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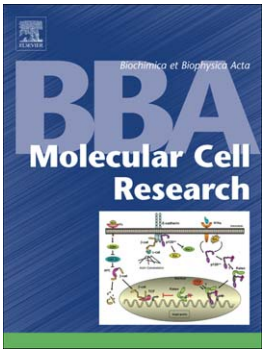

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Signaling pathways of cell proliferation are involved in the differential effect of erythropoietin and its carbamylated derivative

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Abbreviations

βcR, beta common receptor; cEpo, carbamylated erythropoietin; CFU-E, colony forming units-erythroid; DMSO, dimethylsulfoxide; Epo, erythropoietin; EpoR, erythropoietin receptor; FBS, fetal bovine serum; GM-CSF, granulocyte-macrophage colony-stimulating factor; Jak2, Janus kinase 2; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly (ADP-ribose) polymerase 1; PBS, phosphate-buffered saline; PI, propidium iodide; PI3K, phosphatidylinositol 3-kinase; SEM, standard error of the mean; STP, staurosporine.

1. Abstract

It is now recognized that in addition to its activity upon erythroid progenitor cells, erythropoietin (Epo) is capable of stimulating survival of different non-erythroid cells. Since stimulation of erythropoiesis is unwanted for neuroprotection, Epo-like compounds with a more selective action are under investigation. Although the carbamylated derivative of erythropoietin (cEpo) has demonstrated non-hematopoietic tissue protection without erythropoietic effect, little is known about differential mechanisms between Epo and cEpo. Therefore, we investigated signaling pathways which play a key role in Epo-induced proliferation. Here we show that cEpo blocked FOXO3a phosphorylation, allowing expression of downstream target $p27^{kip1}$ in UT-7 and TF-1 cells capable of erythroid differentiation. This is consistent with the involvement of cEpo in slowing down G1-to-S-phase progression compared

with the effect of Epo upon cell cycle. In contrast, similar antiapoptotic actions of cEpo and Epo were observed in neuronal SH-SY5Y cells. Inhibition and competition assays suggest that Epo may act through both, the homodimeric (EpoR/EpoR) and the heterodimeric (EpoR/βcR) receptors in neuronal SH-SY5Y cells and probably in the TF-1 cell type as well. Results also indicate that cEpo needs both the EpoR and βcR subunits to prevent apoptosis of neuronal cells. Based on evidence suggesting that cell proliferation pathways were involved in the differential effect of Epo and cEpo, we went forward to studying downstream signals. Here we provide the first evidence that unlike Epo, cEpo failed to induce FOXO3a inactivation and subsequent $p27^{kip1}$ downregulation, which is clearly shown in the incapacity of cEpo to induce erythroid cell growth.

Keywords:
Erythropoietin, carbamylated erythropoietin, FOXO3a, $p27^{kip1}$, cell proliferation, cell cycle

1. Introduction

Erythropoietin (Epo) is a pleiotropic cytokine originally identified for its role in erythropoiesis. The requirement of Epo starts at the mature burst-forming unit erythroid (BFU-E) stage, and is especially important at the colony-forming unit erythroid (CFU-E). That is why during erythroid differentiation the erythropoietin receptor (EpoR) is expressed at the BFU-E stage, peaks at the CFU-E/ proerythroblast stages, and subsequently decreases to less detectable levels. One of the earliest detectable signaling events elicited upon EpoR activation is the tyrosine phosphorylation of several intracellular proteins. Owing to the fact that EpoR lacks a kinase domain within its cytoplasmic region, the protein tyrosine kinase function is carried out by the Janus kinase 2 (Jak2) associated to the receptor, which serves as the main kinase involved in Epo signal transduction activating survival and proliferation cell programs. Interestingly, EpoR expression and Epo responses were also observed in non-hematopoietic cell types. These findings can explain why, in addition to having activity upon erythroid progenitors, Epo is capable of stimulating survival and/or proliferation of different non-erythroid cells [1-4].

Since stimulation of erythropoiesis is unwanted for neuroprotection, Epo-like compounds with more selective action are under investigation. One such compound, the carbamylated erythropoietin (cEpo), has been experimentally demonstrated to provide non-hematopoietic tissue protection without erythropoietic effect [5-8].

A major discovery concerning extra-hematopoietic actions of Epo was the identification of a different type of receptor in non-erythroid tissues, particularly in nerve cells [5,6]. These reports suggest that in erythroid cells EpoR is a dimer of two identical Epo receptor monomers, whereas in non-erythroid tissues Epo activity is mediated by a heterodimer consisting of one Epo receptor monomer (equivalent to cytokine receptor α-subunit) and CD131, the cytokine β common (βc) subunit, shared by receptors for GM-CSF, IL-3 and IL-5 [5,9]. On the contrary, Um et al [10] reported that the antiapoptotic effect of Epo in neuronal SH-SY5Y and pheochromocytoma PC-12 cell lines was primarily mediated by its binding to the “classical” homodimeric EpoR complex. These authors could not detect βcR expression in differentiated SH-SY5Y and PC-12 cells.

This lack of agreement on the receptors involved in Epo and cEpo actions, together with the limited knowledge available to explain the differential action of cEpo, prompted us to compare Epo and cEpo activities in neuronal cells (SH-SY5Y cell line) and in cells capable of erythroid differentiation with different Epo requirements (UT-7 and TF-1). In addition, we investigated signaling pathways involved in cell activation by Epo in order to explain the differential action of both erythropoietins.

2. Materials and methods

2.1 Materials

All chemicals used were of analytical grade. Alpha minimal essential medium (alpha-MEM), Dulbecco's Modified Eagle Medium (D-MEM), Ham F12 Medium, and Iscove's Modified Dulbecco's Medium (IMDM) were obtained from GibcoBRL. Bovine serum albumin (BSA), 3,4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sodium o-vanadate, phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin, pepstatin A, staurosporine (STP), Hoechst 33258 dye, methylcellulose, dimethylsulfoxide (DMSO), and propidium iodide (PI) were obtained from Sigma-Aldrich; Cytofix/cytoperm, Perm Wash, Annexin V-FITC apoptosis detection Kit II, and purified mouse anti human-PARP antibody were from BD Transduction Laboratories; anti-βactin (sc-47778), anti-Epo-R (M-20 sc-697), and anti-β-common (IL-3/IL-5/GM-CSFRβ sc-21765) antibodies were from Santa Cruz Biotechnology. Anti-phosphorylated FOXO3a, anti-FOXO3a, and anti-phosphorylated Jak2 antibodies were from Cell Signaling Technology. Nitrocellulose (NC) membranes (Hybond), chemiluminescent system kit (ECL) from Amersham Bioscience; acrylamide/bis-acrylamide solution and sodium potassium cyanate from Merck; fetal bovine serum (FBS, Bioser) and penicillin–streptomycin (PAA Laboratories) from GENSA; and Ly294002 and AG490 were obtained from Calbiochem. Recombinant human erythropoietin (rhEpo) was kindly supported by Zelltek (Argentina).

2.2. Preparation of cEpo

Carbamylated erythropoietin (cEpo) was prepared as described by Leist et al [5] with modifications. Briefly, one volume of Epo (0.5 mg/ml) was mixed with one volume of 1 M sodium borate (pH 8.8) and one volume of 3 M potassium cyanate (KCNO). The mixture was incubated at 37 ºC for 48 h. Samples were dialyzed against milli-Q water during 72 h (4 ºC) with frequent changes of liquid, and then concentrated by Centricon (Millipore, 10 kDa cut-off). The decrease in the number of free amino groups measured by its reaction with 2,4,6-trinitrobenzenesulfonic acid [11] was used to determine the efficiency of carbamylation.

Carbamylation of Epo leads to the transformation of lysine residues into homocitrulline. Therefore, characterization of cEpo, analyzed by gel electrophoresis under alkaline non-denaturing conditions and immunoblotting, displayed increased electrophoretic mobility of cEpo with respect to Epo, which is explained by the increase in net electric charge of the molecule.

2.3. Clonal Assay of Hematopoietic Progenitors

Femoral bone marrow cells, obtained from Balb/c mice, were flushed into alpha-MEM containing 2% heat inactivated FBS and cell suspensions were obtained by gentle pipetting. Cultures were performed in semisolid medium as previously described [12,13]. Briefly, $5x10^5$ cells/ml were stimulated with 5

U/ml Epo in a mixture containing alpha-MEM, 0.8% methylcellulose medium, 25% heat inactivated FBS, 2 mM L-glutamine, antibiotics (100 U/ml penicillin–100 g/ml streptomycin), and 0.1 mM 2-mercaptoethanol. Cultures were incubated at 37 ºC for 48 h, in an atmosphere containing 5% CO_2 and 100% humidity. Erythroid colonies were stained with 2,7-diaminofluorene-3% H_2O_2 in 50 mM Tris-HCl buffer, pH 7.6. Small erythroid colonies consisting of eight or more hemoglobinized cells were considered as CFU-E and counted under 400× magnification using an inverted microscope (Zeiss Axiovert 135). Geometric means of CFU-E triplicate cultures were calculated for each assay and expressed per $5x10^5$ mononuclear cells.

2.4. Cell lines and cultures

a) Human UT-7 cell line, kindly provided by Dr. Patrick Mayeux (Cochin Hospital, Paris, France), shows growth dependence on Epo. These cells were maintained in IMDM supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 U/ml Epo [14].

b) Human TF-1 cells (CRL-2003, American Type Culture Collection, ATCC) are completely dependent on interleukin 3 (IL-3) or granulocyte-macrophage colony-stimulating factor (GM-CSF) for long term growth. They can also be stimulated by Epo for short periods (less than 7 days). TF-1 cells were maintained in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml anfotericin, and 4 ng/ml GM-CSF [15].

c) Human SH-SY5Y neuroblastoma cells (CRL-2266, ATCC) were grown in 5 ml of 1:1 D-MEM:Ham F12 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% (v/v) heat-inactivated FBS [16,17].

Cell cultures were developed at 37 °C in an atmosphere containing 5% CO_2 and 100% humidity. The medium was routinely replaced every 2-3 days. Cell viability and proliferation were evaluated by Trypan blue exclusion test and the MTT assay.

Erythroid differentiation of UT-7 and TF-1 cells was induced by 30 µM hemin during 48 h while SH-SY5Y cells were differentiated by treatment with 10 µM retinoic acid (RA) during 4 days.

STP, LY294002, and AG490, dissolved in DMSO, were added to complete medium and adjusted to final concentrations as referred below in text and figures. The vehicle DMSO was added to controls. Neutralizing antibodies anti-Epo-R and anti-β-common were added to cultures at 400 ng/ml final concentration.

2.5. MTT assay

Cells were cultured in 35 mm Petri dishes at a density of $2x10^5$ cells/ml. After the cells had been subjected to appropriate treatments and the medium removed, they were incubated at 37 °C with MTT at a final concentration of 0.5 mg/ml. The supernatant was removed and the pellet washed with phosphate buffer saline (PBS). Finally, 100 µl of 0.04 M HCl in isopropanol was added to dissolve the blue formazan product (reduced MTT), which was quantified by measuring the absorbance at 570 nm test wavelength and 655 nm reference wavelength in a microplate reader (BioRad).

2.6. Fluorescent nuclear staining of apoptotic cells

Cells were cultured on slide covers plated in 35 mm Petri dishes. After fixing with 4% (v/v) p-formaldehyde in PBS for 20 min at room temperature, the samples were exposed to 0.05 g/l Hoechst

33258 dye in PBS for 10 min at room temperature, washed thrice with 18 MΩ water and finally mounted by using 50% glycerol in PBS (v/v). Fluorescent nuclei with apoptotic characteristics were detected by microscope under UV illumination at 365 nm (Zeiss Axiovert 135). Images were photographed by a Nikon Coolpix 5000 equipment and digitalized. Differential cell counting was performed by analyzing at least 500 cells [17].

2.7. Electrophoresis and Western blotting

Cell were washed with ice-cold PBS and lysed with hypotonic buffer (50 mM TRIS pH 8.0, 150 mM NaCl, 1% Triton X-100, 1 mM sodium o-vanadate) containing protease inhibitors (1 mM PMSF, 4 μM leupeptin, 1μg/ml aprotinin, 2 μM pepstatin) in a ratio of 200 μl/10^7 cells. After 30 min of incubation on ice, insoluble material was removed by centrifugation (15,000 g, 15 min). Cell extracts were boiled for 3 min in the Laemmli sample buffer [18] and resolved on 10% polyacrylamide-SDS gel electrophoresis. Electrophoresis under non denaturing conditions was run in a Miniprotean III electrophoretic system (BioRad) using 10% polyacrylamide gels. After electrophoresis, protein samples were electroblotted onto nitrocellulose membrane during 1.5 h (transfer buffer: pH 8.3, 25 mM Tris, 195 mM glycine, 0.05% SDS, pH 8.3, and 20% (v/v) methanol). Membranes were blocked by 1 h-incubation in Tris Buffer Saline (25 mM Tris, 137 mM NaCl, 3 mM KCl, pH 7.4) containing 0.1% Tween 20 and 0.5% skim-milk powder, and then incubated with appropriate concentrations of specific antibodies. After washing with TBS–0.1% Tween 20, the immunoblots were probed with adequate peroxidase-conjugated secondary antibody (1:1000) for 1 h at 20 °C and washed. Antigen-antibody complex signals were detected by enhanced chemiluminiscence, using ECL kit and a Fujifilm Intelligent Dark Box II equipment (Fuji) coupled to a LAS-1000 digital camera. Anti-β-actin polyclonal antibody was used to assess sample loading variations. Densitometry with ImageGauge software was performed to quantify the bands.

2.8. Flow cytometry

2.8.1. Jak2 phosphorylation

After experimental treatments, cells were collected by centrifugation and washed with 2% FBS in PBS. Then, cells were incubated with Cytofix-Cytoperm for 20 min, and following centrifugation (350 g, 10 min) they were incubated with anti-phosphorylated Jak2 antibody (30 min, 4 ºC) and washed with Perm Wash. After that, Alexa Fluor 488-second antibody incubation (30 min, 4 ºC) was made and finally, samples were analyzed in a flow cytometer equipped with a 488 nm argon laser (FACSort, Becton-Dickinson). WinMDI 2.9 software was used to analyze data.

2.8.2. Annexin V binding and propidium iodide analysis

At the end of treatments, the cells were washed with binding buffer and then incubated with Annexin V and propidium iodide (15 min at room temperature in the dark), according to the manufacturer's indications. After this period, cultures were washed with binding buffer and finally, the samples were mounted and analyzed by flow cytrometry as described above.

2.8.3. Cell Cycle Analysis

After UT-7 cells ($5x10^5$ cells/ml) were subjected to appropriate treatments, they were harvested by centrifugation, washed twice with PBS, and fixed with 70% ethanol at 4 °C overnight. Then the cells were washed with PBS. The DNA of ethanol-fixed cells was stained by propidium iodide (0.5 mg/ml)

containing RNase A (120 μg/ml) at 37 °C for 60 min. The cell cycle distribution for single cells was carried out using flow cytometry [19].

2.9. Statistics

Results are expressed as mean ± standard error (Mean ± SEM). Comparison among groups was carried out by the Kruskal-Wallis one-way analysis of variance and the Mann-Whitney U-test when corresponding. Least significant difference with $P<0.05$ was considered as the criterion for statistical significance.

3. Results

3.1. Comparison between the effects of erythropoietin and its carbamylated derivative upon erythroid and neuronal cells

Firstly, we have to demonstrate that the prepared carbamylated erythropoietin (cEpo) has neuroprotective but not erythropoietic ability. In order to do that we made cultures of the SH-SY5Y cell line from neuronal origin and of cells with capacity to erythroid differentiation (physiological murine CFU-E and human UT-7 and TF-1 cell lines). Since Epo has long been considered a specific apoptosis-preventing trophic factor which allows erythroid cells to fulfill their program of proliferation and differentiation [20], we evaluated the differential antiapoptotic effects between Epo and cEpo.

It can be seen in Figure 1 that Epo, but not cEpo, significantly stimulated progenitor cells from murine bone marrow leading to the appearance of CFU-E (Fig. 1.A) and maintained the survival of cells from UT-7 and TF-1 lines with ability to erythroid differentiation (Fig. 1.B and 1.C).

Interestingly, Epo and cEpo simultaneously added to cultures at 1:10 ratio completely inhibited CFU-E growth, suggesting cEpo interference with the proliferative effect of Epo (Fig. 1.A). This yet unreported finding which was not observed when cEpo was added after 60 min of Epo stimulation supports the concept that cells are fully activated by Epo within this period.

Quite opposite was the action of cEpo upon SH-SY5Y cells of neuronal origin induced to apoptosis by staurosporin (STP), which had been previously demonstrated to be overcome by Epo [17]. Before the addition of the proapopotic agent, cells were preincubated in the presence of Epo or cEpo. Figure 1.D shows that both erythropoietins have similar neuroprotective effects since they completely prevented STP-induced apoptosis. Moreover, we add new information about mechanisms of this neuroprotective action. The fact that prevention of programmed cell death by Epo or cEpo was mediated by PI3K and Jak2 signalling pathways in assays with Ly294002 (PI3K inhibitor) and AG490 (Jak2 inhibitor), suggests similar mechanisms for Epo and cEpo neuroprotection (Fig. 1.D).

Similar results between Epo and cEpo were observed when apoptosis of SH-SY5Y cells was induced by the proinflammatory cytokine tumor necrosis factor (data not shown).

3.2. Erythropoietin and beta-common receptors in erythroid and neuronal cell lines. Cell activation by Epo and cEpo

Current knowledge supports the idea that two different receptors, the homodimer EpoR-EpoR and the heterodimer EpoR-βcR, may be involved in the action of Epo and cEpo. We therefore investigated the expression of both receptors in erythroid and neuronal cells under different stages of cell differentiation.

Unlike SH-SY5Y cells differentiated by retinoic acid, undifferentiated cells showed the expression of EpoR and βcR. On the other hand, regardless of their differentiation stage, cells with erythroid differentiation ability (UT-7 and TF-1) express both receptor subunits (Fig. 2.A).

As a consequence of the binding of an appropriate ligand to each of the EpoR-EpoR or the EpoR-βcR dimers, cell activation is initiated by Jak2 phosphorylation. Therefore, this mechanism was evaluated in cell cultures performed in the presence of both inducers. As expected, Epo and cEpo induced Jak2 phosphorylation in SH-SY5Y cells (Fig. 2.B). Interestingly, cEpo also induced Jak2 phosphorylation in TF-1 and UT-7 cell lines which are known to be activated by Epo (Fig. 2.C and D), even though cEpo did not support cell survival. This finding was confirmed in UT-7 culture assays with neutralizing anti-EpoR and anti-βcR antibodies which prevented the cEpo induction of Jak2 phosphorylation (data not shown).

3.3. Receptors involved in the action of erythropoietin and carbamylated erythropoietin

Different authors reported that Epo neuroprotective action would be mediated by the heterodimeric receptor (EpoR-βcR) [5,6,21,22]. On the other hand, it is known that TF-1 cells are dependent on GM-CSF to survive and that the specific receptor of this factor involves the βc receptor subunit.

Results obtained so far suggest that Epo and cEpo have similar behavior upon neuronal SH-SY5Y cells. Nevertheless, Epo and cEpo show differential action upon TF-1 cells capable of erythroid differentiation. We consequently decided to carry out assays of competition and inhibition to investigate the receptors involved in the interaction of Epo and cEpo with both cell lines.

Epo and cEpo neuroprotective effects against STP-induced apoptosis in undifferentiated SH-SY5Y cells were analyzed in the presence of 400 ng/ml neutralizing anti-EpoR or anti-βcR antibodies (Fig. 3.A). The neuroprotective action of Epo against STP induced apoptosis was completely blocked in the presence of anti-EpoR and only blunted by incubation with anti-βcR antibody. In contrast, treatment of cultures with each antibody completely prevented the antiapoptotic action of cEpo. These results agree with those of cells grown in the presence of GM-CSF, which competes for the binding to βcR (Fig. 3.B). It has to be noted that the high concentration of GM-CSF used in this assay did not induce cell apoptosis.

This evaluation of programmed cell death obtained by detection of fluorescent nuclei after Hoechst stain strongly agreed with results of analysis of other sign of apoptosis, such as membrane phosphatidylserine translocation detected by Annexin V binding and propidium iodide nucleus stain (Fig. 3.C).

Regarding TF-1 cells, we found that high concentrations of cEpo blocked the antiapoptotic action of GM-CSF and partially affected the Epo activity (Fig. 4).

The results shown in Figures 3 and 4 suggest that in neuronal cells Epo acts via both, the homodimeric and the heterodimeric receptors whereas cEpo only binds to the latter. However, cEpo is also able to interact with TF-1 cells, which can explain Jak2 phosphorylation induced by cEpo in this cell line.

3.4. Erythropoietin and carbamylated erythropoietin action upon cell proliferation signaling

This work suggests that differential behavior of Epo and cEpo in erythroid cells seems to be principally related to cell proliferation. Based on results of Jak2 activation which confirmed the interaction of both

erythropoietins with erythroid cells, it was interesting to investigate events associated to cell cycle and proliferation. Downstream targets of Epo have been shown to regulate mechanisms which have significant effects on hematopoietic cell proliferation under physiological conditions. Therefore, we analyzed phosphorylation of the forkhead transcription factor FOXO3a and expression of $p27^{kip1}$ in TF-1 and UT-7 cells stimulated by Epo or cEpo. As can be seen in Figure 5 (A and B), activation of cells by Epo led to phosphorylation of FOXO3a whereas this event could not be induced by cEpo. This factor is a known regulator of genes, such as *p27*, thus affecting cell cycle progression. In accordance with this, $p27^{kip1}$ levels in cell cultures treated with cEpo were similar to that observed in non-activated cells (Fig. 5.A, B). These results are consistent with the involvement of cEpo in slowing down G1 to S progression in comparison with the effect of Epo on UT-7 cell cycle (Fig. 5.C).

It was interesting to study $p27^{kip1}$ expression in neuronal SH-SY5Y cells, which are not dependent on Epo or cEpo to proliferate. In this cell line, neither Epo nor cEpo induced changes in $p27^{kip1}$ levels with respect to non-treated controls (data not shown).

We can conclude that FOXO3a inactivation and $p27^{kip1}$ expression induced by cEpo is compatible with cell cycle arrest, and consequently, restrains cell proliferation.

4. Discussion

The results obtained from different *in vitro* assays included in this work agree with the concept that carbamylation of Epo lysine residues leads to a derivative that retains the ability of Epo to protect neuronal cells against apoptosis induced by STP or TNF-α while it is unable to support erythroid cell proliferation.

Interestingly, our unexpected finding was that –similarly to its effect on neuronal cells- cEpo induced Jak2 phosphorylation in UT-7 and TF-1 cells despite its incapacity to act as growth factor in these cell lines. This suggests that the interaction between cEpo and erythroid cells involves a receptor which belongs to the type I cytokine superfamily.

After the initial study by Masuda et al [23], it is currently assumed that the Epo receptor involved in non-hematopoietic tissue protection is different from that participating in signal transduction in the hematopoietic system. Based on different observations, the heterodimeric receptor formed by the EpoR and βcommonR subunits was hypothesized to play a role in the antiapoptotic action of Epo [24,25]. Alternatively, other studies support the notion that at least in some types of neuronal cells the cytoprotective effects of Epo are mediated by the “classical” homodimeric EpoR complex, in a similar way as Epo exerts its antiapoptotic and proliferative effects on erythroid cells. Data from an *in vivo* experiment suggest that most neurons in the rat brain are likely to express high levels of EpoR but low, if not null, levels of βcR [26]. Likewise, Um et al [10] reported that differentiated SH-SY5Y cells do not express a detectable level of βc receptor, even though Epo was able to specifically bind to high-affinity Epo surface binding sites on these cells and to exert neuroprotective activity. In this work, we have also found undetectable βc subunit in SH-SY5Y cells induced to differentiation with retinoic acid (Fig. 2). Instead, both receptors, EpoR and βcR, were found expressed in undifferentiated SH-SY5Y and in undifferentiated or erythroid differentiated UT-7 and TF-1 cell lines. The finding of EpoR and βcR in all cell lines used in this study let us design inhibition experiments with neutralizing antibodies and competition assays between cEpo and other specific ligands. Considering that βc is also a subunit involved in the GM-CSF receptor, this growth factor was chosen for competition assays. In SH-SY5Y

cell cultures, STP-induced apoptosis could not be prevented by cEpo in the presence of GM-CSF or by the βcR inhibition with specific antibodies (Fig. 3). In a reverse experiment, GM-CSF failed to support TF-1 cell growth in the presence of high cEpo concentration (Fig. 4). While further investigation is needed to fully clarify this possibility, our results suggest an interaction between cEpo and the EpoR and βcR subunits, therefore hinting at its binding to the heterodimeric receptor EpoR/βcR in both cell lines. This finding may explain why cEpo competes with the proliferative action of Epo upon murine CFU-E since we demonstrated that cEpo, at tenfold concentration (200 ng/ml) with respect to Epo (20 ng/ml), interferes with the stimulation of colony growth by the latter erythropoietin (Fig.1). The fact that this interference is not observed when cells are stimulated with Epo prior to the addition of cEpo is not unexpected, as a previous study showed that inhibition of CFU-E growth by aluminum only occurred in the presence of Epo at early stages (less than 60 min) during the interaction of the hormone with its target cell [12]. It is known that activation of the EpoR after Epo binding is transient as EpoR appears to be quickly degraded after ubiquitination by two proteolytic systems that proceed successively: the proteasomes remove part of the intracellular domain at the cell surface, and the lysosomes degrade the remaining part of the receptor-hormone complex [27].

On the other hand, it seems that Epo may act through both, the homodimeric (EpoR/EpoR) and the heterodimeric (EpoR/βcR) receptors in neuronal SH-SY5Y cells and probably in erythroid cell type as well. This finding was not unexpected because as early as 1995 Epo was reported to induce the tyrosine phosphorylation of βcR in UT-7 cells [24].

This dual binding capacity of Epo probably depends on density of the receptor subunits. Thus, EpoR may either self-associate to form EpoR/EpoR or participate in the complex EpoR/βcR which can also support signaling. cEpo can only bind to the heteroreceptor in SH-SY5Y cells. Additionally, cEpo binding to the EpoR/βcR in TF-1 cells probably explain Jak2 phosphorylation in these cell lines.

In a review discussing the structure of βcR, special note was made of ongoing debate regarding the activation mechanisms of more complicated class I cytokine receptors in which the receptor systems contain a shared receptor subunit. The authors mentioned the βc subunit of the IL-3, IL-5 and GM-CSF receptors as examples [28] and it is likely that the complex formed by EpoR and βcR can also be a case in point.

The UT-7 and TF-1 cells have been included in this work because both cell lines are stimulated by Epo but they have different dependence on the growth factor. This difference may be explained by the type of EpoR expressed in both cell lines. Chrètien et al [29] reported that TF-1 cells, which overexpress a truncated EpoR, showed an impaired activation of the STAT5 transcription factor when they are stimulated by Epo. However, the EpoR structure in different cells is still open to investigation. Human bone marrow cells express truncated and full-length forms of EpoR being the former predominating in immature progenitors and the latter in the late-state progenitors [30]. In different tissues Arcasoy et al [31] found the expression of EpoR splice variants encoding peptides that are truncated in the carboxy-terminal region of the intra-cytoplasmic domain. Following this paper we investigated these EpoR transcripts in different lines and observed similar expression patterns between UT-7 and TF-1 [unpublished data]. Whether the presence of EpoR isoforms is related with different cell stimulation by Epo remains to be determined. Nevertheless, this point seems not to be

relevant to explain differences between Epo and cEpo action since in the present work we demonstrated similar behavior upon UT-7 and TF-1 cells.

The ability of cEpo to bind to βcR in TF-1 cells and the finding of UT-7 and TF-1 cell activation by cEpo detected by Jak2 phosphorylation (Fig. 2) prompted us to further investigate the step at which the action of the modified erythropoietin differs from that of the native Epo. Jak2 phosphorylation is generally important in transmitting signals from the cell surface to the nucleus. Since cEpo seems to retain the antiapoptotic property of Epo without its ability to promote cell proliferation, we analyzed factors involved in cell growth, such as FOXO3a and $p27^{kip1}$. The activity of FOXO3a, which belongs to the FOXO family of transcription factors, is negatively regulated by the PI3K/Akt cascade, a signaling pathway activated by Epo. Akt can phosphorylate FOXO3a and promote its cytoplasmatic accumulation [32] with decreased transcription of the target genes, among which $p27^{kip1}$ is known to be a key molecule that modulates cell cycle [19]. Therefore, it was also interesting to study cell cycle in cell cultures stimulated either by Epo or by cEpo. Cultures in the presence of Epo increased cell number at S phase, showing that it could promote the G1-S transition whereas cEpo induced cell arrest at G0-G1 phase (Fig. 5). These results are in line with those showing Epo-induced FOXO3a phosphorylation and $p27^{kip1}$ downregulation. It has been reported that Epo controls the phosphorylation of FOXO3a via the PI3K/Akt signaling pathway and its degradation to retain it in the cytoplasm through binding to 14-3-3- protein [33]. Phosphorylated FOXO3a does not translocate to the nucleus, and is consequently unable to stimulate the expression of $p27^{kip1}$, thus allowing the progression of the cell cycle and cell proliferation. In contrast, in cell cultures with cEpo cell cycle progression at the G1/S transition was inhibited, probably due to the translocation of the unphosphorylated forkhead factor FOXO3a to the nucleus and its upregulation of the cyclin-dependent kinase inhibitor $p27^{kip1}$ (Fig. 5).

Present results may explain those reported by Ramirez et al [34] who found phosphorylation of the antiapoptotic Jak2/Akt signal in UT-7 cells induced by carbamylated darbepoietin but had a marginal effect on cell proliferating signals (Erk1/2, NF-κB and Stat-5) in endothelial progenitor cells. Our results went forward to downstream signals, confirming that cEpo failed to activate signaling pathways of cell proliferation.

Regarding neuronal cells, we observed similar Jak2 and PI3K-mediated mechanisms of the antiapoptotic actions of Epo and cEpo, although cEpo seems to bind to the heterodimeric receptor and Epo may do it to both, the EpoR/EpoR and EpoR/βcR (Fig. 3). A mechanism of cEpo action mediated by PI3K/Akt was also reported regarding cardioprotection [35].

The similar Epo and cEpo activities found in our work upon SH-SY5Y cells as well as $p27^{kip1}$ levels after activation of these cells by Epo or cEpo are not unexpected given that cEpo keeps its antiapoptotic ability and the SH-SY5Y neuronal cell line does not depend on these growth factors to proliferate.

In conclusion, here we present an advance to elucidate differential effects between Epo and cEpo upon hematopoietic and non-hematopoietic cells since for the first time we report the inability of the carbamylated erythropoietin to completely activate signaling pathways focused to cell proliferation. Finally, we suggest that Epo may activate neuronal cells through both receptors, the homodimer and the heterodimer, whereas cEpo may only bind to the heterodimer EpoR/βcR. This means that only cells which express EpoR and βcR would be potentially targets for the cEpo antiapoptotic activity

leading to reducing degree of tissue damage. In addition, precise focus upon interference of high doses of cEpo with Epo and GM-CSF is required to elucidate benefits and risks of future therapeutic strategies.

Acknowledgements

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Figure Captions

Fig. 1. Effects of erythropoietin and carbamylated erythropoietin on erythroid progenitors and different cell lines. A) Murine bone marrow cells ($5x10^5$/ml) were stimulated with 20 ng/ml of either Epo (E) or cEpo (cE) for 48 h. CFU-E development was determined by colony count under microscope after reaction with 2,7-diaminofluorene. Unlike Epo, cEpo did not induce significant CFU-E growth

(***P<0.001, n=10). In competition assays, CFU-E growth was decreased at levels of cultures performed without Epo only when 200 ng/ml cEpo concentration (cEpo 10x) was simultaneously added with Epo to the cultures. This cEpo interference disappeared when it was added 60 min after Epo stimulation had started (***P<0.001, n=10). B-C) UT-7 cells were incubated with 20 ng/ml Epo or 20 ng/ml cEpo for 48 h. After 18 h-starvation (Without GF), TF-1 cells were cultured with 4 ng/ml GM-CSF (GM) or 20 ng/ml of either Epo or cEpo. Apoptosis was evaluated by fluorescence microscopy after Hoechst stain, Western blotting of PARP degradation (indicated by the appearance of the 85 kDa b-band; β-actin: internal control of protein loading), and Annexin V binding to phosphatidylserine. In UT-7 and TF-1 cultures, Epo but not cEpo prevented apoptosis (***P<0.001, **P<0.01, *P<0.05; n=6). D) SH-SY5Y neuronal cells were cultured in the presence of 100 ng/ml of either Epo or cEpo during 24 h and then, exposed to 50 nM STP (24 h). In assays with 25 µM Ly294002 or 25 µM AG490, the inhibitors were added 2 h before the addition of Epo or cEpo. Apoptosis induced by STP (**P<0.01, n=5) was prevented by pretreatment with either Epo or cEpo while these protective effects were abrogated by the presence of Ly and AG (**P<0.01, n=3). Each bar represents Mean±SEM of the indicated independent assays.

Fig. 2. Expression of EpoR and βcR under different stages of cell differentiation. A) TF-1 and UT-7 cells were subjected to erythroid differentiation by 30 µM hemin during 48 h (H) while differentiation of neuronal SH-SY5Y cells was achieved by retinoic acid (10 µM RA, 4 days). Cell extracts were resolved by SDS-PAGE (T=10%), electroblotted on a NC membrane and then immunodetected by quemiluminiscence using specific antibodies. Each bar represents Mean±SEM of band density with respect to β-actin, used as control of sample loading. B) SH-SY5Y cell incubations with 250 ng/ml of either Epo (E) or cEpo (cE) for 10 min and p-Jak2 analyzed by flow cytometry. C) After 18 h without growth factors, TF-1 cells were incubated with 20 ng/ml Epo or 20 ng/ml cEpo during 10 min and p-Jak2 was detected. D) UT-7 cell cultures were incubated with 20 ng/ml of either Epo or cEpo (10 min) to determine Jak2 phosphorylation (p-Jak2). Each bar represents Mean±SEM. Significant differences of fluorescence intensity: **P<0.01; * P<0.05 (n=3).

Fig. 3. Receptors involved in the Epo and cEpo actions on neuronal cells. A) SH-SY5Y cells were incubated in the presence of anti-EpoR (EpoR Ab) or anti-βcR (βcR Ab) antibodies during 2 h before the addition of Epo or cEpo. After 24 h, cells were exposed to 50 nM STP for additional 24 h. Percentage of apoptotic cells was determined by fluorescence microscopy after Hoechst stain. The presence of neutralizing antibodies (400 ng/ml) completely blocked the neuroprotective effect of cEpo (**P<0.01; n=3). Anti-EpoR prevented the action of Epo (**P<0.01, n=3) while anti-βcR only blunted the Epo effect (*P<0.05, n=3). B-C) SH-SY5Y cells, pretreated for 24 h with Epo or cEpo (20 ng/ml) in the presence or absence of GM-CSF (40 ng/ml), were exposed to 50 nM STP for additional 24 h. Apoptosis was evaluated by differential count of apoptotic nuclei (B: fluorescence microscopy) and by phosphatidylserine translocation (C: flow cytometry analysis). GM-CSF, which was not toxic for the cells, did not prevent the STP-induced apoptosis (GM-CSF-STP vs. STP, NS) but blocked the protective action of cEpo (**P<0.01, *P<0.05, n=3).

Fig. 4. Receptors involved in the Epo and cEpo actions on erythroid cells. After deprivation of growth factors for 18 h, TF-1 cells were cultured in the presence of Epo (20 ng/ml), cEpo (20 ng/ml) or GM-CSF (4 ng/ml) during 24 h. In other assays, Epo or GM-CSF were simultaneously added with low (20 ng/ml) or high (200 ng/ml) cEpo concentration. Analysis of apoptosis by fluorescence microscopy (A) and flow cytometry (B) revealed partial interference of cEpo with cell protection by Epo and complete interference with the action of GM-CSF. Each bar represents Mean±SEM. Significant differences ***P<0.001, **P<0.01, *P<0.05 (n=3).

Fig. 5. Erythropoietin and carbamylated erythropoietin on events of cell proliferation. A-B) UT-7 cells were incubated with 20 ng/ml Epo or 20 ng/ml cEpo. After 18 h-starvation (Without GF), TF-1 cells were cultured with 4 ng/ml GM-CSF (GM) or 20 ng/ml of either Epo or cEpo. Cultures were performed for 30 min to analyze FOXO3a phosphorylation or 24 h for $p27^{kip1}$ detection by Western blotting. Blots are representative of 3 assays. Each bar represents Mean±SEM of band density with respect to β-actin. Unlike Epo, cEpo was incapable of inducing phosphorylation of FOXO3a, thus leading to $p27^{kip1}$ expression (**P<0.01, *P<0.05, n=3). C) After incubation with Epo or cEpo for 24 h, UT-7 cells were assayed for DNA content by PI staining and flow cytometry. Histograms indicating percentage of cells with respect to fluorescence intensity (DNA content) are representative of 4 independent experiences. Each bar corresponds to positive cells in each cell cycle phase (**P<0.01, N.S.: non significant, n=4).

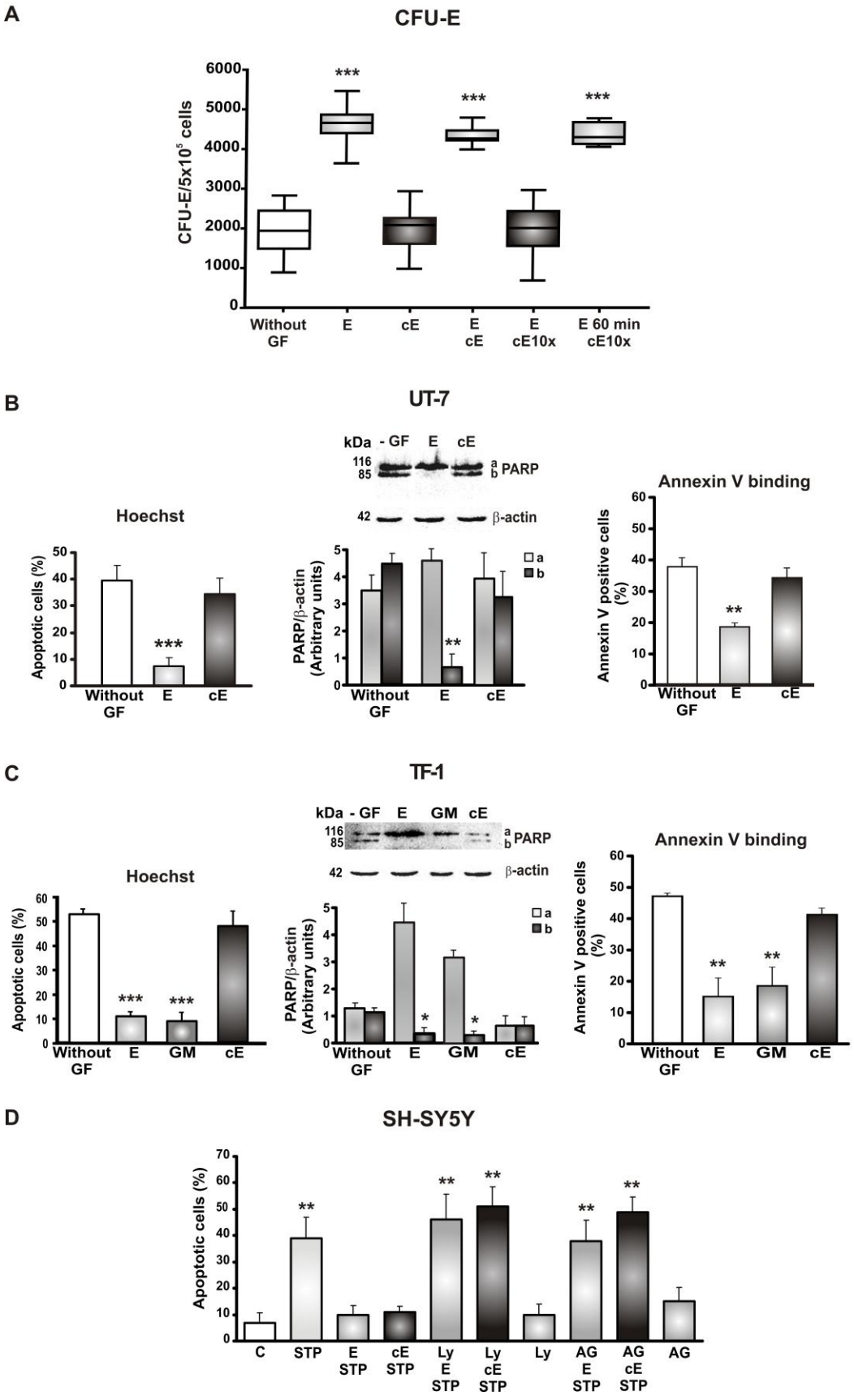
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Figure 1

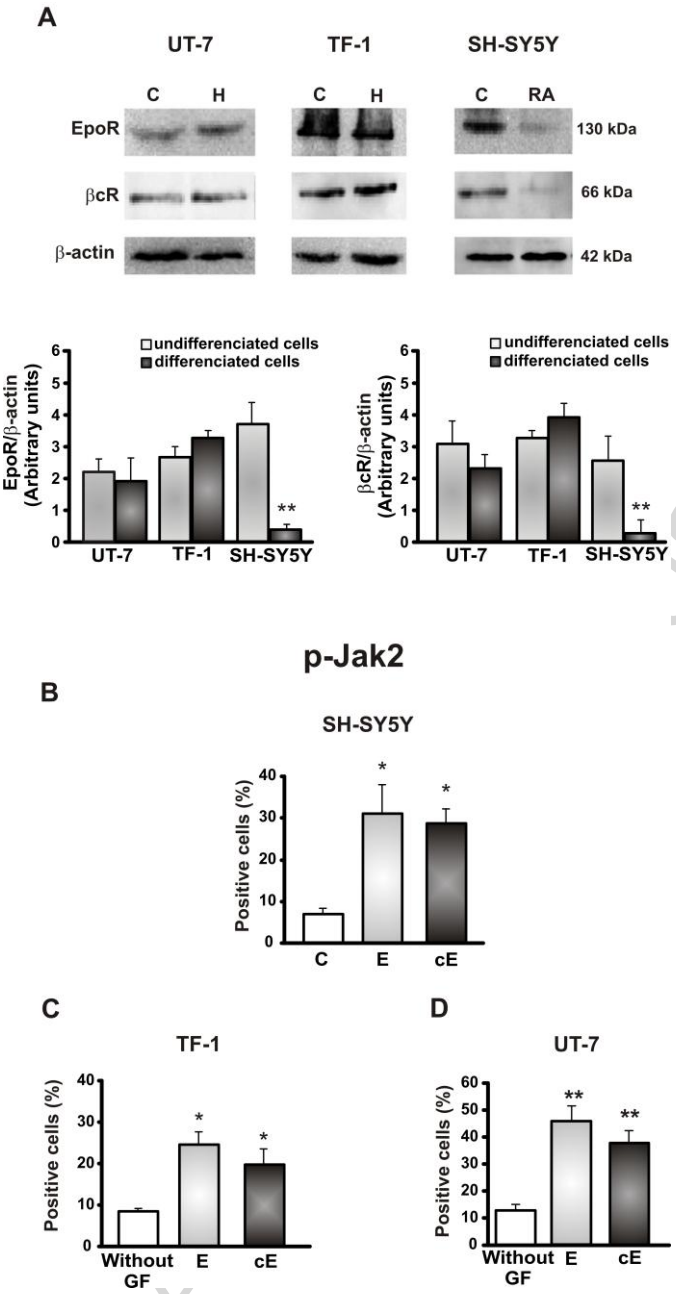


Figure 2

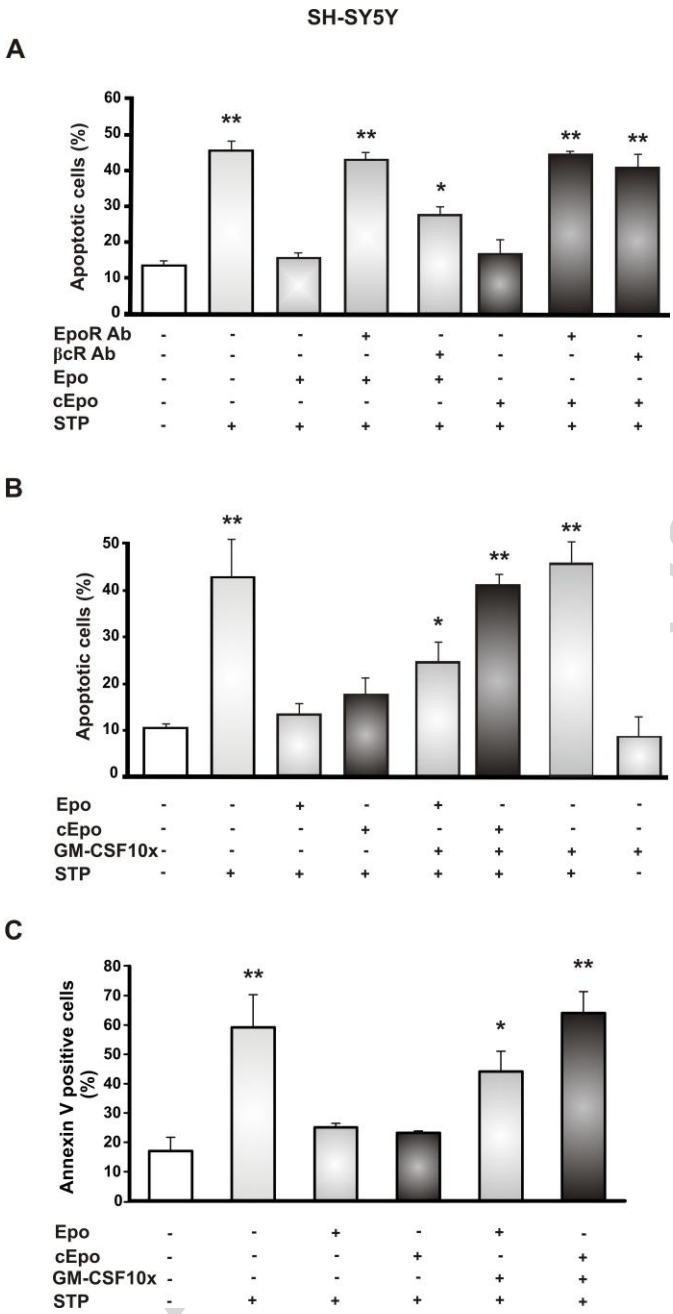


Figure 3

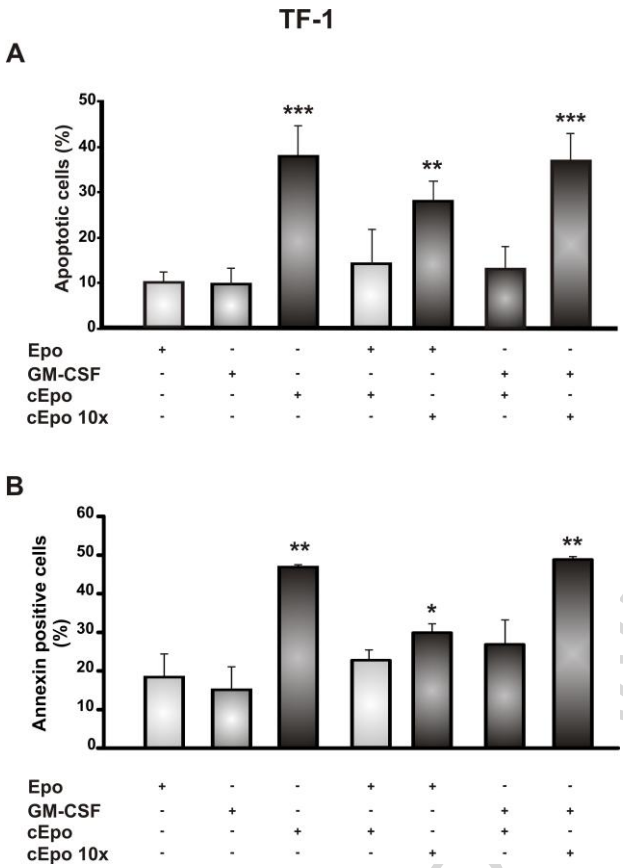


Figure 4

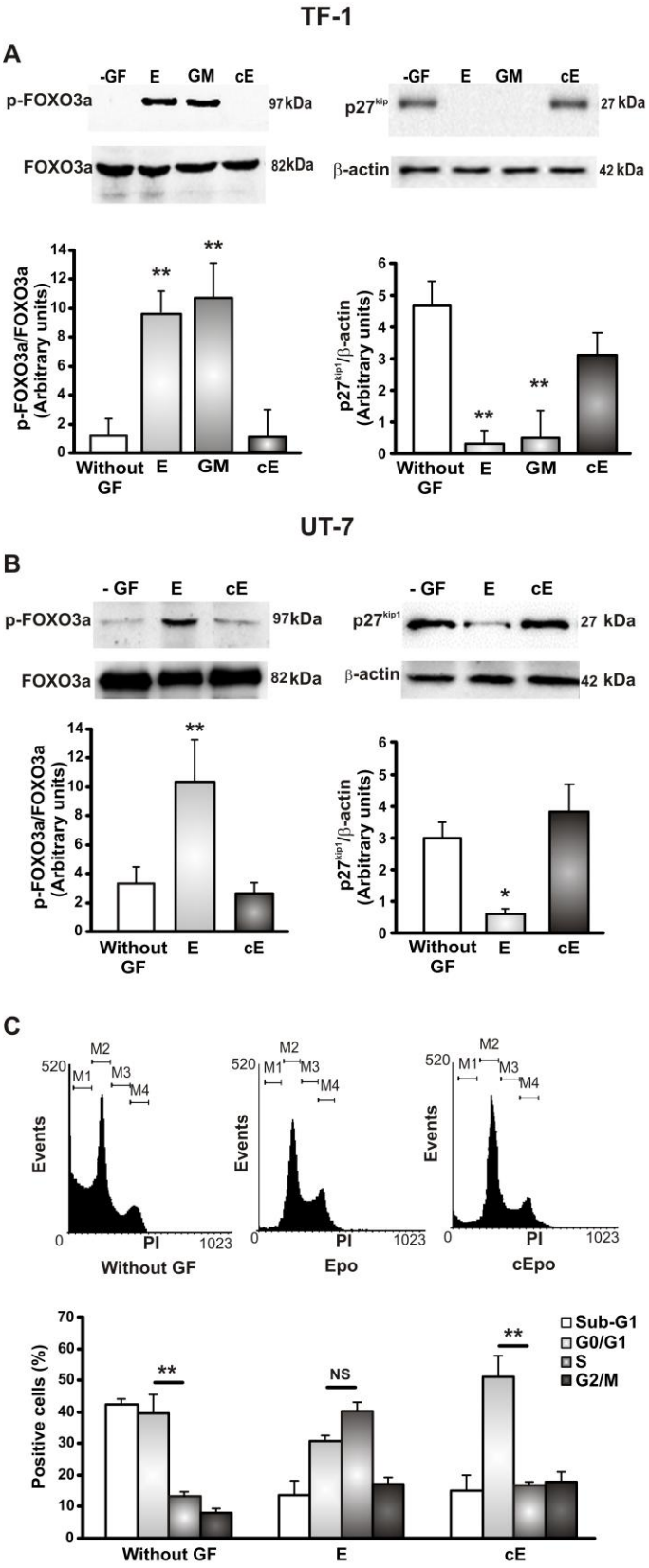
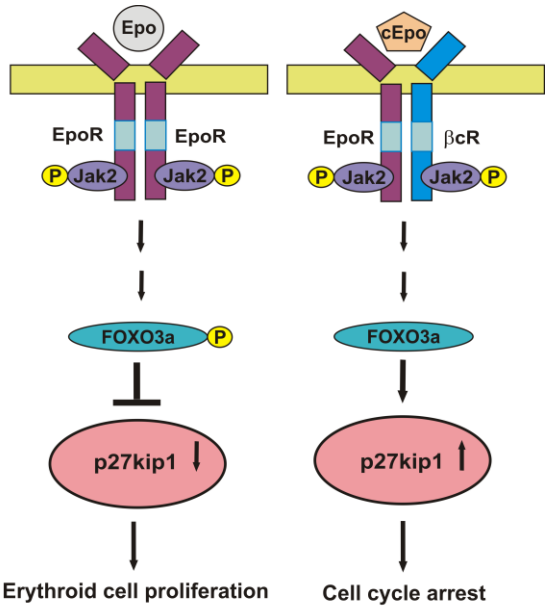


Figure 5



Graphical Abstract

Highlights:

- Differential action of Epo and cEpo is associated to cell proliferation signaling
- EpoR and βcR are expressed in UT-7, TF-1 and SH-SY5Y cell lines
- p-Jak2 was induced by either Epo or cEpo in UT-7, TF-1, and SH-SY5Y cells
- Reciprocal interference was detected between GM-CSF and cEpo
- $p27^{kip1}$ expression and cell cycle arrest were induced by cEpo in erythroid cells