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Research Article

c-FLIP is involved in erythropoietin-mediated protection of erythroid-differentiated cells from TNF-α-induced apoptosis

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Abstract

The TNF-α (tumour necrosis factor) affects a wide range of biological activities, such as cell proliferation and apoptosis. Cell life or death responses to this cytokine might depend on cell conditions. This study focused on the modulation of factors that would affect the sensitivity of erythroid-differentiated cells to TNF-α. Hemin-differentiated K562 cells showed higher sensitivity to TNF-induced apoptosis than undifferentiated cells. At the same time, hemin-induced erythroid differentiation reduced c-FLIP (cellular FLICE-inhibitory protein) expression. However, this negative effect was prevented by prior treatment with Epo (erythropoietin), which allowed the cell line to maintain c-FLIP levels. On the other hand, erythroid-differentiated UT-7 cells – dependent on Epo for survival – showed resistance to TNF-α pro-apoptotic action. Only after the inhibition of PI3K (phosphatidylinositol-3 kinase)-mediated pathways, which was accompanied by negative c-FLIP modulation and increased erythroid differentiation, were UT-7 cells sensitive to TNF-α-triggered apoptosis. In summary, erythroid differentiation might deregulate the balance between growth promotion and death signals induced by TNF-α, depending on cell type and environmental conditions. The role of c-FLIP seemed to be critical in the protection of erythroid-differentiated cells from apoptosis or in the determination of their sensitivity to TNF-mediated programmed cell death. Epo, which for the first time was found to be involved in the prevention of c-FLIP down-regulation, proved to have an anti-apoptotic effect against the pro-inflammatory factor. The identification of signals related to cell life/death switching would have significant implications in the control of proliferative diseases and would contribute to the understanding of mechanisms underlying the anaemia associated with inflammatory processes.

Keywords: cellular FLICE-inhibitory protein (c-FLIP); erythroid differentiation; erythropoietin; K562 cells; tumour necrosis factor-α (TNF-α); UT-7 cell

1. Introduction

Pro-inflammatory cytokines, such as IL-1 (interleukin-1) or TNF-α (tumour necrosis factor-alpha), are among the factors that contribute to the pathogenesis of anaemia in chronic diseases by impairing proliferation and/or differentiation of bone marrow erythroid progenitors (Means, 2003). Several *in vitro* assays have suggested the existence of mechanisms of apoptosis induced by pro-inflammatory cytokines (Roodman et al., 1987; Rusten and Jacobsen, 1995; Jacobs-Helber et al., 2003). Growing evidence indicates that programmed cell death plays a key role in the control of physiological erythropoiesis. However, under pathological conditions, the apoptotic extrinsic pathway may be further induced upon the activation of death receptors – such as those belonging to the TNF superfamily – which transduce apoptotic signals after binding to their natural ligands (Testa, 2004). Modulation of death receptor-mediated apoptosis was observed at the receptor level or further downstream interfering with the apoptotic signalling cascade through the action of anti-apoptotic members of the Bcl-2 family or caspase inhibitors. In this regard, c-FLIP (cellular FLICE-inhibitory protein), which is a catalytically inactive caspase 8 homologue, functions as a caspase 8-blocking apoptosis (Peter, 2004).

The key inflammatory cytokine TNF-α seems to modulate signal transduction cascades, inducing gene expression towards cell life or death depending on cell type and/or environmental conditions. These events may explain the opposite effects of this pleiotropic cytokine in different cells. TNF-α has been reported to suppress erythropoiesis, exerting a direct effect upon erythroid progenitors CFU-E and BFU-E (Rusten and Jacobsen, 1995) or inhibiting the generation of glycophorin A-positive cells derived from $CD34^+$ cells (Xiao et al., 2002). Conversely, TNF-α alone, or acting synergistically with other cytokines, stimulated the growth of several human leukaemia and lymphoma cell lines (Liu et al., 2000).

In animal models, the growth factor Epo (erythropoietin) has been shown to protect several tissues. This glycoprotein, necessary for the production of mature erythrocytes, is now considered not only an anti-apoptotic but also an anti-inflammatory factor (Feng, 2006). It is well known that the erythropoietic action of Epo is mediated by the EpoR (Epo receptor), involving the activation of Jak2 (Janus tyrosine kinase 2), STAT5 (signal transduction and activator of transcription 5) and PI3K (phosphatidylinositol-3 kinase) signalling pathways.

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Abbreviations: Ac-DEVD-pNA, acetyl-aspartic acid-glutamine-valine-aspartic acid *p*-nitroaniline; c-FLIP, cellular FLICE-inhibitory protein; DAF, 2,7-diaminofluorene; DMSO, dimethylsulfoxide; Epo, erythropoietin; EpoR, Epo receptor; EtBr, ethidium bromide; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IMDM, Iscove's modified Dulbecco's medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor κB; NI, non-induced; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; PI3K, phosphatidylinositol-3 kinase; TRAIL, TNF-related apoptosis-inducing ligand; TNF-α, tumour necrosis factor-alpha.

Based on the hypothesis that variable effects of TNF-α might be attributed to differential activation of signalling mechanisms related to cell life/death switching, the current study focused on whether erythroid differentiation or signalling pathway inhibition may cause differential cell activation, making cells more or less sensitive to TNF-α. It also analysed the effect of Epo in cell treatment before TNF-α exposure and the possible mechanisms of Epo action. Pluripotent transformed K562 and UT-7 cell lines were chosen as models of erythroid differentiation based on their ability to synthesize haemoglobin in response to a variety of agents and their differential dependence on the growth factor Epo.

2. Materials and methods

2.1. Reagents and antibodies

All chemicals used were of analytical grade. RPMI-1640 medium, BSA, DAF (2,7-diaminofluorene), Hoechst 33258 dye, hemin, Ac-DEVD-pNA (acetyl-aspartic acid-glutamine-valine-aspartic acid *p*-nitroaniline), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide], PMSF, monoclonal anti-actin (Clone AC-40) and polyclonal anti-cFLIP (F6550) antibodies were obtained from Sigma–Aldrich, IMDM (Iscove's modified Dulbecco's medium), Trizol reagent, and specific primers for c-FLIP$_L$, Bcl-x$_L$ and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were from Invitrogen Life Technologies. Monoclonal anti-TNF-α and anti-PARP [poly(ADP-ribose)] human antibodies, recombinant human TNF-α, Annexin-FITC apoptosis detection Kit II and Cytofix/Cytoperm Kit were purchased from BD Biosciences. Ready To Go T-Primed First-Strand Kit, chemiluminiscent system kit (ECL), NC (nitrocellulose) membranes (Hybond-ECL) and anti-mouse horseradish peroxidase-conjugated antibody were obtained from Amersham Biosciences and Quantitect SYBR Green PCR from Qiagen. Anti-rabbit IgG rhodamine conjugate antibody and Ly294002 were obtained from Calbiochem; EtBr (ethidium bromide) was from Mallinckrodt. FBS (fetal bovine serum) and penicillin-streptomycin (PAA Laboratories GmbH) were purchased from GENSA and human recombinant erythropoietin (rHuEpo, Hemax) was from Biosidus.

2.2. Cell lines and cultures

Human erythroleukaemia K562 cells were purchased from American Type Culture Collection. Cultures were developed in RPMI-1640 medium, supplemented with 10% heat-inactivated FBS and 100 units/ml penicillin/100 μg/ml streptomycin (Pérez et al., 1999).

Human UT-7 cells were kindly provided by Dr Patrick Mayeux (Cochin Hospital, Paris, France). This cell line shows complete growth dependence on Epo (Komatsu et al., 1991). Cell cultures were maintained in IMDM supplemented with 10% heat-inactivated FBS, antibiotics and 1 unit/ml rHuEpo (Vittori et al., 2005).

Media were routinely replaced every 3–4 days and cultures developed at 37°C in a humidified atmosphere containing 5% CO_2.

2.3. Cell viability

Cell viability was estimated by the MTT reduction assay. Cells (1×10^5/ml) were seeded in a 96-microwell flat-bottom plate. MTT solution in PBS was added at 0.5 mg/ml final concentration. After 2 h at 37°C and centrifugation for 10 min at 9500 ***g***, the pellets were washed with PBS. The precipitated formazan (reduced MTT) was dissolved in 0.04 M HCl in propan-1-ol and the absorbance measured at 570 nm test and 655 nm reference wavelengths in a microplate reader (Model 680, BioRad). All assays were run in triplicate.

2.4. Cell differentiation

Fresh 2.5 mM hemin stock solution was prepared in 0.2 M Na_2CO_3. The suspension, protected from direct light, was stirred for 2 h at room temperature and the pH adjusted to 7.4 with 1 M HCl (Pérez et al., 1999).

K562 and UT-7 cells (2×10^5/ml) were induced to erythroid differentiation with 30 μM hemin. After centrifugation, cell pellets were washed with 0.2 M Tris/HCl buffer, pH 7, to avoid interference of residual hemin. Haemoglobin-producing cells were determined by DAF–hydrogen peroxide reaction. From a stock solution of 10 mg/ml DAF in 90% acetic acid, a working solution was freshly prepared by mixing 10 μl of DAF stock, 10 μl of 9% hydrogen peroxide and 100 μl of 0.2 M Tris/HCl buffer. Cell suspensions were diluted 1:2 with this staining solution, and after 10 min, cell differentiation was estimated by counting at least 600 cells under an optical microscope ($\times$400 magnification). Haemoglobinized cells were expressed as a percentage of total cells.

2.5. Fluorescent nuclear staining of apoptotic cells

Apoptotic cells were detected by fluorescence microscopy after being stained with the DNA-binding dye Hoechst 33258. Cells cultured on slide covers were treated with Carnoy solution (methanol/acetic acid, 3:1 v/v) for 2 min, and after complete removal of medium, they were fixed with 2 ml of Carnoy solution for 5 min (step repeated twice) and dried at 20°C. Cell nuclei were stained by incubation for 15 min with 1 μg/ml Hoechst 33258 solution in PBS. After washing thrice with distilled water and mounting by using PBS, differential count of apoptotic cells was performed by analysing at least 700 cells by fluorescent microscopy (Axiovert 135, Zeiss) (Vittori et al., 2005).

2.6. Colorimetric assay for caspase 3 activity

Cells were washed with PBS and treated with lysis buffer (50 mM Tris/HCl pH 7.4, 1 mM EDTA, 10 mM EGTA, 10 μM digitonin and 100 μM PMSF) for 30 min on ice. Cell debris was removed by centrifugation at 10000 ***g*** for 15 min at 8°C. A volume of clear lysate corresponding to 100 μg of protein (Lowry, 1951), was added to the reaction buffer (100 mM Hepes pH 7.5, 0.5 mM EDTA, 5 mM dithiotreitol and 20% glycerol v/v) and incubated in the dark at 37°C with 157 μM final concentration Ac-DEVD-pNA, the chromogenic substrate of caspase 3-like proteases. The amount of released pNA (*p*-nitroanilide) was measured at 415 nm

in an automatic microplate reader, at each hour during a 7-h incubation period. Based on these time-course curves of caspase activity, data at 6-h incubation were selected for comparison among treatments.

2.7. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated by means of the Trizol reagent and its concentration estimated by measuring the optical density at 260 nm (Sambrook and Russell, 2001). RNA (2.5 μg) was reverse transcribed by using the Ready To Go T-Primed First-Strand Kit. Aliquots of cDNA were amplified by PCR using specific primers for Bcl-x_L (Benito et al., 1996), c-$FLIP_L$ (Rippo et al., 2004) and the internal standard GAPDH (McKinney and Robbins, 1992). PCR reactions were performed in a Mastercycler Gradient (Eppendorf) at the following conditions: 30 PCR amplification cycles (each of 94°C for 45 s, 62°C for 30 s, 72°C for 45 s) and a final incubation at 72°C for 10 min. PCR products were analysed by electrophoresis on 1.5% (w/v) agarose gels containing EtBr, visualized under UV light, and photographed with a Kodak DC240 camera. For data analysis, ArrayGauge and ImageGauge softwares were used (Vittori et al., 2005).

2.8. Quantitative real-time PCR analysis

Real-time PCR assays for c-$FLIP_L$ and GAPDH were performed using a 25 μl final volume containing 2 μl of cDNA (1:10 dilution), 0.20 mM dNTPs, 0.25 μM specific primers, 3 mM $MgCl_2$, 2 units of Taq DNA polymerase and 1:30000 diluted SYBR Green stain. Real-time PCR reactions, performed with DNA Engine Opticon equipment (MJ Research Inc.), consisted of an initial denaturing step (94°C for 5 min) followed by 40 cycles (each of 94°C for 45 s, 61°C for 45 s and 72°C for 45 s) and a final elongation at 72°C for 5 min. 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 (untreated cells). Specific amplifications were confirmed by measuring the dissociation curves for the amplified products. All samples were run in triplicate and the experiments repeated with independently isolated RNA.

2.9. Western blotting

Cells were washed with ice-cold PBS solution and lysed in ice-cold hypotonic buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100 and 1 mM PMSF) during 30 min incubation on ice. Lysates were clarified by centrifugation at 15000 ***g*** for 15 min at 4°C. Aliquots of cell extracts were boiled in the sample buffer, subjected to SDS/PAGE (T=8%) using Tris-glycine pH 8.3 [25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS] and transferred electrophoretically onto nitrocellulose membranes during 1.5 h [transfer buffer: 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-buffered saline (25 mM Tris, 137 mM NaCl, 3 mM KCl, pH 7.4), containing 0.1% Tween 20 and 0.5% skimmed milk powder. Blots were then incubated at 4°C overnight with specific monoclonal antibodies, washed three times for 10 min each with TBS-Tween, and probed with a 1:1000 dilution of anti-mouse horseradish peroxidase-conjugated antibody for 1 h at 20°C. Specific signals were visualized using the enhanced chemiluminiscent system ECL kit with Fujifilm Intelligent Dark Box II equipment (Fuji) coupled to a LAS-1000 digital camera. The Image Reader LAS-1000 and LProcess V1.Z2 programs were employed for data analysis.

2.10. Flow cytometry analysis

2.10.1. Apoptosis

Annexin staining in conjunction with the vital dye PI (propidium iodide) allows the detection of different stages of cell death. The assay was performed according to the instructions of the commercial kit. Briefly, cells were washed twice with cold PBS and then suspended (1×10^6/ml) in binding buffer (0.01 M Hepes/NaOH pH 7.4, 0.14 M NaCl, 2.5 mM $CaCl_2$). Annexin V-FITC (5 μl) and PI (5 μl) were added to 100 μl of cell suspension which was incubated for 15 min at 25°C in the dark. After addition of 400 μl of binding buffer, analysis was performed in a flow cytometer PAS III (Partec) within 1 h.

2.10.2. c-FLIP expression

After cells were washed twice with cold PBS, they were fixed and permeabilized for 20 min at 4°C with buffer Cytofix/Cytoperm. Cell suspensions in buffer Perm-Wash (BD Cytofix/Cytoperm Kit) were treated with rabbit anti-human c-$FLIP_L$ antibody and goat anti-rabbit IgG rhodamine conjugate at 4°C for 1 h. c-FLIP expression was determined by flow cytometry, and data were analysed with WinMDI 2.9 program.

2.11. Statistical analysis

Results are expressed as means ± S.E.M. Statistical significance was evaluated by using the non-parametric Mann–Whitney *U* test or Kruskal–Wallis one-way analysis of variance. *P*-values <0.05 were considered statistically significant.

3. Results

3.1. Erythroid differentiation increased sensitivity to TNF-α in K562 cells

TNF-α has been reported to exert both negative and stimulatory effects on *in vitro* cell growth of erythroid progenitors and erythroleukaemic cells (Roodman et al., 1987; Rusten and Jacobsen, 1995; Rae and MacEwan, 2004). The first step was to investigate whether this cytokine could affect K562 cell growth at different stages of cell maturation. This has been suggested by two conflicting reports. One stated that TNF-α exerted a negative action on K562 cells, but that this inhibitory effect was not detected after chronic treatment with hemin, ascribing cell

resistance to erythroid differentiation (Li et al., 1989). On the other hand, TNF-α did not affect survival of quiescent CD34$^+$ erythroid progenitors, but induced apoptosis when cells were stimulated to proliferate/differentiate along the erythroid lineage with the erythropoietin plus kit ligand (Ratajczak et al., 2003). In one set culture, TNF-α was added at final concentrations of 5, 10, 30 or 100 ng/ml. Spectrophotometric measurement of MTT-reduced product showed that TNF-α did not alter cell viability at the concentrations assayed up to 48 h (data not shown).

Since the K562 cell line shows a biochemical erythroid phenotype, 30 μM hemin proved to be an appropriate inducer of erythroid maturation. The percentage of haemoglobin-positive cells significantly increased after 24 h [40.3 ± 5.8% compared with 1.9 ± 0.7% (control), $P<0.05$, $n=4$], reaching approximately 80% after 48 h of hemin induction [79.9 ± 4.3% compared with 2.8 ± 0.7 (control), $P<0.01$, $n=4$]. As in the case of undifferentiated cultures, erythroid differentiated cells were exposed to TNF-α in a dose–response manner, measuring cell viability by the MTT assay. Significant differences between non-induced and hemin-differentiated K562 cells were already observed by incubation with 30 ng/ml TNF-α for 48 h. Optical density values were NI (non-induced) 0.216 ± 0.024; NI-TNF 0.196 ± 0.022 (NI-TNF compared with NI; NS, $n=5$); H: 0.234 ± 0.026; H-TNF 0.128 ± 0.022 (H-TNF compared with H, $P<0.01$, $n=5$).

To further analyse the cause of the decreased cell viability, programmed cell death was evaluated in cultures exposed to 30 ng/ml TNF-α for 48 h. Apoptotic cells were differentially counted, based on their apoptotic features characterized by condensed, glossy and fragmented nuclear chromatin. Figure 1(A) shows that hemin differentiation increased the sensitivity of K562 cells, which were otherwise resistant to TNF-α. Apoptosis significantly increased in erythroid-differentiated cells exposed to TNF-α (H-T) with respect to NI cultures ($P<0.001$), whereas it was not significantly induced by hemin or TNF-α alone (Figure 1A, H and NI-T). The pro-apoptotic effect of TNF-α was partially reversed by the presence of anti-TNF-α antibody.

To exclude the possibility that the increase in K562 cell sensitivity to TNF-α had been caused by direct action of the cytokine on cell differentiation, haemoglobin-containing cells were differentially counted after hemin induction for 48 h in the presence or the absence of TNF-α ($n=7$). The results ruled out a deleterious effect of TNF-α upon the differentiation process, since no significant changes were detected in the number of erythroid-differentiated K562 cells, regardless of the presence (80.2 ± 3.3%) or the absence (80.9 ± 4.6%) of the cytokine during the period of hemin induction.

In an attempt to identify the apoptotic pathway induced by TNF-α on haemoglobinized K562 cells, caspase 3 activation was measured by its ability to cleave the chromogenic substrate Ac-DEVD-pNA. The enzyme activity increased nearly 2-fold after cell exposure to 30 ng/ml TNF-α, when compared with cells cultured without the cytokine (Figure 1B, H-T compared with H, $P<0.05$). Furthermore, the presence of TNF-α resulted in endogenous cleavage of the DNA-repairing protein PARP, one of the caspase 3 substrates, yielding the characteristic 85-kDa fragment (Figure 1C). Anti-TNF antibody, on the other hand, reversed the effect of TNF-α on caspase 3 activation and PARP degradation. Data shown in Figure 1 indicate that caspase 3 and PARP degradation are involved in TNF-α-induced cell death of erythroid-differentiated K562 cells.

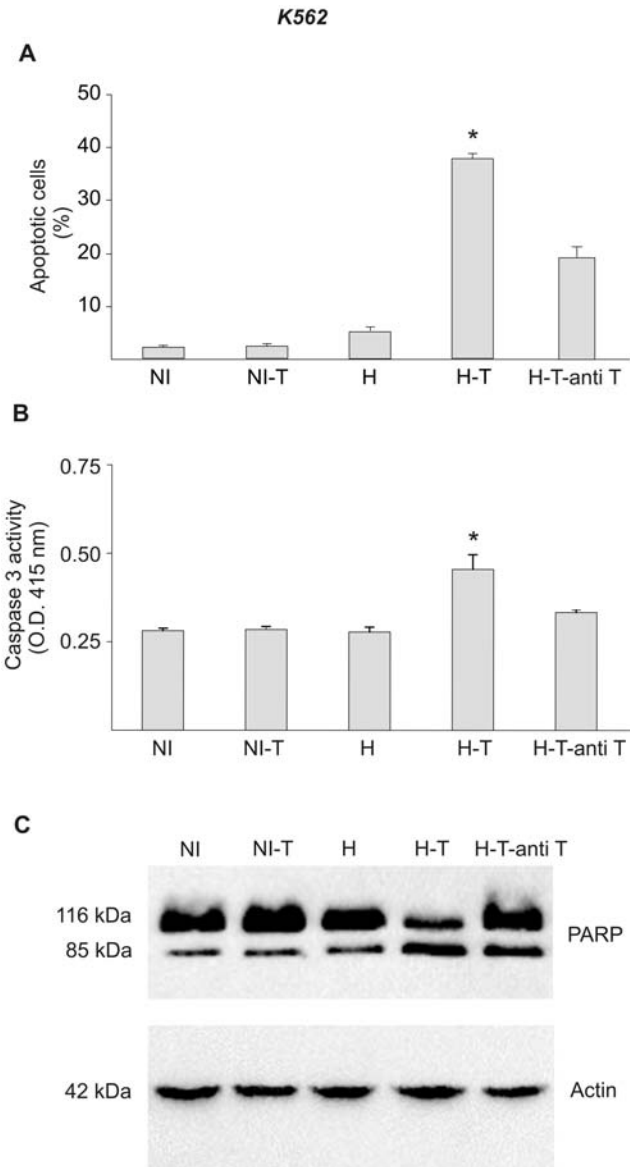

Figure 1 Erythroid differentiation induces K562 cell sensitivity to TNF-α

K562 cells were induced or not to erythroid differentiation by 30 μM hemin and incubated in the absence (NI and H) or the presence of 30 ng/ml TNF-α (NI-T and H-T) for 48 h. In one culture set of H-induced cells, sequential addition of anti-TNF-α antibody and TNF-α was performed (H-T-anti-T). (**A**) TNF-α significantly increased cell apoptosis (fluorescent microscopy) with respect to controls (*H-T compared with NI, NI-T or H, $P<0.001$, $n=5$). (**B**) Caspase 3 activity (Ac-DEVD-pNA cleavage) significantly increased in cell cultures treated with hemin and TNF-α (*H-T compared with H, $P<0.05$, $n=3$) and returned to basal levels in assays with anti-TNF-α antibody (H-T-anti T). Each bar represents the amount of cleaved Ac-DEVD-pNA after 6-h incubation. Results related to caspase 3 activation are in accordance with those of PARP cleavage (**C**), showing an increase in the 85-kDa band, which corresponds to PARP degradation product (Western blotting; actin used as internal control of protein loading). The immunoblot shown is representative of three independent experiments with similar findings.

3.2. Erythroid differentiation did not change UT-7 cell sensitivity to TNF-α

Similar assays to those performed with K562 cells were carried out with UT-7 cells to investigate whether erythroid differentiation may also change the sensitivity to TNF-α of Epo-dependent cells. Hemin induced erythroid differentiation of UT-7 cells cultured in the presence of Epo (E-H: 65.9$\pm$4.4%, NS), and this process was not significantly affected by TNF-α (E-H-T: 70.2$\pm$2.9% compared with E-H, NS: 65.9$\pm$4.4%; E-H-T and E-H compared with E: 6.6$\pm$2.8%, $P<0.001$, $n=4$).

In contrast to K562 cells, Epo-stimulated UT-7 cells did not show signs of apoptosis in the presence of TNF-α in spite of having been induced by hemin to similar levels of erythroid differentiation. There were no significant differences in the percentages of cells with fluorescent nucleus between cultures with or without TNF-α (E: 4.6$\pm$0.4%, E-H: 5.8$\pm$2.1%, E-H-T: 7.1$\pm$1.3, $n=3$).

In order to discard an effect masked by the strong anti-apoptotic action of Epo, 24-h assays with TNF-α were repeated under Epo deprivation. Despite this unfavorable condition, the sensitivity of UT-7 cells to TNF-α was not altered (data not shown).

3.3. Role of c-FLIP in cell sensitivity to TNF-α

3.3.1. c-FLIP expression was reduced in K562 cells after erythroid differentiation

Evidence demonstrated that modulation of c-FLIP is related to changes in cell sensitivity to pro-inflammatory cytokines (Hietakangas et al., 2003; Ratajczak et al., 2003). Therefore, the effect of hemin on the expression of this protein was studied to analyse its role in the differential sensitivity to TNF-α observed between undifferentiated and erythroid-differentiated K562 cells.

Consistent results of mRNA levels and protein expression showed significant c-FLIP$_L$ down-regulation due to hemin treatment of K562 cells (Figures 2A–2C). mRNA levels were almost 60% of values measured in non-differentiated cell cultures, and c-FLIP protein expression, measured by flow cytometry, decreased from 20% (NI) to 5% (H). In agreement with UT-7 cell resistance to TNF-α, hemin differentiation did not induce c-FLIP$_L$ down-regulation in this cell line (Figures 2A–2C), and both mRNA and protein c-FLIP$_L$ expression levels remained unchanged.

3.3.2. Modulation of c-FLIP expression

UT-7 cell activation by Epo is mediated by signalling pathways involving PI3K. Moreover, this kinase pathway was also related to the regulation of c-FLIP levels (Panka et al., 2001; Tucker et al., 2004). Thus, UT-7 cells were cultured during 24 h with or without PI3K inhibitor Ly294002 (Ly) to examine the relationship between this signalling pathway and c-FLIP modulation. Apoptosis was evaluated by differential counting of fluorescent nuclei and by detection of phosphatidylserine translocation. Taking into consideration the dependence of Epo function on PI3K activation, cell death by incubation with Ly was not unexpected (Figure 3A, E-H-Ly compared with E-H, $P<0.05$). However, combined treatment with Ly and TNF-α enhanced apoptosis at a significantly higher level than that observed after the inactivation of Epo/EpoR signals by PI3K inhibition (Figure 3A, E-H-Ly-T compared with E-H-Ly, $P<0.05$). Likewise, Ly treatment significantly decreased c-FLIP$_L$ mRNA level (Figure 3C, $P<0.05$) and protein expression (Figure 3D). On the other hand, the presence of Ly dramatically enhanced erythroid differentiation induced by hemin in this cell line (Figure 3E: E-H-Ly or E-H-Ly-T compared with E-H or E-H-T, $P<0.005$). In fact, after 24 h, the number of haemoglobinized cells was similar to that observed after 48 h of treatment with hemin alone.

Cell sensitivity to TNF-α in K562 cultures exposed to Ly was also higher than in samples treated with hemin (Figure 4A, H-Ly-T compared with H-T, $P<0.05$), even though the inhibition of PI3K signalling pathways did not affect cell differentiation (data not shown). As in UT-7 cells, increased K562 cell sensitivity to the pro-apoptotic effect of TNF-α due to PI3K inhibition, which was reversed with anti-TNF-α antibody, can be explained by c-FLIP down-regulation. In fact, c-FLIP mRNA was found 50% decreased (Figure 4B), and protein expression changed from 20% to 5.5% (Figure 4C).

3.4. Role of Bcl-x$_L$ in cell sensitivity to TNF-α

In many cell types, there is cross-talk between extrinsic and intrinsic pathways (Khosravi-Far and Esposti, 2004). In addition to c-FLIP, it has been reported that the anti-apoptotic factor Bcl-x$_L$ confers resistance to TRAIL (TNF-related apoptosis-inducing ligand) in tumour cell lines. Since this is one of the main factors involved in the mitochondrial anti-apoptotic signalling pathways of erythroid lineages (Benito et al., 1996), we investigated whether an alteration in the molecular mechanism of cell protection mediated by this factor would contribute to changing cell sensitivity to TNF-α.

Results show that high Bcl-x$_L$ basal mRNA levels in K562 and UT-7 cells were not altered by hemin or by TNF-α treatment (data not shown). Therefore, the modulation of this factor does not account for the differences in sensitivity to TNF-α-induced apoptosis observed in both cell models.

3.5. Protective effect of erythropoietin against TNF-α-induced apoptosis

3.5.1. Erythropoietin as an anti-apoptotic factor

In addition to its well-known role in the control of erythropoiesis, Epo also has anti-inflammatory properties (Feng, 2006). Current assays using UT-7 cells suggest that Epo would support cell proliferation and viability, preventing the deleterious action of TNF-α. In a previous report, we have detected the expression of Epo receptors in K562 cells, even though they do not depend on it to survive (Vittori et al., 2005). Thus, to investigate whether this growth factor could counteract the apoptotic effect of TNF-α, cultures were pretreated with Epo for 18 h before addition of hemin and TNF-α. Epo significantly prevented TNF-α-induced apoptosis of erythroid-differentiated K562 cells (Figures 5A and 5B, E-H-T compared with H-T, $P<0.05$). These results, as a whole,

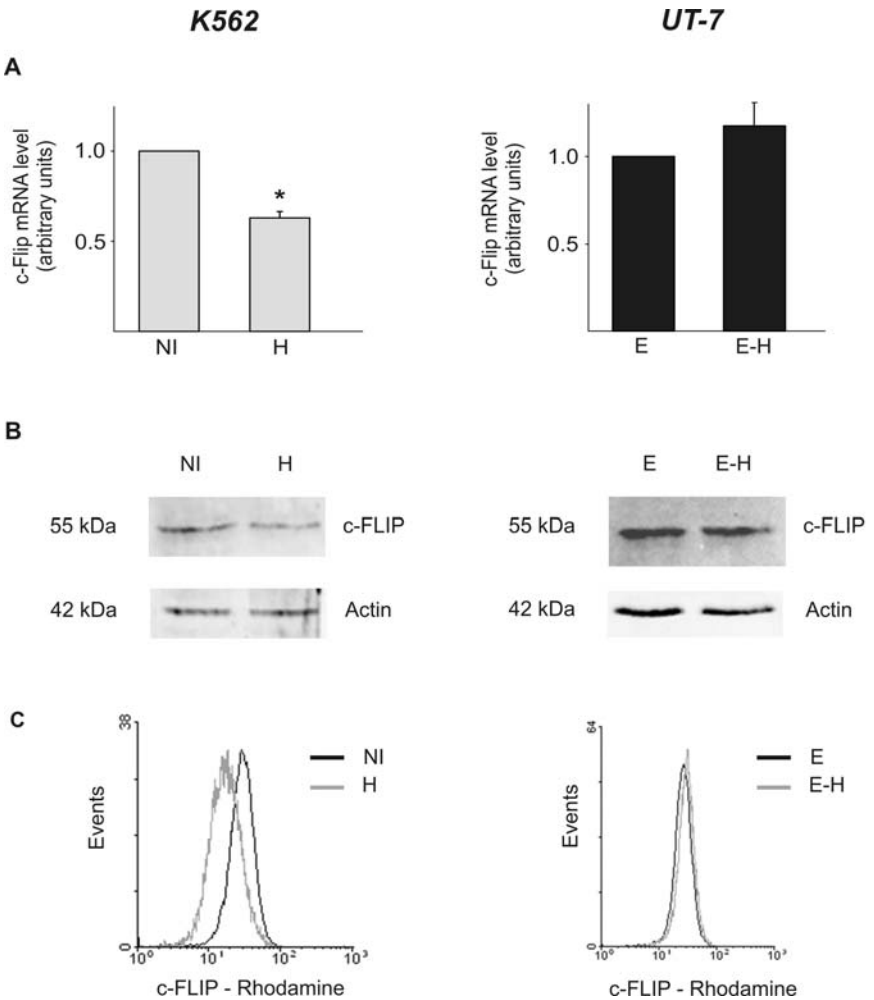

Figure 2 **Hemin-induced erythroid differentiation and c-FLIP expression**
K562 and UT-7 cells were non-induced (NI or E) or induced to erythroid differentiation by 30 μM hemin (H and E-H). (**A**) After 24-h culture, c-FLIP$_L$ mRNA levels were measured by real-time PCR. Data are expressed in arbitrary units with respect to the respective control (means ± S.E.M., $n=4$ independent experiments). Protein expression was analysed by Western blotting (**B**) and by flow cytometry (**C**) after 48-h culture. Under hemin-induced erythroid differentiation, c-FLIP mRNA was found significantly reduced in K562 cells (*H compared with NI, $P<0.01$), whereas it remained unchanged in UT-7 cells (E-H compared with H, NS). These results were consistent with those of c-FLIP protein expression (**B** and **C**, $n=3$). Rhodamine fluorescence intensity measured by flow cytometry changed from 20% (NI) to 5% (H).

support the hypothesis that Epo is able to increase cell resistance to the pro-inflammatory cytokine TNF-α.

3.5.2. c-FLIP mRNA decrease is prevented by Epo

To further define the role of c-FLIP in the mechanism of action of Epo, c-FLIP expression was studied in erythroid differentiated K562 cells previously incubated with Epo. Figures 5(C) and 5(D) show that Epo maintained c-FLIP expression within control levels, clearly preventing the hemin-induced negative modulation of c-FLIP observed in the absence of the growth factor. This would explain why blocking the Epo action by inhibiting the PI3K signalling pathway resulted in enhanced UT-7 cell sensitivity to TNF-α (Figure 3). It can be suggested that c-FLIP plays a critical role in the protection of erythroid-differentiated cells against the pro-apoptotic stimulus of TNF-α.

4. Discussion

In order to study the inhibition of erythropoietic cell growth attributed to TNF-α, this pro-inflammatory cytokine was added to cell cultures able to develop erythroid differentiation. Results show that TNF-α failed to exert a direct inhibitory effect on K562 or UT-7 cell growth and viability. However, hemin-induced differentiation enhanced K562 sensitivity to the cytokine. Conversely, the apoptotic effect of TNF-α was not detected in erythropoietin-dependent UT-7 cells under similar conditions of hemin induction. This was not surprising since TNF-α may not only induce pro-apoptotic signals, but also negatively regulate this ability via activation of nuclear factor κB (NF-κB) (Tucker et al., 2004). According to our data, the effect of TNF-α observed in cultures containing anti-TNF-α antibodies can only be attributed to

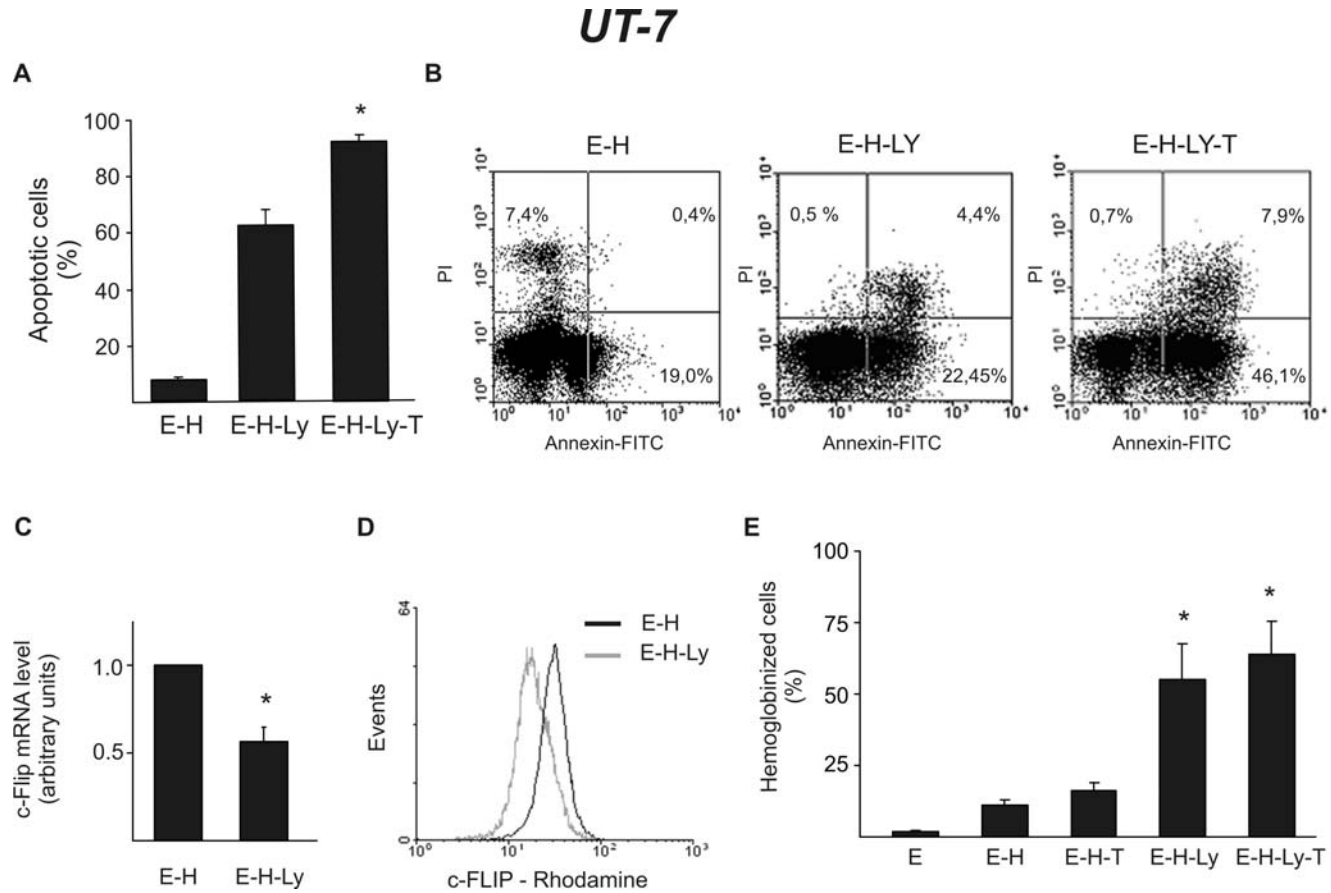

Figure 3 **Relationship between PI3K and c-FLIP expression in UT-7 cells**

Epo-stimulated UT-7 cells, induced to erythroid differentiation by hemin during 24 h (E-H), were treated with 50 μM Ly294002 (E-H-Ly) or with Ly and TNF-α (E-H-Ly-T). Apoptosis was evaluated following standard criteria of DNA fragmentation using Hoechst dye (means ± S.E.M., **A**) and by flow cytometry after annexin V and PI dual staining (representative cytograms, **B**). The increased apoptosis of differentiated cells in the presence of Ly and TNF-α was significantly higher than that due to inactivation of the Epo signalling pathway via PI3K (*E-H-Ly-T compared with E-H-Ly, $P<0.05$, $n=3$). Cytogram shows consistent results obtained by flow cytometry (46.1% compared with 22.5%). c-FLIP mRNA was analysed by real-time PCR (**C**), and protein expression was evaluated by flow cytometry using Rhodamine-conjugated antibody (**D**). Following PI3K inhibition, the level of c-FLIP mRNA was significantly diminished (*E-H-Ly compared with E-H, $P<0.05$, $n=3$) and the reduced fluorescence intensity (from 20% to 5.5%) indicated consistently reduced c-FLIP protein expression. (**E**) Erythroid differentiation of UT-7 cells, evaluated by differential count of haemoglobinized cells after DAF staining, was significantly increased by the presence of Ly (*E-H-Ly or E-H-Ly-T compared with E, E-H or E-H-T, $P<0.01$, $n=3$).

changes in cell conditions during the differentiation process, since TNF-α *per se* did not have any effect upon the proliferation or viability of non-differentiated K562 cells.

Apoptosis is a highly regulated process, and several inhibitors are known to interfere with both extrinsic and intrinsic apoptotic pathways. c-FLIP, a caspase 8 homologue except for its lack of amino acids critical for protease activity, is an endogenous inhibitor at the level of activated death receptors (Peter, 2004). We found that the long splice form c-$FLIP_L$, constitutively expressed in K562 cells, was down-regulated in response to hemin-mediated erythroid differentiation, suggesting that cell protection might be reduced, making cells sensitive to apoptosis by subsequent incubation with TNF-α. Both K562 and UT-7 cell lines showed high c-$FLIP_L$ basal levels. Therefore, a lower level of this protein could explain the higher susceptibility of erythroid-differentiated K562 cells to TNF-induced apoptosis. This hypothesis is supported by the fact that the effect of this cytokine was not observed in hemin-differentiated UT-7 cells, which showed unaltered c-FLIP expression. Moreover, the apoptotic effect of TNF-α on UT-7 cells was only detected when c-$FLIP_L$ expression decreased after PI3K inhibition. Panka et al. (2001) have pointed out that the PI3K/Akt pathway is the predominant regulator of c-FLIP expression in tumour cells, based on results showing that c-FLIP modulation might depend on cell type and/or treatment. In accordance with our results, many other reports have shown the influence of c-FLIP modulation on cell sensitivity to death receptor stimuli (Ratajczak et al., 2003; Hietakangas et al., 2003; Perez and White, 2003; Peter, 2004; Rippo et al., 2004). The differences in behaviour shown by erythroid-differentiated K562 and UT-7 cells towards TNF-α exposure support the notion that decreased protection of erythroid cells, due to lower c-FLIP levels, might account for changes in cell sensitivity to the pro-inflammatory cytokine.

PI3K has been found to be related to cell cycle progression and cell survival. However, its role in cellular differentiation is still unclear, and according to reports, PI3K is involved both in the

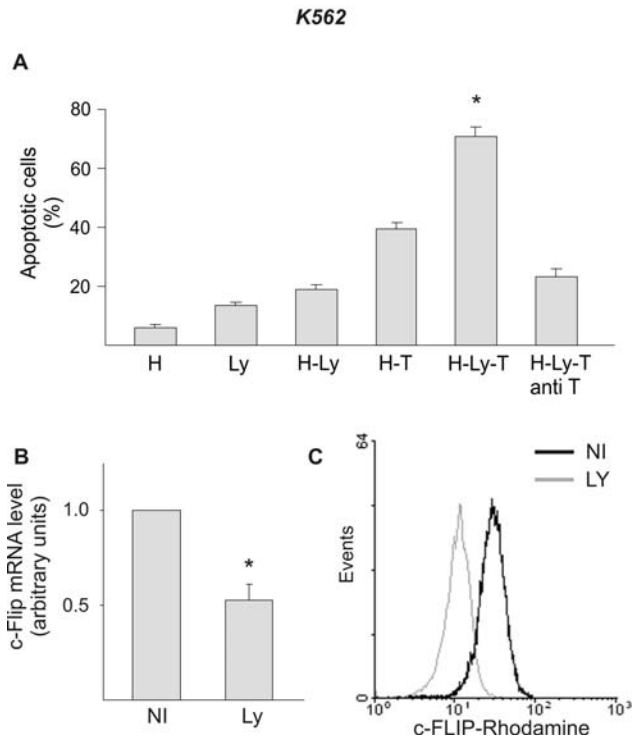

Figure 4 PI3K inhibition and K562 cell sensitivity to TNF-α

K562 cells, cultured at a density of 2×10^5 cells/ml, were induced to cell differentiation by 30 μM hemin in the absence (H) or the presence of 50 μM Ly294002 (H-Ly) or with Ly and TNF-α (H-Ly-T). In one H-Ly set of cells, sequential addition of anti-TNF-α antibody and TNF-α was performed (H-Ly-T-anti T). (**A**) Apoptosis, evaluated by changes in nuclear morphology in 48-h cultures, significantly increased following exposure to Ly and TNF-α (*H-Ly-T compared with H-T or H-Ly, $P<0.05$). Values are given as mean percentage $\pm$ S.E.M. of four independent experiments. c-FLIP expression was analysed by real-time PCR in 24-h cultures (**B**) and flow cytometry in 48-h cultures (**C**) with the PI3K inhibitor. Inhibition of the PI3K pathway induced down-regulation of c-FLIP at mRNA (*$P<0.05$, $n=3$) and protein levels (20% to 5.5%) in K562 cells.

promotion and inhibition of non-erythroid cell differentiation (Lewis et al., 2004). Although previous studies showed that PI3K inhibition suppressed maturation in some haematopoietic cell lines (Haseyama et al., 1999; Lewis et al., 2004), our results demonstrated that the PI3K inhibitor Ly294002 was a strong inducer of UT-7 cell erythroid differentiation. Whereas TNF-α caused apoptosis in UT-7 cultures treated with Ly, cell death was not detected in samples with similar differentiation levels without PI3K inhibition. This would suggest that biochemical changes produced during erythroid differentiation were not enough to modify UT-7 cell sensitivity to TNF-α, in contrast to data observed in K562 cell assays. This is further evidence of the different behaviours exhibited by the two cell lines used in this study. PI3K inhibition in K562 cells also enhanced the sensitivity to TNF-α already observed with hemin treatment, but this effect did not have significant consequences on cell differentiation. As a whole, these results suggest that the high expression of c-FLIP and another unidentified pathway involving PI3K activation contribute to maintaining cell resistance to TNF-α-mediated apoptosis.

Owing to their differential dependence on growth factor Epo, both cell lines (K562 and UT-7) represent good experimental systems to study cell sensitivity to apoptosis during erythroid differentiation. The balance between survival and death inducers in erythroid-differentiated UT-7 cells suggests that Epo would support cell proliferation and viability to counteract the deleterious action of TNF-α. Also consistent with this hypothesis, we demonstrated that the action of Epo on erythroid-differentiated K562 cells prevented TNF-α-induced apoptosis and was associated with the recovery of c-FLIP$_L$ levels. To our knowledge, this report provides the first evidence of the effect of Epo upon modulation of c-FLIP expression. These results let us suggest that Epo prevents the decrease in c-FLIP expression levels during differentiation of UT-7 cells and, therefore, counteracts the apoptotic effect of TNF-α. Owing to the ability of EpoR to induce survival signals through NF-κB activation (Bittorf et al., 2001), this pathway might activate signals that induce c-FLIP expression, as has been found in other cell models (Micheau et al., 2001). In a similar context, stem cell factor increased FLIP expression and reduced interferon-γ-induced apoptosis in human erythroid progenitor cells (Chung et al., 2003). According to our results, Epo would play an anti-inflammatory role in cells able to undergo erythroid differentiation. This effect should be considered in future pharmacological studies related to chronic inflammatory states associated with anaemia.

The complex relationship between maturation and sensitivity to pro-apoptotic stimuli has not been fully elucidated yet. Ratajczak et al. (2003) postulated that quiescent CD34$^+$ erythroid progenitor cells are somehow protected by FLIP from the potential influence of inhibitory cytokines, whereas cell cultures in which growth factors stimulated BFU-E formation are susceptible to TNF-mediated apoptosis. Hietakangas et al. (2003) found that upon hemin-induced erythroid differentiation, K562 cells specifically lose their resistance to TRAIL due to a mechanism independent of mitochondria but dependent on c-FLIP down-regulation. Our results only partially agree with those from Hietakangas et al., who reported that hemin-induced K562 cells were sensitive to TRAIL but nevertheless maintained their resistance to TNF-α.

In addition to death receptors, the intrinsic apoptotic pathway might also be activated to induce programmed cell death. Therefore, it was interesting to investigate the induction of Bcl-x$_L$, an anti-apoptotic factor of the Bcl-2 family constitutively expressed in K562 and UT-7 cells. The sensitivity to TNF-α shown by these cells appeared to be mediated by the activation of apoptotic mechanisms independent of Bcl-x$_L$, since reduced cell resistance was accompanied by high levels of this anti-apoptotic factor, which remained unaltered during erythroid differentiation.

In conclusion, we have demonstrated that cells with different dependence on the growth factor Epo show different sensitivity to TNF-α under similar conditions of erythroid differentiation, suggesting that survival and differentiation programmes depend on cellular type. Hemin-differentiated K562 cells were prone to be affected by the pro-inflammatory cytokine, indicating that the cell differentiation process may deregulate the balance between cell growth and cell death signals induced by TNF-α. It was found that cells rendered highly sensitive to death-ligand apoptosis by hemin differentiation could be rescued by c-FLIP expression. Thus, c-FLIP appeared to be critical in protecting cells from apoptosis or determining cell sensitivity to TNF-mediated programmed cell death. An interesting finding that emerges from this study is that

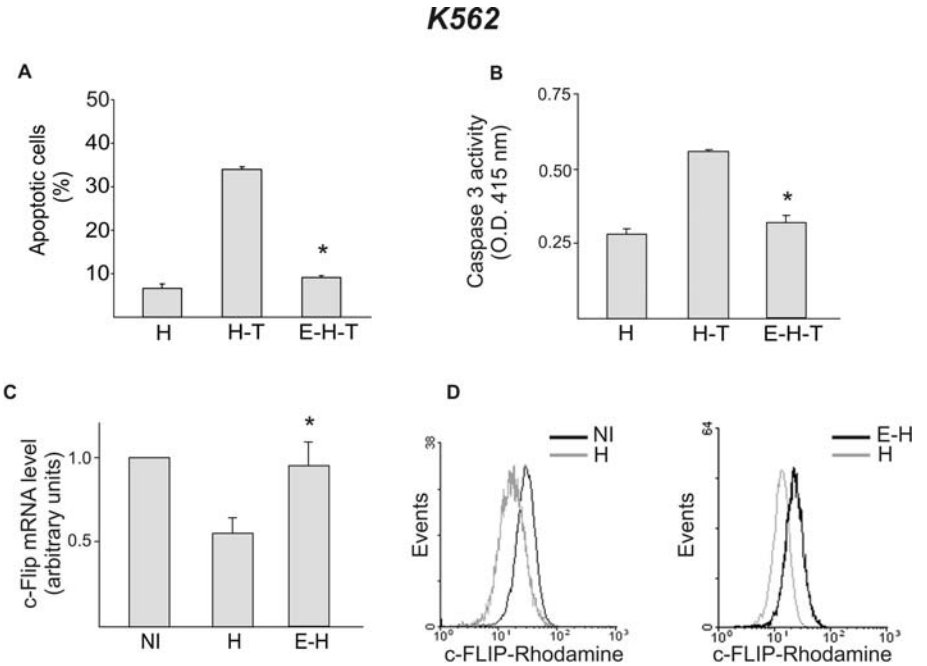

Figure 5 **Protective effect of Epo against TNF-α-induced apoptosis**

K562 cells were pretreated with 10 units/ml Epo for 18 h before TNF-α and hemin exposure (48 h). (**A**) Each bar represents percentage value (means $\pm$ S.E.M., $n=3$) of apoptotic cells with respect to total cell number. (**B**) Caspase 3 activity was estimated by its ability to cleave the Ac-DEVD-pNA chromogenic substrate. Each bar represents the amount of cleaved Ac-DEVD-pNA after 6-h incubation (means $\pm$ S.E.M.). (**C**) c-FLIP$_L$ mRNA levels were measured by real-time PCR in cell cultures pretreated with Epo before the addition of hemin. Quantitative results are expressed in arbitrary units with respect to those in non-induced cultures (NI) (means $\pm$ S.E.M., $n=3$). (**D**) c-FLIP protein expression was analysed by flow cytometry ($n=3$). The TNF-α-induced apoptosis was clearly prevented in Epo-pretreated K562 differentiated cells (**A** and **B**: E-H-T compared with H-T, *$P<0.05$, $n=3$). The hemin-induced c-FLIP down-regulation associated with high apoptosis and caspase 3 activation was counteracted by Epo mRNA (*E-H compared with H, $P<0.05$) and protein levels (change of fluorescence intensity from 5% to 11%). Cells treated with Epo maintained high c-FLIP expression (**C** and **D**: E-H compared with NI, NS).

Epo is able to prevent hemin-induced c-FLIP mRNA down-regulation during erythroid differentiation. This novel effect will contribute to the knowledge of the Epo anti-apoptotic mechanism on erythroid cells.

These results may have potential implications in the understanding of mechanisms through which the process of erythroid differentiation is associated with changes in cell sensitivity to inflammatory cytokines, contributing to our understanding of pathogenesis of several diseases. The fact that different cell conditions may cause deregulation of the strict balance that switches from cell life to death is very important and should be taken into account when designing new strategies in drug therapy.

Author contribution

Daniela Vittori and Daiana Vota took an active part in the experimental development of the work as well as in the discussion of results and writing of the manuscript. Alcira Nesse directed the whole work and discussions. Mariana Callero participated in assays performed by real-time PCR, and María Eugenia Chamorro helped with cell cultures.

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