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Erythroid expansion and survival in response to acute anemia stress: The role of EPO receptor, GATA-1, Bcl-x_L and caspase-3

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Abstract

Erythropoietic stress occurs under conditions of tissular hypoxia, such as anemia. Functional relationships between erythroid bone marrow (BM) proliferation, differentiation, the expression of survival and apoptotic related proteins, as well as the features of the BM microenvironment upon acute anemic stress, are not fully elucidated. To achieve this aim, CF-1 Swiss mice were injected with a single dose of 5-fluorouracil (5-FU, 150 mg/kg ip) and a multiparametric analysis was conducted for 20 days.

Apoptosis (TUNEL assay), BM architecture organization (scanning electronic microscopy), proliferation (DNA assay), differentiation (clonogenic cultures), expression of survival erythroid related proteins (EPO-R, GATA-1, Bcl-x_L) as well as the expression of apoptotic- related proteins (Bax, activated Caspase-3) by Western blotting, were evaluated. Experimental data showed that apoptosis, arrest of cell proliferation and disruptions of BM architecture were maximal within the first period of acute stress (1–3 days). Bax and caspase-3 overexpressions were also coincident during this acute period. Moreover, from day 5 upon drug challenge BM responds to acute stress through the EPO-EPO-R system, prompting expressions of GATA-1 and Bcl-x_L. Erythroid proliferation rates and red-cell-committed progenitors enhanced in a coordinated way to restore the size and function of the red cell compartment. A second overexpression wave of active caspase-3 was noticed during stress recovery.

Together, these results indicate that in response to acute stress a dramatic increase in CFU-E (erythroid colony forming units) population is concomitant with upregulation of EPO-R, GATA-1 and Bcl-x_L in the BM erythroid compartment, and that these concurrent processes are crucial for acquiring proper erythroid cell functionality without delayed response to tissular hypoxia.
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1. Introduction

The production of erythroid cells in the bone marrow to maintain the steady-state levels of circulating cells is continuous throughout life. However, due to tissue hypoxia the rate of red-cell production can increase dramatically under certain physiological and clinical conditions, a process known as stress erythropoiesis (Erslev and Beutler, 1995; Ebert and Bunn, 1999).

Erythropoiesis is a complex multistep process encompassing the differentiation of hematopoietic stem cells (HSC) into mature erythrocytes. The stages of the commitment and differentiation processes are numerous and involve the earliest erythroid-committed progenitors corresponding to erythroid burst-forming units (BFU-E), which further differentiate from erythroid colony-forming units (CFU-E) and proerythroblasts into erythroblasts.

These erythroid progenitors (CFU-E), which account for a few populations of hematopoietic cells under basal conditions, have the potential to proliferate rapidly in response to anemia, hypoxia and acute erythropoietic stimuli through erythropoietin (EPO) (Broudy et al., 1996; Bauer et al., 1999; Vannucchi et al., 2001).

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The receptor for erythropoietin (EPO-R), which is expressed abundantly in bone marrow CFU-E stage progenitors and proerythroblasts (Broudy et al., 1991), plays a crucial role in promoting the erythropoietic response (Socolovsky et al., 2001; Li et al., 2003).

EPO-EPO-R triggers signalling cascades leading to the survival, proliferation and differentiation of erythroid progenitors (Jelkmann, 2004), at least in part by upregulating the erythroid-specific transcription factor GATA-1 (Weiss and Orkin, 1995).

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, and cooperates with EPO signalling pathways to promote erythroid cell survival (Silva et al., 1999; Gregoli and Boundurant, 1997; Gregory et al., 1999). Furthermore, EPO-dependent activation of the factor STAT5 is also involved in the induction of Bcl-x_L expression (Silva et al., 1999; Socolovsky et al., 1999).

Following EPO deprivation or engagement of the death receptor Fas, there is activation of the apoptotic machinery, activation of the caspases and cleavage of GATA-1, with the consequent arrest of erythroid cell maturation or apoptotic cell death (Dai and Krantz, 1999; De Maria et al., 1999).

Several lines of evidence indicate that caspases may also display, in addition to a role in the apoptotic process, a function in the differentiation of normal erythroid cells (Zermati et al., 2001; Carlile et al., 2004; Ribeil et al., 2005).

To address physiological and molecular features in different hemopoietic tissues during the recovery of acute and chronic erythroid response, several murine models have been developed (Reissmann and Samorapoompichit, 1968; Hara and Ogawa, 1976; Ou et al., 1980; Koury et al., 1984).

The timing of each transcriptional factor, as well as EPO-R expressions during hematopoiesis, the cell lineages affected and the existing combination of other factors determines the fate of the erythroid cell (Perry and Soreq, 2002). Elucidation of the functional relationship between erythroid progenitors or precursors, regulatory molecules and their microenvironment, as well as their relative contribution *in vivo* in response to erythropoietic stress remains to be established.

In this study, the use of acute secondary anemia induced by the 5-fluorouracil (5-FU) model provided a useful system for the study of erythroid response to acute stress (Rich, 1991), to evaluate *in vivo* bone marrow changes of erythropoiesis over a period of 20 days.

To achieve this aim, a multiparametric analysis of apoptosis, bone marrow architectural organization, proliferation, differentiation and the expression of erythroid-related proteins in acute response were performed. Our results show that CFU-E compartment and EPO-R expression increase dramatically in bone marrow, acting as primary response to acute anemic stress. This finding was followed by induction of GATA-1, Bcl-x_L and caspase-3 coexpressions, as a rate-determining step required for erythroid cell survival. To meet physiological needs, the interplay of these molecules may be a coordinated response to deal with the acute demands of cell expansion in the erythropoietic compartment.

2. Materials and methods

2.1. Animals and drug treatment

Female CF-1 Swiss mice weighing 26–28 g (age: 8–10 weeks) 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 12-h light:12-h dark cycles. They were allowed to free access to pelleted food and water. All experimental procedures were conducted according to the principles in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD, USA, 1996).

Mice were randomly divided into 2 groups. The control group ($n = 40$) received an ip injection of sterile saline solution. The second group ($n = 110$) was injected with a single dose of 5-fluorouracil (150 mg/kg body weight ip) (5-FU; Sigma, St. Louis, MO, USA) to induce secondary acute anemia. The dose used was selected in accordance with the literature (Rich, 1991). At the end of the experimental period (1, 2, 3, 5, 7, 10, 15 and 20 days), animals were anesthetized with pentobarbital (100 mg/kg body weight ip), bled by cardiac puncture and killed by cervical dislocation. Experimental data for the 5-FU injected groups were compared with the control group (day 0).

Recombinant human erythropoietin (hr-EPO) – Hemax 2000, Biosidus, Argentina – was used for *ex vivo* assay on murine cells. Biological cross reactivity EPOs within the class of mammals has been extensively documented (Wen et al., 1993).

2.2. Hematological and bone marrow parameters

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

Total bone marrow (BM) cells ($\times 10^6$/femur) were determined as previously described (Aguirre et al., 2005). Briefly, BM was expelled from the femurs by flushing with 500 μl of phosphate buffer saline (PBS) and suspended in minimal essential medium (MEM, Alpha Modification, Sigma Co, MO, USA) supplemented with 10% of FBS (Genser, Argentina). Red blood cells were removed by lysis in hypotonic solution (0.85% NH_4Cl, 17 mM Tris–Cl pH 7.4) for 5 min on ice. Total BM nucleated cells were counted in a hemocytometer on the scheduled days. Differential cell determinations were performed counting 500 – 1000 cells in May Grünwald–Giemsa (MGG) stained smears and were classified as erythroid, myeloid and lymphoid. Total percentages for each lineage and differential among populations were determined.

Absolute cellularities that made up each lineage in BM samples were calculated according to the percentages and the total cell femoral counts of each animal. Results were expressed as absolute erythroid, myeloid and lymphoid cells $\times 10^6$/femur.

2.3. Scanning electronic microscopy

Direct observation of inner BM architecture in acute anemic response was essentially performed as previously described (Juaristi et al., 2007). Briefly, samples were dehydrated and critical-point dried (Dento Drier). They were coated with gold-palladium for 3 min. Samples were observed with a scanning electronic microscope (Jepl JSM-5800 LV) and images (2500×) were obtained at different times of the experimental study.

2.4. Detection of apoptosis: TUNEL assay

Apoptosis was evaluated in BM cells of control and anemic mice by TdT-mediated dUTP nick end labeling (TUNEL). Briefly, BM smears were obtained as described above and fixed with paraformaldehyde (4% v/v in PBS pH 7.4) for 30 min at room temperature and incubated in a permeabilizing solution (0.1% Triton, 0.1% sodium citrate) for 2 min on ice. The DNA strand breaks that are characteristic of apoptotic cells were identified using the ApoptoTag fluorescein direct *in situ* apoptosis kit (Intergen Co, N Y, USA), according to the manufacturer's instructions (Gavrieli et al., 1992). Apoptotic nuclei were identified using a fluorescence microscope. Nuclei of apoptotic cells were stained positive for green fluorescence, while counterstaining showed red fluorescence with propidium iodide. The percentage of apoptotic cells was calculated from 5 to 10 randomly selected fields on each slide. One hundred cells were counted in each field. A total of 500 cells were counted for every sample taken. Images of the apoptotic cells were collected using Olympus CX-35 microscope equipped with a Y-FL epifluorescence attachment and an Olympus Coolpix Digital Camera.

2.5. Mitotic index and proliferation assays

Mitotic indexes (MI%) were determined in MGG-stained BM smears by typical morphological features; hematopoietic precursor proliferative response was determined as described before (Juaristi et al., 2001). Briefly, BM cells (4×10^5 cells/well) were incubated for 22 h (5% CO_2 in air) in the presence or absence of human EPO (125 mU/ml). Thereafter, BM cells were incubated 2 h with 0.5 μCi ^{3}H-thymidine (1 mCi/ml, 20 Ci/mM, Dupont NEN, USA). The cells were treated according to standard protocols. The extent of ^{3}H-thymidine incorporation was measured in a liquid scintillation counter. Results were obtained as the differences in isotopic uptake between the presence (+EPO) and the absence of erythropoietin (−EPO), and were expressed as mean cpm $\times 10^2$.

2.6. Hematopoietic progenitors cell assays

The frequency of BM hematopoietic progenitor cells (2×10^5 cells /plate) isolated from either normal or anemic mice was conducted as described previously (Romero-Benitez et al., 2004) in standard methylcellulose cultures (1% w/v; Fisher Co, NJ, USA) supplemented with 20% of FBS (Genser) and 2 U/ml of human EPO. Cultures were incubated at 37 °C in a humidified air containing 5% CO_2. Colonies were scored either on the 2nd day (for colony-forming-units-erythroid [CFU-E] derived colonies) or on the 7th day (for the CFU-granulocyte-erythroid-macrophage colony [CFU-GEM] and burst-forming-unit-erythroid [BFU-E]-derived colonies) of incubation. Results were expressed as mean colonies $\times 10^3$ per femur.

2.7. Cell extract preparation and Western blotting analysis

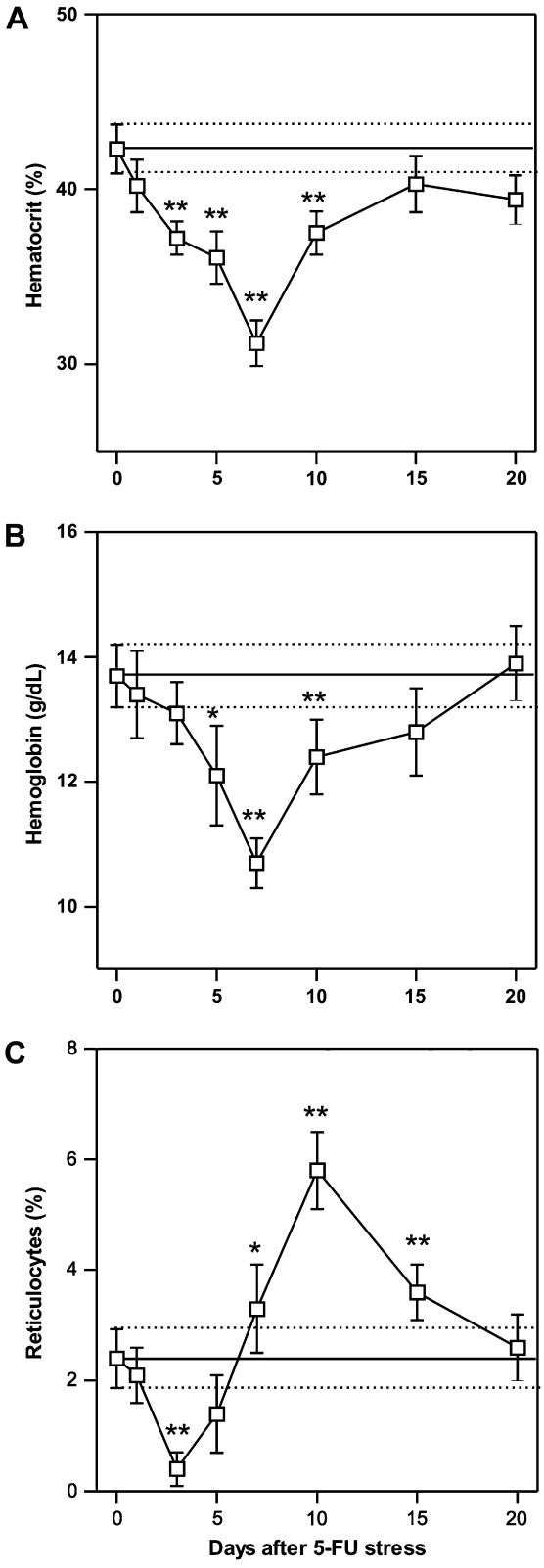
EPO-R, GATA-1, Bcl-x_L and Bax were quantified by Western blotting analysis from whole BM extracts obtained in RIPA buffer (50 mM Tris, 150 mM NaCl, 2.5 mg/ml deoxycholic acid, 1 mM EGTA, 10 μg/ml Nonidet-40 (pH 7.4), supplemented with protease inhibitors: 2.5 μg/ml leupeptin, 0.95 μg/ml aprotinin and 2.5 mM phenylmethylsulfonyl fluoride [PMSF]) as previously described (Aguirre et al., 2005). Cytosolic BM lysates were used for caspase-3 immunoblottings. Briefly, BM 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 (DTT), 0.1% IGEPAL (Sigma Co, MO, USA), 2.5 mM PMSF), supplemented with protease inhibitors − 2.5 μg/ml leupeptin and 0.95 μg/ml aprotinin − 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. Protein concentrations were determined by the Bradford method (Bradford, 1976).

Proteins from whole cell lysates and cytosolic fractions were loaded onto 12% SDS-PAGE (40 μg/well) and transferred to nitrocellulose membranes (Bio-Rad, CA, USA). Membranes were blocked with 5% non-fat dried milk in 0.05% Tris-buffered saline-Tween 20 (TBST). They were incubated overnight at 4 °C with primary antibodies diluted 1:500 for rabbit polyclonal Bax (sc-6236), EPO-R (sc-697), rat monoclonal GATA-1 (sc-265), goat polyclonal Bcl-x_L (sc-7122), and goat polyclonal caspase-3 (sc-1225) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Incubation conditions were optimized for each antibody. Secondary antibodies: IgG goat anti-rabbit (CN 111-035-045), IgG donkey anti-goat (CN 1705-035-147), IgG goat anti-rat (CN 112-035-143) and horseradish peroxidase (HRP) labeled (Jackson Immunoresearch Inc, USA) were diluted 1:2000 in blocking solution and incubated for 2 h at room temperature. Immunocomplexes were detected by an Opti4CN kit (Bio-Rad, CA, USA). Band intensities were quantified using the NIH-image system.

Results were given in arbitrary units (AU) obtained from the ratio between the densitometric units of protein under the study and total μg of protein loaded (Podesta et al., 2000; Juaristi et al., 2007).

2.8. Caspase-3 activity assay

Measurement of caspase-3 activity was done with the commercially available caspase-3 assay kit (Sigma, St. Louis, MO, USA). The caspase-3 colorimetric assay is based on hydrolysis



of the peptide substrate acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide (Ac-DEVD-pNA) by caspase-3, resulting in the release of the *p*-nitroanilide (p-NA) moiety. The p-NA has a high absorbance at 405 nm ($\epsilon^{nm} = 10.5$). Proteolytic reactions were carried out in extraction buffer containing 20 μg of cytosolic protein extract and 40 μM Ac-DEVD-pNA. The reaction mixtures were incubated at room temperature for 2 h, and formation of pNA was measured at 405 nm using a colorimeter. Experiments were performed in triplicate. Caspase-3 activity was calculated as fold increase of untreated control.

2.9. Statistical analysis

Experimental values were expressed as mean ± SEM. Data represent results from at least three separate experiments, each performed in triplicate. Hematological results from treated and control groups were statistically analyzed using a one-way ANOVA test corrected by Bonferroni. Data from all the other experiments were compared to ANOVA 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. Development of acute anemia

Secondary anemia was induced by a single dose of 5-FU (150 mg/kg), and the changes in hematocrit, hemoglobin content and peripheral reticulocytes were monitored over 20 days. Hematocrits decreased significantly between 3 and 10 days ($P < 0.01$) and normality was achieved on day 15 (Fig. 1A). The hemoglobin content exhibited a similar pattern to that of the hematocrit and returned to normal values at the end of the observation period (Fig. 1B).

The reticulocyte profile is shown in Fig. 1C. Reticulocyte counts diminished on the 3rd day and remained low until day 8. On day 10 this parameter was enhanced 2.4 -fold over basal values. Thereafter, reticulocytes declined and returned to normal levels at the end of the observation period. This finding is characteristic of acute erythropoietic stress.

3.2. Bone marrow cellularities and erythroid precursor subsets

As shown in Table 1, BM cellularities decreased drastically to minimal values between 2 and 3 days post-acute anemic

Fig. 1. Peripheral parameters during the acute anemic stress recovery. Hematocrits (%), hemoglobin (g/dl) and reticulocytes (%) were monitored over a 20 day period. (A) Hematocrits and (B) hemoglobin showed maximal decrease on day 7, whereas reticulocyte counts (C) diminish drastically on day 3 post-stress showing a progressive enhancement, maximal on the 10th day. All parameters reached normality at the end of the experiment. Data were obtained from three different assays. Results were expressed as mean ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate significant differences between control and 5-FU treated animals.

Table 1
Bone marrow cellularities following 5-FU dosing

Day of sampling	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)	
	Control	5-FU	Control	5-FU	Control	5-FU	Control	5-FU
1	13.9 (0.8)	5.0** (0.8)	3.2 (0.4)	1.0** (0.4)	6.6 (0.7)	1.9** (0.6)	4.1 (0.5)	2.1** (0.3)
2	13.5 (0.6)	1.4** (0.5)	3.0 (0.2)	0.2** (0.1)	6.7 (0.5)	0.5** (0.3)	3.8 (0.2)	0.7** (0.2)
3	14.7 (0.4)	1.9** (0.9)	2.7 (0.5)	0.3** (0.1)	7.0 (0.6)	0.8** (0.2)	5.0 (0.1)	0.8** (0.4)
5	16.2 (0.7)	4.8** (0.6)	3.6 (0.5)	0.8** (0.3)	7.2 (0.4)	1.9** (0.2)	5.4 (0.4)	2.1** (0.5)
7	15.2 (0.8)	7.7** (0.8)	3.4 (0.4)	1.3** (0.3)	7.8 (0.3)	3.7** (0.3)	4.0 (0.4)	2.7** (0.6)
10	13.9 (1.1)	11.2 (1.2)	3.1 (0.4)	2.0** (0.5)	6.9 (0.6)	5.3 (0.5)	3.9 (0.5)	3.8 (0.3)
15	14.1 (0.9)	12.4 (1.0)	2.8 (0.7)	2.6 (0.4)	6.4 (0.4)	5.8 (0.4)	4.9 (0.6)	3.9 (0.5)
20	15.5 (0.8)	13.9 (0.8)	3.5 (0.6)	2.9 (0.3)	7.6 (0.8)	6.3 (0.6)	4.4 (0.3)	4.7 (0.4)

Absolute cell numbers from hematopoietic lineages in bone marrow were calculated from total femoral cell counts and different percentages at the scheduled times. Differential cell determinations were performed counting 500–1000 cells in May Gründwald–Giemsa (MGG) stained bone marrow smears as described in Section 2. Hematological results were expressed as mean $\times$ 10^6 cells/femur $\pm$SEM. ($n = 4$–6 for controls group and 5-FU treated groups) at each time points of the study for three independent experiments. **$P < 0.01$ indicates significant differences between the control group and the 5-FU treated group.

induction (seven times below control, $P < 0.01$), and returned to the normality on the 10th day. The acute stress is characterized by marked depletions of all bone marrow lineages (erythroid, myeloid and lymphoid), and as was expected the red cell compartment was the most noticeably affected.

Erythroid absolute BM cellularities decreased on the 2nd day almost 16 times compared to control values ($P < 0.01$); meanwhile the myeloid and lymphoid cells diminished 12 and six times, respectively, compared to normal parameters ($P < 0.01$).

Differential absolute cell counts of the erythroid precursor subset revealed that anemic induction affected mature as well as early populations of the erythroid compartment (Fig. 2), throughout a 10 day period of the experimental schedule ($P < 0.01$). At this particular time, proerythroblasts were the only erythroid precursor cells that reached normal values, with a delayed response of basophilic erythroblasts on the 12th day. This increase may be secondary to the proerythroblasts. The lack of significant increase in polychromatic and orthochromatic erythroblasts on day 12 might reflect the time required for erythroid early precursor maturation, and seems to contribute to the accelerated release of late erythroblasts from bone marrow tissue in response to acute anemia.

3.3. Acute anemic stress on bone marrow architecture

To evaluate the effect of acute stress on BM architecture, femurs obtained through the experimental protocol were examined under scanning electronic microscope. The whole BM architecture was strongly affected on the 2nd day and the recovery towards normality began from day 7 following acute anemic induction.

A direct loss of membrane integrity was assumed to be necrosis damage of the cell. Apoptotic cells with characteristic plasma membrane blebbing are shown in a depleted background of hematopoietic cells. This stage shows BM depletion and loss of the optimal microenvironment for hematopoiesis.

The results are in agreement with these considerations, since BM electronic scanning microscopic images clearly show the necrosis/apoptosis induced by 5-FU dosing. Representative images on different days of the experimental study are shown in Fig. 3.

3.4. Apoptotic assay

Apoptotic bone marrow from mice with secondary anemia induced by 5-FU was examined using the TUNEL technique. The number of apoptotic cells increased in a time-dependent manner as shown in Fig. 4 A,B.

Apoptosis was detected from the 1st day. However, apoptosis values increased dramatically on the 2nd day ($9.1 \pm 0.6\%$ *vs.* $19.2 \pm 0.5\%$, $P < 0.01$), showing its maximal levels of seven times over control ($2.0 \pm 0.60\%$). Apoptotic indexes decreased progressively on the 3rd day ($14.7 \pm 0.7\%$, $P < 0.01$) and returned almost to normality on day 7 post-dosing ($3.1 \pm 0.5\%$).

Data show maximal apoptosis with minimal cellularities between days 1 and 2 post-dosing. A direct correlation between BM cellularities and apoptosis experimental data was extremely significant ($r = 0.92$, $P = 0.0001$).

3.5. Proliferation assays and mitotic index

Proliferative capacity was determined by ^{3}H-thymidine incorporation; mitotic indexes were assessed by morphological examination and assayed throughout response to acute anemia.

EPO stimulates cell proliferation on erythropoietic tissue (Brandan et al., 1981). The effect of 5-FU on rate of DNA synthesis was determined by measuring the difference of ^{3}H-thymidine incorporation into BM cells cultures with and without EPO (125 mU/ml).

The proliferation response changed throughout the whole experiment. A remarkable proliferation was observed between the 7th and 10th day (6–10 -fold over the control, $P < 0.01$, respectively), which was preceded by a period of reduced proliferative response (1–5 days).

Afterwards, the proliferative response decreased from the 15th day until the end of the experiment (2.5-fold over the control, $P < 0.05$; Fig. 5A).

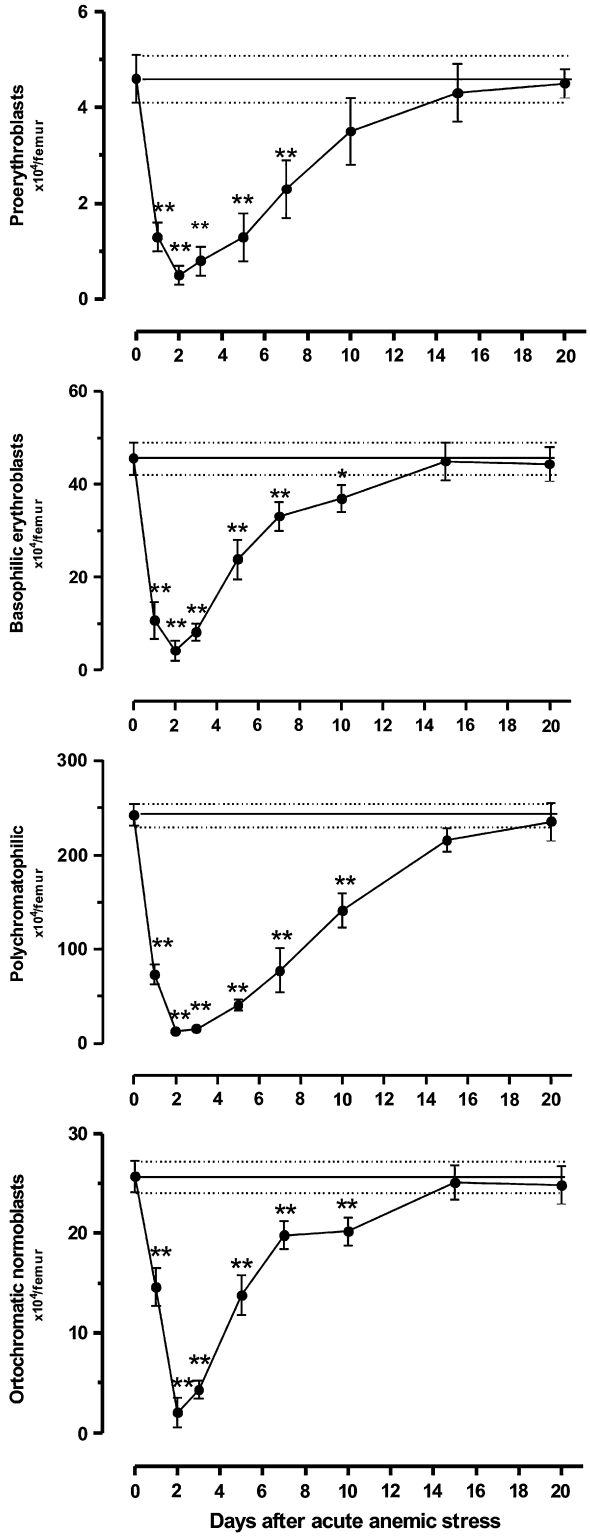


Fig. 2. Changes in erythroid bone marrow precursors after acute anemic stress. Erythroid precursors were morphologically identified following standard criteria at the scheduled times, counting 500–1000 cells in May Gründwald–Giemsa stained bone marrow smears done in triplicate. Cells were classified as proerythroblasts, basophilic erythroblasts, polychromatophilic erythroblasts and orthochromatic normoblasts. Data were obtained from three different assays. Results were represented as mean (SEMs) $\times 10^4$ cells/femur. $^*P < 0.05$ and $^{**}P < 0.01$ indicate significant differences between control groups and the 5-FU treated group.

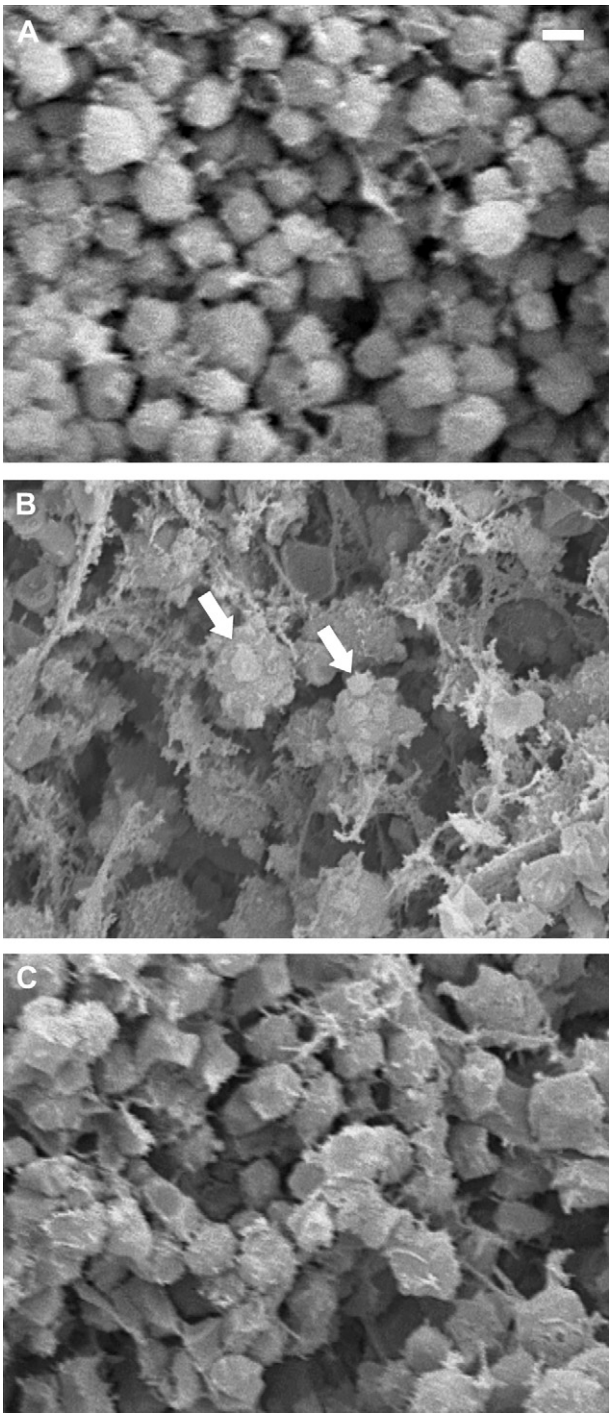


Fig. 3. Bone marrow architecture after stress induced by 5-FU. The figures show representative scanning electron microscopic images (2500×) of bone marrow samples from control (day 0, A) and 5-FU-treated mice on 2 (B) and 7 (C) days. The outstanding features of cytotoxic injury are the cellular depletion and bone marrow architecture alterations. Apoptotic cells with characteristic plasma membrane blebbing are shown on day 2 (arrows). An apparent morphological recovery of the hematopoietic niche was noticed from the 7th day onwards. Scale bar represents 20 μm length.

On the other hand, the BM mitotic index was greatly affected between days 2 and 3 ($P < 0.01$). Seven days after post-anemic induction, mitotic percentages were enhanced two times over control values ($4.2 \pm 0.3\%$ to $1.1 \pm 0.6, P < 0.01$, respectively).

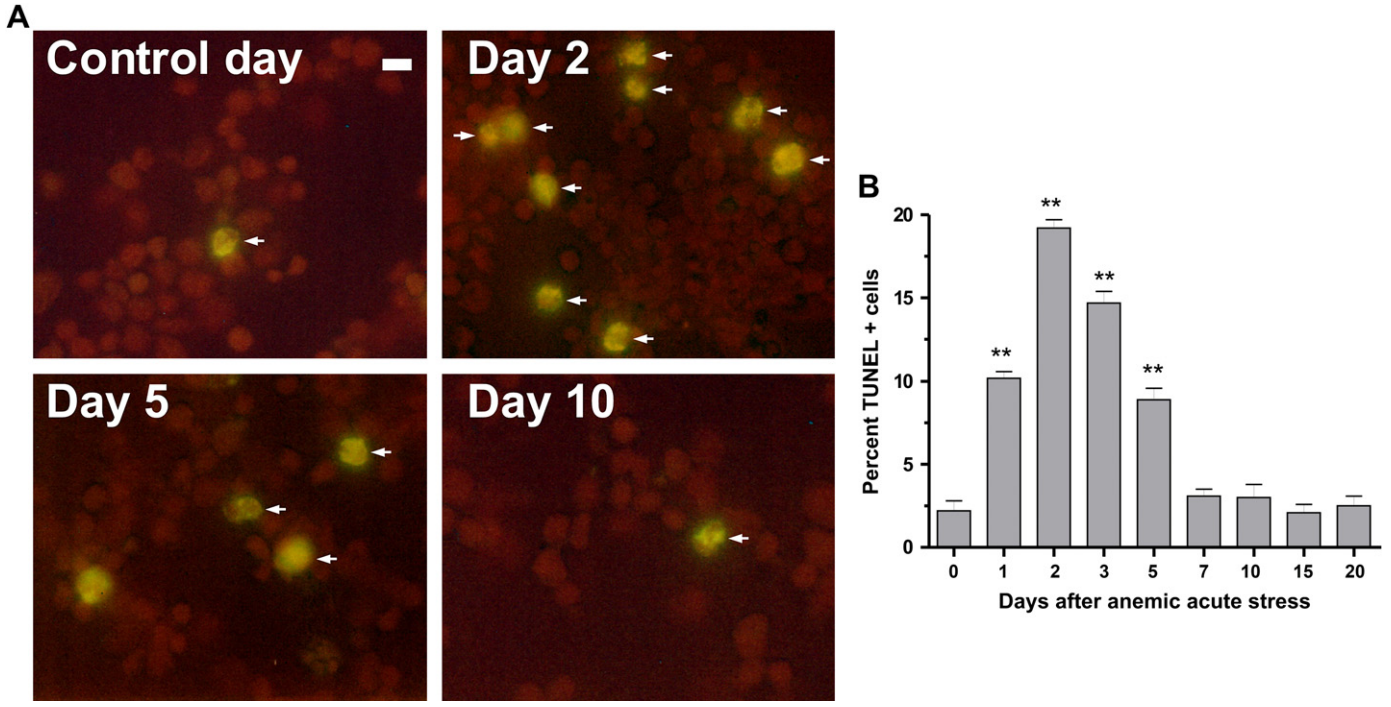


Fig. 4. Bone marrow apoptosis (TUNEL positive cells) after acute anemic stress. Murine bone marrow cells were isolated from control and treated animals and fixed as described in Section 2. The extent of DNA fragmentation in these cells was determined by the TUNEL assay. A total of 500 cells were counted for every sample taken at the scheduled times. (A) Representative images (400 ×) from the control group (day 0) and the 5-FU-treated group (2, 5 and 10 days) are shown. Arrows indicate apoptotic cells. Scale bar represents 30 μm lengths. (B) Percentages of TUNEL positive cells (mean ± SEM) from three different assays in triplicate are represented. $^{**}P < 0.01$ indicates significant differences between apoptotic percentages from the control group (day 0) and the 5-FU treated group.

They were significantly enhanced on the 10th day (6.5 ± 0.5% $P < 0.01$). Thereafter, these values decreased progressively by the end of the study (Fig. 5B). A direct correlation between BM proliferation *vs*. mitotic index was extremely significant ($r = 0.92$; $P = 0.0001$).

Altogether, these results clearly indicate the occurrence of a low mitotic index, high apoptotic rate and reduced cell proliferative EPO response in this erythropoietic situation. Thereafter, the population of BM erythroid cells proliferates vigorously in response to EPO, in order to restore the diminished erythroid pool.

3.6. Hematopoietic progenitor assays

Semisolid methylcellulose cultures were performed to assess the frequency of BM erythroid progenitors throughout the study. In order to evaluate differences in the occurrence of the hematopoietic populations committed to erythropoietic pathways, CFU-GEM, BFU-E and CFU-E erythroid colony forming units were identified. As shown in Fig. 6A, the numbers of CFU-GEM showed great inhibition between the 2nd (seven times less than controls, $P < 0.01$) and 5th days ($P < 0.05$), reaching maximal values on the 10th day ($P < 0.01$). The numbers of BFU-E and CFU-E colonies decreased to minimal values on day 2 (five- and four-times under the control, respectively, $P < 0.01$). The numbers of BFU-E progenitors returned to control values on day 7 ($P < 0.01$) (Fig. 6B), while the number of BFU-E and CFU-E progenitors increased dramatically on the 10th day ($P < 0.01$) (Fig. 6 C).

The increment of erythroid-committed colonies in BM was time coincident (day 10) in the restoration of the early erythroid precursors (proerythroblasts) in response to acute anemia.

3.7. EPO-R expression

EPO and EPO-R are crucial to the proliferation, survival and differentiation of CFU-E progenitors into definitive erythrocytes (Wu et al., 1995; Lin et al., 1996). The EPO-R was quantified by Western blotting analysis to observe changes in its expression in BM cells during acute stress response.

EPO-R was over-expressed from the 7th day until the end of the experiment (between four- and six-times over the control, $P < 0.01$). In addition, the EPO-R expression was more strongly upregulated on the 10th day (six times over the control, $P < 0.01$), as shown in Fig. 7. In contrast, changes on EPO-R expression in the 1st days after anemic induction could not be observed.

Interestingly, the EPO-R upregulation seen on day 10 was time coincident with the significant increase of the absolute number proerythroblast precursors, the highest proliferation rate ($r = 0.085$; $P = 0.001$) and enhancement of the CFU-E progenitors ($r = 0.92$; $P = 0.0001$). These results suggest that these concurrent processes play a significant key role in contributing to the erythroid stress response.

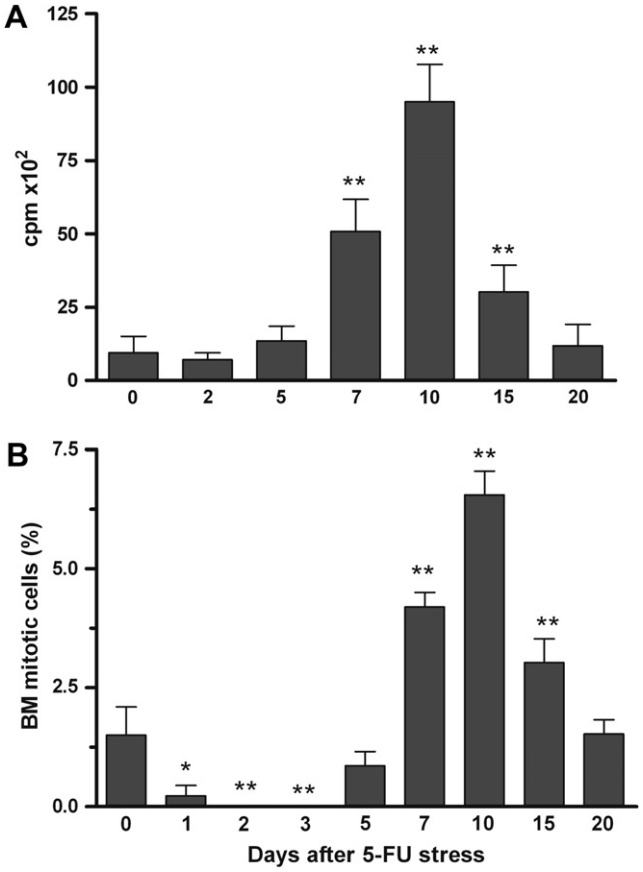


Fig. 5. Bone marrow proliferative assays and mitotic indexes during erythropoietic stress. (A) Murine bone marrow cells were pooled on the scheduled days and ^{3}H-thymidine incorporation was measured. DNA synthesis increased drastically from the 7th day after a period of reduced response (2–5 days post-5-FU). Day 10 showed the maximum proliferation (about 10-fold over control). Proliferative rates decreased from day 15 onwards, returning to basal values by the end of the schedule. Data were obtained as the differences of the isotopic uptake between the presence (+EPO) and the absence of erythropoietin (−EPO). Results were expressed as cpm $\times 10^2$ (mean ± SEM). (B) Mitotic indexes (mean percentage ±SEM) determined by light microscopy in May Gründwald–Giemsa stained bone marrow smears evidenced no dividing cells until the 5th day, followed by a marked increment between days 7 and 15 post-5-FU dosing. Normal percentages of mitotic cells were found by the end of the study. Three different assays in triplicate were performed for both experiences. $^*P < 0.05$ and $^{**}P < 0.01$ indicate significant differences between control group and 5-FU treated group.

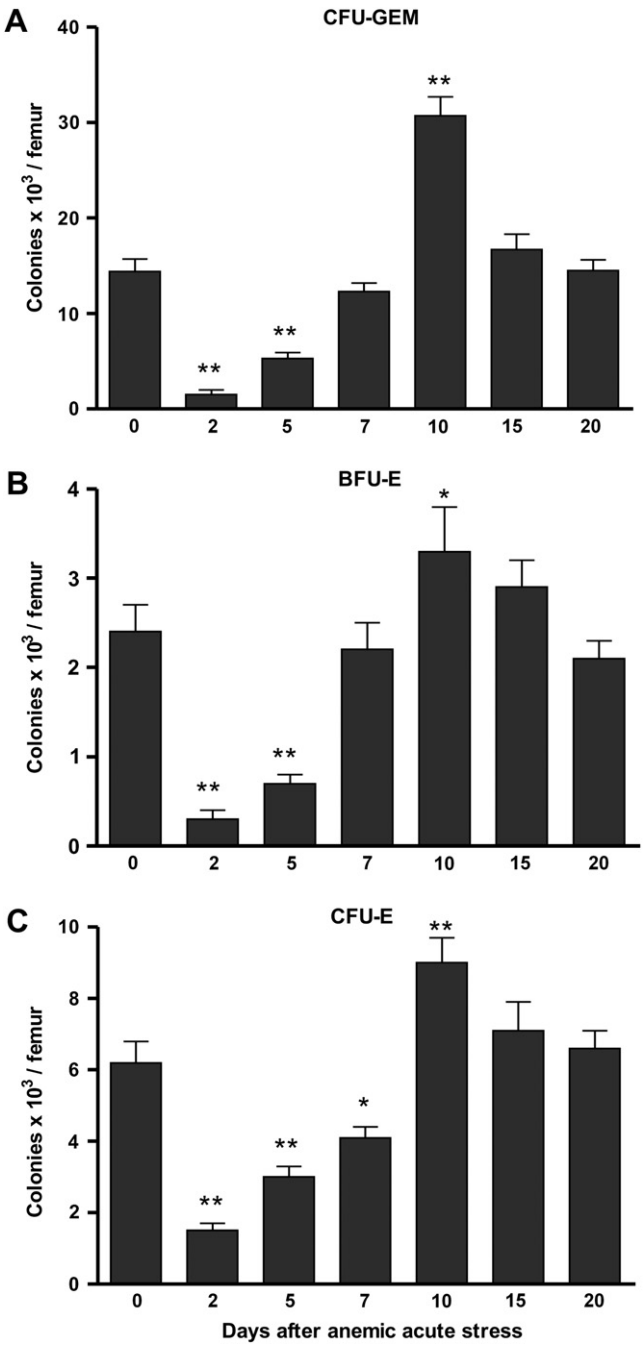


Fig. 6. Analysis of bone marrow erythroid progenitors during acute anemic stress recovery. Single-murine bone marrow cells (2×10^5 cells/ml) were obtained from 5-FU treated mice at the indicated time points and they were cultured as described in Section 2. (A) CFU-GEM and (B) BFU-E colonies were counted on day 7 of incubation. (C) CFU-E colonies were scored on the 2nd day of culture. Data were presented as the number of colonies per femur $\times 10^3$ (mean ± SEM) from three different assays in triplicate. CFU-GEM, BFU-E and CFU-E colonies diminished drastically between the 1st and the 5th day. More immature progenitor colonies (CFU-GEM and BFU-E) gradually enhanced from the 7th day onwards, reaching the maximum scores by day 10. All kind of colonies returned to normality from day 15 of the study. $^*P < 0.05$ and $^{**}P < 0.01$ indicate significant differences between the untreated control group (day 0) and the 5-FU treated group.

3.8. GATA-1 expression

Transcription factor GATA-1 can act as a survival factor in committed erythroid cells (Weiss and Orkin, 1995). BM GATA-1 expression in response to acute anemia was examined by immunoblotting.

GATA-1 was clearly noticed from day 7 to day 15 ($P < 0.01$; Fig. 8). In addition, it was noticed that GATA-1 was strongly overexpressed on the 10th day (2.5 times the control); thereafter this expression fell to control level. In contrast, GATA-1 showed a strong downregulation between 1 and 3 days (∼2 times less than the control, $P < 0.01$). This result shows that after *in vivo* anemic induction, BM cells expressing small amounts of GATA-1 exhibit reduced proliferation rates

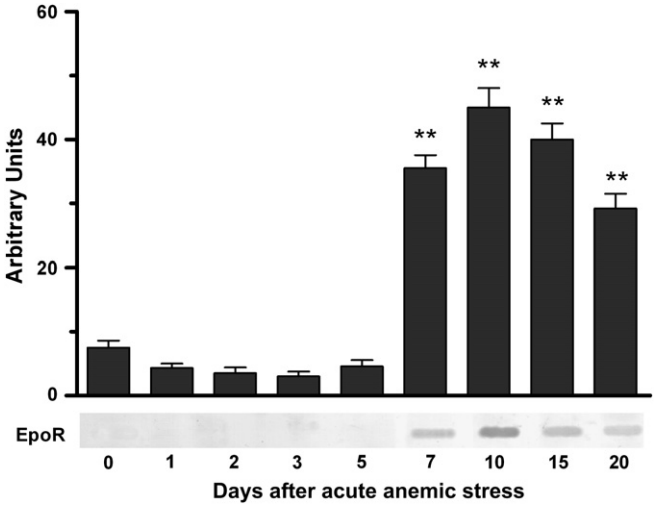


Fig. 7. EPO-R expression in bone marrow through stress response. Murine bone marrow cells were isolated from 5-FU treated mice at the indicated time points. Cell suspensions were lysed and subject to Western blotting analyses as described in Section 2. A representative blot of three independent experiments is shown. EPO-R was overexpressed from the 7th day until the end of the experience. Results represent mean ± SEM of three mice per group. $^{**}P < 0.01$ indicates significant differences between protein expressions from the control group (day 0) and the 5-FU treated groups.

and are introduced into the apoptotic pathway. In contrast, up-regulations of GATA-1 are concurrent with EPO-R expression acting as survival factors.

3.9. Bcl-x_L expression

Members of the Bcl-2 family, such as Bcl-2, Bcl-x_L and Mcl-1, function as antiapoptotic proteins (Adams and Cory, 1998). Among these, Bcl-x_L is required for the survival and normal maturation of erythroid cells, especially at the late stage of erythroid differentiation (Gregoli et al., 1997; Motoyama et al., 1999). Moreover, Bcl-x_L is the major late target of EPO-R signaling (Dolzing et al., 2001).

To determine the survival profile of BM cells during acute response, Bcl-x_L expression by Western blotting was studied (Fig. 9A). This Bcl-2 member was upregulated from the 5th day until the end of the experiment. In addition, it was noticed that Bcl-x_L was strongly overexpressed on the 10th day (12.1- fold over the control, $P < 0.01$). Data collected revealed that Bcl-x_L upregulation was time coincident with EPO-R ($r = 0.8810$, $P < 0.001$) and GATA-1 ($r = 0.9429$, $P < 0.001$) expression, and they were necessary to induce the enhancement of early erythroid precursors and the terminal

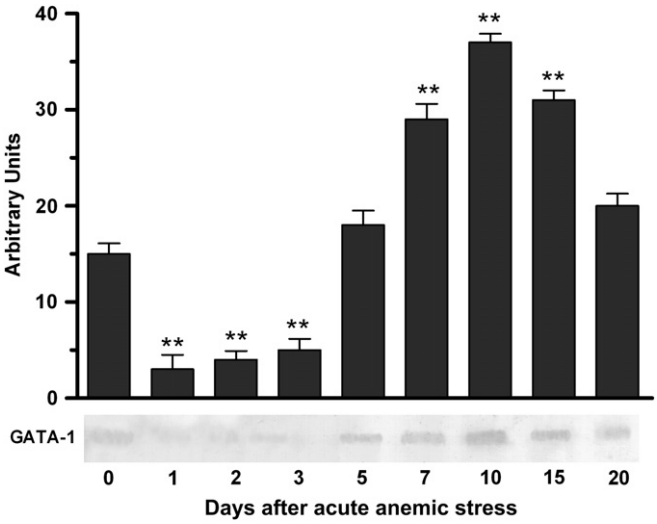


Fig. 8. GATA-1 expression during acute anemic stress. Murine bone marrow cells were isolated from 5-FU treated mice at the indicated time points. Cell suspensions were lysed and subject to Western blotting analyses as described in Section 2. A representative blot of 3 independent experiments is shown. GATA-1 decreased significantly from days 1 to 3 followed by over expression between the 7th and the 15th day. Results represent mean ± SEM of 3 mice per group. $^{**}P < 0.01$ indicates significant differences between protein expressions from the control group (day 0) and the 5-FU treated group.

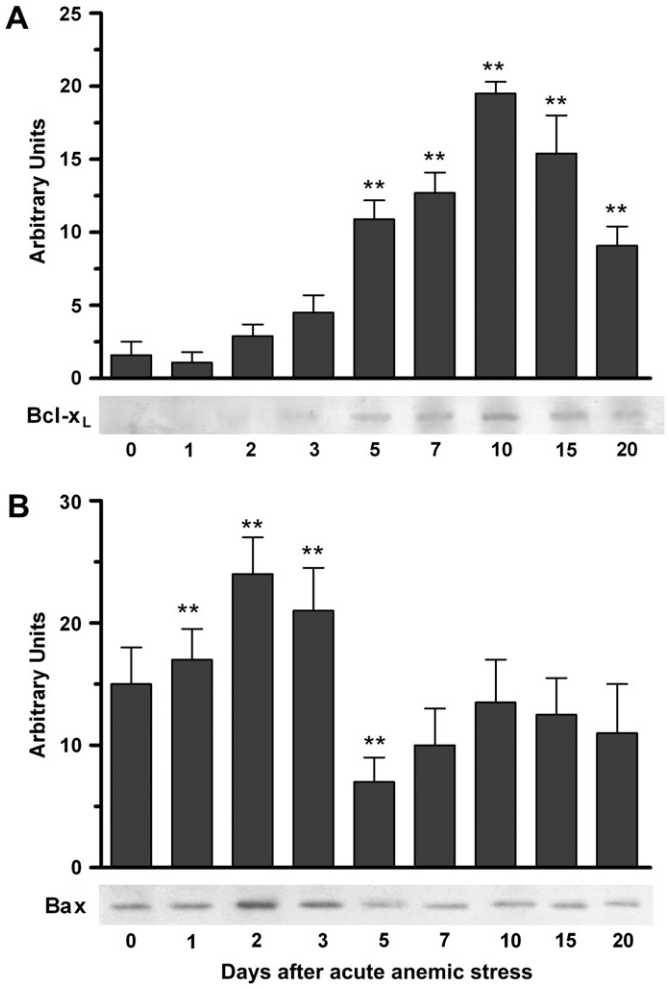


Fig. 9. Bax and Bcl-x_L expressions during acute anemic stress. Murine bone marrow cells were isolated from 5-FU treated mice at the indicated time points. Cell suspensions were lysed and subject to Western blotting analyses as described in Section 2. Representative blots of 3 independent experiments are shown. (A) Bcl-x_L expression was noticeable from day 5 post-dosing until the end of the experience. (B) Bax expression showed overexpression in the acute period of 5-FU stress induction (from days 1 to 3). Results represent mean ± SEM of 3 mice per group. $^{**}P < 0.01$ indicates significant differences between protein expressions from the control group (day 0) and the 5-FU treated group.

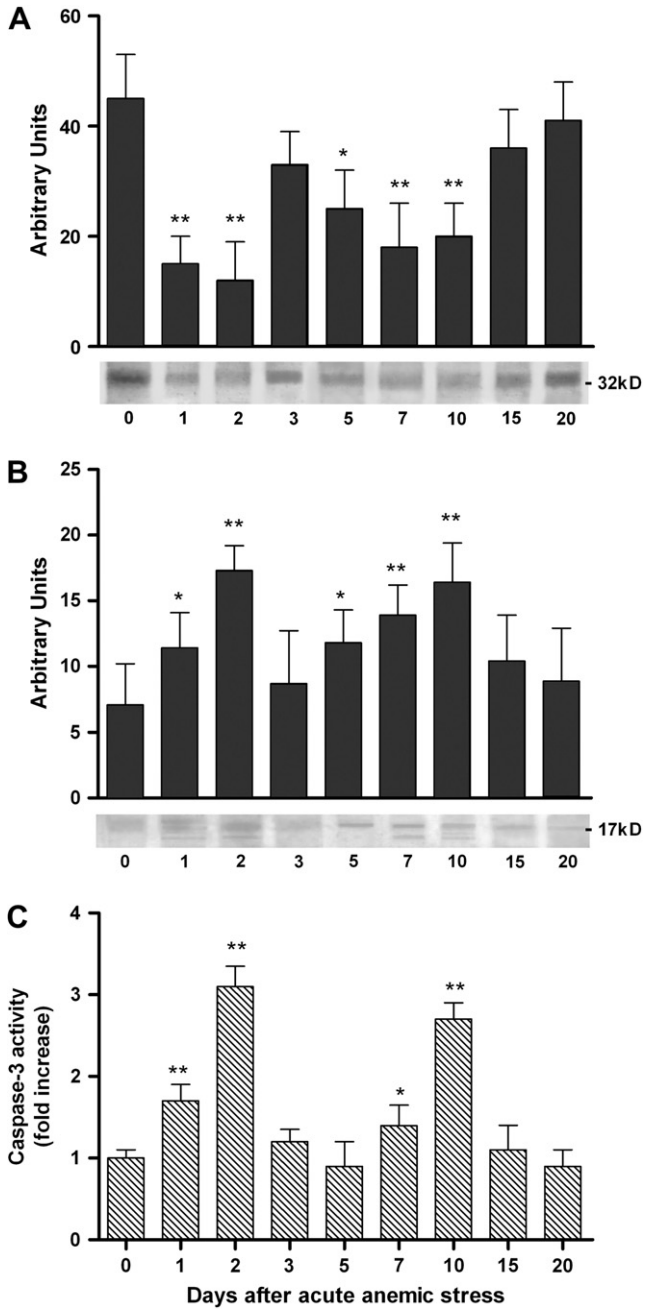


Fig. 10. Caspase-3 expression and enzyme activity in bone marrow during acute anemic stress. Bone marrows were obtained from 5-FU treated mice at the scheduled times, cell suspensions were lysed. Western blottings and caspase-3 activity assay were performed as described in Section 2. (A) Uncleaved caspase-3 expression. Cleavages of the inactive form of caspase-3 (32 kD) were noticed between 1 and 2 days post 5-FU injection. A further 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 kD) were noticed concomitantly with the diminution of the inactive caspase-3 expression. Representative blots of three independent experiments are shown. $*P < 0.05$ and $**P < 0.01$ indicate significant differences between protein expressions from the control group (day 0) and the 5-FU treated group. (C) Caspase-3 activity assay. Caspase 3 activity was measured with a colorimetric assay kit that relies on caspase-mediated cleavage of *p*-nitroanilide (pNA) from a synthetic caspase substrate peptide (DEVD). The kit was used in accordance with the manufacturer's guidelines (data points represent mean fold induction ±SEM). $*P < 0.05$ and $**P < 0.01$ significantly different from control group (day 0).

differentiation/survival of the erythroid cells. These results strengthen the crucial role of Bcl-x_L in BM erythroid cells and may be critical in preventing apoptosis in cooperation with EPO-R and GATA-1 in response to acute anemia.

3.10. Bax expression

Damage signals activate the proapoptotic Bcl-2 family proteins, such as Bax and Bak which are required for drug-induced apoptosis (Wei et al., 2001).

The participation of Bax in the apoptotic pathway in BM cell after stress induction was analyzed by Western blotting. The expression of this proapoptotic protein was incremented between the 1st and the 3rd days (1.6- and 1.5 -fold over the control, $P < 0.01$ respectively). In contrast, its expression fell below that of the control ($P < 0.01$) from 5th day until the last day of the experiment (Fig. 9B).

These results suggest that increased expression of Bax after anemic induction is required to trigger BM cell death program, in agreement with reduced proliferation and low expression of EPO-R, GATA-1 and Bcl-x_L. This process is concomitant with the low number of erythroid progenitors. In contrast, downregulation of this proapoptotic protein was accompanied by a bone marrow erythroid response.

3.11. Caspase-3 expression and activity assay

Caspases, a family of cysteine proteases, are critical for programmed cell death (Budihardjo et al., 1999; Fadeel et al., 2000). Several studies suggest that caspase-3 may also function in erythroid differentiation and maturation (Zermati et al., 2001; Carlile et al., 2004).

To determine the involvement of caspase-3 in bone marrow upon acute anemic erythropoiesis, caspase-3 immunoblottings and an enzymatic activity assay were performed. The activation of the caspase-3 was indicated by the disappearance of the 32 kD pro-enzyme form (Zhang et al., 2000). Control values of inactive caspase-3 (45.2 ± 4.1) showed a remarkable decrease from the 1st (15.3 ± 5.2, $P < 0.01$) to 2nd day (12.0 ± 6.5, $P < 0.01$), as shown in Fig. 10A. Moreover, the cleaved active forms of caspase-3 (17 kD) were overexpressed between the 1st and the 2nd day (11.4 ± 2.7 and 17.3 ± 1.9, $P < 0.01$), coincident with apoptosis experimental data. A direct correlation between apoptosis *vs*. cleaved caspase-3 expression was extremely significant ($r = 0.9572$, $P = 0.0001$).

Interestingly, an unexpected overexpression of activated caspase-3 was noticed between days 5 and 10 (from 11.8 ± 2.5 to 16.4 ± 3.0, $P < 0.05$ and $P < 0.01$, respectively; Fig. 10B).

Changes in caspase-3 activity in BM cell lysates were assayed using a colorimetric method (Ac-DEVD-pNA). Fig. 10C shows a 2.4 -fold increase in caspase-3 activity between the 1st and 2nd day ($P < 0.01$) compared to untreated cells. These results are in agreement with the increment of the cleaved active form (17 kD) and the apoptotic process. Hence, as expected following acute anemic induction, activated-caspase-3 was involved in the apoptotic period (1–2 days).

Furthermore, caspase-3 activity increased ~2 -fold between 7 and 10 days ($P < 0.05$ and $P < 0.01$, respectively), concomitant with the cleaved active caspase-3 overexpression. However, cell death enhancement failed to be detected in this period.

On day 10, BM exhibited the maximal proliferation (correlation between proliferation *vs.* caspase-3 expression: $r = 0.8716$, $P = 0.001$), enhancement of CFU-E progenitors (correlation CFU-E *vs.* activated caspase-3 expression: $r = 0.9468$, $P = 0.001$), concomitant with the highest expressions of EPO-R and GATA-1.

Thereafter, the protease overexpression and the increment of caspase-3 activity during the restorative erythropoietic phase (7–10 days) might link this protein to the onset of the erythroid differentiation.

Thus, the results obtained are in accordance with other reports; we propose that activated caspase-3 expression appears to play a key role in the maintenance of an adequate CFU-E differentiating pool for optimal acute erythroid response.

4. Discussion

Bone marrow erythropoiesis is a highly efficient system that tunes the rate of erythropoietic production to supply physiologic needs. A well-established *in vivo* experimental model of acute secondary anemia induced by 5-FU was used (Rich, 1991; Takatsuki et al., 1996), in order to study some molecular functions that are modified specifically within the erythroid bone marrow compartment as a result of stress signalling.

This study revealed that, in bone marrow cells, the acute stress response corresponds to the emergence of a particular relationship between the expression of some erythroid lineage-related and antiapoptotic proteins. Moreover, the intracellular targets in bone marrow of EPO-R, such as GATA-1 and Bcl-x_L, are unknown during acute anemia response, thus the targets in which they act are not fully defined.

The immediate effect after acute anemic induction is a decrease in hemoglobin, hematocrit and reticulocyte counts, as well as depletion of bone marrow cellularity. This massive reduction of hematopoietic cell progenitors is concomitant with a reduction in the number of mature erythroid precursors.

This quantitative deficit of red cells clearly decreases the rate at which they are produced, thus resulting in a severe anemia. This process is followed by the ability of erythroid cells compartment to generate high erythropoietic rates in response to stress. Once secondary acute anemia induced by 5-FU is established, the system triggers a compensatory erythropoietic response through serum EPO level increments.

In post-anemic induction, EPO levels increase progressively from the 2nd day, reaching 100 -fold basal values on the 10th day (Rich, 1991; Gao et al., 2004). As a consequence, EPO promotes recovery from anemia by acting on erythroid progenitors/early erythroid precursors in bone marrow, stimulating them to increase the rate at which red cells are generated.

On day 10, an increase in proerythroblasts and basophilic erythroblasts – to a lesser extent – in response to anemia induction was observed, while on day 15 the number of polychromatic and orthochromatic normoblasts reached normal level. This substantial increase in erythroid precursors presumably reflects a compensatory response to anemia, driven in part by the raised serum EPO.

It is well known that interactions among erythroid progenitors and their microenvironment play critical roles in promoting acute erythropoietic responses (Lenox et al., 2005; Scott et al., 2003). The most direct and dramatic consequence of acute response induced by 5-FU is the loss of cycling hematopoietic progenitor cells within the bone marrow, following an altered homing pattern that affects the proper microenvironment (Morrison et al., 1996; Randall and Weissman, 1997).

In accordance with these studies, bone marrow electronic scanning microscopic images clearly showed the architectural disruption of the microenvironment interactions as well as necrotic/apoptotic cells. This process was maximal on the 2nd day as a consequence of the abrupt loss of most of the hematopoietic precursors. Bone marrow architecture returned to normality from day 7 onwards, providing an adequate erythropoietic niche.

Therefore, the increment of apoptosis was observed between days 1 and 5 following acute stress, with maximal values on day 2. This process was concomitant with a low mitotic index and a large decay in bone marrow cellularities, particularly in the erythroid lineage. Our observations were concurrent with the reduced magnitude response in proliferation assays, and the growth inhibition of the multipotent progenitors CFU-GEM, BFU-E, and CFU-E between days 2 and 3 following acute anemia.

However, the bone marrow erythroid compartment displayed different proliferative and differentiation potentials during stress erythropoiesis. The number of BFU-Es was restored to normal levels on the 7th day, while CFU-E population had already expanded dramatically over the control by day 10, coincident with previous findings (Socolovsky et al., 2001; Li et al., 2003).

This fact might indicate that under stress BFU-E/CFU-E progenitors increase selectively for prompt erythroid restitution. In addition, CFU-E amplification occurs within the bone marrow compartment, either by acquisition of proliferative potential or by increased differentiation of the expanded BFU-E population, giving adequate erythroid output in these emergency conditions.

These findings could be explained in view of the role of EPO-EPO-R required for the proliferation and cell survival *in vitro* (Koury and Boundurant, 1990; Lin et al., 1996), suggesting a similar function for CFU-E expansion *in vivo* during the stress acute response in agreement to previous reports (Gregory et al., 1974; Peschle et al., 1977; Koury and Boundurant, 1990). In this case, the early erythroblast progeny of CFU-E express EPO-R, and is a potential EPO target during erythropoietic stimuli (Wickrema et al., 1991; Broudy et al., 1991).

EPO-EPO-R signaling results in the survival, proliferation, and differentiation of erythroid progenitors (Jelkmann, 2004). Like EPO and EPO-R, GATA-1 serves an antiapoptotic function by regulating the expression of Bcl-x_L required for erythroid cell survival (Gregory et al., 1999).

Our studies reveal that after induction of acute stress, reduced bone marrow erythroid progenitors/precursors are concomitant with increased expression of proapoptotic proteins such as Bax and activated caspase-3, in agreement with a reduced proliferation, higher apoptotic rate and downregulation of EPO-R, GATA-1 and Bcl-x_L. Furthermore, the absence of an EPO-R-Bcl-x_L pathway implies activation of the apoptotic pathway, leading to increased destruction of erythroid precursors, responsible for the development of anemia. After this acute stress, we observed that the maximum number of erythroid progenitors were rescued by simultaneous activation of EPO-R signalling and GATA-1, which cooperate to induce the antiapoptotic Bcl-x_L.

Thus, expressions of these proteins are involved in cell survival and proliferation of bone marrow progenitor cells with erythroid differentiation potential during the stress response. The striking positive correlation observed among EPO-R, GATA-1, and Bcl-x_L are in agreement with a previous report (Gregory et al., 1999): this suggests that a particular intrinsic recovery program is suitable in bone marrow erythroid cells.

EPO-R expression was upregulated from day 7 and maximal at day 10. Thereafter, it was over-expressed through the entirely experiment. These results are in agreement with erythroid progenitor proliferation followed by a dramatic expansion of CFU-E compartment in order to restore the different subsets of erythroid precursor cells.

Moreover, the enhanced GEM, BFU-E and CFU-E colonies growth reflected strong erythroid amplification in response to EPO levels and EPO-R upregulation. There is clear evidence that, under stress, EPO-R exerts its effect on a broader progenitor spectrum, extending to early hematopoietic progenitors including HSC and cells with BFU-E activity (Miyamoto et al., 2002; Forsberg et al., 2006).

Our findings suggest that the requirement for EPO-R signaling may be more stringent during acute stress than during basal erythropoiesis, playing a principal role during stress response *in vivo*, in agreement with a previous report by Socolovsky (2007).

The present results clearly show the coexistence of increased expressions of EPO-R, GATA-1, and Bcl-x_L from the 7th day onwards with their maximal overexpression on day 10. They remained expressed over control group levels until the last day of the experience, except GATA-1, which is essential for mature erythroid cell functions, which returned to control values by day 15.

Our findings agree with a previous *in vitro* communications about the role of GATA-1 in erythroid proliferation and differentiation by different mechanisms (Zheng et al., 2006).

Moreover, at this particular time, total erythroid precursors reached almost the control values while the numbers of CFU-E increased dramatically with the high proliferative/differential potential of erythroid-committed progenitors. These results strengthen the crucial role of EPO-R, GATA-1 and Bcl-x_L in bone marrow erythroid cell response under acute stress.

In response to anemia, activated caspase-3 is involved in the apoptotic pathway. In contrast, during the late stage of erythroid differentiation caspase-3 activation neither GATA-1 down regulation nor apoptosis was observed. The positive correlation between CFU-E population and caspase-3 activation seems to play a crucial role in maintaining an adequate pool of CFU-E under acute stress erythropoiesis, encompassing CFU-E differentiation to proerythroblasts. These observations fully agree with previous findings (Ribeil et al., 2005; Krauss et al., 2005), and might represent a new insight for *in vivo* caspase-3 activation to erythropoietic stress response.

In summary, our data reveal that a dynamic induction of EPO-R, GATA-1 and the antiapoptotic protein Bcl-x_L, were differentially activated in the bone marrow erythroid compartment in response to erythropoietic stress. These processes are likely to be controlled by the integrated output of multiple signaling pathways, triggered by high erythropoietin levels and the suitable microenvironment, necessary for acquiring proper erythroid cell function without delay to respond adequately to tissue hypoxia.

These *in vivo* studies will greatly facilitate the understanding of the homeostatic mechanisms regulating stress erythropoiesis.

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