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Role of a Truncated Erythropoietin Receptor for Erythroid Differentiation

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Erythropoietin (EPO) is a cytokine that regulates erythropoiesis through the EPO receptor (EPOR). We reported previously that erythroid progenitor cells express both a full-length and a truncated form of EPOR (EPOR-F and EPOR-Tph). EPOR-Tph cannot transmit growth signals and acts as a dominant negative regulator against EPOR-F-mediated signals for cell survival and growth. Upon transfection of EPOR-F in a cell line, Ba/F3, β-globin accumulation, which is considered to be a marker of erythroid-differentiation, is inducible in the transformants. We show here that the co-expression of EPOR-Tph in EPOR-F-transformants does not inhibit and rather upregulates the β-globin induction while inhibiting survival and growth of the transformants. These data suggest that, in contrast to survival and growth signals, EPOR-Tph acts as a positive regulator for erythroid-differentiation signals in erythroid progenitor cells.

Erythropoietin (EPO) is a glycoprotein essential for erythropoiesis (1,2). EPO exerts its action by binding to homodimer of the EPO receptor (EPOR) (3–5). After the cloning of the EPOR cDNA (6–9), the functions of the receptor have been analyzed in cell lines transformed by the EPOR gene (10–14). The studies using mutant EPORs have revealed that the proximal cytoplasmic region is responsible for transmitting growth signals (10–14). A protein tyrosine kinase, JAK2, was found to associate with this region and to be phosphorylated after EPO-stimulation (15,16).

We have previously reported that human bone marrow cells express both a full-length and a truncated form of the EPOR (EPOR-F and EPOR-Tph) (17). EPOR-Tph is predominating in immature erythroid progenitor cells, while EPOR-F is predominating in mature progenitors (17). EPOR-Tph alone can not transmit growth signals and acts as a dominant negative regulator against EPOR-F mediated signals for cell growth and prevention of apoptosis (18).

Ba/F3 is an interleukin-3 (IL-3) dependent mouse cell line that was originally described as a lymphoid cell line (19). But after the transformation by the EPOR-F β-globin mRNA expression is inducible in this cell line (20–24). We transformed Ba/F3 cells with EPOR-F, EPOR-Tph alone or both (18). The effect of the expression of EPOR-Tph on β-globin induction was examined in these Ba/F3 transformants.

MATERIALS AND METHODS

Cell Lines and Cytokines

Ba/F3 cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and 50 unit/ml recombinant mouse IL-3. Recombinant mouse IL-3 and recombinant human EPO were provided by Kirin Brewery Company (Tokyo, Japan).

Northern Blot Analysis

Poly (A)-RNAs were prepared by the Quickprep (Pharmacia, Milwaukee, WI). 1μg of each poly (A)-RNA was fractionated in 1.0% agarose gel containing 2.2 M formaldehyde. The fractionated RNAs were transferred to a nitrocellulose filter and hybridization was performed with a ^{32}P labeled probe. Probes were a genomic DNA fragment of mouse β-globin and a cDNA of mouse GATA-1, labeled with Ready-To-Go DNA labeling kit (Pharmacia, Milwaukee, WI). The hybridized bands were visualized with the BAS-2000 Bio-Image analyzer (Fuji Photo Film Company, Kanagawa, Japan).

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Detection of mRNA by Polymerase Chain Reaction (RNA-PCR)

Total RNAs were prepared with Isogen (NipponGene, Tokyo, Japan). 5 μg of total RNA was reverse transcribed into cDNAs using oligo-dT primers. 1/10 volume of each cDNA product was used in each PCR. PCR for elongation factor-lα (EF-1α) gene was performed for 17 cycles in all experiments to confirm the presence of cDNA. To detect β-globin mRNA, the first PCR was performed for 15 cycles and then the second PCR with nested primers was done with 1/100 volume of first PCR products for either various cycle numbers (Fig. 2) or 19 cycles (Fig. 3, 4). Each PCR cycle was performed as follows; 94°C-30 seconds, 60°C-30 seconds, and 72°C-30 seconds. Primers used are as follows; first PCR for β-globin with upstream primer (5′-GATGAAGTTGGTGGTGAGGC-3′) and downstream primer (5′-TAGTGGTACTTGTGAGCCAG-3′); second PCR for β-globin with upstream primer (5′TTGTCTACCCTTGGACCCAG-3′) and downstream primer (5′-ACTCCAGCCACCACCTTCTG-3′) (313 bp is the expected length of product); PCR for EF-lα with upstream primer (5′-CTGGCTTCACTGCTCAGGTG-3′) and downstream primer (5′TAGCCTTCTGAGCTTTCTGG-3′) (372 bp). 1/10 volume of first (EF-lα) or second (β-globin) PCR products was fractionated on a 1.5% agarose gel and visualized after ethidium bromide staining.

RESULTS AND DISCUSSION

We established Ba/F3 transformants expressing EPOR-F,-Tph, or both (Ba-F, Ba-Tph, or Ba-F + Tph) (18). After IL-3 deprivation, Ba-F and Ba-F + Tph were cultured with 10 unit/ml EPO for 7 days and the induction of β-globin mRNA was examined by Northern blot analysis (Fig. 1). Unexpectedly, β-globin induction was observed more clearly in Ba-F + Tph (lane 2) than in Ba-F (lane 1).

The result raised the possibility that EPOR-Tph mediated β-globin induction more efficiently than EPOR-F. However, Ba-Tph could not survive with EPO alone (18) and the co-existence of IL-3 inhibited β-globin induction (22–24). Furthermore, it was reported that long time culture of the transformants in the presence of EPO changed the cells to express β-globin mRNA constitutively even after EPO-deprivation (20,21). We therefore performed a polymerase chain reaction assay with RNA (RNA-PCR) at earlier times after EPO-stimulation.

Also by RNA-PCR analysis, β-globin induction could be detected more clearly in Ba-F + Tph than in Ba-F even 24 hours after EPO-stimulation (Fig. 2). Time course analysis revealed that β-globin was induced as early as 6 hours after EPO-stimulation in Ba-F + Tph and Ba-Tph cells (Fig. 3, lane 2), while never in Ba-mock (mock transformants) and Ba-F cells even at 24 hours (Fig. 3, lane 5). It is highly unlikely that a trace amount of endogenous EPORs (18) induced β-globin mRNA, since they should be equally expressed on all transformants including Ba-mock cells. The

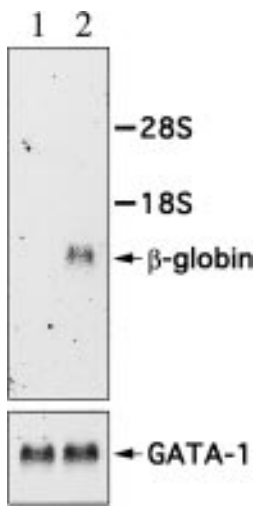

FIG. 1. Northern blot analysis of β-globin and GATA-1 mRNA in Ba/F3 cells expressing either EPOR-F (Ba-F) (lane 1) or both EPOR-F and -Tph (Ba-F + Tph) (lane 2) cultured with 10 units/ml EPO for 7 days.

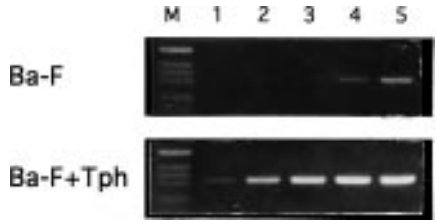

FIG. 2. Polymerase chain reaction with RNA (RNA-PCR) for β-globin mRNA in Ba-F or Ba-F + Tph cultured with 10 unit/ml EPO for 24 hours. The first PCR was performed for 15 cycles and then a second PCR with nested primers was performed for 15 (lane 1), 17 (lane 2), 19 (lane 3), 21 (lane 4), or 23 (lane 5) cycles. M; 1 kb ladder marker (GIBCO BRL, Grand Island, NY) (each band indicates approximately 500, 400, 350, or 300 bp).

result strongly suggests that not only the homodimer of EPOR-F but also the heterodimer of -F and -Tph and/or the homodimer of -Tph can induce β-globin mRNA.

Next, we studied the effect of EPO-concentration on β-globin induction 12 hours after IL-3 deprivation, since majority of cells were viable at this time point (17). Surprisingly, β-globin was induced in Ba-F, Ba-F + Tph, and Ba-Tph cells even without both EPO and fetal calf serum (FCS), while never in Ba-mock (Fig. 4, lane 1). Moreover strikingly and contrary to our expectations, EPO rather inhibited β-globin induction in a dose-dependent manner in Ba-F and Ba-F + Tph (Fig. 4, lane 2-4). These results are in agreement with the results reported recently during the preparation of this manuscript (23,24). One group showed the β-globin induction in Ba/F3 cells expressing EPOR-F even without EPO (23). Although even EPOR-F transformants could not survive in the presence of FCS alone, very low levels of EPO involved in FCS may have transformed cells to express β-globin mRNA only with IL-3-deprivation. Another group showed that β-globin was inducible only in the presence of low concentration of EPO and high EPO-concentration rather inhibited β-globin induction (24). Both groups propose that EPO-induced erythroid-differentiation requires prolongation or arrest of the G1 phase of the cell cycle. In this regard, the co-expression of EPOR-Tph may act positively for erythroid-differentiation with decreasing EPO-induced signals

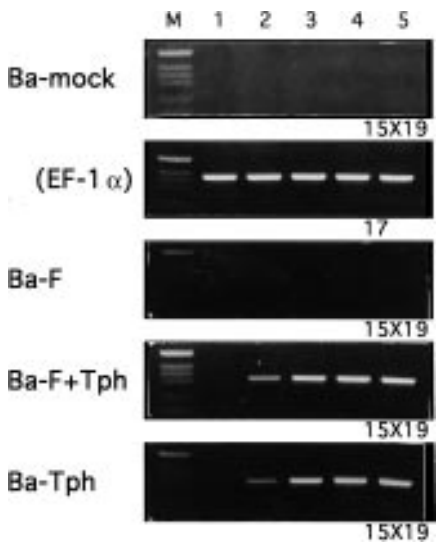

FIG. 3. Time course of β-globin induction in Ba-mock (mock transformants), Ba-F, Ba-F + Tph, or Ba-Tph (Ba/F3 expressing EPOR-Tph) cultured in 10 units/ml EPO for 0 (lane 1), 6 (lane 2), 12 (lane 3), 18 (lane 4), or 24 (lane 5) hours. The second row shows the results of RNA-PCR for 17 cycles to detect elongation factor-lα (EF-1α) mRNA in Ba-mock cells at each time point. This assay was used as a control in all other experiments (data not shown).

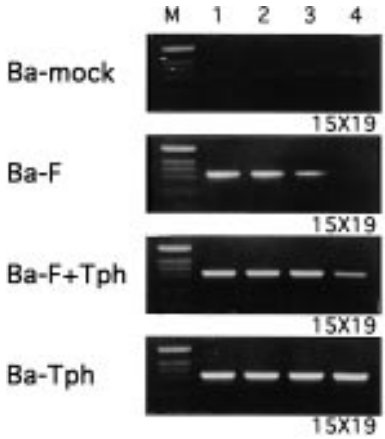

FIG. 4. Analysis of EPO-dose dependency of β-globin induction. β-Globin induction in transformants cultured with 0 (lane 1), 0.001 (lane 2), 0.1 (lane 3), or 10 (lane 4) unit/ml EPO for 12 hours, without both IL-3 and FCS.

that results in prolongation or arrest of G1 phase. Collectively, all these data indicate that erythroid-differentiation signals through EPOR, at least in part, can be mediated with very lower levels of EPO-stimulation than the levels required for survival and/or growth signals.

It has been remained unclear that signals for growth and differentiation through each cytokine/receptor system are mediated simultaneously or independently. The data described here indicate that EPO-induced growth and differentiation signals are mediated independently at least in part, and at certain circumstance(s) they are regulated reciprocally. In such mechanisms, EPOR-Tph seemingly has a very important biological role in erythropoiesis regulating both growth and differentiation of erythroid progenitor cells.

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