of [³H]thymidine (American Radiolabeled Chemicals Inc, St Louis, MO). After 4 hours of culture, the cells were harvested with an automatic multiple cell harvester (Labo Mash; Labo Science Co, Ltd, Tokyo, Japan) onto glass fiber papers, and the radioactivity of the four replicate wells was measured with a scintillation counter.

Immunofluorescence and flow cytometry. TR and glycophorin-A expressions were analyzed by flow cytometry (Ortho Cytron; Ortho Diagnostic Systems Inc, Raritan, NJ) using fluorescein-labeled (MoAbs). Fluorescein isothiocyanate-conjugated anti-CD7 antibody (anti-TR antibody; OKT9) was purchased from Becton Dickinson Immunocytometry System, CA, and anticyclophilin-A antibody, from Immunotech, SA, Marseille, France.

Immunocytochemical staining. Cells collected by cytopsin were incubated with anti-CD7 antibody. Avidin-conjugated goat antimouse IgG antibody and biotinylated alkaline phosphatase (Vector Laboratories, Inc, Buringame, CA) were used for staining.

Metabolic labeling with [³H]methionine and immunoprecipitation. The cells were suspended in methionine-free Eagle’s MEM (Nissui Co, Tokyo, Japan) supplemented with glutamine (2 mmol/L) and 5% dialyzed FCS at a density of 1 X 10⁶ cells/mL, and incubated for 30 minutes. Then, the cells were further incubated for 5 hours with 3.7 MBq/L X10⁶ cells of [³H]methionine (American Radiolabeled Chemicals Inc, St Louis, MO). All the cultures were incubated at 37°C in a humidified atmosphere containing 5% O₂ and 5% CO₂. The cell suspensions were centrifuged at 300g for 15 minutes, and the supernatant and the cell pellets were recovered separately. The cells were washed thoroughly with 10 mmol/L KPO₄, 150 mmol/L NaCl, pH 7.4 buffer (phosphate-buffered saline [PBS]), immediately resuspended in PBS containing 0.1% triton X-100 and a cocktail of protease inhibitors (Protease inhibitors set; Boehringer Mannheim Biochemica, Mannheim, Germany) consisting of leupeptin (100 μg/mL), pepstatin (50 μg/mL), EDTA-Na₂ (10 mmol/L), aprotinin (10 μg/mL), antipain-dihydrochloride (50 μg/mL), bestatin (40 μg/mL), phosphoramidon (100 μg/mL), and phenylmethylsulfonyl fluoride (1 μmol/L) at a density of 1 X 10⁶ cells/mL, and disrupted by sonication. After this treatment, membranes were removed by several centrifugations at 30,000g for 30 minutes each) and the solubilized cell extract was suspended. In the parallel experiment, the culture supernatant was mixed with a cocktail of protease inhibitors containing 0.05% tween 20 and passed through a 0.2-μm Nalgene filter. Samples derived from both the cells and the culture supernatant were immunoprecipitated with OKT9 and Protein-A Sepharose (Pharmacia Fine Chemicals) as described elsewhere. Normal mouse IgG (Zymed Laboratories Inc, San Francisco, CA) was used instead of OKT9 as control. All the procedures after metabolic labeling were performed at 4°C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. Immune complexes were analyzed by SDS-PAGE under reducing conditions. The dried gel was exposed to Kodak XAR-5 film (Eastman Kodak, Rochester, NY) at −70°C for 10 to 14 days.

Densitometric analysis. Using the Image software package (National Institutes of Health Research Services Branch, Bethesda, MD), the intensity of the bands on the autoradiogram of the culture supernatant was analyzed by a Macintosh Centris 650 computer (Apple Computer, Japan, Inc, Tokyo, Japan) equipped with a scanner (model JX-32F3, SHARP Corp, Osaka, Japan).

Semi-quantification of TR mRNA by reverse transcriptase-polymerase chain reaction (RT-PCR) assay. Total cellular RNA was extracted by acid guanidinium thiocyanate-phenol-chloroform from the cultured cells. Total cellular RNA extracted from K562 cells was used as a positive control.

The oligonucleotide primers were synthesized using a DNA Synthesizer (Biorsearch, San Rafael, CA). The primers used for RT-PCR reaction were TR-1: 5'-CTATGAGAGGTACAACAGCC, beginning at base position 14 of the TR-I primer, 1 mmol/L deoxyribonucleotide triphosphate, 20 U of avian myeloblastosis virus reverse transcriptase (US Biochemicals, Cleveland, OH), and 20 U of RNasin (Promega, Madison, WI). The mixture was incubated at 37°C for 90 minutes. The resulting DNA templates were resuspended in 80 μL of 100 mmol/L Tris/HCl, pH 8.3, 10 mmol/L MgCl₂, 50 mmol/L KCl, and 10 mmol/L dithiothreitol containing 300 ng of the TR-2 primer, 1 mmol/L deoxyribonucleotide triphosphate, 20 U of EPO (Promega, Madison, WI) and 2.5 U of Taq polymerase (Promega-Elmer Cetus, Norwalk, CT). PCR (denaturation at 94°C for 1 minute, annealing at 55°C for 2 minutes and extension at 72°C for 1.5 minutes) was then performed for 30 cycles.

RESULTS

Proliferation and differentiation of erythroid progenitor cells in a liquid-culture system. Table 1 shows the serial changes in cell number, differential counts, BFU-E and CFU-E, [³H]thymidine incorporation, glycophorin-A expression, and Hb concentration of cultured cells days after EPO stimulation. The blasts grown by IL-3 were further proliferated and differentiated into erythroid cells by EPO addition. The cell number started to increase immediately and reached a plateau on day 5 after EPO addition. Immature erythroblasts (basophilic and polychromatic erythroblasts), mature erythroblasts (orthochromat erythroblasts), and denucleated erythrocytes (reticulocytes) appeared serially after EPO stimulation. The ratio of the mature cells increased with incubation time. The sequential morphologic changes of cells were essentially similar to those noted by the previous report. The colony assay showed BFU-E colony at maximum on day 0, and gradually
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Table 1. Serial Changes in Cell Number, Morphologic Profile, BFU-E and CFU-E, \(^{3}H\)Thymidine Incorporation, Glycophorin A Expression, and Hb Concentration on Cultured Cells After Erythropoietin Addition

<table>
<thead>
<tr>
<th>Days After EPO Addition</th>
<th>0</th>
<th>3</th>
<th>5</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell no.*</td>
<td>5.0 ± 0.0</td>
<td>7.1 ± 0.8</td>
<td>10.1 ± 0.7</td>
<td>10.2 ± 1.0</td>
</tr>
<tr>
<td>Morphologic profile†</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blastic cells</td>
<td>98.0 ± 0.3</td>
<td>89.5 ± 1.5</td>
<td>52.0 ± 6.5</td>
<td>9.4 ± 2.0</td>
</tr>
<tr>
<td>Immature erythroblasts</td>
<td>1.6 ± 0.2</td>
<td>9.6 ± 1.4</td>
<td>41.2 ± 4.5</td>
<td>13.6 ± 1.6</td>
</tr>
<tr>
<td>Mature erythroblasts</td>
<td>0.0 ± 0.0</td>
<td>5.9 ± 0.8</td>
<td>59.1 ± 3.0</td>
<td></td>
</tr>
<tr>
<td>Denucleated RBCs</td>
<td>0.4 ± 0.1</td>
<td>0.9 ± 0.3</td>
<td>0.4 ± 0.2</td>
<td>17.1 ± 1.5</td>
</tr>
<tr>
<td>Myeloid cells</td>
<td>0.5 ± 0.1</td>
<td>1.0 ± 0.2</td>
<td>7.5 ± 0.5</td>
<td>15.5 ± 0.5</td>
</tr>
<tr>
<td>No. of colonies formed‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BFU-E</td>
<td>220 ± 20</td>
<td>40 ± 20</td>
<td>10 ± 2</td>
<td>0</td>
</tr>
<tr>
<td>CFU-E</td>
<td>314 ± 40</td>
<td>250 ± 50</td>
<td>130 ± 40</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>(^{3}H)thymidine incorporation§</td>
<td>6782.7 ± 53.03</td>
<td>2,914.15 ± 174.90</td>
<td>5,085.60 ± 249.80</td>
<td>3,053.70 ± 188.64</td>
</tr>
<tr>
<td>Glycophorin A expression]</td>
<td>9.6</td>
<td>6.0</td>
<td>39.2</td>
<td>66.8</td>
</tr>
<tr>
<td>Hemoglobin concentration</td>
<td>0.5 ± 0.1</td>
<td>1.0 ± 0.2</td>
<td>7.5 ± 0.5</td>
<td>15.5 ± 0.5</td>
</tr>
</tbody>
</table>

Abbreviation: RBCs, red blood cells.

* Values represent the number of total viable cells each day (×10^6 cells/mL; mean ± SD).
† Values are percentage of differential counts each day (mean ± SD). Immature erythroblasts represent basophilic and polychromatic erythroblasts, and mature erythroblasts orthochromatic ones.
‡ Values represent the numbers of BFU-E and CFU-E colonies formed by 10^6 cultured cells each day (mean ± SD).
§ Values represent the radioactivity of \(^{3}H\)thymidine of three experiments (cpm/10^6 cell; mean ± SD). The radioactivity of four replicate wells was measured for each experiment.
† Values are percentage of positive cells detected by MoAb and flow cytometry each day.
† Values are Hb content (μg) per 10^6 cultured cells detected by cyanmethemoglobin method each day (mean ± SD).

decreasing thereafter. CFU-E colony also showed a maximum at day 0 but its disappearance rate was slower than that of BFU-E. \(^{3}H\)Thymidine incorporation increased immediately after EPO stimulation, peaked at day 5 to approximately 7.5-fold of that on day 0 and was followed by a decrease on day 8. The radioactivity on day 8 was almost equal to that on day 3. On the other hand, both the expression of glycophorin A and the Hb concentration showed time-dependent and consistent increase from day 5.

Therefore, we chose three sets of cultured cells at days 0, 5, and 8 for the analysis of TR expression. The cells at day 0 were mainly made up of blastic cells and proerythroblasts (the early phase of maturation). Those at day 5 were rich in immature erythroblasts (the middle phase of maturation). The sample from day 8 was rich in mature erythroblasts and denucleated erythrocytes (the terminal phase of maturation).

Expression of TR during erythroid maturation. The expression of TR in different phases of maturation was studied. As shown in Fig 1, the percent of positive cells for surface TR analyzed by flow cytometry was 7.3%, 50.1%, and 37.9% on days 0, 5, and 8 after EPO stimulation, respectively. The maximum expression was obtained from the cells on day 5, a mixture mainly made up of immature erythroblasts. We compared the results of May-Giemsa staining with those of the immunocytochemical staining of TR; cells exhibiting the maximum expression of TR on day 5 mainly consisted of polychromatich erythroblasts as shown in Fig 2.

We labeled cultured cells with \(^{35}S\)methionine in an attempt to determine whether this increased receptor expression on erythroid cells at the middle maturation phase was brought about by the increase in receptor synthesis. When \(^{35}S\)-labeled TR of the cell lysate was analyzed by immunoprecipitation using OKT9 and SDS-PAGE/autoradiography, one major band of molecular mass 95-kD, corresponding to an intact monomer TR, was detected at day 5 (Fig 3, lane 3), whereas less intense bands corresponding to this peptide were detected in samples from days 0 and 8 (Fig 3, lanes 2 and 4) than that from day 5.

Expression of TR mRNA on cultured cells. We used the RT-PCR procedure to estimate the receptor-mRNA expression, because the amount of mRNA obtained from donors' PBMCs was too small for the conventional Northern technique.

As shown in Fig 4, a single band of 442 bp, which was the expected size of the deduced TR mRNA, was obtained in...
both K562 cells (Fig 4, lane 1; as a positive control) and the cultured cells (Fig 4, lanes 2 through 4). The intensity was maximum at day 5 after EPO addition (Fig 4, lane 3), whereas rather weak at days 0 and 8 (Fig 4, lanes 2 and 4).

Extracellular release of TR on cultured cells. When the primary culture supernatant (containing 35S-labeled TR released from cells) was solubilized with tween 20 and analyzed by immunoprecipitation using OKT9 and SDS-PAGE/autoradiography as described before, two major bands were observed on days 5 and 8 (Fig 5, lanes 3 and 4) that were not observed on day 0 (Fig 5, lane 2). One was the 95-kD band of the intact monomer TR and the other was an 85-kD band corresponding to the truncated form of TR (Fig 5, lanes 3 and 4). The 95-kD polypeptide detected in the culture supernatant was more intense at day 5 than that at day 8. We further compared the intensity of the two major bands on days 5 and 8 by densitometric scanning; the relative intensities of 95-kD and 85-kD bands were 2.75 and 1.00 at day 5, respectively, and 2.28 and 1.68 at day 8, respectively, showing that the proportion of the truncated form of soluble TR was larger at day 8 than at day 5. There were also a few lighter bands below the 85-kD band on days 5 and 8, which may represent degraded fragments of TR proteins.

DISCUSSION

It is noted that erythroid cells have a special requirement for iron in the synthesis of Hb beyond that for rapid expansion in hematopoietic progenitor cells. This extraordinary requirement is supplied by the rapid influx of transferrin-bound iron through the surface TR.1,3,4

The present study details the expression of TR on erythroid cells throughout their maturation process. To examine the expression of TR on erythroid progenitor cells, flow cytometric analysis has been used. By flow cytometry, TR was found to be expressed highly on BM erythroid cells that were sustained until a relatively late stage of maturation as compared with myeloid cells.20,21 However, further biochemical and molecular biologic analysis of TR expression of differentiating erythroid cells could not be conducted, because enough amounts of starting material cells for such experiment were not available. In the present experiment, we used two-phase liquid-culture system to provide a relatively homogeneous population of erythroid cells with a sequential differentiation.22 As confirmed by morphologic examination, flow cytometric analysis of glycophorin A and determination of Hb contents (Table 1), this culture system is a considerable improvement over methods requiring BM aspirates4,20,21 and will be useful in future studies of these cells.

As shown in Fig 1, the cell-surface TR expression was rather weak on the early phase of erythroid maturation, and maximum at the middle phase; it was most numerous on polychromatic erythroblasts (Fig 2), and gradually decreased in the subsequent phases through the terminal phase. As shown in Table 1, both the total viable cell number and [3H]thymidine incorporation of cultured cells increased linearly from day 0 to 5 after EPO addition, and Hb content showed a rapid increase from day 3. It is noteworthy that the increase of cell-surface TR expression from day 0 to 5 (Fig 1) seemed to be biphasic consisting of a gradual increase from day 0 to 3 and a rapid one from day 3 to 5. Thus, it is suggested that TR expressed on erythroid cells might
Fig 3. Autoradiograph of immunoprecipitates from \[^{[35S]}\text{-methionine-labeled cell lysate. The cultured cells were metabolically labeled with \[^{[35S]}\text{methionine and the cell lysate was immunoprecipitated with anti-TR MoAb (OKT9) and Protein-A Sepharose. The immune complex thus obtained was analyzed by SDS-PAGE (12.5%) under reducing conditions and autoradiography on day 0 (lane 2), day 5 (lane 3), and day 8 (lane 4) after EPO addition. Instead of OKT9, normal mouse IgG was also used for immunoprecipitation on day 5 as a control (lane 1). Molecular weight of intact monomer TR is indicated by an arrow with 95 kD at left.}

contribute to the iron uptake, mainly for their proliferation in the early phase, and for both cell proliferation and Hb production in the middle to late phases of erythroid maturation. By metabolic labeling (Fig 3) and semiquantification of receptor mRNA (Fig 4), it was confirmed that the increase of receptor-protein synthesis as well as receptor mRNA give rise to the augmented receptor expression. The increase of receptor-mRNA expression could be explained by the increased transcript level of the receptor gene and/or stability of receptor mRNA. Chan et al\(^1\) showed that TR and its mRNA are hyperepressed on chicken embryonic erythroid cells as compared with embryonic fibroblasts, and suggested that the receptor mRNA expression is regulated to a significant degree at the transcriptional level. We found that IRE-BP activity was increased and stabilized receptor mRNA during the middle phase of erythroid differentiation (in preparation).

Recently, we have shown that immunoreactive TR is present in human serum and reflects the rate of BM erythropoiesis,\(^7,8\) but there remain some controversies regarding the source and release manner of soluble TR from erythroid cells. Beguin et al\(^12,33\) reported that the immunoreactive TR in rat and human serum is identical to the intact receptor of the placenta. This is consistent with previous reports that sheep or rat reticulocytes shed intact receptor as a vesicular form.\(^13-16\) However, Shih et al pointed out that the main compartment of TR in normal human sera consists of smaller truncated receptors; the intact ones precipitable by ultracentrifugation is negligible.\(^12,34\) It is also noted that some cell lines such as K562 cells and HL60 cells, both of which express TR highly, externalize the immunoreactive receptors both in an intact vesicular and a truncated form.\(^17-19\) Because TR is expressed highly in erythroblasts as well as in such cultured cell lines, it is possible that soluble TR is derived from differentiating erythroblasts less mature than reticulocytes.

Our findings provide the evidence for the extracellular release of TR from erythroblasts less mature than reticulocytes. The culture supernatant of erythroid cells, which was solubilized by Tween-20, exhibited two major immunoprecipitable bands of 95-kD and 85-kD (Fig 5, lanes 3 and 4), corresponding respectively to the intact monomer and the truncated form of TR.\(^12\) It is clear that soluble receptors are actually released from erythroid cells at the middle to late phases of maturation as vesicular and/or truncated form, mainly from polychromatic erythroblasts. Other important issues to be resolved are which phase of erythroid differentiation is responsible for the externalization of soluble TR and how the soluble TR has appeared. It was noteworthy that the 95-kD band was more intense at day 5 than that at day 8, whereas the proportion of the truncated form of soluble TR was larger at day 8 than at day 5. There were also some less intense immunoprecipitable bands of smaller molecular weights. Taken together, it is assumed that the synthesis of intact TR is at a maximum at the middle phase of maturation (day 5) and the truncation of TRs concomitantly occurs from the middle to late maturation stages by

Fig 4. Southern blot analysis of RT-PCR products deduced from TR mRNA on K562 cells (lane 1) and the cultured cells on day 0 (lane 2), day 5 (lane 3), and day 8 (lane 4) after EPO addition.
proteolytic cleavage of the intact TRs that originally had been exocytosed as a vesicle (exosome). Our data is compatible with a very recent report that TR of native size appears in exosomes before the truncated form of TR is detected in the culture medium of sheep reticulocytes. When a patient with iron deficiency anemia was treated with intravenous iron administration, serum TR level showed a transient rise just after the initiation of iron supplementation, and then returned to normal levels with the improvement of anemia. The reticulocyte counts also increased after iron supplementation and returned to normal levels. However, the peak of reticulocyte counts should precede that of serum TR level. This discrepancy could be explained by our data, which indicate that polychromatic erythroblasts are actually an important source of serum TR; these cells will mature into reticulocytes in a few days in BM.

It still remains to be determined whether some parts of soluble TR are truly secretory forms, and how the novel form of TR is responsible for this process.

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