An extra high dose of erythropoietin fails to support the proliferation of erythropoietin dependent cell lines

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Abstract Erythropoietin is responsible for the red blood cell formation by stimulating the proliferation and the differentiation of erythroid precursor cells. Erythropoietin triggers the conformational change in its receptor thereby induces the phosphorylation of JAK2. In this study, we show that an extra high dose of erythropoietin, however, fails to activate the erythropoietin receptor, to stimulate the phosphorylation of JAK2 and to support the cell proliferation of Ep-FDC-P2 cell. Moreover, high dose of EPO also inhibited the proliferation of various erythropoietin-dependent cell lines, suggesting that excess amount of EPO could not trigger the conformational change of the receptor. In the presence of an extra high dose of erythropoietin as well as in the absence of erythropoietin, the cells caused the DNA fragmentation, a typical symptom of apoptosis. The impairment of cell growth and the DNA fragmentation at the extremely high concentration of EPO was rescued by the addition of erythropoietin antibody or soluble

form of erythropoietin receptor by titrating the excess erythropoietin. These results suggest that two erythropoietin binding sites on erythropoietin receptor dimer should be occupied by a single erythropoietin molecule for the proper conformational change of the receptor and the signal transduction of erythropoietin, instead, when two erythropoietin binding sites on the receptor are shared by two erythropoietin molecules, it fails to evoke the conformational change of erythropoietin receptor adequate for signal transduction.

Keywords Erythropoietin · Erythropoietin receptor · JAK2 · Apoptosis · Antibody · Phosphorylation · Proliferation · Fragmentation

Abbreviations

EPO	Erythropoietin
EPOR	EPO receptor
sEPOR	Soluble form of EPOR
CFU-E	Colony-forming unit erythroid
IL-3	Interleukin-3
MEM	Minimum essential medium
FCS	Fetal calf serum
MTX	Methotrexate
MTT	3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide
JAK	Janus family of nonreceptor-type protein tyrosine kinase
STAT	Signal transducers and activators of transcription

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Introduction

Red blood cell formation depends on the differentiation and proliferation of erythroid precursor cells, CFU-E, by the stimulation of EPO (Jelkmann 2004, 2007; Sasaki 2003; Sasaki et al. 2000, 2001). EPO supports the cell growth through its specific EPOR on the cell surface. The murine EPOR contains a single hydrophobic membrane-spanning domain (D'Andrea et al. 1989). Its cytoplasmic portions do not contain tyrosine kinase domain but associates with the tyrosine kinase protein JAK2.

The crystal structural analysis has shown that EPOR forms a dimer in the presence or absence of EPO. Unliganded receptor dimer exists in a conformation that prevents the activation of JAK2 (Ebie and Fleming 2007; Livnah et al. 1998, 1999; Remy et al. 1999; Seubert et al. 2003; Syed et al. 1998). Two JAK2 molecules associated with each receptor molecules are separated by 73 Å in the absence of ligand. In contrast the binding of EPO mimicking peptide triggers the receptor conformational change and shortens the inter-EPORs distance to 39 Å and promotes the JAK2 activation (Remy et al. 1999). Similarly, the binding of EPO induces receptor conformational change, which results in activation of JAK2. Thus the conformational change of the receptor is essential for EPO signal transduction. The structure of EPO with EPO binding protein, an extracellular portion of EPOR, shows that one molecule of EPO binds two receptors, indicating that EPO has two receptor binding sites (Syed et al. 1998). In agreement with the above findings, EPO has been shown to have two binding sites to receptor, one with higher affinity to EPOR ($K_d = 1$ nM) and the other one with much lower affinity (1 μ M) (Philo et al. 1996).

In response to EPO, tyrosine phosphorylation of the receptor itself and of JAK2 occurs within a minute of stimulation (Witthuhn et al. 1993). The activated JAK2 phosphorylates STAT5 (Damen et al. 1995; Gobert et al. 1996; Klingmuller et al. 1996). Tyrosine phosphorylation at the carboxy-terminal site in STAT results in its dimerization through the SH domains, translocation to the nucleus and activating the target genes. JAK-STAT pathway is thought to have an important role for EPO signal transduction (Socolovsky et al. 1999). EPO supports survival of erythroid cells by preventing their apoptotic death

(Gregoli and Bondurant 1997). In EPO-dependent cell lines, EPO induces the expression of Bcl-x, a Bcl-2 family member that acts as an anti-apoptotic protein and this induction is achieved by the binding of STAT5 to an upstream cis-element in the Bcl-x promoter (Silva et al. 1999).

Here we examined whether excess amount of EPO sustain the viability of EPO-dependent cells. The data show that extra high amount of EPO does not trigger the phosphorylation of EPOR itself and JAK2, and can not support cell proliferation probably due to the failure of invoking the conformational change of EPOR.

Materials and methods

Materials

Anti EPO monoclonal antibodies, R2 and R6 were produced in mouse and purified by protein G. Anti N-terminal EPOR antibody was produced by immunizing rabbit. Anti phosphotyrosine 4G10 and PY20, JAK1 and JAK2 antibodies were purchased from Upstate biotechnology, Santa Cruz Biotechnology, Upstate biotechnology, and Upstate biotechnology, respectively.

The purification of sEPOR

The preparation of sEPOR was described previously (Nagao et al. 1992). Briefly, cells producing sEPOR were cultured to confluence with nucleoside-deprived MEM, 10% dialyzed FCS and 100 nM MTX. The medium was replaced with OPTI-MEM containing 0.5% FCS to secrete sEPOR into culture medium. Used culture medium was centrifuged to remove cells and precipitates, and was mixed with a gel with immobilized EPO in 15 mL tube. The gel was washed thoroughly with PBS. Then sEPOR was eluted by PBS containing 1.5 M $MgCl_2$ and dialyzed with PBS. Protein concentration was determined by Bradford assay (Bio-Rad).

Cell proliferation assay using the EPO-dependent cell lines

EPO-dependent cell lines were used. Ep-FDC-P2 cells were used, when not otherwise indicated, to

measure the EPO biological activity as previously published (Yamaguchi et al. 1991). Briefly, the cells were maintained with RPMI1640 containing 10% FCS and 1 U mL^{-1} EPO were washed four times with RPMI1640 containing 10% FCS to remove EPO and were suspended with the same medium. The cells ($4 \times 10^5 \text{ mL}^{-1}$) were cultured for 20 h in microtiter wells containing various concentrations of EPO in a total of $100 \mu\text{L}$. Cell proliferation was assayed colorimetrically by the use of MTT. BaF/ER and UT-7 cells were maintained with RPMI1640 containing 10% FCS and 1 U mL^{-1} EPO. TF-1 was maintained with RPMI1640 containing 10% FCS and 10 ng mL^{-1} human GM-CSF.

DNA fragmentation assay

Ep-FDC-P2 cells were cultured with or without EPO and recovered at the times indicated and washed twice with PBS. Cells were digested with 50 mM Tris containing 10 mM EDTA, 0.5% *N*-laurylsarcosine and 0.5 mg mL^{-1} Proteinase K at 50°C for 1 h. Then, Ribonuclease A was added to make 0.25 mg mL^{-1} and further incubated for 1 h. The digested solution was diluted with 2.5 volume of 10 mM Tris, 1 mM EDTA and the DNA from the cells was precipitated by ethanol. Chromosomal DNA was resuspended with 10 mM Tris, 1 mM EDTA and loaded into 1% agarose gel.

Immunoprecipitation and detection

Immunoprecipitation was done as described previously (Masuda et al. 1993). The cells ($1.4 \times 10^7 \text{ mL}^{-1}$) were incubated with EPO for 10 min. Cells were quickly washed twice with PBS and lysed with PBS containing 1% Triton X100 and a protease inhibitor cocktail (purchased from Roche, Tokyo, Japan) for 20 min on ice. The solubilized proteins were obtained by centrifugation. EPOR was immunoprecipitated using anti EPOR antibody attached on protein A beads or beads with immobilized EPO. After washing the sample, EPOR was eluted with sample loading buffer and boiled for 2 min. To detect the phosphorylated proteins induced by EPO addition, anti phosphotyrosine antibody, PY20 or 4G10 was used for immunoprecipitation. Immunoprecipitated proteins were separated by SDS–polyacrylamide gel and detected with 4G10 antibody and peroxidase-fixed secondary antibody. To identify the

phospho-protein induced by EPO stimulation, we used JAK1 or JAK2 antibody, used as a negative control, antibody for immunoprecipitation.

Results and discussion

An extra high dose of EPO impairs cell growth

EPO stimulates the proliferation of erythroid precursor cells as well as erythroid committed cell lines. The full growth was achieved by the addition of 1 U (10 ng mL^{-1}) EPO using the mouse EPO-dependent cell line, Ep-FDC-P2 (Fig. 1a). The addition of EPO antibody completely inhibits cell growth in the presence of 300 U mL^{-1} EPO or less. To investigate the effect of high dose of EPO addition on the proliferation of Ep-FDC-P2 cells, we added a high dose of EPO to the cell culture media. The cells fully proliferated at concentrations ranging from 1 U to $1 \times 10^3 \text{ U mL}^{-1}$ EPO (Fig. 1b). However, cell growth was slightly inhibited at $1 \times 10^4 \text{ U mL}^{-1}$ EPO and was impaired when the concentration of EPO was more than $3 \times 10^4 \text{ U mL}^{-1}$. As a result, the cell growth curve shows a bell-like shape.

The growth impairment by high dose of EPO is rescued by EPO antibody

We examined to exclude the possibility that this bell-shaped growth curve is due to the toxicity of contaminants in the EPO preparation. We then characterized the EPO action at high concentrations. If apparent concentration of EPO become less than $3 \times 10^3 \text{ U mL}^{-1}$ by the addition of EPO antibody into the cell culture media, cells may proliferate as much as in presence of 1 U mL^{-1} EPO. To examine this possibility, R2 or R6 antibody was added to the cell culture media to capture EPO. In the absence of EPO antibody but treated with $1 \times 10^4 \text{ U mL}^{-1}$ EPO, cell growth was partially impaired (Fig. 1b). In contrast, cell growth was recovered to fully proliferative state by the addition of EPO antibody to $1 \times 10^4 \text{ U mL}^{-1}$ EPO cell culture, but the addition of unrelated antibody did not rescued cell growth. These results indicate that the EPO antibody titrated the EPO in the culture media thereby the apparent EPO concentration becomes adequate for fully supporting cell growth.

Fig. 1 High dose of EPO fails to support cell proliferation in EPO dependent Ep-FDC-P2. **a** EPO dependent growth curve. *closed circle* EPO only; *open circle* EPO plus anti EPO antibody R2 (1 mg mL⁻¹), **b** EPO dependent growth curve. *closed circle* EPO only; *open triangle* EPO plus anti EPO antibody R2 (1 mg mL⁻¹); *closed triangle* EPO plus anti EPO antibody R6 (1 mg mL⁻¹); *open square* EPO plus unrelated antibody CX-C (1 mg mL⁻¹). **c** purification of sEPOR from

culture supernatant. *lane 1* culture supernatant, *lane 2* purified sEPOR by EPO immobilized to a column. +CHO shows *N*-linked glycosylation of sEPOR; -CHO shows lack of glycosylation of sEPOR. **d** EPO dependent growth curve. *closed circle* EPO only; *closed triangle* EPO plus 1 mg mL⁻¹ sEPOR; *open triangle* EPO plus 3 mg mL⁻¹ sEPOR. Each point is the mean $\pm$ S.D. of triplicate assay (**a**, **b** and **d**)

The addition of sEPOR to high dose of EPO culture media rescued cell growth

We also examined the addition of sEPOR to titrate EPO from the culture media. To isolate sEPOR, we cultured sEPOR producing CHO cells and purified sEPOR by the use of beads with immobilized EPO (Fig. 1c). Soluble EPOR contains one consensus *N*-linked glycosylation site. Purified sEPOR consists of glycosylated and nonglycosylated molecules. The presence of the sugar chain in sEPOR does not alter the ligand binding characteristics (data not shown).

The addition of sEPOR (1 mg mL⁻¹) inhibits cell growth in the presence of low EPO concentrations (less than 1×10^3 U mL⁻¹ EPO) by titrating EPO as expected (Fig. 1d). In contrast, the presence of 1×10^4 U mL⁻¹ EPO inhibits cell growth but the addition of sEPOR rescues cell growth. However, the

cell growth recovery by the addition of sEPOR was not effective at 3×10^4 U mL⁻¹ EPO or more, probably sEPOR did not titrate excess EPO to lower the apparent EPO concentration to less than 1×10^4 U mL⁻¹. We then added more concentrated sEPOR (3 mg mL⁻¹) to the culture medium in the presence of 3×10^4 U mL⁻¹ EPO or more. The addition of concentrated sEPOR mostly recovered the cell growth. These results indicate that high dose of EPO fails to support Ep-FDC-P2 cell proliferation.

An extra high dose of EPO impairs growth of mouse and human EPO-dependent cell lines

To examine whether high dose of EPO also impairs the growth of other EPO-dependent cell lines, we used the mouse BaF/ER, human UT7 and human TF-1 cells. As shown in Fig. 2, all cell lines tested could

not proliferate at the high amounts of EPO and showed the bell-shaped growth curve as Ep-FDC-P2 cells do. The addition of sEPOR to culture medium at high EPO concentration rescued cell growth. These results indicate that the growth curve of EPO-dependent cell lines is bell-shaped and impairment of cell growth at high dose of EPO is general in EPO-dependent cell lines.

Excess amount of EPO induces the DNA fragmentation

Ep-FDC-P2 cells showed apoptosis when EPO was depleted from culture medium. Chromosomal DNA fragmentation is one of the most typical phenotype of apoptosis. To examine that high dose of EPO also caused apoptosis, we prepared chromosomal DNA from the cells treated with 10 U mL^{-1} or $1 \times 10^4 \text{ U mL}^{-1}$ EPO. DNA ladder formation was first observed at 4 h after EPO depletion but was not observed in the presence of 10 U mL^{-1} EPO (Fig. 3a). In addition, DNA ladder formation was also observed for cells treated with a high EPO concentration. By 8 h, DNA fragmentation of cells treated without EPO as well as with $1 \times 10^4 \text{ U mL}^{-1}$ EPO became massive and this continued up to 12 h, indicating Ep-FDC-P2 cells treated with high EPO concentration undergo apoptosis.

To examine whether the addition of sEPOR to medium with high concentration of EPO also rescued DNA fragmentation, agarose gel electrophoresis was done to observe the DNA ladder formation. Addition of sEPOR to medium with high concentration of EPO clearly inhibited the fragmentation of DNA (Fig. 3b). Taken together with Fig. 1, we conclude that the excess amount of EPO does not support proliferation of Ep-FDC-P2 cells.

Addition of IL-3 to high amounts of EPO rescues the cell proliferation

We next examined whether Ep-FDC-P2 cells still had potency to proliferate even in the presence of excess amounts of EPO. As Ep-FDC-P2 cell is dependent on IL-3 as well as EPO, the effect of IL-3 was observed in the presence or absence of excess EPO. The addition of IL-3 supported cell growth and prevented DNA fragmentation (Fig. 4) even in the presence of

Fig. 2 EPO dependent growth curve. A high dose of EPO does not support murine and human EPO-dependent cell lines. **a** BaF/ER; **b** UT-7; **c** TF-1; cells were cultured with EPO and with or without sEPOR, respectively. *closed circle* EPO only; *closed triangle* EPO plus 1 mg mL^{-1} sEPOR; *open triangle* EPO plus 3 mg mL^{-1} sEPOR. Each point is the mean $\pm$ S.D. of triplicate assay

Fig. 3 High dose of EPO induced electrophoresis DNA fragmentation. Chromosomal DNA was prepared at times as indicated and separated by agarose gel. **a** time course of DNA fragmentation. + cells were cultured with 10 U mL⁻¹ EPO or

100 μg mL⁻¹ R2, ++ with 1 × 10⁴ U mL⁻¹ EPO, **b** DNA fragmentation analysis. Cells were cultured with or without EPO. + 10 U mL⁻¹ EPO or 1.5 mg mL⁻¹ sEPOR; ++ 1 × 10⁴ U mL⁻¹ EPO

high concentrations of EPO. These results indicate that IL-3 elicits its own downstream signal even in the presence of high concentrations of EPO and that proliferation defect at high EPO concentration is probably due to the impairment of EPO signal transduction pathway.

High dose of EPO does not induce phosphorylation of EPOR and JAK2

The high concentration of EPO impairs cell growth but not the general signal transduction pathway of

Ep-FDC-P2 because IL-3 rescues the growth defect of Ep-FDC-P2 cell treated with high dose of EPO. We then investigated the signal transduction pathway of EPO. The binding of EPO to its receptor quickly induced phosphorylation of EPOR. To examine whether the high dose of EPO can stimulate the downstream signal, especially the phosphorylation of EPOR, immunoprecipitation was done using anti EPOR antibody or beads with immobilized EPO. The addition of 10 U mL⁻¹ EPO promoted tyrosine phosphorylation of EPOR (Fig. 5a). In contrast, the addition of 1 × 10⁴ U mL⁻¹ EPO failed to induce

Fig. 4 IL-3 supports cell proliferation in the presence of high dose of EPO. **a** IL-3 dependent growth curve at high EPO concentration. closed circle EPO only; closed square EPO plus murine IL-3 (100 U mL⁻¹), each point is the mean ± S.D. of triplicate assay. **b** time course of DNA fragmentation. + cells were cultured with 10 U mL⁻¹ EPO or 100 U mL⁻¹ IL-3, ++ with 1 × 10⁴ U mL⁻¹ EPO

Fig. 5 A high dose of EPO fails to activate the phosphorylation of EPOR and JAK2 kinase.

a phosphorylation of EPOR by EPO. **b** high dose of EPO inhibits the phosphorylation of EPOR. **c** phosphorylation of p130. **d** high dose of EPO fails to activate the phosphorylation of p130. **e** p130 is JAK2. + Ep-FDC-P2 cells were cultured with 10 U mL^{-1} ; ++ with $1 \times 10^4 \text{ U mL}^{-1}$ EPO

phosphorylation of EPOR (Fig. 5b). We detected a band at 130 kDa appearing only when cells were treated with 10 U mL^{-1} EPO using anti-phosphotyrosine antibody (Fig. 5c). This band disappeared by the addition of high dose of EPO as in the case of EPOR (Fig. 5d). Because JAK2 is a well known substrate stimulated by EPO and migrated as a band of about 130 kDa (Witthuhn et al. 1993), we predicted that this 130 kDa band represented a phosphorylated form of JAK2. Indeed, immunoprecipitation using JAK2 specific antibody revealed that the 130 kDa phospho-protein was JAK2 (Fig. 5e). These results indicate that high dose of EPO did not activate the downstream event at all.

High dose of EPO does not activate signal transduction

The half maximal response in a cell proliferation assay using Ep-FDC-P2 cells is evoked at an EPO concentration of 5 mU mL^{-1} ($=1.5 \text{ pM}$) (see Fig. 1a). A scatchard analysis of the EPO binding data indicated that Ep-FDC-P2 cell bound EPO with a dissociation constant (Kd) of 580 pM and contained 320 binding sites per cell (data not shown), which agrees with various EPO-dependent cell lines. These findings indicate that only several % ($\sim$ few sites) of the receptor was occupied with EPO in the presence of 1.5 pM EPO and such few binding was enough for the half maximal signal transduction strength. When

EPO was added at 1 U mL^{-1} ($=300 \text{ pM}$) to 4×10^5 cells mL^{-1} in a total of 100 μL , the calculated number of EPO was 2×10^{10} molecules and the number of EPOR was 1.3×10^7 sites, indicating 1.5×10^3 fold excess of EPO over EPOR than EPOR was in the culture medium and almost all of the receptors were occupied with EPO. This condition supported the full growth of Ep-FDC-P2 cell. In contrast, when EPO was added at $1 \times 10^4 \text{ U mL}^{-1}$ ($=3 \mu\text{M}$), a 1.5×10^7 folds excess of EPO over EPOR was in the medium. Apparent EPO activity, however, was as much as 5 mU mL^{-1} EPO ($=1.5 \text{ pM}$) (see Fig. 1b) even in the presence of a 1.5×10^7 fold excess of EPO, suggesting $1 \times 10^4 \text{ U mL}^{-1}$ of EPO gave a signal transduction strength similar to 5 mU mL^{-1} EPO. This idea was supported by the failure of phosphorylating EPOR and JAK2 in the presence of high concentrations of EPO (Fig. 5). To explain how excess EPO inhibits the signal transduction, we raised one possibility that there were at least two forms of EPO-EPOR complexes. One EPO molecule binds to one receptor dimer using two binding sites within EPO (we referred this form as 1–2 Form hereafter) at relatively low EPO concentration ($1\text{--}1 \times 10^3 \text{ U mL}^{-1}$), in contrast, two EPO molecules bind to one receptor dimer (as 2–2 Form) at high EPO concentration ($1 \times 10^4 \text{ U mL}^{-1}$ or more). The 1–2 Form evokes the conformational change of EPOR adequate for signal transduction (Livnah et al. 1999; Remy et al. 1999; Syed et al.

1998), on the other hand, the 2–2 Form does not. There is increasing amount of 2–2 Form and decreasing amount of 1–2 Form as EPO concentration increases. The addition of EPO antibody or sEPOR to high dose of EPO aids the transition from 2–2 Form to 1–2 Form, an adequate structure for EPO signal transduction, by titrating excess EPO. At the high concentration of EPO as 3×10^4 U mL⁻¹, almost all EPO-EPOR complexes should exist in 2–2 Form. As stated above, an half maximal signal transduction strength is evoked when only a few of 1–2 Form are produced, suggesting that almost all EPO-EPOR complexes should be in the 2–2 form to shut down EPO signal transduction. Thus, this possibility may explain why such an extra amount of EPO was required to erase the downstream signal.

There is another possibility that SHP1, which are the general negative feedback regulators, inhibit the cytokine signal transduction (Jiao et al. 1996; Klingmuller et al. 1995). However, this is unlikely because the addition of excess EPO did not stimulate JAK2 (Fig. 5) although the activation of JAK2 is first required for the SHP1 action. Detailed analysis is still required to elucidate the question on what kind of receptor structure is formed in the presence of excess amount of EPO and how excess amount of EPO fails to elicits the EPOR conformational change.

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