European Journal of Pharmacology 636 (2010) 42–51

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Molecular and Cellular Pharmacology

Murine erythropoietic impairment induced by paclitaxel: Interactions of GATA-1 and erythroid Krüppel-like transcription factors, apoptotic related proteins and erythropoietin receptor

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ARTICLE INFO

Article history:
Received 6 September 2009
Received in revised form 30 January 2010
Accepted 15 February 2010
Available online 29 March 2010

Keywords:
Paclitaxel
Erythropoiesis
Apoptosis
EKLF
EPO receptor
GATA-1

ABSTRACT

Paclitaxel, an antitumoral drug, was used in a single dose (29 mg/kg i.p.) as an injury agent for inducing transient suppression of hematopoiesis in a murine experimental model during 10 days. The aim of this study focuses on erythropoietin (EPO) receptor, GATA binding protein 1 (globin transcription factor 1) (GATA-1) and erythroid Krüppel-like factor (EKLF) expressions related to the apoptotic events triggered by paclitaxel in bone marrow and the subsequent in vivo erythropoietic recovery. Results showed a massive impairment of erythropoiesis early post paclitaxel administration (1–2 days), which involved induction of high Bax/Bcl-x_L ratio, caspase-3 activation, disruptions of the medullar niche and cell death by both apoptosis and necrosis. EPO receptor over-expression was noticed from day 3 onwards. It prompted the subsequent up-regulations of GATA-1 and EKLF transcription factors as well as of the anti apoptotic protein Bcl-x_L, crucial proteins in driving erythropoiesis. This study suggests that EPO receptor recovery is necessary for the subsequent bone marrow ability to accomplish the erythroid program through the modulation of apoptotic and survival events after a single paclitaxel insult. These findings contribute to new insights into the molecular mechanisms involved during in vivo erythropoiesis post paclitaxel administration. Therefore, the detailed knowledge of the injury elicited by this drug on red blood cell production may have clinical relevance to explore new therapeutic approaches.

1. Introduction

Erythropoiesis is a multi-step process involving commitment, proliferation, and differentiation of hematopoietic progenitors to mature erythrocytes. Erythropoietin (EPO) is the principal regulator of this process interacting with its receptor on the surface of erythroid progenitor cells (Jelkmann, 2004).

The normal homeostasis of the erythropoietic system requires a balance between the rate of erythroid cell production and red blood cell destruction. The hematopoietic steady state may be disturbed by numerous injuries such as those produced by cytotoxic drugs. Although myelosuppression is the most common dose-limiting toxicity for clinical treatment (Guest and Uetrecht, 2000), erythropoiesis is usually affected by several chemotherapeutic agents (Juaristi et al., 2007; Aispuru et al., 2008).

Paclitaxel, is the anticancer drug of choice of many solid malignancies and leukemia. It inhibits microtubule depolymerization, causes blockage of the cell cycle progression, activates apoptosis and induces the secretion of several pro-inflammatory cytokines and cyclooxygenase-2 (Rowinsky, 1997; Moos and Fitzpatrick, 1998). Although paclitaxel is usually indicated in a multidose administration in chemotherapy patients; a single dose paclitaxel (29 mg/kg i.p.) was used in this study to induce a temporary hematopoietic disturbance in an experimental model.

Several factors contribute to maintain the red cell phenotype by activating erythroid related genes. Among them, GATA binding protein 1 (globin transcription factor 1) (GATA-1), the founding member of the GATA family of transcription factors, is essential for erythroid differentiation (Shimizu et al., 2001).

The gene encoding the EPO receptor is a downstream target of GATA-1 (Zon et al., 1991).

Moreover, EPO also induce the expression of EPO receptor and Bcl-x_L, an antiapoptotic protein of Bcl-2 family, crucial for the survival of erythroid cells (Kapur and Zhang, 2001; Silva et al., 1999). Therefore, the withdrawal of EPO or the stimulation of death receptors triggers the caspases cascade activation (Testa, 2004), with the consequent arrest of erythroid maturation by GATA-1 cleavage and the demise of cells through apoptosis (De Maria et al., 1999). In addition, the erythroid Krüppel-like factor (EKLF) is an important activator of β-globin gene expression. It also coordinates the expression of several proteins involved in erythroid differentiation and in the erythrocytes maturation (Drissen et al., 2005; Hodge et al., 2006).

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doi:10.1016/j.ejphar.2010.02.056

We have previously reported that paclitaxel disturbs murine erythropoiesis (Juaristi et al., 2001) by rearrangements of critical protein expressions (Romero Benítez et al., 2004). Nevertheless, the specific molecular interplay between bone marrow apoptosis and the molecular events that govern erythropoietic recovery post paclitaxel administration remains to be fully elucidated.

Therefore, the aim of this study is to evaluate the underlying mechanisms of murine erythropoiesis post paclitaxel dosing over a period of 10 days with regard to EPO receptor, transcriptional factor expressions (GATA-1 and EKLF) and apoptotic events.

To achieve these purposes, a multiparametric analysis of apoptosis, bone marrow architectural organization, bone marrow progenitor's proliferation and differentiation, as well as the expression of erythroid related proteins was performed.

Findings suggest that the erythroid recovery post paclitaxel injury involves the coordinated expressions of EPO receptor, crucial antiapoptotic proteins and erythroid related transcription factors.

2. Materials and methods

2.1. Animals and drug treatment

Female CF-1 Swiss mice (8–10 weeks, 26–28 g) were provided by the Animal Center at the National Northeast University, Argentina. Animals were housed in cages in an air-conditioned room (23 ± 1.0 °C) for 5 days before the experiment and were maintained in a 12-light:12-h dark cycles. They were allowed free access to pelleted food and water. All experimental procedures were conducted according to the principles outlined in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD, 1996).

Mice were randomly divided into two groups. Control group ($n = 24$) received an i.p. injection of sterile saline solution for peripheral hematological and bone marrow cellular determinations. The second group ($n = 100$) was divided in 9 subgroups of 10–12 mice for all the other experiments at each time of the experience (0, 1, 2, 3, 4, 5, 6, 7, and 10 days post paclitaxel administration). Animals were injected with a single dose of paclitaxel (Taxol, Bristol Myers, Squibb; 29 mg/kg i.p.) to induce erythropoietic injury. Mice were anesthetized (pentobarbital, 60 mg/kg i.p.) and euthanized by cervical dislocation.

The paclitaxel dose used in this study was selected in accordance with previous reports from our laboratory (Juaristi et al., 2001). Experimental data from paclitaxel injected group was compared to the control (saline injected) or day 0 (paclitaxel untreated) groups.

Human recombinant EPO (Hemax 2000, Biosidus, Argentina) was used for ex-vivo assay on murine cells.

2.2. Hematological and bone marrow parameters

Hematocrits and hemoglobin concentrations were determined by standard methods. Peripheral reticulocyte counts (%) were determined using cresyl brilliant blue (1%) staining.

Total bone marrow cells were determined as described previously (Aguirre et al., 2005). Briefly, bone marrows were expelled from the femurs and total nucleated cells were counted in a hemocytometer on the scheduled days. Differential cell determinations were performed in May Gründwald–Giemsa (MGG) stained smears. Results are expressed as absolute mean $\times 10^6$ cells/femur ± S.E.M.

2.3. Scanning electronic microscopy

A direct observation of the inner bone marrow architecture post paclitaxel recovery was essentially performed as previously described (Juaristi et al., 2007). Briefly, samples were dehydrated and dried to the critical point (Dento Drier). They were exposed to gold–palladium coating for 3 min. Samples were observed under a scanning electronic microscope (Jepl JSM-5800 L.V.) and images (original magnification 2500×) were obtained at different time points of the experimental study.

2.4. Methodological aspects of apoptosis detection

2.4.1. May Gründwald–Giemsa staining

Methanol-fixed cytospin preparations were stained with MGG to identify apoptosis according to morphological criteria (Kerr, 2002). Apoptotic indexes were evaluated from three different assays in triplicate by dividing the number of recognizable apoptotic cells to 1000 cells counted randomly (400×).

2.4.2. Double fluorescent staining

Acridine orange/ethidium bromide (AO/EB) staining is an exclusion fluorescent dye method which enables differentiation between live, early-apoptotic, late-apoptotic, and necrotic cells (Lopes et al., 2001). Briefly, 500 µl of bone marrow cell suspensions (5×10^6 cells/ml) were incubated with 20 µl of AO/EB dye mix (one part each of 100 µg/ml AO and 100 µg/ml EB in PBS) just before microscopic observation at each time of the experimental protocol.

Slides were examined under fluorescence microscope (Olympus CX-35 equipped with a Y-FL epifluorescence attachment and an Olympus Coolpix Digital camera).

Late-apoptotic cells (bright orange chromatin which is highly condensed or fragmented) were counted in several randomly selected independent fields (x 400). A total of 500 cells were counted to determine the percentage of apoptosis at each point of the experimental protocol.

2.4.3. TUNEL assay

Apoptotic percentages of bone marrow cells post drug treatment were confirmed by TdT-mediated dUTP nick-end labelling (TUNEL) assay as described previously (Romero Benítez et al., 2004). Briefly, samples fixed in 4% paraformaldehyde were assayed using the ApoptoTag fluorescein direct in situ apoptosis kit (Intergen Co., New York) according to manufacturer's instructions. The assay was also performed in control slides. Apoptotic nuclei were identified under a fluorescence microscope. Nuclei of apoptotic cells were stained positive for green fluorescence, while counterstaining showed red fluorescence with propidium iodide. Results are expressed as mean percentage of TUNEL positive apoptotic cells ± S.E.M.

2.5. Radioiron uptake assay

The percentage of [^{59}Fe] incorporation into hemoglobin synthesizing bone marrow cells was determined as described before (Aguirre et al., 2005). Briefly, control and paclitaxel treated mice were i.p. injected with 0.5 µCi of sterile ferrous citrate solution diluted in saline (0.15 mCi/ml Dupont NEN Products, Boston, USA). Radioiron incorporation in bone marrow cell suspensions was determined after 24 h with a gamma counter (Alfanuclear). Experimental data are expressed as mean percentage of [^{59}Fe] uptake of the initial injected dose ± S.E.M.

2.6. Hematopoietic progenitors cell assays

The frequency of bone marrow hematopoietic progenitor cells at each time of the experimental protocol was determined as described previously (Aguirre et al., 2005). Briefly, bone marrow cells (2×10^5 cells/plate) were cultured in methylcellulose (1% w/v, Fisher Co., USA) supplemented with 20% of fetal bovine serum and 2 U/ml of human recombinant EPO. Cultures were incubated at 37 °C in a humidified air containing 5% CO_2. Colonies were scored on the second day (colony-forming-units-erythroid [CFU-E]) and on the seventh day of incubation (burst-forming-unit-erythroid [BFU-E]). Results are expressed as mean colonies/femur ± S.E.M.

2.7. Western blot analysis

Expressions of EPO receptor, GATA-1, EKLF, Bcl-x_L and Bax were determined by immunoblotting from whole bone marrow extracts obtained in RIPA buffer (50 mM Tris, 150 mM NaCl, 2.5 mg/ml deoxycholic acid, 1 mM EGTA, 10 µg/ml Nonidet-40 (pH 7.4), supplemented with protease inhibitors: 2.5 µg/ml leupeptin, 0.95 µg/ml aprotinin and 2.5 mM phenylmethylsulfonyl fluoride [PMSF]) as previously described (Aguirre et al., 2005).

Cytosolic bone marrow lysates were used for caspase-3 immunoblottings. Briefly, single cell suspensions were rinsed twice with ice-cold PBS and then lysed with ice-cold buffer (10 mM HEPES pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1% IGEPAL) (Sigma Co, MO, USA), supplemented with the cocktail of protease inhibitors for 30 min. Cell lysates were briefly sonicated, centrifuged at 14,000 *g* for 20 min at 4 °C and the supernatant was used as cytosolic fraction.

Proteins (40 µg) were separated on 12% SDS-PAGE, blotted onto nitrocellulose membranes (Bio-Rad) and probed with 1:500 dilutions of primary anti-Bax, anti-EPO receptor, anti-GATA-1, anti-Bcl-x_L, anti-caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-β actin (Sigma Aldrich, USA), followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Jackson Immunoresearch Inc, USA).

Immunocomplexes were detected by an Opti4CN kit (Bio-Rad, CA, USA). Band optical density (OD) was analyzed using NIH-image software and results are expressed as the ratio: (protein of interest OD/β-actin OD) × 100 (mean arbitrary units ± S.E.M).

2.8. Caspase-3 activity assay

Enzymatic activity of caspase-3 was measured with a commercially available caspase-3 assay kit (Sigma, St. Louis, MO, USA) as previously described (Aispuru et al., 2008). Proteolytic reactions were carried out in extraction buffer containing 20 µg of cytosolic protein extract and 40 µM Ac-DEVD-*p*NA. The reaction mixtures were incubated at room temperature for 2 h, and the formation of *p*NA was measured at 405 nm using a colorimeter. Experiments were performed in triplicate. Caspase-3 activity was calculated as fold increase of untreated control at each time points of the study.

2.9. Statistical analysis

Experimental values were expressed as mean ± S.E.M. Data represents results from a least three separate experiments, each performed in triplicate. Differences among means were tested for statistical significance using a one-way ANOVA test followed by a Dunnett's *t*-test (Graph Pad Software Inc, San Diego, CA, USA). A P value <0.05 was considered statistically significant. The correlations between different variables were performed using the Spearman rank correlation test.

3. Results

3.1. Hematological and bone marrow parameters

The hematocrits declined 27% on the first day post paclitaxel administration (from 40.23 ± 0.43% to 29.48 ± 0.88%, $P<0.01$) and then started recovering on the following days (Fig.1A). This parameter almost achieved normality at the end of the study (38.80 ± 0.58%).

The initial gradual decline in hemoglobin content reached a minimum value of 9.50 ± 0.6 g/dl on day 2, resulting in an approximately 29% decrease from its normal baseline of 13.40 g/dl. The hemoglobin concentration returned to normal values at the end of the observation period, revealing that paclitaxel caused a secondary mild transitory anemia (Fig. 1B).

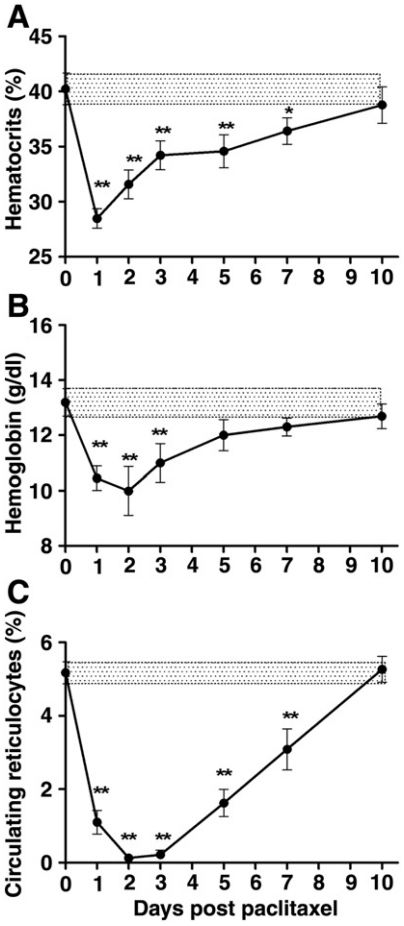

Fig. 1. Changes in peripheral hematological parameters post paclitaxel treatment. (A) Hematocrits (%), (B) hemoglobin (g/dl) and (C) circulating reticulocytes (%) were monitored after single i.p. administration of either saline (shadowed area) or 29 mg/kg paclitaxel (filled circle) in a 10 day period. Results are expressed as mean ± S.E.M. ($n=3$). Data were obtained from three different assays. $^{*}P<0.05$ and $^{**}P<0.01$ indicate significant differences between control (saline) and paclitaxel treated mice.

The minimal percentage of peripheral reticulocytes was noticed on the second day (0.13 ± 0.10% *vs.* control 5.17 ± 0.60%, $P<0.01$). Subsequently, reticulocytes exhibited a similar restoration profile compared to the other hematologic parameters (Fig. 1C).

As shown in Table 1, paclitaxel caused about a 35% of decrease in total bone marrow cellularities between 1 and 2 days post administration. Erythroid absolute bone marrow cells declined on the first day almost 4.3 times compared to control values ($P<0.001$); meanwhile the myeloid and lymphoid cells diminished 3.8 and 1.7 times, respectively, compared to normal parameters ($P<0.001$ and $P<0.05$). The bone marrow red cell compartment was the most noticeably affected in degree and extent. Erythroid absolute total bone marrow counts only reached control values by the end of the study (day 10) whilst myeloid and lymphoid cellularities recovered to baseline values from the fourth day onwards.

Paclitaxel affected the mature as well as the early populations of the erythroid compartment throughout the time course study (Fig. 2). However, the delayed response of polychromatic and orthochromatic erythroblasts might imply the time required for their maturation from earlier precursors.

3.2. Paclitaxel effects on bone marrow architecture

Axial cuts of femurs obtained all through the experimental protocol were examined with a scanning electron microscopy for evaluating the effect of the cytotoxic injury. The whole inner bone marrow architecture

Table 1
Bone marrow cellularities post paclitaxel treatment.

Cell type	Total bone marrow cells ($\times 10^6$/femur)		Erythroid cells ($\times 10^6$/femur)		Myeloid cells ($\times 10^6$/femur)		Lymphoid cells ($\times 10^6$/femur)	
Day of sampling	Control	Paclitaxel	Control	Paclitaxel	Control	Paclitaxel	Control	Paclitaxel
1	14.4	5.1[c]	3.0	0.7[c]	6.9	1.8[c]	4.5	2.6[a]
	(0.8)	(0.2)	(0.2)	(0.01)	(0.6)	(0.1)	(0.4)	(0.8)
2	13.5	5.6[c]	3.1	0.8[c]	6.5	2.9[c]	3.9	1.9[b]
	(0.5)	(0.3)	(0.2)	(0.1)	(0.5)	(0.2)	(0.2)	(0.7)
3	14.4	6.8[b]	2.8	0.7[b]	6.7	3.5[a]	4.9	2.6[a]
	(0.5)	(1.3)	(0.4)	(0.2)	(0.6)	(1.6)	(0.2)	(0.9)
4	16.4	8.7[b]	3.4	0.9[c]	6.6	5.5	4.4	2.3
	(0.8)	(1.4)	(0.4)	(0.5)	(0.5)	(0.2)	(0.4)	(1.1)
5	15.2	12.8	3.5	1.02[b]	8.0	8.2	4.9	3.6
	(1.0)	(0.4)	(0.4)	(0.9)	(0.5)	(0.3)	(0.5)	(0.6)
6	15.8	13.5	3.5	1.18[b]	7.0	9.1	4.7	3.6
	(0.8)	(0.4)	(0.5)	(0.2)	(0.6)	(0.2)	(0.4)	(0.1)
7	17.4	13.9	3.3	1.3[b]	6.8	8.9	5.5	3.8
	(0.7)	(0.2)	(0.5)	(0.5)	(0.3)	(0.1)	(0.5)	(1.3)
10	16.4	14.9	3.6	2.04	7.8	8.0	5.0	4.86
	(0.8)	(1.2)	(0.5)	(0.9)	(0.5)	(0.2)	(0.3)	(0.1)

Absolute cell numbers from hematopoietic lineages were calculated from total femoral cell counts and different percentages on the scheduled days of the study. Differential cell determinations were performed counting 500–1000 cells in May Gründwald–Giemsa (MGG) stained bone marrow smears done in triplicates. Results are expressed as mean ± (S.E.M.) × 10^6 cells/femur ($n=3$). [a]$P<0.05$, [b]$P<0.01$ and [c]$P<0.001$ indicate significant differences between control (saline) and paclitaxel injected mice.

was strongly affected. Cell death events induced by the drug injury were noticeable on day 1. Apoptotic cells with characteristic plasma membrane blebbing within a depleted background of hematopoietic cells were noticed. Disruptions of the plasma membrane integrity were assumed as necrotic cellular damages. A drastic reduction of the bone marrow cellularity, a severe decrease of cell–cell and cell–stroma contacts and, therefore, the loss of the optimal microenvironment for hematopoiesis were noticed at this time point of the study. A progressive morphological recovery of the hematopoietic niche was noticed from the fifth day until the end of the study. Representative images at different days of the study are shown in Fig. 3.

3.3. Apoptotic evaluations

Apoptosis was assessed using MGG staining (Fig. 4A), double fluorescent (AO/EB) staining (Fig. 4B) and finally, by TUNEL assay (Fig. 4C) as described in Section 2.4. Percentages of apoptotic cells were obtained by TUNEL, a widely used confirmatory assay for programmed cell death (Fig. 4D). Apoptotic indexes increased noticeably on the first day post paclitaxel injection (24.00 ± 0.50% *vs.* 4.85 ± 0.74%, $P<0.01$). Apoptotic percentages decreased progressively from the second day (12.32 ± 0.70%, $P<0.01$) and returned to control values on day 3 post paclitaxel administration (3.10 ± 0.50%).

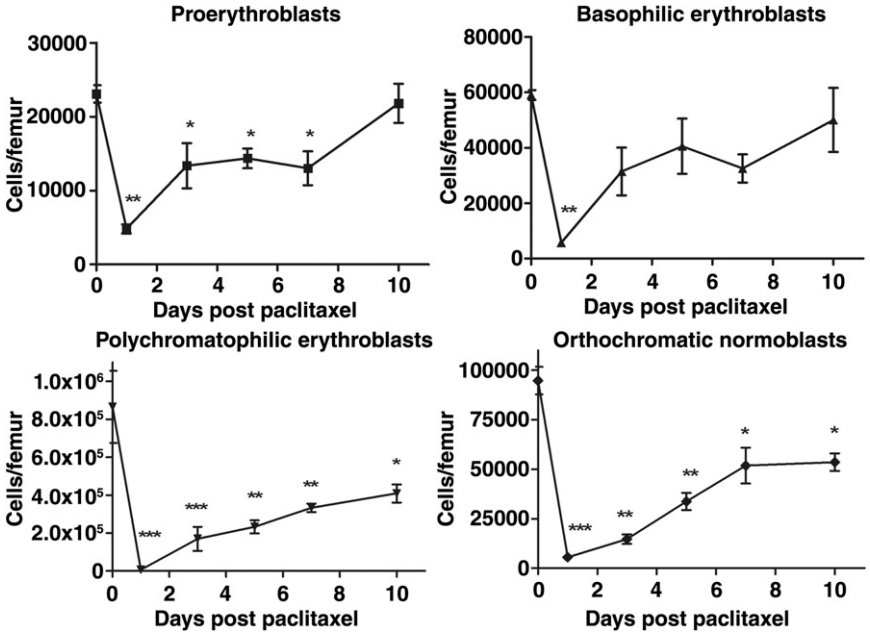

Fig. 2. Changes in erythroid bone marrow precursors after paclitaxel treatment. Erythroid precursors (proerythroblasts, basophilic erythroblasts, polychromatophilic erythroblasts and orthochromatic normoblasts) were morphologically identified following standard criteria at the scheduled times, counting 500–1000 cells in May Grünwald–Giemsa stained bone marrow smears done in triplicate. Data were obtained from three independent assays. Results are represented as mean cells/femur ± S.E M. ($n=3$). *$P<0.05$, **$P<0.01$ and ***$P<0.001$ indicate significant differences between control (saline) and paclitaxel treated mice.

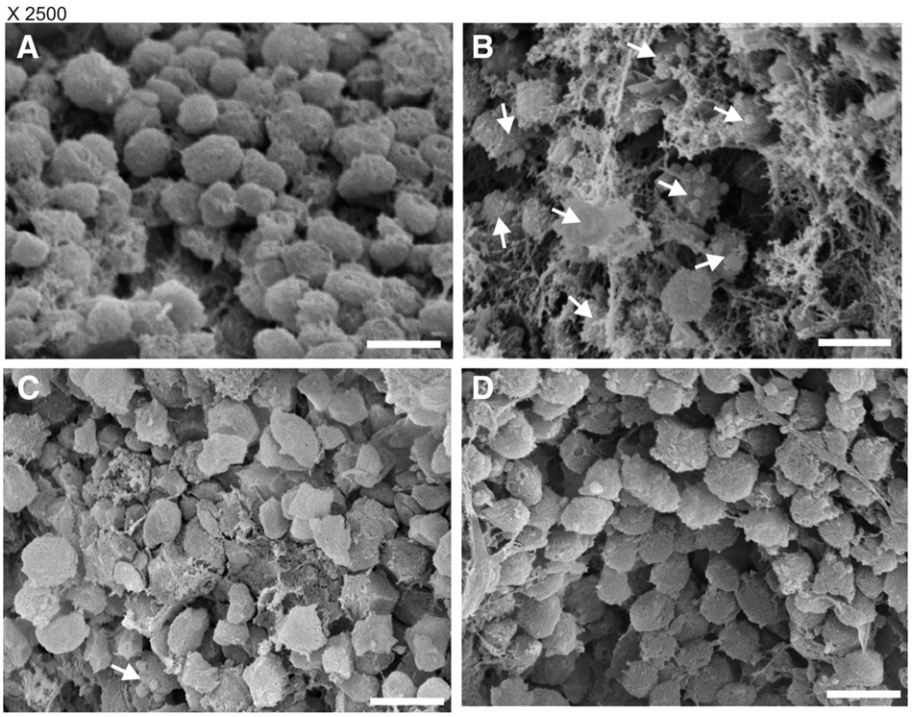

Fig. 3. Effects of paclitaxel on bone marrow architecture. The figure shows representative scanning electron microscopic images of bone marrow samples from (A) control (day 0) and paclitaxel treated mice on 1 (B), 5 (C) and 10 (D) days post drug dosing. Original magnifications are indicated on top of the photograph set (2500×). Depletion of bone marrow cells and loss of cell–cell contact were clearly noticed on the first day (B). Arrows indicate apoptotic cells. An apparent morphological recovery of the bone marrow microenvironment was seen from day 5 (C) to day 10 (D) of the study. Scale bars represent 10 μm length.

Data show coincidence among maximal apoptotic percentages, minimal erythroid cellularities and deep bone marrow structural changes on day 1 post-dosing.

3.4. Radioiron incorporation

Hemoglobin synthesizing cells were monitored through the [^{59}Fe] incorporation in bone marrow cells from control and paclitaxel treated mice.

Radioiron uptake decreased sharply from 1 to 5 days ($P<0.01$). This finding suggests an erythroid maturation interruption in bone marrow compartment. The recovery towards the normality began from day 6 post-dosing as shown in Fig. 5.

Altogether, these results clearly indicate the coexistence of high apoptotic rate, bone marrow microenvironment disruption and reduced maturation of the erythroid cell precursors that contribute to the impaired erythropoiesis post paclitaxel administration.

3.5. Hematopoietic progenitor assays

Clonogenic assays were performed to assess the frequency of bone marrow erythroid progenitors: BFU-E and CFU-E erythroid colony forming units all throughout the study. As shown in Fig. 6, BFU-E and CFU-E colony counts decreased to minimal values on day 1 ($P<0.01$). Both kinds of colonies, increased since day 5 until the end of the study ($P<0.01$). The increment of erythroid progenitor populations (CFU-E and BFU-E) on day 5 preceded the restoration of immature bone marrow erythroid precursors (proerythroblasts and basophilic erythroblasts) at the end of the experience.

3.6. EPO receptor expression

EPO and EPO receptor are crucial to the proliferation, survival and differentiation of CFU-E progenitors into definitive erythrocytes (Jelkmann, 2004). The EPO receptor was quantified by Western blot analysis to study the changes in its bone marrow expression during paclitaxel recovery. EPO receptor was over-expressed from the third day until the end of the experience ($P<0.05$), as shown in Fig. 7.

Interestingly, the over-expression of EPO receptor observed from day 3 was coincident with the end of the apoptotic process and preceded the ulterior recovery of the erythroid colonies from day 5. These results suggest that these concurrent processes play a significant key role in contributing to the erythroid response following paclitaxel injury.

3.7. GATA-1 expression

Transcription factor GATA-1 could act as a survival factor in committed erythroid cells (Cantor and Orkin, 2002). Bone marrow GATA-1 expression in response to paclitaxel was examined by immunoblottings.

As shown in Fig. 8, GATA-1 showed a notable down-regulation on the first day (almost 2 times under control, $P<0.01$). Conversely, GATA-1 was over-expressed from 4 to 10 days ($P<0.01$). The maximal expression of this transcription factor was noticed on the fifth day (2.5 times over control). These findings suggest that under paclitaxel injury, the bone marrow cells expressing small amounts of GATA-1 exhibited arrest of erythroid maturation rates whereas they were forced to enter into the apoptotic pathway.

Conversely, over-expressions of GATA-1 were concurrent with high EPO receptor levels acting as survival factors.

3.8. Erythroid Krüppel-like factor expression

EKLF is an erythroid cell-specific transcription factor required for the activation of erythroid cell-specific genes that are important for hemoglobin metabolism and stabilization of the cells (Hodge et al., 2006).

As shown in Fig. 9 the expression of EKLF was clearly noticed from 5 to 10 days ($P<0.01$) of the experimental schedule. Interestingly, bone marrow exhibited the maximal enhancement of CFU-E progenitors, concomitant with of EKLF expression (correlation CFU-E *vs.* EKLF

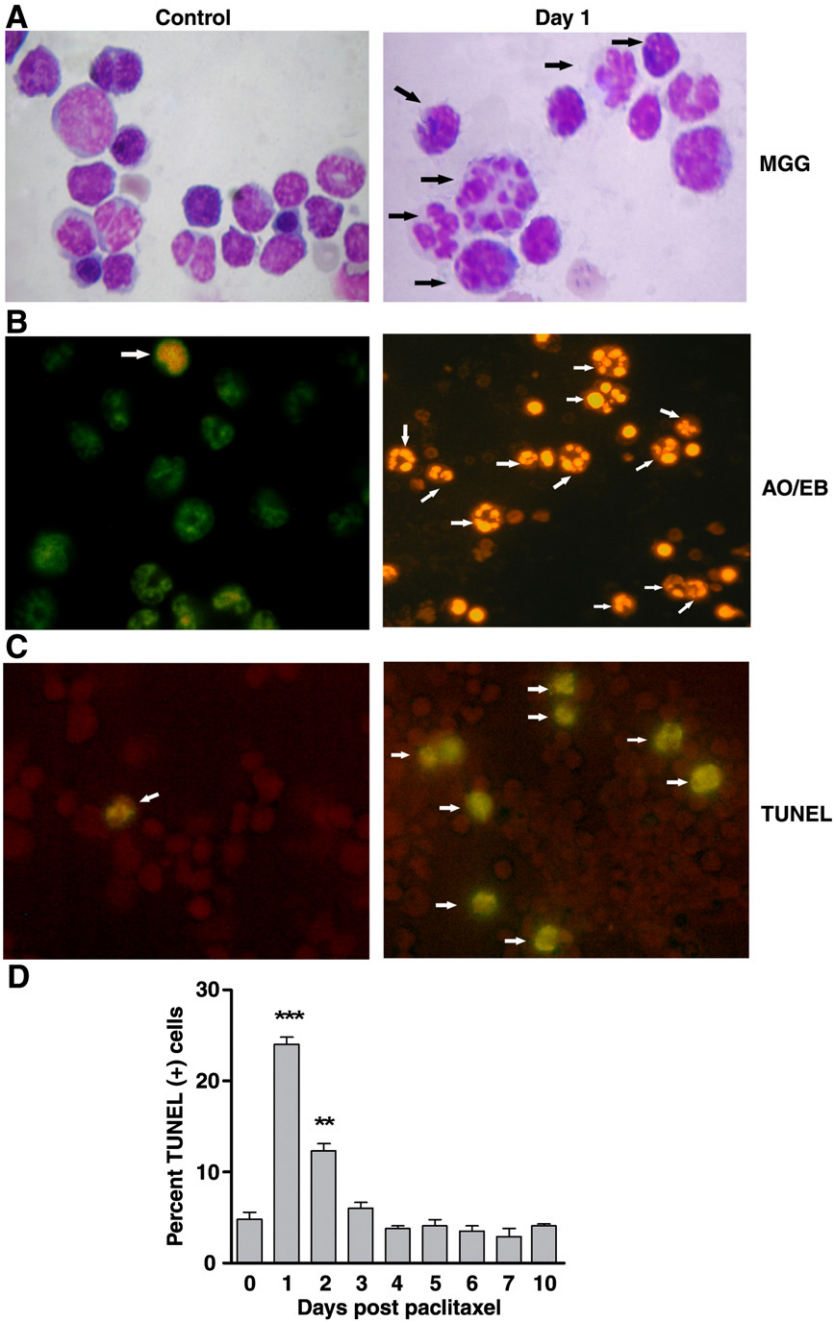

Fig. 4. Apoptosis in murine bone marrow post paclitaxel dosing. The apoptotic biochemical and morphological changes were assessed under light and fluorescence microscopes as well as TUNEL assay. Representative images were obtained from control (day 0) and paclitaxel treated mice 1 day post injection. (A) May Grünwald–Giemsa (MGG) staining of apoptotic cells exhibit body shrinkage, with nuclear condensation and fragmented nuclei. (B) Fluorescence assays with acridine orange and ethidium bromide (AO/EB), show viable cells with homogeneous green fluorescence. In contrast, apoptotic cells show nuclei with irregular bright red fluorescence as a result of chromatin condensation and nuclear fragmentation. Necrotic cells denote uniform red bright fluorescence. (C) TUNEL assay was used as a confirmatory technique to assess bone marrow apoptosis. Nuclei of apoptotic cells were stained positive for green fluorescence. Arrows indicate apoptotic cells in each panel. (D) Percentages of apoptotic cells related to total cells determined by TUNEL assay were represented. Five hundred cells were counted for each sample taken on the scheduled days. Results are expressed as mean ± S.E.M. ($n = 3$). $^{**}P<0.01$ and $^{***}P<0.001$ indicate significant differences between apoptotic percentage differences between control (day 0) and paclitaxel treated mice.

expression, $r = 0.9468$, $P = 0.001$) by the end of the study. EKLF overexpression during the restorative erythropoietic period (5–10 days) might indicate the functional relationship of this factor with the onset of the erythroid differentiation.

3.9. Bcl-x_L and Bax expressions

Bcl-x_L is a member of the Bcl-2 family with antiapoptotic function required for the survival of erythroid cells, especially at the late stage of erythroid differentiation (Gregoli and Boundurant, 1997). Moreover, Bcl-x_L is the major late target of EPO receptor signalling (Dolzing et al., 2001).

On the other hand, damage signals activate pro-apoptotic Bcl-2 family proteins such as Bax and Bak which are required for drug-induced apoptosis (Wei et al., 2001; Zhang et al., 2000).

To gain insight into the roles of Bcl-x_L and Bax in the survival and the apoptotic pathway in bone marrow cells after paclitaxel injury, the expression of these proteins were analyzed by inmunoblottings (Fig. 10).

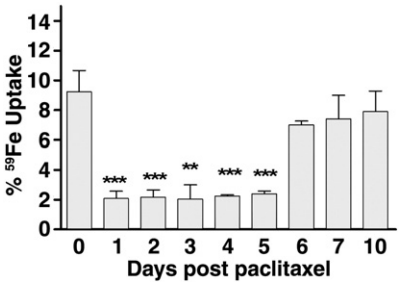

Fig. 5. Radioiron uptake in bone marrow cells post paclitaxel treatment. Percentages of radio iron incorporation in bone marrow were measured at each day of the experimental protocol. Data were obtained from three different assays. Bars represent the mean percentage of [^{59}Fe] uptake ± S.E.M. of the initial dose injected ($n=3$). Isotopic incorporation was significantly reduced from 1 to 5 days post drug administration and returned to basal values from day 6 until the end of the experience. $^{**}P<0.01$ and $^{***}P<0.001$ indicate significant differences between control (day 0) and paclitaxel treated mice.

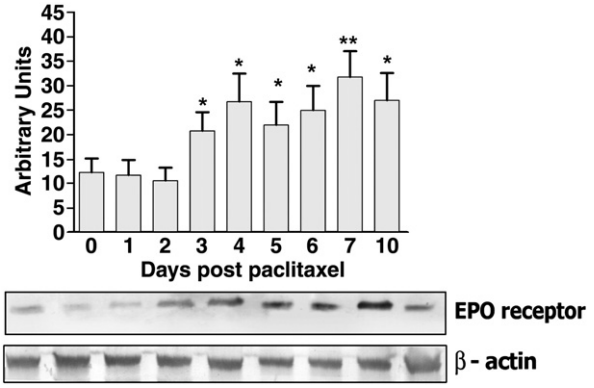

Fig. 7. EPO receptor expression in bone marrow post paclitaxel treatment. Murine bone marrow cells were isolated from drug treated mice at the indicated time points. Cell suspensions were lysed and subjected to immunoblottings. β-actin was used as an internal control. EPO receptor was over-expressed from the third day until the end of the experience. A representative blot of three experiments is shown. Data represent mean arbitrary units ± S.E.M. ($n=6$). $^{*}P<0.05$ and $^{**}P<0.01$ indicate significant differences between control (day 0) and paclitaxel treated mice.

Bcl-x_L was over-expressed from 7 to 10 days ($P<0.01$). Data revealed that Bcl-x_L up-regulation was timely coincident with EPO receptor over-expression ($r=0.8810$, $P=0.001$). Both proteins were necessary to induce the enhancement of early erythroid precursors and the terminal differentiation/survival of erythroid cells. These results strengthen the crucial role of Bcl-x_L in preventing apoptosis in cooperation with EPO receptor as a response to paclitaxel-induced injury.

Conversely, Bax expression was enhanced between 1 and 2 days (1.6 and 1.3 fold above control, $P<0.01$ respectively) and decreased from the fifth day until the end of the experience.

These results suggest that the increased expression of Bax post paclitaxel dosing is required to trigger bone marrow cell death program. A direct correlation between apoptotic indexes *vs.* Bax expression was extremely significant ($r=0.9572$, $P=0.0001$). The lowest counts of erythroid progenitors were noticed concomitantly with Bax over-expression and the decrease of erythroid maturation rate. These experimental evidences were also concurrent with the minimal EPO receptor, GATA-1 and Bcl-x_L expressions. Conversely, the recovery of bone marrow erythroid compartment was coincident with the down-regulation of Bax.

3.10. Caspase-3 expression and enzymatic activity assay

In order to confirm whether pro-caspase 3 was activated during paclitaxel treatment, the cleavages of this precursor were detected by immunoblottings. It is well known that the activation of pro-caspase 3 is indicated by the disappearance of the 32 kDa pro-enzyme form (Chang and Yang, 2000). In this study, control values of the inactive caspase-3 showed a significant decrease ($P<0.01$) from the first to the third day post drug administration (Fig. 11A). Moreover, the cleaved active forms of caspase-3 (20 and 17 kDa) were over-expressed from the first ($P<0.001$) to the third day ($P<0.05$) after drug treatment (Fig. 11B). In addition, the changes of caspase-3 activity in bone marrow cell lysates were assayed using a colorimetric method (Ac-DEVD-*p*NA). Fig. 11C shows an increment of approximately 3 fold increase in caspase-3 activity between the first and second day ($P<0.001$) compared to untreated cells.

These results agree with the increased expression of the cleaved active forms of caspase-3 and the maximal apoptotic period. Hence, as expected, activated-caspase-3 was involved in the apoptotic period (1–2 days) post paclitaxel dosing. In addition, this pattern is coincident with the increment of the apoptotic percentages, Bax over-expression and Bcl-x_L decrease, which might reflect the delicate relations between these proteins during the induction and accomplishment of the apoptotic process.

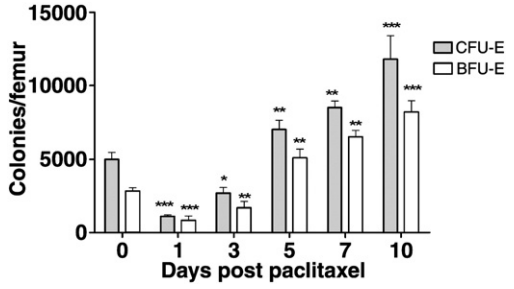

Fig. 6. Erythroid bone marrow colonies post paclitaxel treatment. Single bone marrow cell suspensions (2×10^5 cells/ml) were obtained from drug treated mice at the indicated time points for clonogenic assays. Colonies were scored on the second day (colony-forming-units-erythroid [CFU-E]) and on the seventh day of incubation (burst-forming-unit-erythroid [BFU-E]). Data were presented as the number of colonies per femur (mean ± S.E.M.) from three different assays in triplicate ($n=3$). $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ indicate significant differences between control (day 0) and paclitaxel treated mice.

4. Discussion

The acquisition of red cell phenotype involves the coordinated expression of regulatory and structural proteins acting in concert to

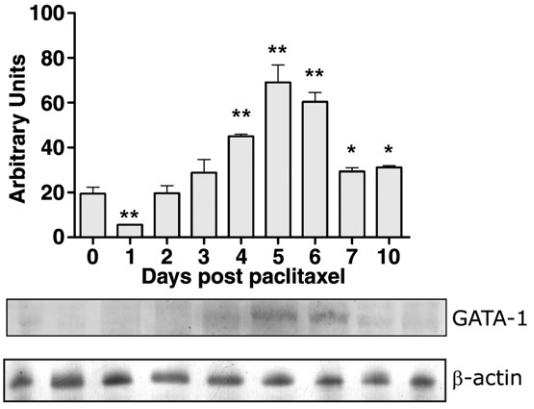

Fig. 8. GATA-1 expression in bone marrow post paclitaxel treatment. Murine bone marrow cells were isolated from drug treated mice at the indicated time points. Cell suspensions were lysed and subjected to immunoblottings. The β-actin was used as an internal control. A representative blot of three experiments is shown. GATA-1 expression decreased significantly on the first day post drug dosing. It was over-expressed at maximal levels on the fifth day of the time course experience. Data are expressed as mean arbitrary units ± S.E.M. ($n=6$). $^{*}P<0.05$ and $^{**}P<0.01$ indicate significant differences between control (day 0) and paclitaxel treated mice.

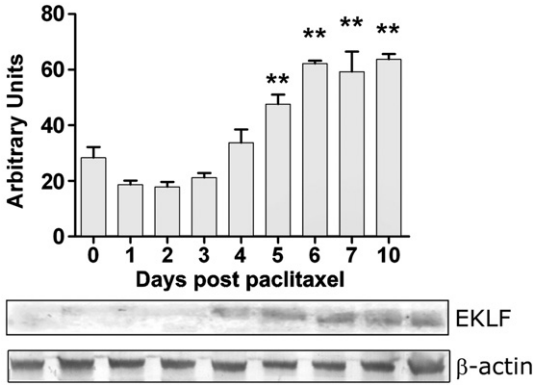

Fig. 9. EKLF expression in bone marrow post paclitaxel treatment. Murine bone marrow cells were isolated from drug treated mice at the indicated time points. Cell suspensions were lysed and subjected to immunoblottings. The β -actin was used as an internal control. A representative blot of three experiments is shown. EKLF exhibited remarkable over-expression from the fifth day until the end of the time course experience. Data are expressed as mean arbitrary units ± S.E.M. ($n = 6$). $^{**}P<0.01$ indicates significant differences between control (day 0) and paclitaxel treated mice.

direct the development of progenitor cells into mature erythrocytes (Koury et al., 2002; Perry and Soreq, 2002).

Most investigations into cell growth, survival and apoptosis have focused on single cells using mammalian cultures. However, the situation in a tissue or in an organ is far more daunting. This report describes in vivo bone marrow interactions between apoptotic events and the expression of EPO receptor, GATA-1 and EKLF following a paclitaxel single administration.

The contribution of the intrinsic cell death pathway in paclitaxel-induced apoptosis has been well documented (Moos and Fitzpatrick, 1998). The mitochondrial pathway is triggered by cell stress, which leads to activation of pro-apoptotic members of the Bcl-2 family (e.g. Bax, Bad, Noxa and Bid). These events, in turn, sequestrate antiapoptotic proteins including Bcl-2 and Bcl-x_L, thereby enabling Bax and Bad oligomerization. Indeed, activated Bax forms pores in the mitochondrial membrane, inducing the release of pro-apoptotic molecules from this organelle, such as cytochrome *c* and apoptosis inducing factor. When cytochrome *c* enters in the cytosol, it is able to

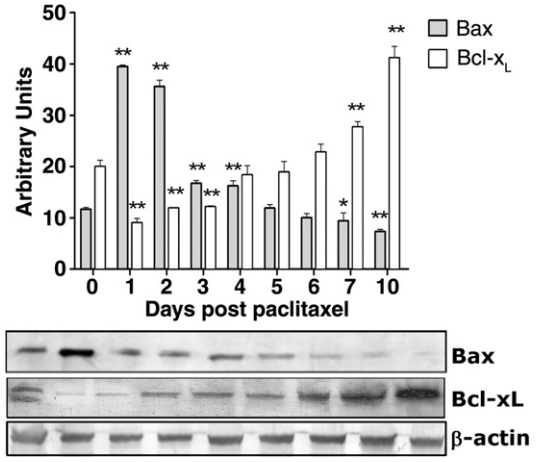

Fig. 10. Bcl-x_L and Bax expressions in bone marrow post paclitaxel treatment. Murine bone marrow cells were isolated from drug treated mice at the indicated time points. Cell suspensions were lysed and subjected to immunoblottings at each time of the experience. The β-actin was used as an internal control. Bax (grey bars) and Bcl-x_L (white bars) expressions are represented along the experience. High ratios Bax/Bcl-x_L were coincident with the maximal percentages of bone marrow apoptosis (1–2 days). Representative blots of three experiments are shown. Data are expressed as mean arbitrary units ± S.E.M. ($n = 6$), $^{*}P<0.05$ and $^{**}P<0.01$ indicate significant differences between control (day 0) and paclitaxel treated mice.

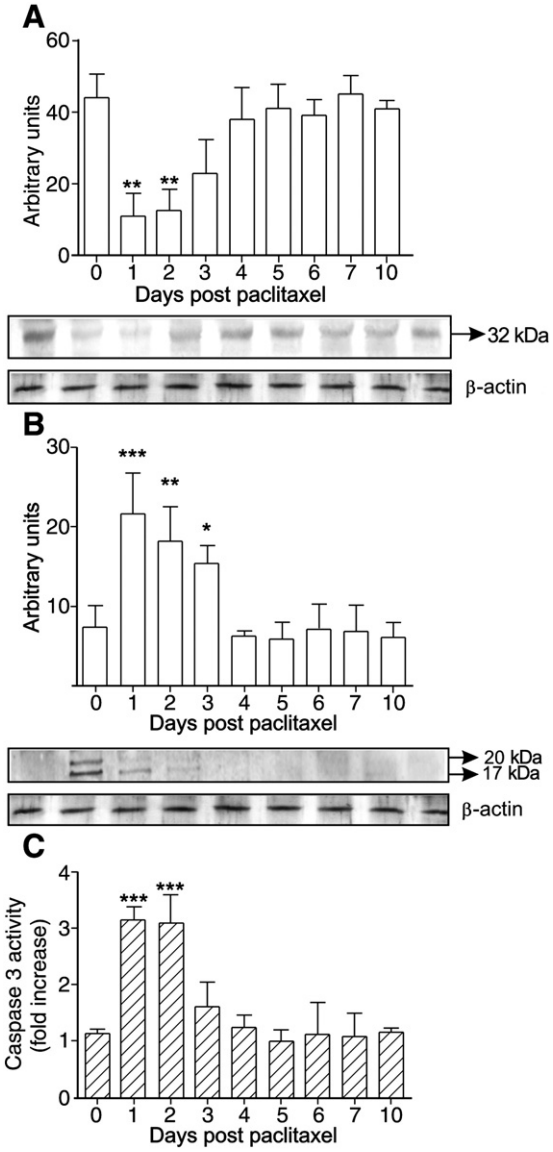

Fig. 11. Caspase-3 expression and caspase-3 activity assay in bone marrow post paclitaxel treatment. Bone marrow cell suspensions were obtained from drug treated mice at the scheduled times. Western blottings from cytosolic extracts were performed for evaluating: (A) uncleaved caspase-3 expression. Cleavages of the inactive form of caspase-3 (32 kDa) were noticed between 1 and 2 days post paclitaxel injection. A subsequent decrease of its expression was noticed at the beginning of the erythroid recovery (5–10 days). (B) Cleaved caspase-3 expression. Over-expressions of the cleaved active forms of caspase-3 (17 kDa and 20 kDa) were noticed concomitantly with the diminution of the inactive caspase-3 expression. The β-actin was used as an internal control in Western blottings. Representative blots of three experiments are shown. Data are expressed as mean arbitrary units ± S.E.M. ($n = 6$), $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ indicate significant differences between control (day 0) and paclitaxel treated mice. (C) Caspase-3 activity assay. Caspase-3 activity was measured with a colorimetric assay kit that relies on caspase-mediated cleavage of p-nitroanilide (*p*NA) from a synthetic caspase substrate peptide (DEVD). The kit was used in accordance with the manufacturer's guidelines. Data are expressed as mean fold induction ± S.E.M. ($n = 6$), $^{***}P<0.001$ significantly different from control group (day 0).

activate caspase 9 and recruit other molecules to form a complex called the apoptosome, which finally activates caspase-3, the most important apoptotic executioner (Elmore, 2007).

The susceptibility of cells to apoptosis is determined in part by the relative concentrations of pro-and antiapoptotic Bcl-2 family members. Immunoblotting analysis showed that Bax/Bcl-x_L ratio, which is associated with the activation of caspase-3, was significantly increased between 1 and 3 days of drug recovery concomitant with the acute apoptotic period. No caspase-3 activity was observed during the next

days of the experience. Even though, it has been reported that caspase-3 is involved in erythroid differentiation under physiological and pathological situations (Zermati et al., 2001, Aispuru et al., 2008). In addition, TUNEL assays revealed a remarkable increment of apoptotic indexes by day 1. These results along with the fluorescent images (AO/EB) and the decrease of the total bone marrow cellularity suggest that paclitaxel induces necrosis as much as apoptosis within the first days after cytotoxic administration in agreement with a previous report (Yeung et al., 1999). Moreover, paclitaxel also induced a decrease of Bcl-x_L expression within this period, which is in line with previously reported observations (Liu and Stein, 2003).

It is well known that the survival of hematopoietic progenitor cells is regulated both positively and negatively by a complex, interacting network of cytokines and adhesion molecules (Janowska-Wieckzorec et al., 2001). The harmful effect of paclitaxel in the bone marrow inner structure was revealed by the cellular depletion and the microenvironment disruption from 1 to 3 days with an apparent recovery from the fifth day. These observations are in accordance with a published study on the effects of several chemotherapeutic agents, including paclitaxel, on mesenchymal stem cells, important components of bone marrow microenvironment for supporting hematopoiesis (Li et al., 2004).

Data show that erythroid committed progenitor colonies (CFU-E and BFU-E) were depleted, EPO receptor expression was down regulated and there was a noticeable decrease in [^{59}Fe] bone marrow uptake, suggesting a blockage in erythropoietic program from 1 to 3 days post drug injection.

Interestingly, several pro-inflammatory cytokines induced by paclitaxel (e.g. tumor necrosis factor α and transforming growth factor β) are negative modulators of erythropoiesis that cause both decrease of EPO production and reduction of hormone bone marrow responsiveness (Jelkmann, 1998). Therefore, the acute apoptotic events observed in this study might be caused by a direct paclitaxel cytotoxicity, the diminution of erythroid progenitor's responsiveness to EPO and/or by a dysfunction of the regulatory processes in bone marrow microenvironment.

Moreover, a recent study on mice revealed that a single higher dose of paclitaxel (40 mg/kg i.p.) induces moderate mitogenic, apoptogenic and mutagenic effects and causes reversible hypoplasia of the bone marrow, with mild hypoplastic anemia, thrombocytopenia and neutropenia (Churin et al., 2008) in accordance with our results. In spite of the different doses used in both studies, the outcomes were similar, probably due to the unlike sensitivities in murine strains. The mild transitory anemia induced by paclitaxel might be attributed to several effects: the decline of the erythropoietic rate, the demise of bone marrow erythropoietic progenitors and the accelerated clearance of circulating erythrocytes by eryptosis (Lang et al., 2006).

EPO receptor expression from day 3 caused the subsequent over-expressions of GATA-1, Bcl-x_L and EKLF, with the further expansion and differentiation of erythroid progenitor cells.

Committed erythroid progenitor cells require exposure to EPO for their survival and for their quantitatively regulated transition to red blood cells. Besides EPO secretion by the kidneys in response to the acute anemic stress as the main control of erythroid recovery, there are complex autocrine/paracrine erythropoietic regulations in bone marrow niche. It has been reported that numerous growth factors, cytokines and chemokines are secreted at physiological concentrations in the bone marrow microenvironment by several cells (Majka et al., 2001) In addition, an endogenous EPO production from mature erythroid progenitors is involved as a part of an intrinsic program of the erythroid development (Sato et al., 2000).

It has been stated that EPO/EPO receptor system triggers signalling cascades leading to the erythroid cell survival (Jelkmann, 2004) at least in part by upregulating the erythroid-specific transcription factor GATA-1 (Zon et al., 1991; Weiss and Orkin, 1995). Moreover, in addition to activating a program of erythroid gene expression (Welch et al., 2004), GATA-1 serves an antiapoptotic function by regulating the expression of Bcl-x_L in erythroid cells (Gregoli and Boundurant, 1997). Furthermore, EPO dependent activation of the STAT5 factor is also involved in the induction of Bcl-x_L expression (Silva et al., 1999; Socolovsky et al., 2001).

In this study, GATA-1 expression drastically diminished on the first day, showing its maximal level by the fifth day. Bcl-x_L expression decreased from 1 to 3 days, and exhibited a striking enhancement from day 7 until the end of the experience, suggesting a coordinated interplay between EPO receptor and GATA-1 effects.

It has been reported that GATA-1 is barely expressed in quiescent erythroid progenitors, but is rapidly induced when the cells are stimulated by EPO to erythroid differentiation. Its expression declines with red blood cell maturation, implying that it is a key factor in the maintenance of the potential erythroid recovery (Cantor and Orkin, 2002). On the other hand, there is clear evidence that, under stress, EPO receptor exerts its effect on a broader progenitor spectrum, extending to early hematopoietic progenitors including hematopoietic stem cells and cells with BFU-E activity (Miyamoto et al., 2002).

In this study, the experimental results support this notion, since the over-expression of GATA-1 observed on day 5 was concurrent with the beginning CFU-E and BFU-E progenitor's expansion. Moreover, it was reported that GATA-1 binds Sp1 and EKLF transcription factors and their DNA binding sites can coreside with GATA motifs (Gregory et al., 1996; Merika and Orkin, 1995).

EKLF was clearly noticed from day 5 until the end of the study, this enhancement was concurrent with the explosion of erythroid compartment and the increment of hemoglobin synthesizing cells. In addition, EKLF over-expression preceded the restitution of bone marrow architecture and the restoration of pronormoblasts and basophilic erythroblasts precursors by the end of the experience. These findings are in accordance with another study about EKLF as a dispensable transcription factor for erythropoiesis up to the proerythroblast stage, but essential for completing the terminal differentiation program (Drissen et al., 2005).

The striking positive correlations observed among the sequential and coordinated over-expressions of EPO receptor, GATA-1, and Bcl-x_L that have been previously reported (Gregory et al., 1999; Aispuru et al., 2008) were confirmed and extended in the current study with EKLF pattern, suggesting the existence of a particular erythroid recovery program in this experimental system.

EKLF location has been extensively described in embryonic tissues, and it was reported to be present solely within the red pulp of the adult mouse spleen (Bieker, 2005). Interestingly, as far as we know, this is the first in vivo report about EKLF expression in bone marrow following a cytoreductive injury.

Collectively, the analysis of experimental data suggests that the impaired erythropoiesis post paclitaxel dosing is associated with a remarkable bone marrow injury through apoptotic and necrotic events within 1 to 2 days of the study, that affect the bone marrow cellularity, the microenvironmental architecture and the erythroid subsets composition, involving a high Bax/Bcl-x_L ratio and caspase-3 activation. Moreover, this time course study provides evidence that EPO receptor expression rules the sequential over-expressions of GATA-1, Bcl-x_L and EKLF; which determine the onset of erythroid bone marrow proliferation and differentiation.

These findings may stimulate the development of new therapeutic approaches to ameliorate bone marrow erythroid impairment after paclitaxel administration.

Acknowledgments

This work was supported by a grant from the SeGCyT-UNNE and by funds from CONICET, Argentina. The authors thank Ms. Mirta Alba Alvarez for her technical assistance.

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