

Differential Antiapoptotic Effect of Erythropoietin on Undifferentiated and Retinoic Acid-Differentiated SH-SY5Y Cells

Shirley D. Wenker,^{1,2*} María E. Chamorro,¹ Daiana M. Vota,^{1,2} Mariana A. Callero,^{1,2} Daniela C. Vittori,^{1,2} and Alcira B. Nesse^{1,2}

¹Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Pabellón II, Piso 4, Ciudad Universitaria, Ciudad Autónoma de Buenos Aires C1428EHA, Argentina

²Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Avda. Rivadavia 1917, C1033AAJ, Ciudad Autónoma de Buenos Aires, Argentina

ABSTRACT

Erythropoietin (Epo) is known to have a significant role in tissues outside the hematopoietic system. In this work, we investigated the function of Epo in cells of neuronal origin subjected to differentiation. Treatment of SH-SY5Y cells with all-*trans*-retinoic acid (atRA) generated differentiated neuron-like cells, observed by increased expression of neuronal markers and morphological changes. Exposure of undifferentiated cells to proapoptotic stimuli such as staurosporine, TNF- α , or hypoxia, significantly increased programmed cell death, which was prevented by previous treatment with Epo. In contrast, atRA-differentiated cultures showed cell resistance to apoptosis. No additional effect of Epo was detected in previously differentiated cells. The inhibition of the PI3K/Akt pathway by Ly294002 abrogated the protective effects induced by either Epo or atRA. The effect of atRA was mediated by an increased expression of Bcl-2 whereas the Epo treatment upregulated not only Bcl-2 but also Bcl-xL. This upregulation by Epo was not detected in atRA-differentiated cells, thus confirming the lack of the protective effect of Epo. As expected, assays with AG490, an inhibitor of Jak2, blocked the Epo action only in undifferentiated cells. This reduced neuroprotective function of Epo on SH-SY5Y-differentiated cells could be explained at least in part by downregulation of the Epo receptor expression, which was observed in atRA-differentiated cells. This study shows differential cellular protection induced by Epo at two stages of SH-SY5Y differentiation. The results allow us to suggest that this differential cell behavior can be ascribed to the interaction between atRA and the signaling pathways mediated by Epo. *J. Cell. Biochem.* 110: 151–161, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: APOPTOSIS; DIFFERENTIATION; ERYTHROPOIETIN; ALL-*trans*-RETINOIC ACID; SH-SY5Y CELLS; ERYTHROPOIETIN RECEPTOR; NEUROPROTECTION

Neuronal differentiation and cellular homeostasis are fundamental events in the development of the nervous system as well as in the regeneration of damaged nervous tissue. Differentiation is regulated by a complex mechanism, in which the result of several interactions between the cell and the extracellular medium determines the fate of the cellular function. Extracellular signaling molecules, including hormones, growth factors, cytokines, and trophic factors, modulate this process through the interplay between cellular pathways, finally resulting in the transcriptional regulation of several genes. On the other hand, a balance between life and death is a key factor in cell development. Cellular homeostasis is regulated

by a genetically planned mechanism called programmed cell death or apoptosis, which results crucial for the normal development of the nervous system [Benn and Woolf, 2004]. The disruption in the balance between antiapoptotic and proapoptotic signals can cause several diseases, which in the case of the nervous system can be related to neurodegenerative processes.

Postmitotic neurons have adopted multiple strategies to avoid inappropriate apoptotic death, such as the induction or activation of intrinsic antiapoptotic brakes. The deficits of these molecules increase cell vulnerability and any toxic stimulus could thus lead to neuronal damage [Benn and Woolf, 2004].

Grant sponsor: University of Buenos Aires (Argentina); Grant sponsor: National Agency for Scientific and Technologic Promotion (ANPCYT, Argentina); Grant sponsor: CONICET (Argentina).

*Correspondence to: Shirley Wenker, Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Pabellón II, Piso 4, Ciudad Universitaria, Ciudad Autónoma de Buenos Aires C1428EHA, Argentina. E-mail: swenker@qb.fcen.uba.ar

Received 21 August 2009; Accepted 7 January 2010 • DOI 10.1002/jcb.22521 • © 2010 Wiley-Liss, Inc.

Published online 11 March 2010 in Wiley InterScience (www.interscience.wiley.com).

Among the factors that support cell survival, erythropoietin (Epo) is well known as a growth factor that maintains the number of circulating erythrocytes primarily by preventing apoptosis of erythroid progenitors. However, its biological role has been expanded by the finding of specific receptors in non-hematopoietic tissues, such as endothelial [Anagnostou et al., 1994] and neuronal [Masuda et al., 1993] tissues. Evidence has shown that Epo exhibits neuroprotective effects *in vitro* against proapoptotic agents such as glutamate, hypoxia, tumor necrosis factor- α (TNF- α), and glucose/serum deprivation [Marti et al., 2000; Buemi et al., 2002; Celik et al., 2002; Pregi et al., 2009]. *In vivo* studies in adult and neonatal animal models have revealed a neuroprotective action of exogenous Epo administration [Sola et al., 2005; Noguchi et al., 2007]. When Epo binds to its specific receptor EpoR, it causes dimerization of the receptor, which activates signaling pathways mediated by the Janus family protein tyrosine kinase (Jak2). This event recruits multiple signaling molecules that contain SH2 domains, including signal transducers and activators of transcription 5 (STAT5), phosphatidylinositol 3-kinase (PI3K), and phospholipase C- α [Silva et al., 1996; Chong et al., 2002; Um and Lodish, 2006].

Epo and EpoR have been detected in specific areas of the embryonic, fetal, and adult brain of rodents, non-human primates, and humans. In addition, EpoR has been found to be necessary during brain development to maintain cell survival [Genc et al., 2004]. However, its role in differentiated neurons remains to be clarified.

We have used the human SH-SY5Y neuroblastoma cell line as a model of neuronal differentiation since it has been recognized that several agents can induce *in vitro* differentiation characterized by typical morphological and biochemical features [Prince and Orelund, 1997; Lopez-Carballo et al., 2002; Yuste et al., 2002; Pregi et al., 2006].

In this work, we compared cell sensitivity to proapoptotic agents between undifferentiated and all-*trans*-retinoic acid (atRA)-differentiated SH-SY5Y cells and investigated cellular and molecular mechanisms involved in the Epo neuroprotective action upon cells under different stages of differentiation.

MATERIALS AND METHODS

ANTIBODIES AND REAGENTS

All chemicals used were of analytical grade. Dulbecco's Modified Eagle (D-MEM) and Ham F12 culture media were obtained from GibcoBRL. Trizol reagent, goat anti-rabbit (IgG)-Alexa Fluor 488 (Molecular Probes), and specific primers for microtubule-associated protein 2 (MAP-2), B cell lymphoma 2 (Bcl-2), Bcl-xL, EpoR, growth axonal protein 43 (Gap-43), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were from Invitrogen Life Technologies. Anti-Bcl-xL mAb was obtained from Chemicon International, and Protein A-agarose, anti-PARP, and anti-Bcl-2 mAbs were purchased from BD Pharmingen. Staurosporine (STP), atRA, EDTA, Nonidet P40, AG490, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), L-glutamine, *p*-formaldehyde, phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin, pepstatin A, Hoechst

33258 dye, and anti-actin mAb were obtained from Sigma-Aldrich. Ready To Go T-Primed First-Strand Kit, chemiluminiscent system kit (ECL), anti-mouse and anti-rabbit horseradish peroxidase-conjugated Abs, and nitrocellulose membranes (Hybond-ECL) were purchased from Amersham Biosciences; Anti-MAP-2 pAb was obtained from Cell-Signalling; Ly294002 was from Calbiochem; and Sybr Green I nucleic acid stain was from Roche. Anti-NF-L pAb was purchased from Synaptic Systems and anti-EpoR pAb (M-20) was from Santa Cruz Biotechnology. Human recombinant TNF- α and agarose were obtained from Promega; ethidium bromide (EtBr) was from Mallinckrodt; and fetal bovine serum (FBS, PAA Laboratories GmbH), penicillin, and streptomycin were from Gensa. Sodium dodecylsulfate (SDS), acrylamide, bis-acrylamide, Folin-Ciocalteu's reagent, Tween-20, Anaerocult C, glutaraldehyde, and dimethylsulfoxide (DMSO) were purchased from Merck. Recombinant human erythropoietin (Hemax) was from Biosidus.

CELL CULTURES

Human SH-SY5Y neuroblastoma cells (CRL-2266, American Type Culture Collection, ATCC) have been used along this work. Cells, at a density of 4×10^5 cells/ml, were routinely grown in 25-cm² plastic tissue-culture flasks (Falcon; BD), containing 4 ml of 1:1 D-MEM:Ham F12 supplemented with 2 mM L-glutamine, antibiotics (100 U/ml penicillin, 100 μ g/ml streptomycin), and 10% (v/v) heat-inactivated FBS in a humidified incubator at 37°C containing 5% CO₂. The medium was replaced every 2 days and the cells were split before they reached confluence. For neurite outgrowth and fluorescent analysis, cells were cultured on slide covers plated in 35-mm Petri dishes (Cellstar; GBO).

STP, atRA, LY294002, and AG490, dissolved in dimethyl sulfoxide, were added to complete medium and adjusted to final concentrations as referred below in text and figures. The vehicle DMSO was added to controls. Hypoxia was established by using an anaerobic 2.5-L culture jar (BD Biosciences) in the presence of a reagent mixture (Anaerocult C) as oxygen-deficient generating agent (5–7% O₂).

DIFFERENTIATION ANALYSIS

SH-SY5Y cells (2×10^5 cells/ml) were seeded and, after 48 h incubation, they were cultured in the presence of atRA. Cell morphology was observed under phase contrast in an inverted microscope at 400 \times magnification (Axiovert 135; Carl Zeiss) and photographed by using a Nikon Coolpix 5000 digital camera. For neurite outgrowth analysis, multiple independent images were taken. At least 10 neurites per field were selected for neurite outgrowth measurement and means of neurite length were calculated for each assay. This neurite tracing technique was implemented in the form of a plug in (NeuronJ) for ImageJ (National Institutes of Health, Bethesda), the computer-platform-independent public domain image analysis program inspired by NIH-Image [Meijering et al., 2004]. Cell differentiation was determined in independent experiments by counting differentiated cells based on morphological criteria [Lopez-Carballo et al., 2002] and expressed as percentage of total cells (at least 500 cells).

ASSESSMENT OF CELLULAR MORPHOLOGY BY SCANNING ELECTRON MICROSCOPY

Samples were prepared as previously reported [Vittori et al., 1999]. Briefly, cells (2×10^5 cells/ml) were seeded on a glass support (50 mm² area), and after exposure to different treatments, were fixed for 20 min with 3% (v/v) glutaraldehyde in 0.1 M-pH 7.4 phosphate buffer. Samples were washed three times in the same buffer, subsequently dehydrated through successive washes in graded acetone (from 25% to 100%, v/v), and dried directly from acetone in a Balzers CPD 030 Critical Point Bomb using carbon dioxide as a transition fluid. Samples were then coated with a thin layer of gold (Balzers Union SCD 040) and examined by using a scanning electron microscope (Phillips 515).

FLUORESCENCE ANALYSIS

Cells were cultured (2×10^5 cells/ml) on slide covers plated in 35-mm Petri dishes. After obtaining 50% confluence (48 h), cell cultures were subjected to different treatments.

Neuronal marker detection. After culture medium removal, cells were fixed with methyl alcohol during 10 min at -20°C . Samples were then blocked during 1 h with 1% albumin in PBS and then incubated for 1 h at 37°C with the specific primary antibody anti-neurofilament L (1:50 dilution). An Alexa Fluor 488 dyelabeled secondary antibody was used (1 h incubation at 37°C) and nuclei were stained with Hoechst. After mounting, immunofluorescence microscopy observation was carried out on Axiovert 135 (Zeiss) equipment, under UV illumination at 365 nm. A Canon Power Shot G9 equipment and Axiovision Rel 4.6 software were used for imaging acquisition and analysis. During the entire following procedure, dishes were washed with cold PBS for 10 min between the different procedure steps.

Nuclear staining of apoptotic cells. After fixing with 4% (v/v) *p*-formaldehyde in phosphate-saline buffer (PBS) for 20 min at 4°C , the samples were exposed to 0.05 g/L Hoechst 33258 dye in PBS for 30 min at room temperature, washed thrice with PBS, and finally mounted by using mounting buffer (50% glycerol in PBS, v/v).

Fluorescent nuclei with apoptotic characteristics were detected by microscopy under UV illumination at 365 nm. Differential cell counting was performed by analyzing at least 500 cells [Pregi et al., 2006].

DETERMINATION OF CELL VIABILITY

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide] reduction assay was used to assess cell viability. Cells were cultured in 35-mm Petri dishes at a density of 2×10^5 cells/ml. After the cells had been subjected to appropriate treatments and the medium removed, they were incubated for 4 h at 37°C with MTT at a final concentration of 0.5 mg/ml. The supernatant was removed and the pellet washed with 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).

ANALYSIS OF mRNA LEVELS

Transcriptase-polymerase chain reaction (RT-PCR). Total RNA was isolated by means of the Trizol reagent. Its amount and the purity of the extraction were evaluated by measuring absorbance at 260 and 280 nm. Complementary DNA was prepared by reverse transcription using Ready-to-Go T-Primed First Strand Kit, starting from a sample of 2 μg total RNA.

Aliquots of cDNA were amplified by PCR using specific primers. PCR reactions were performed in a Mastercycler Gradient (Eppendorf) at the following conditions: 30 amplification cycles (94°C for 45 s, 60°C for 30 s, and 72°C for 45 s) with an initial incubation at 94°C for 5 min and a final incubation at 72°C for 10 min for Bcl-xL and Bcl-2. For MAP-2, a program of 30 amplification cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s, with an initial incubation at 94°C for 5 min and a final incubation at 72°C for 10 min, was used.

The specific primer sequences were as follows:

Bcl-2 [Benito et al., 1996]:

Forward, 5'-AGATGTCCAGCCAGCTGCACCTGAC-3';

Reverse, 5'-AGATAGGCACCAGGGTGAGCAAGCT-3'.

Bcl-xL [Benito et al., 1996]:

Forward 5'-CGGGCATTTCAGTGACCTGAC-3';

Reverse, 5'-TCAGGAACCAGCGGTTGAAG-3'.

MAP-2 [Kalcheva et al., 1995]:

Forward, 5'-GACGGAGTAACCAAGAGCCCA-3';

Reverse, 5'-GTGTGCGTGAAGAATAACTTGGTG-3'.

GAPDH [McKinney and Robbins, 1992]:

Forward, 5'-TGATGACATCAAGAAGTGGTGAAG-3';

Reverse, 5'-TCCTGGAGCCATGTAGGCCAT-3'.

The resulting PCR fragments were analyzed by electrophoresis on 2% agarose gels (w/v) containing 0.5 mg/L EtBr. Bands observed under an UV transilluminator (Hoefer; Amersham-GE) were digitalized with a Kodak DC240 equipment. Densitometric analysis was performed using the GelPro 4.0 software.

Quantitative real-time PCR. Real-time PCR assays for EpoR were performed in a 25- μl final volume containing 2 μl of cDNA (1:10 dilution), 0.20 mM dNTPs, 0.25 μM specific primers, 3 mM MgCl₂, 0.625 U Taq DNA polymerase, and 1:30,000 dilution of Sybr Green, using a DNA Engine Opticon (MJ Research, Inc.). The program consisted in an initial denaturing step (94°C for 5 min), followed by 40 cycles (each of 94°C for 60 s, 59°C for 60 s, and 72°C for 60 s). Sample quantification was normalized to endogenous GAPDH. Each assay included a DNA minus control and a standard curve performed with serial dilutions of control cDNA coming from SH-SY5Y cells maintained in D-MEM:Ham F12 with 10% FBS. All samples were run in duplicate and the experiments repeated with independently isolated RNA.

The primers used for EpoR analysis were those reported by Arcasoy et al. [2003]:

Forward: 5'-GTGGAGATCCTGGAGGGCCG-3';

Reverse: 5'-ACTCCAGGGAAGCAGGTGG-3'

PROTEIN ANALYSIS

Cellular extract. Cells were washed with ice-cold PBS solution and lysed with hypotonic buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P 40, 0.1% SDS and 0.5% sodium deoxycholate, with protease inhibitors: 1 mM PMSF, 4 μ M leupeptin, 2 μ M pepstatin A, and 1 μ g/ml aprotinin), in a ratio of 100 μ l/10⁷ cells. After 30-min incubation on ice, insoluble material was removed by centrifugation at 15,000g for 15 min and protein concentration was measured in the supernatant [Lowry et al., 1951].

Immunoprecipitation. Cell extracts were incubated with specific antibodies during 1 h at 4°C. Protein A-agarose was added and, after overnight incubation at 4°C, immunoprecipitates were collected by centrifugation at 15,000g during 15 min and washed with the lysis buffer.

Western blotting. Lysates or immunoprecipitates were boiled for 3 min in sample buffer (625 mM Tris-HCl, pH 6.8; 5% 2-mercaptoethanol, 2% SDS, bromophenol blue, glycerol), resolved by SDS-polyacrylamide gel electrophoresis (T=8%) using Tris-glycine pH 8.3 (25 mM Tris; 192 mM glycine; 0.1% SDS) as the running buffer. Then, proteins were electroblotted onto a Hybond nitrocellulose membrane (Amersham Biosciences) using 25 mM Tris, 195 mM glycine, 0.05% SDS, pH 8.3, and 20% (v/v) methanol, as the transfer buffer. Non-specific binding sites on the membrane were blocked with 5% ECL membrane blocking agent in Tris-buffered saline-Tween (TBS-Tween: 25 mM Tris, 137 mM NaCl, 3 mM KCl, pH 7.4, 0.1% Tween 20) for 1 h at room temperature. The blots were briefly rinsed using two changes of TBS-Tween buffer, and washed three times for 10 min each with fresh changes of the washing buffer. Then, the blots were incubated overnight at 4°C with appropriate concentration of each antibody (1:100 anti-Bcl-xL, 1:200 anti-Bcl-2, 1:500 anti-MAP-2, 1:200 anti-EpoR, or 1:200 anti-actin), washed three times for 10 min each with TBS-Tween, and probed with a 1:1,000 dilution of anti-mouse or anti-rabbit horseradish peroxidase-conjugated antibody for 1 h at 20°C. After washing, the blots were incubated with enhanced chemiluminescence substrate (ECL kit) and the bands detected by using Fujifilm Intelligent Dark Box II (Fuji) equipment coupled to a LAS-1000 digital camera. To visualize the bands, the Image Reader LAS-1000 and LProcess V1.Z2 programs were employed.

STATISTICAL ANALYSIS

Results are expressed as mean \pm standard error of the mean (SEM). When corresponding, the non-parametric Mann-Whitney *U*-test or the Kruskal-Wallis one-way analysis of variance test was employed. At least differences with *P* < 0.05 were considered the criterion of statistical significance.

RESULTS

EFFECT OF PROAPOPTOTIC AGENTS UPON UNDIFFERENTIATED AND atRA-DIFFERENTIATED CELLS

The main aim of this study was to investigate a possible neuroprotective role of Epo against injury in cells under different stages of differentiation. In previous works, we demonstrated

the role of Epo in preventing apoptosis induced by STP [Pregi et al., 2006] or TNF- α [Pregi et al., 2009] in undifferentiated SH-SY5Y cells. Different cell responses upon the differentiation agent have been described [Tieu et al., 1999]. Therefore, we decided to induce cell differentiation with atRA, a derivative form of vitamin A with physiological action in the development of the nervous system. The differentiation process was analyzed and characterized in a time and concentration manner. SH-SY5Y cells were exposed to different doses of atRA (from 10 to 30 μ M), varying the incubation periods (1, 4, or 7 days). In this range of concentrations, retinoic acid proved to be an appropriate inducer of neuronal differentiation without changing cell survival, as studied by the Trypan blue and MTT assays (data not shown).

Neuronal SH-SY5Y cells differentiated by atRA displayed morphological features similar to those observed when this cell line was induced by STP [Pregi et al., 2006]. Morphological changes were followed by phase-contrast microscopy, and thereafter, neuronal cell differentiation was characterized by scanning electron microscopy (Fig. 1A), neurite outgrowth (Fig. 1B), and neuron-like cell score (Fig. 1C). Cell differentiation induced by atRA was detected as neuritogenesis combined with subsequent cell body condensation. According to the morphological criteria of Lopez-Carballo et al. [2002], we observed that 10 μ M atRA significantly induced cell differentiation at 4-day incubation (Fig. 1). To further study the expression pattern of differentiation markers, the MAP-2 and Bcl-2 were analyzed by RT-PCR and Western blotting. As shown in Figure 1D, the expression of both markers increased during the differentiation phase induced by 10 μ M atRA. In addition, the expression of the recognized neural cell marker neurofilament L (NF-L) [Paterno et al., 1997; Messi et al., 2008] was detected in SH-SY5Y cells after atRA treatment, thus confirming its mature state (Fig. 1E). We also detected an increment in mRNA levels of another neuronal marker, GAP-43 (data not shown).

In order to analyze SH-SY5Y cell susceptibility to apoptosis at immature and mature stages, cultures in the absence or presence of atRA were exposed to proapoptotic agents, such as TNF- α , STP, or hypoxia environment (H). Cell viability was evaluated by the MTT assay and apoptosis was detected by chromatin condensation (Hoechst staining) and PARP cleavage (Fig. 2). The experimental conditions in undifferentiated cell cultures were established on the basis of our own previous results [Pregi et al., 2006; Pregi et al., 2009] as well as on dose-response and time-response curves. Cultures were either exposed to 100 nM STP or 25 ng/ml TNF- α for 12 h, or incubated under an atmosphere of 5–7% O₂ for 16 h. A significant decrease in cell viability, parallel to an increase in the apoptotic cell number, suggested that undifferentiated SH-SY5Y cells were highly sensitive to the proapoptotic procedures applied. In contrast to undifferentiated SH-SY5Y cells, atRA-induced cultures proved to be more resistant to the same proapoptotic agents, which showed significantly decreased apoptotic effects. Only STP at a 100-nM concentration induced a mild but significant decrease in viability due to cell death by apoptosis (Fig. 2A–C).

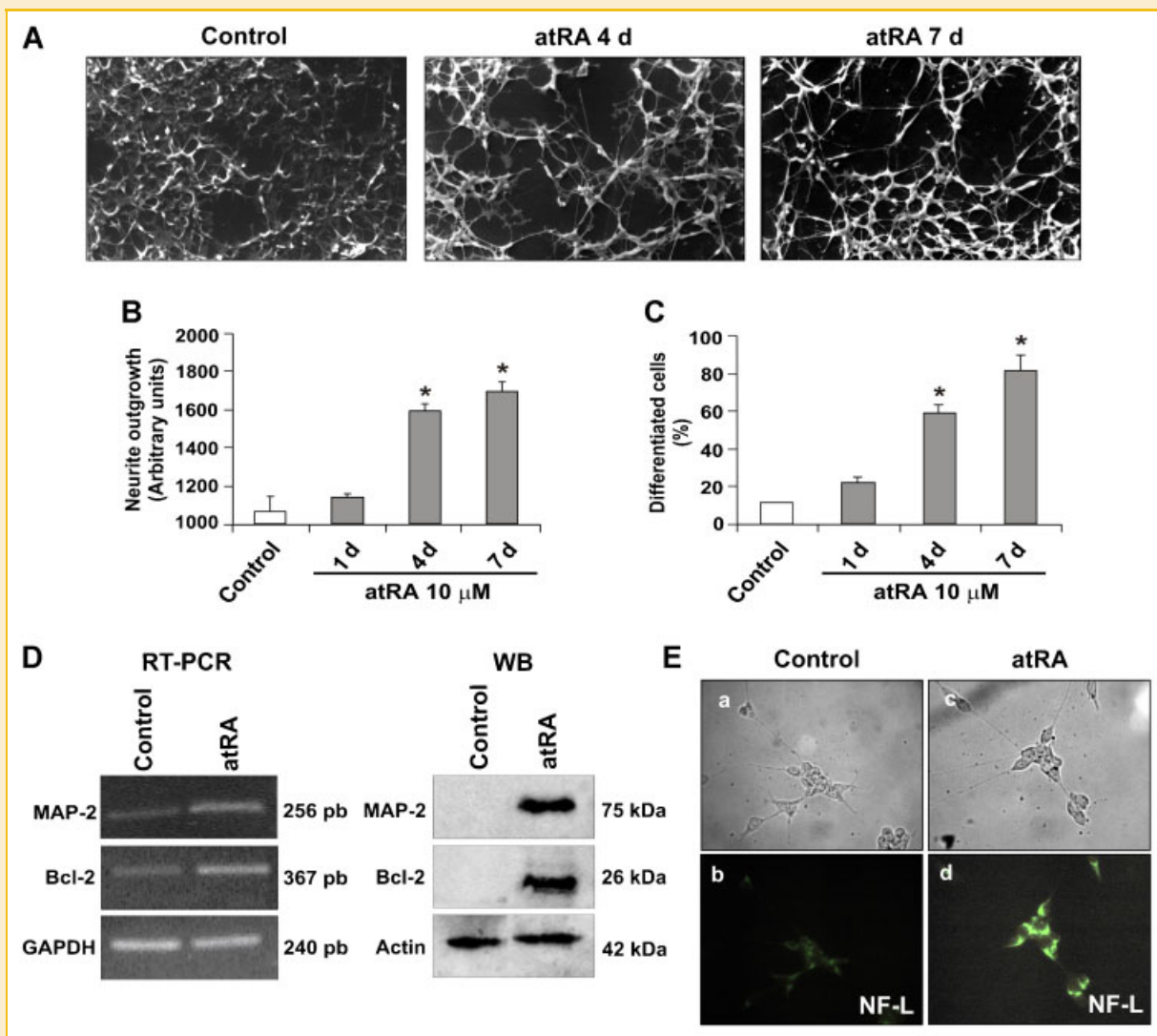


Fig. 1. Effect of atRA upon cell differentiation. SH-SY5Y cells (10^5 cells/ml) were treated with different concentrations of atRA for 1, 4, or 7 day-periods. Morphological changes due to cell differentiation were detected by scanning electron microscopy (A). For semi-quantitative analysis, photographs from independent experiments were analyzed to determine neurite outgrowth (B) and neuron-like cell count (C), as described in the section Materials and Methods. Each bar represents mean \pm SEM. Significant differences of these parameters were observed at 4 as well as at 7 days of cell differentiation with respect to control (undifferentiated cultures) ($*P < 0.001$, $n = 5$). The expression of the differentiation markers MAP-2 and Bcl-2, analyzed by RT-PCR and Western blotting, were found increased after atRA treatment for 4 days (D). Cell differentiation induced by atRA was confirmed by the presence of the neural marker NF-L (E.d). Comparison of the same field observed under visible (E.a and c) and UV lights (E.b and d) are shown. Representative results of three independent experiments with similar results are shown in (D) and (E).

CELL PROTECTION BY ERYTHROPOIETIN

Taking into consideration the different cell survival responses to proapoptotic agents, we then investigated whether a pretreatment with Epo could affect undifferentiated and atRA-differentiated cells in a similar way. Prevention of apoptosis was observed by a 12-h pretreatment with 25 U/ml Epo before the exposure of immature cells to TNF- α , STP or hypoxia (Fig. 3A-C), although Epo did not induce SH-SY5Y cell differentiation. Unlike that expected, no synergistic antiapoptotic effect was detected when atRA-differentiated cells were treated with Epo before being induced to apoptosis (Fig. 3).

ERYTHROPOIETIN AND RETINOIC ACID: CELL SURVIVAL AND ANTIAPOPTOTIC FACTORS

Among the signaling pathways involved in cell survival, PI3K is recognized by its relevance. Pathways mediated by PI3K have been reported to be involved in the activation of erythroid cells as well as in neuronal cell protection by Epo [Masuda et al., 1993; Anagnostou et al., 1994; Maiese et al., 2005]. To evaluate whether PI3K-mediated activation was also involved in the protective effect of atRA, assays were carried out in the presence of Ly294002, an inhibitor of the PI3K signaling pathway. After 60 min in culture medium containing 25 μ M Ly294002, the cells were cultured in the presence of either

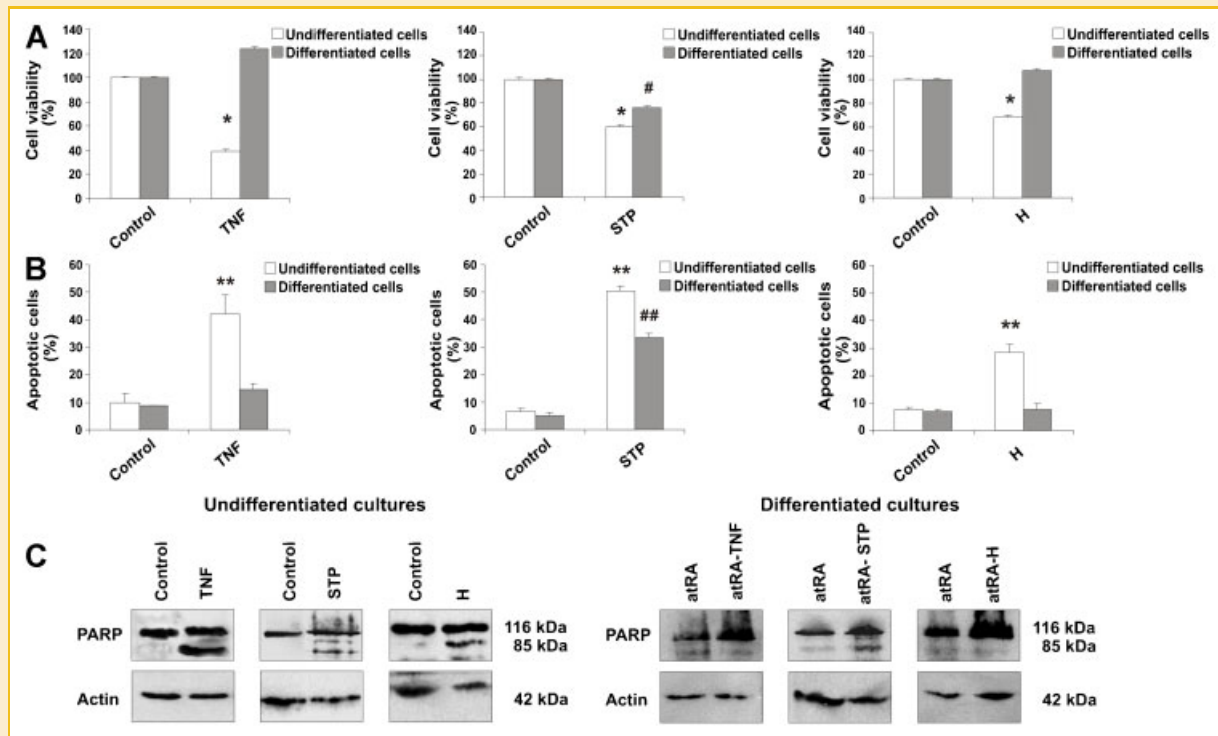


Fig. 2. atRA-differentiated SH-SY5Y cells are more resistant to proapoptotic agents than undifferentiated cells. Undifferentiated SH-SY5Y cells and cells treated with 10 μ M atRA for 4 days were cultured in the presence of 25 ng/ml TNF- α or 100 nM STP for 12 h, or exposed to hypoxia during 16 h (H). Cell viability/proliferation was determined by the MTT assay (A). Apoptotic cells were detected by fluorescence microscopy after Hoechst 33258 staining (B) and by PARP degradation analyzed by Western blotting (C). Each bar represents mean \pm SEM of the indicated n independent assays. A significant decrease in the viability of undifferentiated cells exposed to each proapoptotic agent (* P < 0.05 vs. C, n = 3) was parallel to the increase in the percentage of apoptotic cells (** P < 0.005 vs. C, n = 6). Instead, atRA-induced cells proved to be resistant to TNF- α or hypoxia, since no significant change in cell viability or apoptosis with respect to controls was observed. Only 100 nM STP reduced cell viability (# P < 0.05) and induced apoptosis (## P < 0.01, atRA-STP vs. atRA, n = 6) of differentiated cells, but in a milder form in comparison with its effect upon undifferentiated cells. These results are in accordance with those of PARP degradation (C). The presence of an 85-kDa band indicates PARP cleavage. Actin was used as the control of protein loading. The immunoblots shown are representative of 3 experiments with similar results.

25 U/ml Epo for 12 h or 10 μ M atRA for 24 h, and then exposed to STP for additional 12 h (Fig. 4).

The inhibition of PI3K prevented the protective effect of both factors—Epo (Fig. 4A) and atRA (Fig. 4B)—against the programmed cell death induced by STP. The magnitude of apoptosis induced by STP in atRA-differentiated cultures run in the presence of the PI3K inhibitor resembled that observed in undifferentiated cells. These results suggest an important role for PI3K-mediated signaling pathways in cell survival supported by atRA-induced cell differentiation.

Since some members of the protein family Bcl-2 afford resistance to cells facing apoptotic stimuli, we decided to investigate whether Epo and/or atRA protective effects are associated with the modulation of the expression of antiapoptotic factors of this family. The modulation of Bcl-xL and Bcl-2 was analyzed at the mRNA and protein levels. In accordance with previous reports [Lasorella et al., 1995], we found that cell differentiation by atRA increased the expression of Bcl-2 (Fig. 5A,B) whereas cell activation by Epo was associated with Bcl-2 and Bcl-xL upregulation (Fig. 5C,D). Interestingly, we also found that Epo was unable to modulate the expression of these antiapoptotic factors when the cells had been previously differentiated by atRA (Fig. 6A,B).

CELL DIFFERENTIATION BY RETINOIC ACID AND DOWNREGULATION OF MECHANISMS INVOLVED IN THE PROTECTIVE EFFECT OF ERYTHROPOIETIN

In order to explain the differential response to Epo observed between immature and mature cultures, we investigated whether atRA interferes with mechanisms involved in cell activation by Epo.

The EpoR does not contain intrinsic tyrosine kinase activity. Instead, the signal is mediated by Jak2. To explore the contribution of this signaling pathway in differentiated SH-SY5Y cells exposed to Epo, assays were carried out in the presence of AG490, an inhibitor of Jak2. Cells differentiated by 10 μ M atRA for 4 days were then subjected to sequential incubation with 25 μ M AG490 for 2 h, with 25 U/ml Epo for 12 h, and finally exposed to 100 nM STP for additional 12 h. Undifferentiated cells received similar sequential treatment. As shown in Figure 7A, Jak2 was required for the Epo antiapoptotic effect upon undifferentiated cells. Since Epo was unable to increase the protective effect of atRA (Fig. 3), it was not surprising that the inhibition of Jak2 had no effect on differentiated cells. This lack of Epo neuroprotective effect upon differentiated cells was in accordance with the impairment of the modulation of Bcl-xL and Bcl-2, both genes related to the Epo protective effect (Fig. 6).

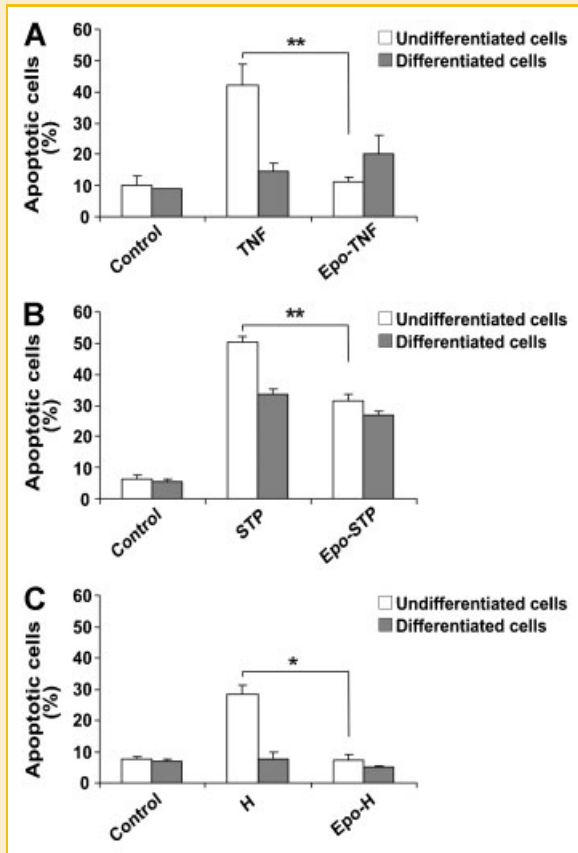


Fig. 3. Differential protective effect of Epo at different stages of cell differentiation. Undifferentiated and atRA-treated (10 μ M, 4 days) SH-SY5Y cells were incubated with 25 U/ml Epo for 12 h and then exposed to 25 ng/ml TNF- α or 100 nM STP during 12 h, or cultured in an oxygen-deficient atmosphere during 16 h (H). Fluorescent apoptotic nuclei after Hoechst staining were differentially counted to evaluate apoptosis. The antiapoptotic effect of Epo was clearly observed in undifferentiated cell cultures ($^*P < 0.05$ Epo-H vs. H, $n = 4$; $^{**}P < 0.01$ Epo-TNF vs. TNF, $n = 3$; and Epo-STP vs. STP, $n = 7$). However, no additional protective effect to that induced by atRA alone could be attributed to the Epo treatment of differentiated cells.

The results obtained up to here allow us to suggest an impediment of Epo to activate atRA-differentiated cells. In order to explain this reduced action of Epo, we hypothesized that atRA could affect normal modulation of the EpoR expression. In fact, 4-day treatment of SH-SY5Y cells with atRA significantly downmodulated the EpoR expression at both mRNA and protein levels, analyzed by real-time PCR and Western blotting, respectively (Fig. 7C).

DISCUSSION

Cell differentiation is a complex process regulated by the interplay among cellular programs and cell-substrate interactions that involve signaling pathways and diverse regulatory factors.

Despite their tumoral origin, neuroblastoma cell lines can be induced to differentiate in vitro by several agents. In this work, we used the SH-SY5Y neuroblastoma cell line, which showed

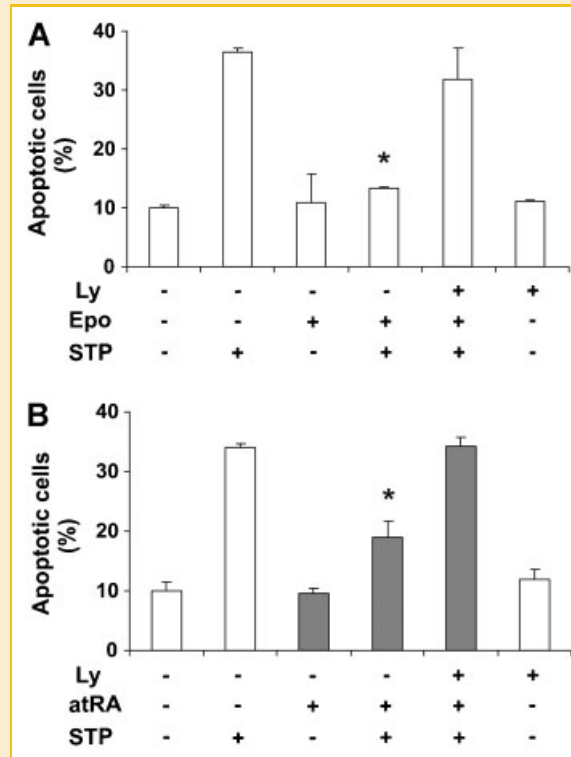


Fig. 4. Cell protective effects are mediated by PI3K-signaling pathways. SH-SY5Y cells were treated with 25 U/ml Epo for 12 h (A) or atRA for 24 h (B). In the indicated sets, cells received 1 h pretreatment with 25 μ M Ly294002 (PI3K inhibitor) prior to the addition of either Epo or atRA. Some cultures were then exposed to 100 nM STP for additional 12 h. Cell apoptosis, evaluated at the end of the whole treatment period by fluorescence microscopy (Hoechst staining), showed significant changes in nuclear morphology. Cell protection by Epo was observed in undifferentiated cells (A) ($^*P < 0.01$ Epo-STP vs. STP, $n = 6$) whereas the protective effect of atRA was observed after cell differentiation (B) ($^*P < 0.01$ atRA-STP vs. STP, $n = 6$). These effects were abrogated by pretreatment with Ly (Ly-Epo-STP or Ly-atRA-STP vs. STP, NS). Each bar represents mean \pm SEM of the indicated n independent assays.

homogeneous populations of differentiated cells due to STP [Yuste et al., 2002; Pregi et al., 2006] or retinoic acid. Retinoic acids are natural and synthetic derivatives of vitamin A that, together with their nuclear control genetic programs, are essential for embryonic development, organ homeostasis, cell growth, differentiation, and apoptosis [Lopez-Carballo et al., 2002].

We observed that cell treatment with 10 μ M atRA for 4 days induced neuronal morphological changes (Fig. 1) similar to those described by other authors [Ronca et al., 1999; Encinas et al., 2000; Lopez-Carballo et al., 2002]. To confirm that the morphological changes observed actually resulted from the neuronal differentiation of SH-SY5Y cells, the expression of several neuronal markers was explored. We observed that the typical morphological feature of differentiation was accompanied by biochemical changes. In agreement with previous reports, the treatment with atRA induced upregulation of cell expression of MAP-2 and Bcl-2 (Fig. 1), the latter of which has been described as an important factor involved in regulation of neural differentiation [Zhang et al., 1996]. In agreement with other reports, the expression of NF-L confirmed

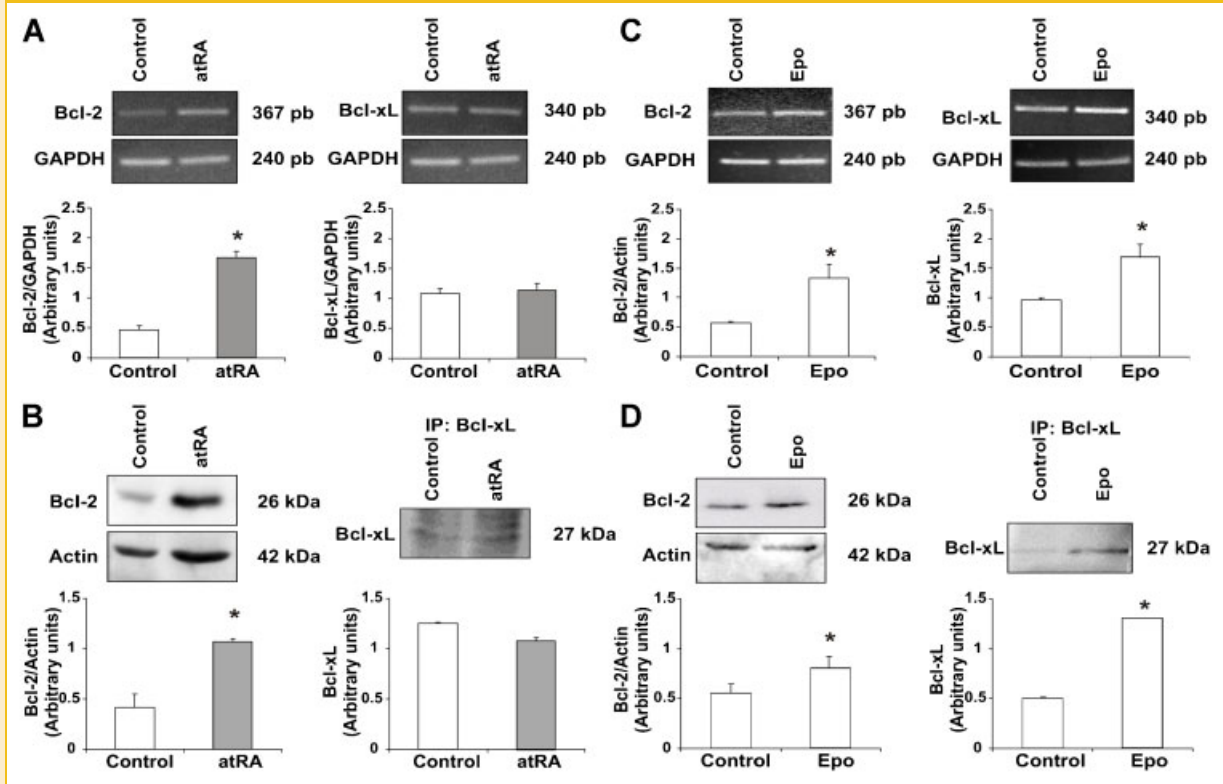


Fig. 5. Expression of antiapoptotic factors in undifferentiated and differentiated cultures SH-SY5Y cells were either differentiated by atRA (10 μ M, 4 days) (A, B) or treated with Epo (25 U/ml, 12 h) (C, D) in order to investigate changes in the expression of factors of the Bcl-2 family at mRNA (RT-PCR) and protein (Western blotting) levels. For immunoblotting, Bcl-xL was immunoprecipitated and Bcl-2 was analyzed in total cell extracts. Cell differentiation induced by atRA was associated with a significantly ($^*P < 0.05$, $n = 5$) increased Bcl-2 expression (A, B), whereas Epo induced not only Bcl-2 but also Bcl-xL upregulation ($^*P < 0.05$, $n = 5$) (C, D).

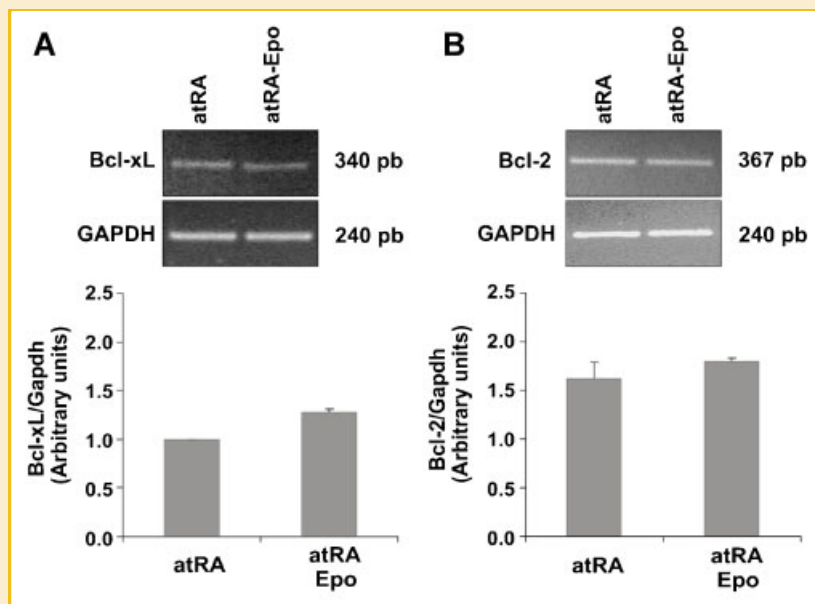


Fig. 6. Impairment of antiapoptotic factor modulation by Epo in differentiated cells. The expression of antiapoptotic factors of the Bcl-2 family at mRNA level (RT-PCR) was analyzed in atRA-differentiated (10 μ M, 4 days) cells treated with Epo (25 U/ml) for additional 12 h. Under this cell condition, no modulation of Bcl-xL (A) or Bcl-2 (B) could be detected due to the Epo treatment. Bars represents mean \pm SEM ($n = 3$).

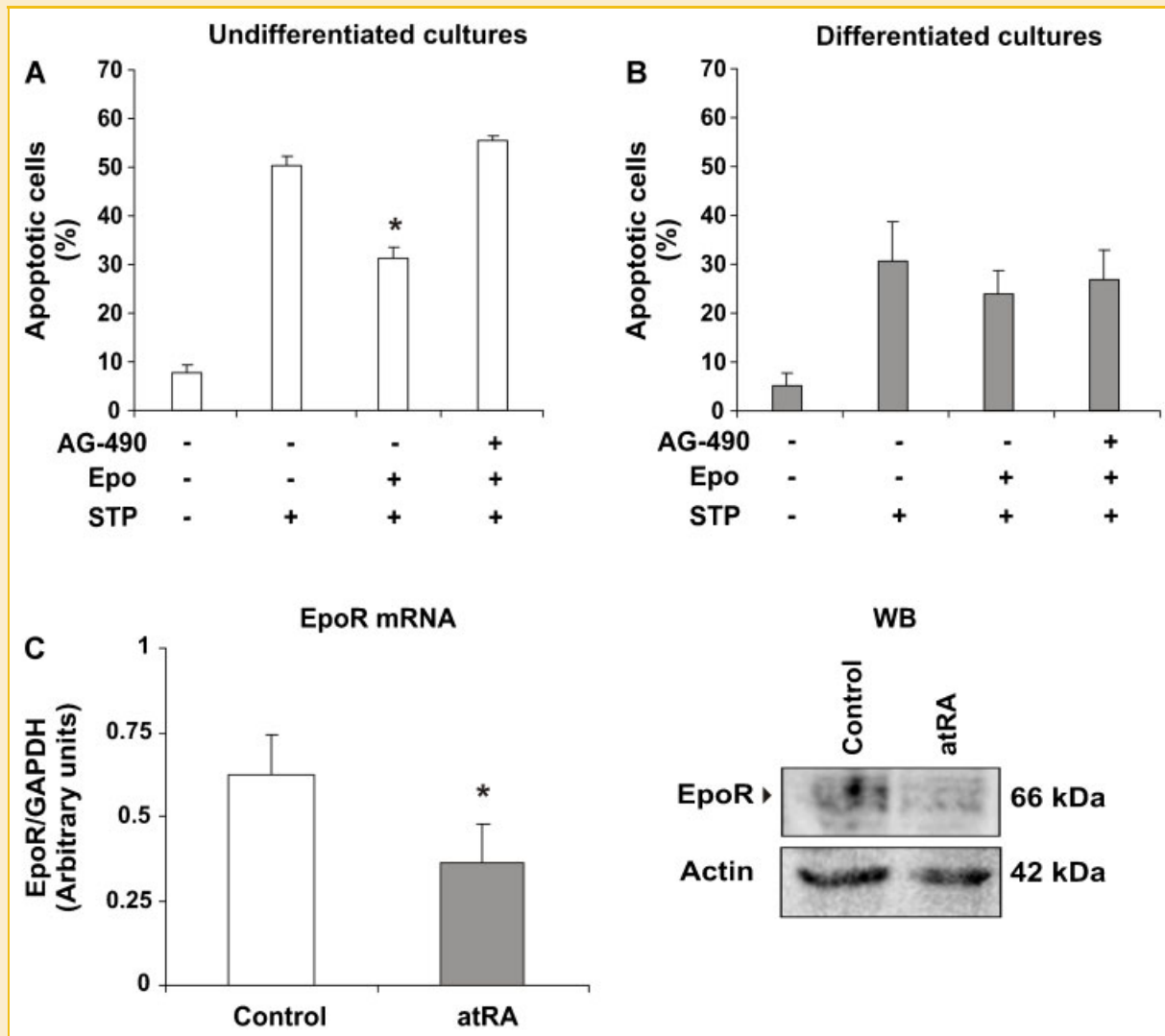


Fig. 7. Downregulation of mechanisms involved in the Epo protective effect. Undifferentiated (A) and atRA-differentiated (B) SH-SY5Y cells were used to investigate the contribution of the Jak2-signaling pathway to cell activation by Epo. Cultures were subjected to sequential treatment with 25 μ M AG490 for 2 h, with 25 U/ml Epo for 12 h, and then exposed to 100 nM STP during additional 12 h, as indicated in the figure. At the end of the whole period, apoptotic nuclei were counted by fluorescent microscopy. Inhibition of the Jak2 pathway by AG490 blocked the antiapoptotic effect of Epo upon undifferentiated cells (* $P < 0.05$ Epo-STP vs. STP; AG-Epo-STP vs. STP, NS, $n = 5$). Instead, no significant change in the action of Epo was detected after inhibition of the Jak2-mediated signaling pathway in cells that had been previously differentiated by atRA. SH-SY5Y cells were subjected to 10 μ M atRA differentiation during 4 days, and EpoR at mRNA and protein levels were analyzed by real-time PCR and Western blotting, respectively (C). Results of EpoR mRNA, expressed in arbitrary units normalized to GAPDH, show significant downregulation induced by the atRA treatment (* $P < 0.05$, $n = 3$). In accordance, EpoR protein was also decreased after 4 days of cell differentiation by atRA. Actin was used as the control of protein loading. The immunoblot shown is representative of three independent assays.

neuronal maturation induced by retinoic acid [Paterno et al., 1997; Messi et al., 2008].

STP is a potent and non-specific inhibitor of protein kinases, which has a key role in cellular differentiation. However, together with the STP capacity to trigger neuronal maturation, it has been recognized as one of the best inducer of apoptosis in many neuronal cell types [Yuste et al., 2002; Pregi et al., 2006]. Using undifferentiated SH-SY5Y cells, we have previously demonstrated proapoptotic effects of STP and the cytokine TNF- α that exerts pleiotropic functions related to immunity and inflammation [Pregi et al., 2006; Pregi et al., 2009]. The treatment with 25 ng/ml TNF- α

resulted in apoptosis as indicated by decreased cell viability, development of typical nuclear morphology, and increased activity of caspase 3 and 8 [Pregi et al., 2009]. In order to compare cell sensitivity to apoptosis at two stages of cellular differentiation we initially confirmed programmed cell death induced by STP and TNF- α (Fig. 2). Besides, undifferentiated SH-SY5Y cells exposed to hypoxia for a short period (16 h) also suffered apoptosis (Fig. 2). In contrast to these results, the atRA-differentiated cells proved to be significantly protected from the proapoptotic stimuli applied. Published data indicate that atRA and many of its natural and synthetic derivatives have been extensively investigated as potential

therapeutics for human cancer because of their effects of inhibition of cell proliferation, induction of cell differentiation, and promotion of apoptosis in many types of cancer cell lines [Nagy et al., 1995; Niizuma et al., 2006]. On the other hand, differentiation of neuroblastoma cells induced resistance to drug therapy [Lasorella et al., 1995] and to apoptosis triggered by chemotherapeutic agents [Ronca et al., 1999; Lombet et al., 2001]. In our experiments, atRA-treatment did not induce apoptosis but effectively conferred resistance to the apoptosis induced by STP, TNF- α , or hypoxia upon SH-SY5Y cells (Fig. 2).

The Bcl-2 family, via its regulation of both caspase-dependent and caspase-independent cell death pathways, seems to be critical to control cell survival [Akhtar et al., 2004]. This can explain why overexpression of Bcl-2 appears to be one of the mechanisms to give neuroblastoma cell resistance against atRA-induced apoptosis [Okazawa et al., 1996; Niizuma et al., 2006]. The inhibition of apoptosis due to atRA cell differentiation observed in the present work seemed to be closely related to high levels of Bcl-2 but not of Bcl-xL protein (Fig. 5). This enhanced Bcl-2 expression after SH-SY5Y cell differentiation could represent a developmental phenomenon functioning physiologically to maintain neuronal survival.

Epo, initially identified as a hematopoietic cytokine acting as a survival and erythroid differentiated factor, is now recognized as a growth factor with pleiotropic function. In brain tissue, there is evidence that Epo is involved in neurodevelopment and neuroprotection [Buemi et al., 2002]. Here we show that the apoptosis induced by STP, TNF- α , or hypoxia in undifferentiated cultures could be prevented by a previous treatment with 25 U/ml Epo for 12 h whereas no significant synergism was detected between atRA and Epo when both protective agents were assayed together (Fig. 3). In analogy with the well-known antiapoptotic action that Epo exerts on erythroid progenitor cells, present results add more evidence that Epo might repress neuronal apoptosis by upregulating the expression of the antiapoptotic factors Bcl-2 and Bcl-xL (Fig. 5). These results are in agreement with our previous reports in which we demonstrated the involvement of the antiapoptotic factors Bcl-xL and Bcl-2 in cell protection by Epo against STP- and TNF- α -induced programmed cell death [Pregi et al., 2006, 2009].

Taking into consideration the protective effects of Epo and atRA upon undifferentiated SH-SY5Y cell cultures, we further compared the mechanisms involved in these processes. Many reports have demonstrated that the Epo antiapoptotic action upon both erythroid and non-erythroid cells is mediated by activation of PI3K-signaling pathways. In this work, no differences between the antiapoptotic effects of Epo and atRA were observed in cell cultures run in the presence of the specific PI3K inhibitor Ly294002 because both factors repressed the apoptosis induced by STP in a PI3K dependent manner (Fig. 4). In accordance with the identification of PI3K/Akt as a signaling pathway involved in regulating differentiation in many cell types, other authors have demonstrated impairment in retinoic acid-induced differentiation by inhibition of this pathway with Ly294002 [Lopez-Carballo et al., 2002]. Moreover, our results indicate that activation of the PI3K-signaling pathway by retinoic acid is also required for the atRA neuroprotective effect.

We demonstrated that Epo is unable to increase the antiapoptotic effect of atRA. One possible explanation is that atRA differentiation

prevented a subsequent antiapoptotic action of Epo. Consistent with this hypothesis, Epo was incapable of inducing the upregulation of Bcl-xL and Bcl-2, target genes required for the protective action of Epo, in cells that had been previously subjected to atRA differentiation (Fig. 6). The Jak2-signaling pathway is also known to be involved in cell activation by Epo. However, when grown in the presence of Epo, differentiated SH-SY5Y cells showed no differences in the levels of apoptosis between cultures run in the presence or absence of the inhibitor AG490 (Fig. 7). This lack of Epo effect in comparison with the Epo behavior in undifferentiated cultures also suggests inactivation of the Epo pathway in atRA-differentiated neuronal cells (Fig. 7). This finding agrees with the results observed in interferon- γ -treated astrocytes, suggesting an anti-inflammatory function of atRA by a suppression of activation of Jak/STAT pathways [Choi et al., 2005].

We then tried to determine whether the resistance of atRA-differentiated cells to Epo was specifically associated with a cross-talk between signaling pathways activated by both factors atRA and Epo. Taking into account the pleiotropic role of EpoR and its requirement for the antiapoptotic effect of Epo during the development of neuronal tissues, we decided to investigate whether atRA is capable of regulating the expression of the EpoR. Interestingly, the EpoR expression was found significantly down-modulated in cells subjected to atRA differentiation (Fig. 7). This finding suggests that the inhibition of the action of Epo coupled to atRA-induced neuronal differentiation may be at least in part mediated by the blockade of EpoR expression. This is also in agreement with the study by Labbaye et al. [1995], which showed that retinoic acid-induced peripheral blood progenitor cells suffer a shift from the erythroid to the granulocytic differentiation pathway, coupled with a sharp inhibition of EpoR expression. However, it may be that in addition to the direct effects of atRA on Epo receptor, signal transduction pathways may also play important roles in mediating the effects induced by atRA-cell differentiation.

In conclusion, our results compare the protective action of Epo between immature and mature cultures of neuroblastoma cells. Besides the increase in cell survival against apoptosis induced by STP, TNF- α , or hypoxia, they also provide evidence that atRA differentiation causes cell resistance to the Epo action. Together, the results describing modulation of the signaling pathways activated by Epo and atRA suggest that differentiated cell resistance to Epo protection can be coupled to an altered response at the EpoR level.

Since brain inflammation is a risk factor of neurodegenerative diseases, the interaction between signaling pathways induced in atRA-differentiated cells and those mediated by Epo should be taken into consideration when new therapeutic strategies are designed.

ACKNOWLEDGMENTS

Dr. A. Nesse and Dr. D. Vittori are research scientists at the National Council of Scientific and Technical Research (CONICET, Argentina). Lic. S. Wenker, D. Vota, and M. Callero are recipients of fellowships from CONICET, and Lic. M.E. Chamorro is a fellow of the National Agency for the Promotion of Science and Technology (Argentina).

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