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Erythropoietin-induced erythroid differentiation of the human erythroleukemia cell line TF-1 correlates with impaired STAT5 activation

Stany Chrétien, Paule Varlet[1], Frédérique Verdier[1], Stéphanie Gobert[1], Jean-Pierre Cartron, Sylvie Gisselbrecht[1], Patrick Mayeux[1] and Catherine Lacombe[1,2]

Institut National de la Transfusion Sanguine (INTS), 6 rue Alexandre Cabanel, F75015 Paris and [1]Institut Cochin de Génétique Moléculaire (ICGM), Institut National de la Santé et de la Recherche Médicale (INSERM U363), Hopital Cochin, Université René Descartes, 27 rue du Faubourg Saint Jacques, F75014 Paris, France

[2]Corresponding author

The TF-1 cell line has been established from a patient with erythroleukemia. While various cytokines induce TF-1 cell proliferation, erythropoietin (Epo) only sustains the short-term growth of these cells and induces their differentiation along the erythroid lineage. A truncated Epo receptor (EpoR) is overexpressed in these cells. The truncation removed the 96 C-terminal amino acids, including seven tyrosine residues. An additional single mutation at position +3 of Tyr344 led to the replacement of leucine 347 by proline. Stimulation by Epo induced an impaired activation of the STAT5 transcription factor in these cells. The same defect in STAT5 activation was found in the murine FDCP-1 cell line transfected with a chimeric EpoR containing the abnormal TF-1 EpoR cytoplasmic domain. Infection of TF-1 cells with a retrovirus containing a normal murine EpoR was able to restore both Epo-induced STAT5 activity and cellular proliferation. In contrast, Epo-induced differentiation was reduced strongly in infected TF-1ER cells. These results suggest that Epo-induced differentiation correlates with impaired Epo-induced STAT5 activation.

Keywords: differentiation/erythroleukemia/erythropoietin/proliferation/STATs

Introduction

The molecular mechanisms that regulate the proliferation and differentiation of primitive erythroid progenitors are still poorly understood. The generation of erythroid progenitors does not require erythropoietin (Epo), but Epo is absolutely necessary for the terminal maturation stages of the erythroid lineage (Wu *et al.*, 1995). It has been suggested that Epo could be required for erythroid progenitor proliferation or differentiation, or to prevent the apoptosis of differentiated cells (for a review see Koury and Bondurant, 1992). Populations of normal Epo-responsive erythroid progenitors are difficult to obtain because of the scarcity of these cells. Human erythroleukemia cell lines expressing high numbers of endogenous Epo receptors (EpoRs) are useful tools to study the proliferative response triggered by Epo stimulation (Broudy *et al.*, 1988; Chrétien *et al.*, 1994). However, most of these cell lines either do not differentiate or differentiate poorly in the presence of Epo, harboring only some erythroid differentiation features (Hermine *et al.*, 1992; Komatsu *et al.*, 1993). TF-1 cells have been derived from a patient with erythroleukemia (Kitamura *et al.*, 1989) and express high levels of an abnormal truncated EpoR (Winkelmann *et al.*, 1995). The growth of these cells is dependent on the presence of granulocyte-macrophage colony stimulating factor (GM-CSF) or interleukin (IL)-3, while they proliferate only transiently in response to Epo which induces an erythroid differentiation program after 1 week of culture (Hoang *et al.*, 1996).

EpoR belongs to the large cytokine receptor superfamily (Bazan, 1990; Kishimoto *et al.*, 1994). Stimulation of these cytokine receptors by their cognate ligands induces the tyrosine phosphorylation of numerous cytoplasmic proteins, including the tyrosine kinases of the Jak family (Ihle and Kerr, 1995). Tyrosine phosphorylation and activation of Jak2 have been shown to be critical to Epo-induced signaling in EpoR-transfected DA-3 murine cells (Witthuhn *et al.*, 1993). Signal transducers and activators of transcription (STAT) proteins are latent cytoplasmic transcription factors. After receptor stimulation and tyrosine phosphorylation, STATs associate with the receptors through phosphotyrosine binding SH2 domains, become phosphorylated, dimerize and translocate to the nucleus where they activate transcription (Shuai *et al.*, 1992; Heim *et al.*, 1995). Seven members of the STAT family have been identified so far (Ihle, 1995). STAT5 is activated by Epo (Pallard *et al.*, 1995a; Wakao *et al.*, 1995) and by several other cytokines: IL-2, IL-3, IL-15, GM-CSF, Thrombopoietin (TPO), growth hormone and prolactin (Wakao *et al.*, 1994; Azam *et al.*, 1995; Fujii *et al.*, 1995; Johnston *et al.*, 1995; Pallard *et al.*, 1995b; Wood *et al.*, 1995). Two genes regulated by STAT5 activation in hematopoietic cells have been identified recently (Yoshimura *et al.*, 1995, 1996). The role of STAT5 activation in signal transduction and, especially, in cytokine-induced mitogenic signaling is controversial. We and others have shown recently that in murine hematopoietic cell lines transfected with several EpoR mutants possessing variable numbers of tyrosine residues, STAT5 activation correlates with Epo-induced cellular proliferation (Damen *et al.*, 1995; Gobert *et al.*, 1996). In contrast, Quelle *et al.* (1996) have suggested that STAT5 activation is not required for the mitogenic response.

Here, we have studied the activation of STAT5 following stimulation of the TF-1 cells by Epo. We have shown that an abnormal EpoR gene caused by a deletion of the 96 C-terminal amino acids is overexpressed in TF-1 cells. It contains only one of the two tyrosine residues identified in the mouse model to be crucial for STAT5 activation. An additional single mutation in the sequence surrounding

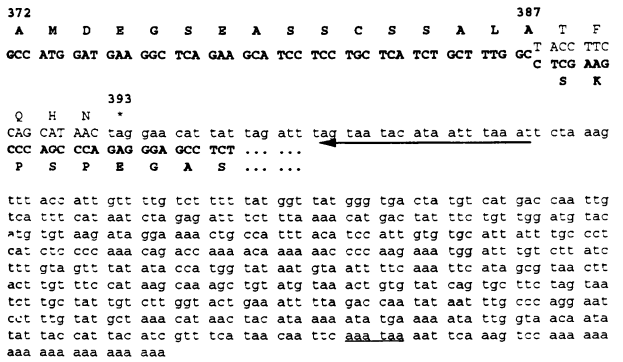

Fig. 1. Cloning of the rearranged EpoR gene in TF-1 cells. The sequence of the normal EpoR gene is represented in bold letters; the sequence of the rearranged EpoR gene in TF-1 cells is depicted in normal letters. The breakpoint is located at amino acid 387. The asterisk, at amino acid 393, indicates the stop codon of the rearranged sequence. The polyadenylation signal is underlined. The oligonucleotide (5′-ATTTAAATTATGTATTACT-3′) specific for the new EpoR TF-1 sequence is shown by an arrow. The sequence is deposited in the EMBL Nucleotide Sequence Database: accession number X97671.

Tyr344 led to an impaired Epo-induced activation of STAT5 in these cells. TF-1ER cells, obtained after the infection of TF-1 cells with a retrovirus carrying a normal murine EpoR, showed normal STAT5 activation and long-term proliferation *in vitro* in response to Epo stimulation. In contrast, the capacity of TF-1ER cells to differentiate along the erythroid pathway in response to Epo was decreased significantly, suggesting that Epo-induced differentiation was related inversely with STAT5 activation in these cells.

Results

Cloning of the rearranged EpoR gene in TF-1 cells

We reported previously that TF-1 cells highly express a rearranged EpoR gene with a breakpoint located 5′ to the translation termination codon leading to the accumulation of a truncated protein (Winkelmann *et al.*, 1995). This rearranged EpoR cDNA was cloned using the 3′ RACE PCR technique (see Materials and methods). The sequence surrounding the rearrangement indicated that the abnormal EpoR was interrupted in the cytoplasmic domain at amino acid 387 in exon 8 (Figure 1). The cDNA sequence fused to EpoR did not correspond to any known sequence. By a Northern blot analysis of different human erythroid cell lines, a probe derived from this sequence hybridized only to TF-1 mRNA, and a Southern blot analysis revealed the same rearranged band which had been detected previously with an EpoR probe in TF-1 DNA (data not shown). The rearranged EpoR of TF-1 cells possessed a substitution of the C-terminal 96 amino acids of the wild-type protein with a stretch of five amino acids (TFQHN). The deduced molecular mass of the resulting protein was 43 kDa, compatible with the estimated molecular weight of 46 kDa measured previously by PAGE (Winkelmann *et al.*, 1995). This abnormal TF-1 EpoR allele contained only two tyrosine residues: Tyr285 (Y0), which is not found in the murine EpoR protein, and Tyr344 (Y1), corresponding to Y343 in the murine EpoR sequence (Maouche *et al.*, 1991) (Figure 2). Thus, this rearrangement led to the deletion of seven tyrosine residues of the EpoR cyto-

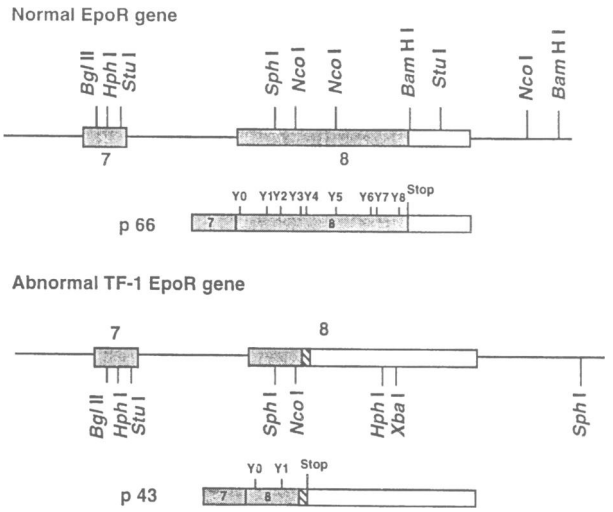

Fig. 2. Restriction endonuclease map of the EpoR translocation breakpoint in TF-1 DNA. The maps of the normal and abnormal EpoR genes and of the resulting proteins are summarized schematically. Coding exons are indicated by shaded boxes; the rearranged TF-1 coding regions in the gene and the protein are indicated by hatched boxes. The location of the tyrosine residues (Y0–Y8) is represented in the EpoR protein.

plasmic domain. The structure of this abnormal TF-1 EpoR allele was quite similar to a murine truncated EpoR mutant, retaining only Tyr343, which we and others generated previously (Damen *et al.*, 1995; Gobert *et al.*, 1995, 1996). Interestingly, this murine EpoR mutant, when transfected into two murine IL-3-dependent cell lines, Ba/F3 and FDCP-1, was shown to confer a normal growth in response to Epo (Gobert *et al.*, 1995). Therefore, the lack of long-term proliferation of the TF-1 cells in response to Epo might be the result of either the lack of a cytoplasmic component specifically required for Epo- but not for GM-CSF-induced proliferation or the expression by TF-1 cells of an inhibitory molecule hampering EpoR signal transduction. Alternatively, an additional alteration of the overexpressed truncated EpoR could be considered as being responsible for the absence of growth in response to Epo.

Infection of TF-1 cells with a normal murine EpoR restored the ability of these cells to proliferate with Epo

To discriminate between these three possibilities, a normal murine EpoR was introduced into the TF-1 cell line via a recombinant retrovirus (Dubart *et al.*, 1994). Infected cells were selected in liquid culture for G418 resistance in the presence of GM-CSF. Infected TF-1ER cells were obtained and used in this study.

To assess the presence of EpoRs of murine origin at the cell surface of the infected TF-1ER cells, we performed binding experiments with [^{125}I]Epo in the presence of a monoclonal anti-EpoR antibody (D'Andrea *et al.*, 1993) which only recognizes human EpoR and specifically inhibits Epo binding to endogenous human EpoRs, but not to exogenous murine EpoRs (Hermine *et al.*, 1996). As shown in Figure 3, the number of Epo binding sites was increased on the TF-1ER cell surface compared with parental TF-1 (6000 versus 3000, respectively), and 80% of these EpoRs were of murine origin. TF-1ER cells were

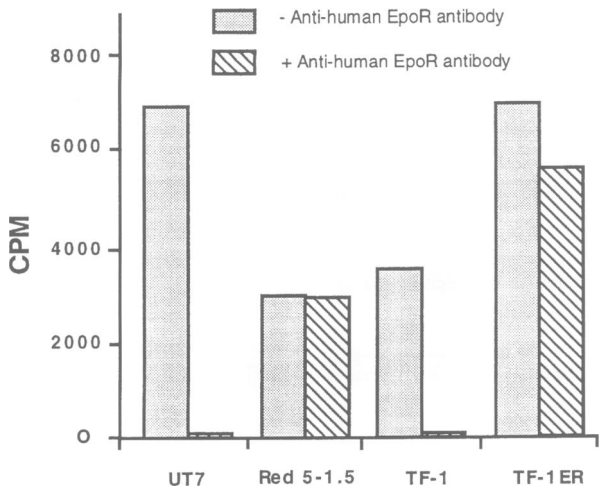

Fig. 3. Murine EpoRs are expressed on the surface of TF-1ER-infected cells. Binding experiments were performed with [^{125}I]Epo on parental TF-1 and infected TF-1ER cells. Two erythroid cell lines, UT7 and Red 5-1.5, having human and murine EpoR at their surface, respectively, were used as controls. [^{125}I]Epo binding sites were determined in the absence (shaded columns) or presence (hatched columns) of anti-human EpoR monoclonal antibody.

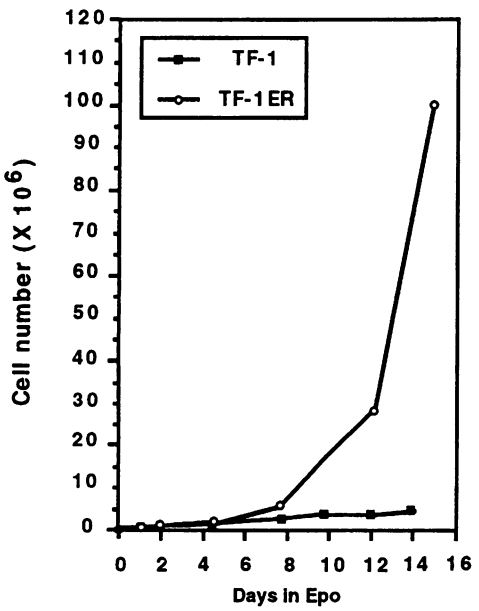

Fig. 4. Infected TF-1ER cells proliferate in the presence of Epo. TF-1 and TF-1ER cells were grown in the presence of 2.5 ng/ml GM-CSF, washed and thereafter 2×10^5 cells were incubated in the presence of 2 IU/ml Epo for 14 days. Cell numbers and viability were determined each day by the trypan blue dye exclusion test. During the culture, the cells were maintained at between 2×10^5 and 8×10^5 cells/ml by dilution.

then tested for their ability to grow in the presence of Epo after GM-CSF removal. Long-term proliferation of the TF-1ER cells was obtained in the presence of Epo treatment alone (Figure 4). The removal of Epo and GM-CSF led to cell death, demonstrating that the proliferation of these cells was not growth factor-independent (data not shown).

To compare the response of TF-1 and TF-1ER cells to Epo, we studied the overall tyrosine phosphorylation pattern induced by Epo stimulation in these cells. As

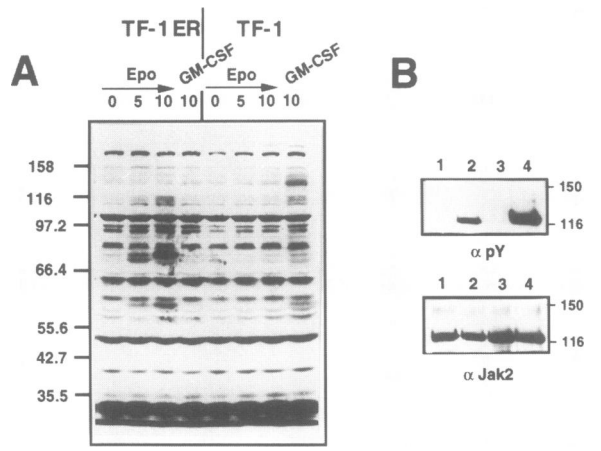

Fig. 5. Epo-induced tyrosine phosphorylation of TF-1 and TF-1ER cells. TF-1 and TF-1ER cells were grown in the presence of 2.5 ng/ml GM-CSF, growth factor starved for one night and stimulated by either 5 IU/ml Epo (5 and 10 min) or 25 ng/ml GM-CSF (10 min). Whole-cell extracts corresponding to 5×10^5 cells were analyzed by a Western blot using monoclonal antiphosphotyrosine antibody (**A**). In a second experiment, extracts from 2.0×10^7 cells (TF-1, lanes 1 and 2; TF-1ER, lanes 3 and 4), unstimulated (lanes 1 and 3) or stimulated 10 min by 5 IU/ml Epo (lanes 2 and 4), were immunoprecipitated using anti-Jak2 antibodies, as described previously (Gobert *et al.*, 1995). Immunoprecipitated materials were analyzed by a Western blot using anti-phosphotyrosine and anti-Jak2 antibodies, successively (**B**).

shown in Figure 5A, Epo was able to induce the tyrosine phosphorylation of a greater number of proteins in TF-1ER cells (TF-1ER Epo 10, lane 3). Immunoprecipitation with specific antibodies followed by a Western blot analysis with anti-phosphotyrosine antibody confirmed that at least part of the 58, 70 and 120 kDa tyrosine-phosphorylated bands corresponded to Shc, EpoR and Jak2 respectively (Figure 5B and data not shown). The 145 kDa phosphorylated protein is probably SHIP (Damen *et al.*, 1996).

Impaired activation of STAT5 in TF-1 cells and restoration of STAT5 activation in infected TF-1ER cells

STAT5 activation has been described recently to correlate with cellular proliferation induced by Epo (Damen *et al.*, 1995; Gobert *et al.*, 1996). Therefore, we investigated the induction of STAT5 DNA binding activity by Epo in parental TF-1 cells and in infected TF-1ER cells. Nuclear extracts prepared from cells starved of growth factors and stimulated with Epo or GM-CSF were tested in an electrophoresis mobility shift assay (EMSA); (Wakao *et al.*, 1994). No electrophoretic shift of the radiolabeled probe was observed in unstimulated cells. In contrast, a DNA–protein binding complex was induced by GM-CSF (Figure 6). A weak DNA–protein binding complex was induced by Epo in TF-1 cells, whereas this complex was much stronger in TF-1ER cells stimulated with Epo. Nuclear extracts from Epo- and GM-CSF-stimulated TF-1ER cells were incubated with antisera specific for STAT5 protein before an analysis by EMSA. As shown in Figure 6, STAT5-specific antibodies supershifted the DNA–protein complexes induced by Epo and GM-CSF, confirming that STAT5 was activated by these two cytokines.

Thus, STAT5 activation by Epo was impaired greatly in TF-1 cells, despite the presence of Tyr344 in the

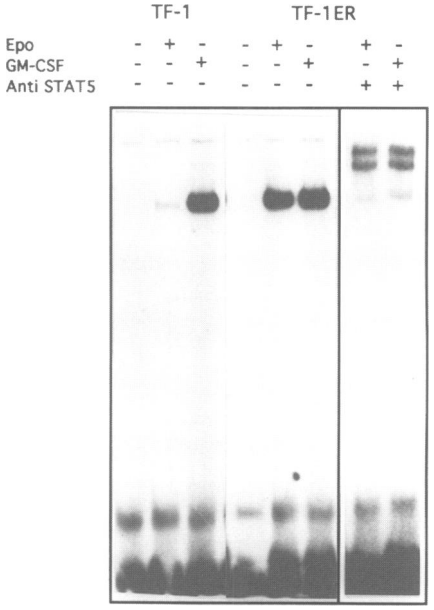

Fig. 6. STAT5 activation in TF-1 and TF-1ER cells. The cells were deprived of growth factor and stimulated with 5 IU/ml Epo for 30 min, or with 25 ng/ml GM-CSF for 30 min as a control. Nuclear extracts were then prepared and tested for EMSA using a β-casein probe. Right panel: nuclear extracts were incubated with antibodies against STAT5 before EMSA.

intracellular part of the EpoR, which we and others identified previously as a crucial docking site necessary for STAT5 activation (Damen *et al.*, 1995; Gobert *et al.*, 1996; Quelle *et al.*, 1996).

Abnormal STAT5 activation in the TF-1 cell line is explained by a single mutation surrounding Tyr344 in the overexpressed EpoR gene

The very weak level of STAT5 activation by Epo in TF-1 cells led us to examine the sequence surrounding Tyr344 of the abnormal TF-1 EpoR. This sequence is depicted in Figure 7A and shows a single mutation (T → C) leading to the replacement of a leucine by a proline at position +3 relative to the tyrosine residue 344 (mutation L347P). To confirm this result, we verified the sequence of the abnormal EpoR TF-1 cDNA by PCR amplification of the intracellular part of the abnormal TF-1 EpoR cDNA using a 5′ EpoR-specific oligonucleotide in exon 7 (see Materials and methods) and a 3′ oligonucleotide in the rearranged TF-1 sequence (oligonucleotide 3′ TF-1 described in Figure 1). The sequence of this amplified fragment confirmed the localization of the EpoR breakpoint in TF-1 and the single L347P mutation.

The functional significance of this L → P mutation was examined after transfection of the murine myeloid FDCP-1 cell line with a chimeric EpoR containing the murine EpoR extracellular and transmembrane domains and the abnormal TF-1 EpoR intracellular part (Figure 7B). The resulting cell line (FDCP-1–EpoR–TF-1) showed reduced growth in the presence of different concentrations of Epo (data not shown). STAT5 activation was then compared in FDCP-1 cell lines transfected with the mutant EpoR–TF-1, normal murine EpoR, or two EpoR mutants described recently: Y1, which retained Tyr343, and F1, devoid of any tyrosine in its intracytoplasmic domain (Gobert *et al.*, 1996; Figure 7B). In FDCP-1–EpoR–TF-1 cells, Epo-induced STAT5 activation was reduced strongly in comparison with the activity observed in FDCP-1–mEpoR and FDCP-1–Y1 cells under identical treatment conditions. The impairment of STAT5 activation was similar to that observed in FDCP-1–F1 cells. The lack of STAT5 activation was not the result of a low level of EpoR expression because selected FDCP-1 cells transfected with the F1 or TF-1–EpoR mutants expressed 6000 and 10 000 EpoRs, respectively. In contrast, FDCP-1 cells transfected with the normal or Y1 mutants expressed ~2000 EpoRs at their cell surface (data not shown). These results indicate that the L347P substitution may be responsible for the impaired activation of STAT5 in TF-1 cells.

Impaired STAT5 activation in the TF-1 cell line correlated with Epo-induced erythroid differentiation

The overexpression of a normal murine EpoR in infected TF-1ER cells restored a long-term proliferation capacity in the presence of Epo (Figure 4). In contrast, parental TF-1 cells cultured in Epo under the same conditions did not sustain long-term growth with this cytokine, but were able to survive over 1 week (Kitamura *et al.*, 1989). To test for differentiation along the erythroid pathway, the presence of hemoglobin was assessed by measuring benzidine-positive cells. Figure 8 shows that after 1 week of Epo induction, TF-1 cells were able to differentiate with ~65% benzidine-positive cells. In similar culture conditions, TF-1ER cells never harbored >20% benzidine-positive cells. These results were confirmed by a cytological examination: smears colored with May Grunwald Giemsa showed that the majority of TF-1ER cells resembled immature proerythroblasts, whereas TF-1 cells were able to differentiate to more mature stages, such as basophilic and polychromatophilic erythroblasts (data not shown). Thus, there is a striking correlation between STAT5 activation and cell proliferation, whereas the impairment of STAT5 activity seemed to favor differentiation along the erythroid pathway.

Discussion

In this report, we present evidence that TF-1 cells overexpressed a C-terminal truncated EpoR protein containing only two of the nine tyrosine residues present in the intracellular part of the normal human EpoR domain. These cells also expressed a low level of normal EpoRs, whose tyrosine phosphorylation could not be detected (Winkelmann *et al.*, 1995; P.Mayeux and C.Lacombe, unpublished results), probably because of the very low level of expression.

Recently we showed that two tyrosine residues of the murine EpoR (Tyr343 and Tyr401) were independently necessary for STAT5 activation (Gobert *et al.*, 1996). Similar results were found for Tyr343 by Damen *et al.* (1995). In both studies, truncated EpoR mutants (Y1 or d392) which possessed Tyr343 were able to efficiently phosphorylate and activate STAT5 in response to Epo. Furthermore, a correlation was found between the ability of these mutant receptors to activate STAT5 and to induce cell proliferation.

We suspected that the EpoR expressed in TF-1 cells

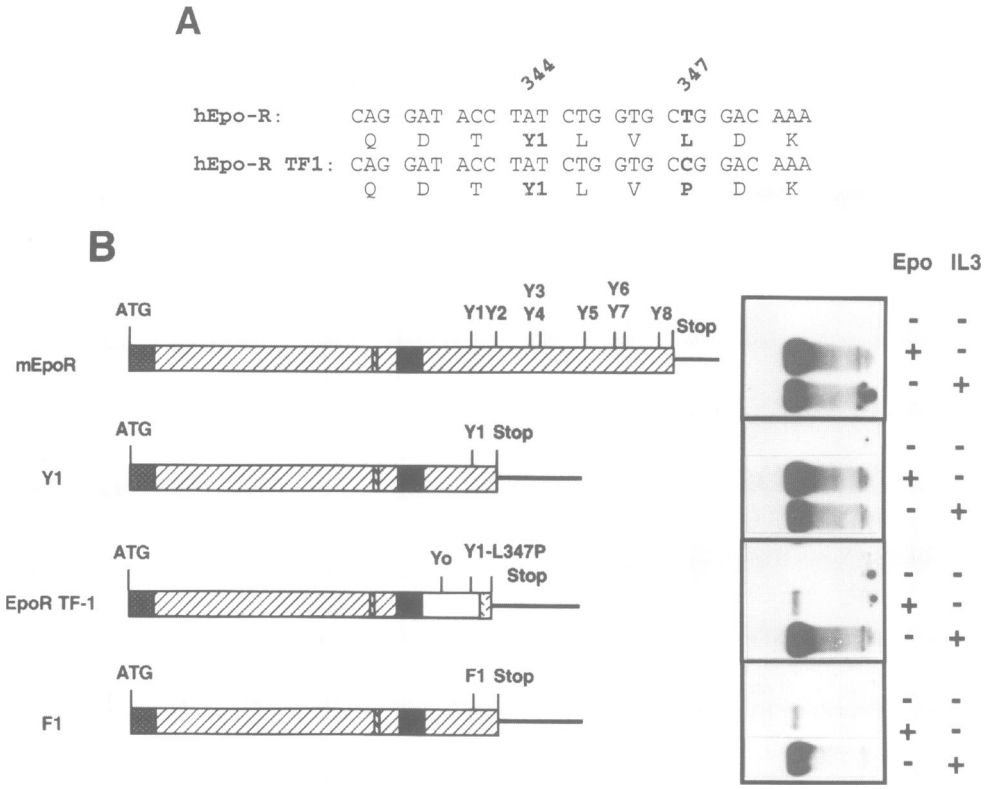

Fig. 7. A single mutation at amino acid 347 of the abnormal TF-1 EpoR gene induced the impairment of STAT5 activity after Epo stimulation. (**A**) Comparison of sequences surrounding Tyr344 in the normal EpoR and in the abnormal EpoR TF-1 cDNA and protein. (**B**) Functional significance of the single mutation in position +3 of Tyr344 (Y1–L347P). FDCP-1 cells were stably transfected with wild-type murine EpoR, with mutant EpoRs (Y1 or F1) (Gobert *et al.*, 1996) or with a chimeric EpoR possessing the abnormal EpoR TF-1 cytoplasmic domain. Left: schematic representation of these EpoR mutants. Right: STAT5 activation mediated by these EpoR mutants after transfection in FDCP-1 cells, analyzed by EMSA. FDCP-1 cells were deprived of growth factor for 4 h and stimulated with 5 IU/ml Epo for 30 min or 10 ng/ml IL-3 for 30 min.

could have an additional defect because, in contrast to GM-CSF, Epo stimulation neither activated STAT5 nor allowed the long-term proliferation of these cells, despite an efficient Jak2 activation (Figure 5B; Wakao *et al.*, 1995). In the truncated EpoR, the sequence surrounding Tyr344 was found to be modified with a single L347P mutation. In the cytoplasmic domain of the cytokine receptors, some degree of specificity in the activation of one STAT by one specific cytokine seems to be conferred by the amino acid sequences surrounding the tyrosine residues (Heim *et al.*, 1995; Stahl *et al.*, 1995). Moreover, the study of specific phosphopeptide sequences recognized by SH2 domains has shown that selectivity is provided by the three amino acids C-terminal to the pTyr residue (Songyang *et al.*, 1993). The L347P substitution could modify the STAT5 docking site at Tyr344 of the human EpoR. Indeed, the transfection of FDCP-1 cells with an expression vector containing a chimeric EpoR with the cytoplasmic domain of TF-1 EpoR allowed confirmation of the impaired STAT5 activation as a consequence of the L347P mutation in the EpoR. Thus, the impairment of STAT5 activation was probably the result of a modification of the Tyr344 environment which inhibited the correct docking of STAT5 SH2. Another hypothesis is that this single mutation at position +3 could inhibit the phosphorylation of Tyr344 and hence its association with STAT5 SH2. Direct testing of this hypothesis is difficult because we (Gobert *et al.*, 1996) and others (Damen *et al.*, 1995; Quelle *et al.*, 1996) have shown that available

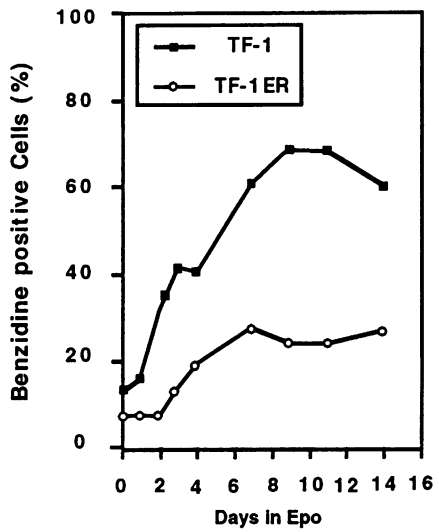

Fig. 8. Benzidine staining of TF-1 and TF-1ER cells cultured in Epo. TF-1 and TF-1ER cells were switched from GM-CSF to Epo (2 IU/ml) at day 0, and plated at 2×10^5 cells/ml in αMEM medium containing 10% FCS. Cells were harvested every 2 days for benzidine staining. Data are the mean of three counts for 200 cells.

reagents do not allow the detection of the phosphorylation of this tyrosine residue. Alternatively, the stretch of five amino acids (TFQHN) located at the C-terminal end of the TF-1 EpoR protein could contribute to the reduced STAT5 activation.

There is a correlation between the impairment of Epo-induced STAT5 activation and the lack of long-term proliferation of TF-1 cells in response to Epo. Furthermore, FDCP-1–EpoR–TF-1 cells, as with FDCP-1–F1 cells, showed a reduced proliferation in response to Epo and a very low level of STAT5 activation. A similar role for STAT5 has been described in the IL-2-induced signaling pathway. Tyr392 and Tyr501 of the cytoplasmic domain of IL-2-Rβ chain have been demonstrated as essential for STAT5 protein docking and activation (Friedmann *et al.*, 1996). The replacement of Tyr392 and Tyr501 by phenylalanine residues induced a substantial decrease in cell proliferation (Goldsmith *et al.*, 1995; Friedmann *et al.*, 1996). Furthermore, a constitutive STAT5 activation has been shown to correlate with cellular transformation by human T-lymphotropic virus type I (Migone *et al.*, 1995) and v-abl (Danial *et al.*, 1995). These data and our results in TF-1 cells strengthen the role of STAT5 in cell proliferation. In contrast, other studies did not find a correlation between STAT5 activation and cell proliferation (Fujii *et al.*, 1995; Quelle *et al.*, 1996). Whether this discrepancy is caused by the different cell lines used or the different experimental conditions remains to be determined.

The infection of TF-1 cells with a retrovirus containing a normal murine EpoR led to the expression of murine EpoR at the cell surface of TF-1ER cells. Hermine *et al.* (1996) recently showed that such murine EpoRs, when introduced into human cells, were able to stimulate cell proliferation and differentiation as efficiently as endogenous EpoRs. In TF-1-infected cells, we obtained simultaneously the restoration of Epo-induced STAT5 activation and cellular proliferation. However, we cannot rule out the fact that, in addition to STAT5 activity, other signaling molecules were activated and participated in Epo-induced proliferation in TF-1ER cells. As seen in Figure 5A, the number of tyrosine-phosphorylated proteins was higher in TF-1ER cells. It is possible that Epo-induced tyrosine phosphorylation of Shc and activation of the Ras pathway were facilitated by EpoR tyrosine phosphorylation, as suggested in the murine model (Damen *et al.*, 1995).

TF-1 cells derived from human erythroleukemia seem to be appropriate for the study of the molecular mechanisms of Epo-induced erythroid differentiation. Little is known about the molecular basis leading to cell differentiation along the erythroid pathway. The inhibitory effect of herbimycin on the proliferation of fetal liver-derived erythroid progenitors but not on Epo-induced differentiation suggests that the signals for differentiation and proliferation are separately controlled (Noguchi *et al.*, 1988). More recently it was reported that the induction of erythroid differentiation by Epo required a delay in cell growth and a prolongation of the G_1 phase of the cell cycle (Carroll *et al.*, 1995). Finally, inducers of erythroid differentiation, such as dimethylsulfoxide, hexamethylene bisacetamide (HMBA) and transforming growth factor-β, while increasing the rate of differentiation, concomitantly decrease the rate of proliferation of erythroid progenitors and inhibit cells from entering the S phase. Interestingly, human bone marrow cells highly enriched in burst forming unit-erythroid enhanced their rate of differentiation after the omission of either Steel factor (SF) or Epo, when compared with cultures containing IL-3, SF and Epo. Thus, it was postulated that Epo could delay the terminal erythroid differentiation program of the most primitive progenitors, while promoting this same program in more mature precursors (Krystal *et al.*, 1994). It is possible that the predominant action of Epo on immature erythroid cells, like TF-1 cells, would rather lead to the induction of a proliferative signal. The abnormalities of the over-expressed EpoR could explain the impaired Epo-induced signal transduction in these cells, which may result in preferential differentiation along the erythroid pathway.

Materials and methods

Reagents

The monoclonal anti-phosphotyrosine antibody 4G10 was a generous gift from Dr B.Drucker. Chicken anti-STAT5 antibodies (Gouilleux *et al.*, 1995) and monoclonal anti-human EpoR antibodies were kindly provided by Dr B.Groner and Dr S.Jones (Genetics Institute), respectively. Highly purified recombinant human Epo (specific activity 200 000 IU/mg) was a generous gift from Dr M.Brandt (Boehringer Mannheim); recombinant murine IL-3 was obtained from Pepro Tech Inc. Anti-Jak2 antibodies were purchased from Upstate Biotechnology Inc. All other reagents were purchased from Sigma.

Cloning of the rearranged EpoR TF-1 cDNA

The abnormal TF-1 EpoR cDNA was cloned by the technique of 3′ RACE–PCR. Poly(A)-selected mRNA from TF-1 cells was isolated by poly(T) affinity binding magnetic beads (Dynal) and reverse transcribed with oligo(dT) tailed with an anchor primer (Clonetech). The normal and abnormal EpoR cDNAs were cloned by PCR amplification using an EpoR 5′ specific oligonucleotide in exon 7 (5′-TCCCACCGCCGGGC-TCTGAAG-3′) and the anchor primer. The PCR products, which contained both normal and rearranged EpoR cDNAs, were cloned in pUC vector. To select only the rearranged EpoR cDNA, colonies were hybridized with a 3′ EpoR oligonucleotide (5′-TCAGGGATCCAA-TATG-3′) specific for the normal EpoR. Negative clones were subsequently isolated and sequenced.

DNA constructs and expression vectors

The chimeric EpoR–TF-1 construct was made by exchanging the normal murine EpoR sequence located 3′ to the *Bgl*II site by the corresponding restriction fragment containing the abnormal TF-1 EpoR cDNA (Figure 2). The normal murine EpoR and the mutant receptor were cloned into a modified pcRSV expression vector and transfected in FDCP-1 cells, as described previously (Gobert *et al.*, 1996).

Retroviral infection of TF-1 cells (TF-1ER cells)

The ϕ2 EpoR packaging cell line containing a retroviral vector (pBTZen–SVNeo) encoding the murine EpoR was kindly provided by A.Dubart and D.Duménil (Dubart *et al.*, 1994). Supernatants of ϕ2 EpoR-producing clones were used to infect the amphotropic GP+ env Am12 cells (Markowitz *et al.*, 1988). After selection with 1 mg/ml G418, supernatants were collected and used to infect TF-1 cells. Selection was performed in liquid culture in the presence of 1 mg/ml G418 and 2.5 ng/ml GM-CSF.

Cell cultures

TF-1 cells were maintained in αMEM medium containing 10% fetal calf serum (FCS) and 2.5 ng/ml GM-CSF. After retroviral infection and G418 selection, TF-1ER cells were passaged in αMEM supplemented with 10% FCS and either 2 IU/ml Epo or 2.5 ng/ml GM-CSF. After transfection with EpoR constructs, Epo-sensitive FDCP-1 cells were cultured in the same medium supplemented with Epo. For signal transduction experiments, the cells were deprived of growth factor for 4 h (FDCP-1) or one night (TF-1 and TF-1ER) by incubation in the Iscove modification of Dulbecco's minimum essential medium containing 0.4% bovine serum albumin and 20 μg/ml iron-saturated transferrin.

Receptor measurement

Epo was iodinated using Iodogen (Pierce) with specific radioactivities ranging from 3.0 to 6.0×10^7 c.p.m./μg. Epo binding experiments were carried out as described previously (Mayeux *et al.*, 1991).

Immunoprecipitation and Western blotting

Immunoprecipitations and Western blots were performed as described previously (Dusanter-Fourt *et al.*, 1992; Gobert *et al.*, 1995). Enhanced chemiluminescence (Amersham Ltd, Les Ullis, France) was used for the Western blot detection.

Preparation of nuclear extracts

Starved cells were stimulated at 37°C with either Epo or IL-3 and chilled quickly with ice-cold PBS. The cells were pelleted and solubilized with buffer A [buffer A: 20 mM HEPES, 10 mM KCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM Na_2VO_4, 0.2% NP40, 10% glycerol and 1 μg/ml each of aprotinin, pepstatin and leupeptin, pH 7.9]. The lysates were centrifuged at 20 000 *g* for 2 min and the pellets extracted with buffer B (buffer B: 20 mM HEPES, 350 mM NaCl, 10 mM KCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.1 mM Na_2VO_4, 20% glycerol and 1 μg/ml each of aprotinin, pepstatin and leupeptin, pH 7.9) using 1 ml of buffer B for 5×10^7 cells. The extracts were centrifuged at 20 000 *g* for 5 min and supernatants were quickly frozen and stored at –80°C.

EMSAs

The STAT5 binding site from the bovine β-casein promoter (5′-AGATTT-CTAGGAATTAAATC-3′; 'β-casein probe') was used as a probe. 2 μl nuclear extracts were mixed with 20 μl binding buffer containing 60 000 c.p.m. end-labeled probe (binding buffer: 10 mM Tris–HCl, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1% NP40, 5% glycerol, 1 mg/ml bovine serum albumin and 2 mg/ml poly(dI–dC), pH 7.5) and the mixture was incubated for 30 min at 4°C. Complexes were separated on 4 or 6% non-denaturing polyacrylamide gels in 0.25× TBE and detected by autoradiography.

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