S. Richards
R. A. Gibbs
Institute for Molecular Genetics and Human Genome Center, Baylor College of Medicine
T. Ashizawa
Department of Neurology, Baylor College of Medicine, and Veterans Administration Medical Center, Houston, TX 77030, USA
G. Scarlato
Istituto di Clinica Neurologica, Universita di Milano, Centro Dino Ferrari Via F. Sforza 35, 20122 Milan, Italy

C. T. Caskey
Institute for Molecular Genetics and Howard Hughes Medical Institute, Human Genome Center, Baylor College of Medicine

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A Truncated Erythropoietin Receptor and Cell Death: A Reanalysis

We reported that human bone marrow cells express both a truncated and a full-length form of erythropoietin receptor (EPOR-T and EPOR-F, respectively), with the truncated form predominating in immature progenitors and the full-length form predominating in late-state progenitors (*1*). Transfection experiments revealed no difference in binding affinity to erythropoietin (EPO) between the two, and showed that both EPOR-F and EPOR-T could transduce mitogenic signals. However, when compared with cells expressing EPOR-F, those expressing EPOR-T were more likely to undergo apoptosis at lower concentrations of EPO. On the basis of these data, we speculated that the signaling pathway for prevention of apoptosis is different from the one for mitogenesis (*1*). R. Schwall proposed a different interpretation of the data, that is, EPOR-T does not selectively fail to prevent programmed cell death, but it transduces EPO actions inefficiently (*2*).

Our experiments, like many others, were performed with a mouse pro-B cell line, Ba/F3, as recipient cells. Recently, it was reported that a small amount of endogenous EPOR is expressed in Ba/F3 cells (*3*). Other investigators reported that JAK2 kinase has a critical role in EPOR-mediated signal transduction by binding to the cytoplasmic portion of the receptor (*4*). These observations prompted us to rule out the possibility that the endogenous mouse EPOR is responsible for EPO-responsive cell proliferation in EPOR-T expressing transfectants, Ba/F3-(EPOR-T). Unexpectedly, RT-PCR of mRNA obtained from EPOR-T–transfected Ba/F3 cells showed presence of both EPOR-T and EPOR-F messages (Fig. 1A, lane 2). When these transfectants were cultured under EPO for a week, the amount of EPOR-F messages increased (Fig. 1A, lane 3). Subcloning and sequencing of the PCR products identified this fragment as human EPOR-F rather than mouse endogenous EPOR. The complementary DNA (cDNA) clone that we used for construction of an expression vector for EPOR-T (pEPOR-TF) contained an intron 7 and the entire exon 8 (Fig. 1B). Since transfection of the same construct into L cells resulted in production of only a trace amount of EPOR-F (Fig. 1A, lane 5), it seems that the intron 7 of EPOR-T type mRNA transcribed from pEPOR-TF is spliced out by some cell-specific mechanism to produce EPOR-F in Ba/F3 cells. Thus, the response of Ba/F3-(EPOR-T) to EPO shown in our previous report (*1*) could have been mediated by coexisting EPOR-F. To rule out this possibility, we constructed an expression vector with EPOR-T7 (pEPOR-T7) (Fig. 1B), which does not contain exon 8 and thus never produces EPOR-F. When this construct was transfected, Ba/F3 cells did not respond to EPO (Fig. 2). Expression of endogenous mouse EPOR (Fig. 1A, lane 1) seems to be responsible for low-grade response at higher concentrations of EPO. From these data, we conclude that EPOR-T cannot transduce mitogenic signals.

How, then, does the expression of EPOR-T affect apoptosis of cells under lower concentrations of EPO? It is believed that EPOR acts as a homodimer to bind with EPO and to transduce signals (*5*). Several experiments using mouse EPOR deletion mutants showed that cytoplasmic portion of EPOR is not essential for cell surface expression and EPO binding (*6*). Therefore, it is possible that EPOR-T forms not only a homodimer, but a heterodimer with EPOR-F. To investigate the influence of EPOR-T co-expression on EPOR-F–expressing cells, we transfected Ba/F3-(EPOR-F) cells with pEPOR-T7 (Ba/F3-EPOR-(F+T7)). The response of Ba/F3-EPOR-(F+T7) was lower than that of Ba/F3-(EPOR-F) (Fig. 2). When Ba/F3-EPOR-

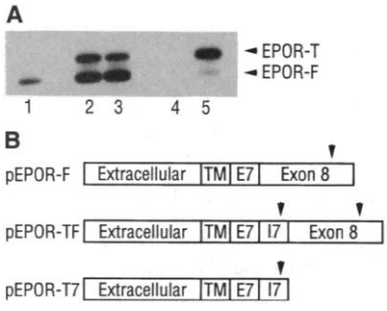

Fig. 1. (**A**) Southern (DNA) hybridization of the reverse transcriptase polymerase chain reaction (RT-PCR) products. mRNA obtained from Ba/F3 parent cells (lane 1), Ba/F3 cells transfected with pEPOR-TF and cultured under IL-3 and G418 (lane 2), Ba/F3 cells transfected with pEPOR-TF and cultured under EPO for a week (lane 3), L cells (lane 4), and L cells transfected with pEPOR-TF (lane 5) were subjected to RT-PCR and Southern blot analysis after gel electrophoresis (*8*). Both human and mouse EPOR cDNA were used as probes. The shorter band (457 bp) represents the full-length EPOR (EPOR-F) and the longer band (552 bp) represents the truncated form (EPOR-T). (**B**) Structure of cDNAs used for construction of an expression vector. pEPOR-F has all exons and encodes full-length EPOR. pEPOR-TF is an alternatively spliced form of EPOR that contains intron 7 and exon 8. pEPOR-T7 contains intron 7 but exon 8 is deleted at the exon-intron junction by PvuII restriction enzyme treatment. Arrows indicate stop codon. Extracellular, extracellular domain; TM, transmembrane region; E7, exon 7; I7, intron 7.

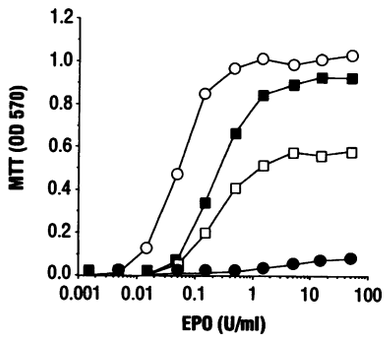

Fig. 2. Cell growth studies on Ba/F3 transfectants expressing different human EPORs. Over-transfection and selection of Ba/F3-(EPOR-F) by pEPOR-T7 was analyzed. The transfectants were cultured in the presence of different concentrations of EPO (*9*): Ba/F3-(EPOR-F) (○), Ba/F3-[EPOR-F(Hygro)+EPOR-T7(G418)] just after the F418 selection (□), Ba/F3-[EPOR-F(Hygro)+EPOR-T7(G418)] after 1 week culture in the presence of EPO (■) and Ba/F3-(EPOR-T7) (●). U, unit.

(F+T7) cells were cultured under EPO for a week, the response to EPO increased (Fig. 2), probably as a result of selection. The growth curve of these cells was similar to that of Ba/F3-(EPOR-T), and their susceptibility to cell death under low concentrations of EPO was similar to that of Ba/F3-(EPOR-T), as we previously described (*1*). These data strongly suggest that what we have observed with Ba/F3-(EPOR-T) is a dominant negative effect of co-expressed EPOR-T over EPOR-F, most probably caused by formation of a heterodimer with EPOR-F.

Although we have to make a correction with regard to our earlier report (*1*) that EPOR-T could transduce mitogenic signals, the data described here indicate that co-expression of EPOR-T makes the cell more susceptible to cell death under lower concentrations of EPO. As Schwall proposed, this susceptibility could be a result of insufficient signaling by a heterodimeric EPOR formation. Alternatively, it could be that a signal to prevent apoptosis, which is different from a mitogenic signal, exists and is mediated by an EPOR-F homodimer, but not by a heterodimer. The answer to this question remains to be determined. In either event, we would like to emphasize the role of EPOR-T as a dominant negative regulator for cell growth and prevention of apoptosis. We have found the existence of a truncated form of EPOR and similar differentiation stage–specific changes in gene expression in mouse (*7*). The fact that the same truncated form of EPOR is produced by the same splicing mechanism in different variants of the species suggests that EPOR-T is important in the regulation of erythropoiesis.

Yukio Nakamura
Laboratory of Cell Growth and Differentiation, Institute of Physical and Chemical Research (RIKEN), 3-1-1 Koyadai, Tsukuba, Ibaraki 305, Japan
Hiromitsu Nakauchi
Department of Immunology, Institute of Basic Medical Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305, Japan

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8. One microgram of total RNA from each sample was subjected to reverse transcriptase reaction with antisense primer 5′-GAGCTGGGGTCCAGGAT-3′ (in exon 8). A portion (1/5) of each product after reverse transcriptase reaction was used for the PCR (30 cycles of denaturing at 94°C, annealing at 60°C, and polymerase reaction at 72°C) with sense 5′-GGACGCGCTACACCTTCGC-3′ (in exon 5) and antisense 5′-TGGGCATGCTCACTGCCCAC-3′ (in exon 8). All three primers used in this assay matched both human and mouse EPOR cDNA completely, so they could detect both human and mouse cDNA. After PCR 1/10 of each product was subjected to electrophoresis in a 1.5% agarose gel, and then Southern (DNA) blot analysis was performed with both human and mouse EPOR cDNA as probes.
9. Cell proliferation was measured by a colorimetric assay using CellTiter 96 (Promega) (MTT assay). Cells were washed twice with RPMI-1640, and then 5000 cells suspended in 100 μl of culture medium in the absence or presence of various concentrations of EPO were plated in each well of a 96-well, flat-bottom microtitre plate and cultured at 37°C for 48 hours. All experiments were performed in triplicate.

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