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Cyclic Nucleotide Response Element Binding (CREB) Protein Activation Is Involved in K562 Erythroleukemia Cells Differentiation

Roberta Di Pietro,[1] Viviana di Giacomo,[1,2] Luciana Caravatta,[1] Silvia Sancilio,[1] Rosa Alba Rana,[1] and Amelia Cataldi[1,2]*

[1]Dipartimento di Biomorfologia, Chieti-Pescara, Italy
[2]Cattedra di Anatomia Umana, Facoltà di Farmacia, Università G. d'Annunzio, Chieti-Pescara, Italy

Abstract K562 are human erythroleukemia cells inducible to differentiate into megakaryocytic or erythroid lineage by different agents. Cyclic nucleotide Response Element Binding (CREB) protein, a nuclear transcription factor which mediates c-AMP signaling, is a potential candidate involved in the occurrence of erythroid differentiation and adaptive response. Here we investigated signaling events in K562 cells induced with 30 μM hemin to undergo erythroid differentiation. CREB activation was detected early 1 h after hemin treatment and up to 4 and 6 days of treatment, when K562 terminal differentiation occurs together with caspase-3 maximal activation and PARP degradation. It was interesting to note that after hemin treatment in the presence of SB203580, p38 MAP kinase specific inhibitor, a reduced rate of CREB phosphorylation as well as a lower percentage of CD71/Gly+ (Glycophorin A) cells were detectable, demonstrating the p38 MAP kinase dependency of these phenomena. All in all these results document a novel relationship between CREB activation and differentiation-related apoptotic cell death and assign a role to p38 MAP kinase pathway in determining these events in K562 erythroleukemia cells. J. Cell. Biochem. 100: 1070–1079, 2007.

Key words: p38 MAP kinase; CREB; K562 cells; differentiation

Cell differentiation involves the interaction of intrinsic cellular programs and extracellular signaling stimuli, such as hormones, growth factors, cytokines, trophic factors and morphogens. K562 are human erythroleukemia cells derived from a patient with chronic myelogenous leukemia in blastic crisis [Fuchs et al., 1995]. A peculiar characteristic of this cell line is the ability to differentiate into different lineages: the megakaryocytic lineage following induction with PMA (Phorbol-myristate-13-acetate) [Herrera et al., 1998] and the erythroid lineage following induction with herbymycin [Kawano et al., 2004], hemin [Woessmann and Mivechi, 2001] or butyrate [Feriotto et al., 1988]. Depending on the differentiation inducer, different proteins can be phosphorylated leading to the activation of multiple signaling pathways. Among these proteins Cyclic nucleotide Response Element Binding (CREB) protein was found to be involved in neuronal [Tucholski and Johnson, 2003; Andreatta et al., 2004; Canon et al., 2004; Shiga et al., 2005] and in "in vivo" hemopoietic differentiation [Kang et al., 2004; Kinjo et al., 2005] and linked to a poorer prognosis and a greater incidence of acute myeloid leukemia relapse [Shankar et al., 2005a]. CREB is a 43–46 kDa nuclear transcription factor which recognizes the highly conserved sequence, known as c-AMP responsive element (CRE), 5′-TGACGTCA-3′, which can be phosphorylated on serine-133 (Ser-133) by several factors [Yamamoto et al., 1988; Brindle et al., 1993], such as PKC, Erk/MAPK, and ATM [Xing et al., 1996; Cohen-Jonathan et al., 1999; Blois et al., 2004; Shi et al., 2004]. In this study, we investigated the possible role of CREB in the early (1 and 3 h) and late (4 and 6 days) events related to hemin-induced

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*Correspondence to: Amelia Cataldi, Dipartimento di Biomorfologia, Via dei Vestini, 6,66100 Chieti, Italy.
E-mail: cataldi@unich.it

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differentiation of K562 cells. The choice of K562 myeloid leukemia cells for present experiments was based on susceptibility to erythroid differentiation by hemin, an agent with redox potential as reported in the literature [Chenais et al., 2000; Di Pietro et al., 2006]. A specific p38 MAP kinase inhibitor, SB203580, was used to evaluate the possible involvement of this enzymatic activity in the control of CREB signaling pathway leading to erythroid differentiation of human K562 neoplastic cells [McCubrey et al., 2000; Pearson et al., 2001; Stork and Schmitt, 2002]. Interestingly, in other experimental systems, SB 203580 was found to target CREB activity by inhibiting the dynamic CREB/p300 assembly [Smith et al., 2003].

METHODS

Cell Culture and Induction of Differentiation

K562 human erythroleukemia cells were grown in suspension in HEPES-buffered RPMI 1640 supplemented with 10% fetal calf serum (FCS), glutamine and penicillin/streptomycin in a controlled atmosphere (5% CO_2 in air). Hemin (30 μM) (Sigma-Aldrich, St. Louis, MI) and SB203580 (20 μM) (Calbiochem, Darmstadt, Germany) were added at the same time to the medium. SB203580 was washed out after 1 h of treatment, while hemin was left for the entire culture period.

For Western blotting analysis cells were recovered after 1, 3, 4, and 6 days of hemin plus or minus SB203580 treatment; for caspase-3 and p38 MAP kinase assays the cells were recovered after 1 h, 3 h, 4 and 6 days, while for phenotypic analysis the cells were recovered after 4 and 6 days of treatment.

Phenotypic Analysis

Surface erythroid differentiation markers were analyzed on K562 cells with flow cytometry using an EPICS-XL cytometer and the EXPO32 analysis software (Beckmann Coulter, FL).

Briefly, 5×10^5 K562 cells, suspended in PBS containing 1% Fetal Bovine Serum (FBS), were stained with PE (Phycoerythrin) conjugated anti-CD71 and Fluorescein Isothiocyanate (FITC) conjugated anti-Glycophorin A monoclonal antibodies (Beckmann Coulter, FL). All antibody incubations were carried out for 30 min on ice. An aliquot of cells was stained with mouse IgG conjugated to FITC and to PE as an isotype control (not shown).

Cell Cycle Analysis

Approximately 0.5×10^6 cells for each experimental condition were harvested, fixed in 70% cold ethanol and kept at 4°C overnight. Cells were then resuspended in 20 μg/ml PI (Propidium Iodide) and 100 μg/ml RNAse, final concentrations. Cell cycle profiles (10,000 events) were analyzed with an EPICS-XL cytometer using the EXPO32 software (Beckmann Coulter, FL). Data were analyzed with Multicycle software (Phoenix Flow Systems, CA).

Immunofluorescence Microscopy

K562 cells were cytocentrifuged at 250 rpm for 5 min, fixed with 3.7% paraformaldehyde for 10 min, and blocked with 10% normal donkey serum for 20 min. Samples were then incubated with rabbit pCREB polyclonal antibody (Cell Signalling Technology Inc., Beverly, MA) diluted 1:500 in 1× PBS plus 0.5% Tween-20, 2% BSA for 60 min at 37°C. After three washings with PBS, samples were incubated with goat anti-rabbit IgG FITC (Jackson Immuno Research, West Grove, PA) diluted 1:100 in 1× PBS plus 0.5% Tween-20, 2% BSA for 45 min at 37°C. After nuclei counterstaining with DAPI mounting medium (Vector Laboratories, Inc. Burlingame, CA), the smears were dehydrated and mounted with Glycerol/DABCO (Sigma-Aldrich, St. Louis, MI). The observations were carried out by means of a ZEISS AXIOSKOP light microscope. Acquisition and analysis of computerized images were done by means of the MetaMorph® 6.1 Software (Universal Imaging Corp, Downingtown, PA).

Morphological Assays

Diaminobenzidine staining solution was freshly prepared by adding 5 ml of 30% hydrogen peroxide to 1 ml of stock solution of 0.2% benzidine/0.5% acetic acid. Cells, cytospinned for 5 min at 250 rpm, were incubated for 25 min with the staining solution and observed in light microscopy. May Grunwald Giemsa method (Sigma Chemical, St. Louis, MO) was used for the analysis of morphological patterns.

Protein Samples Preparation

Cells, lysed in RIPA buffer (1×PBS, 1% NP-40, 0.55 sodium deoxycholate, 0.1% SDS, 10 μg/ml aprotinin, 10 μg/mL leupeptin, 1 μg/mL PMSF, 1 mM DTT, 1 mM Na_3VO_4), were passed 10 times through a 25-gauge needle and

centrifuged at 10,000*g* at 4°C. The supernatant was recovered and stored at −80°C.

For immunoprecipitation whole cell lysate (500 μg) was incubated in the presence of 50 μl of the suspended IP matrix (Exacta CRUZ, Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 30 min at 4°C. The matrix was pelleted at 14,000 rpm for 30 s at 4°C. Then 50 μl of the suspended IP matrix, 3 μg of mouse pCREB^{Ser133} monoclonal antibody, 500 μl of PBS were added to the supernatant and incubated at 4°C on a rotator for 1 h. The matrix was then pelleted and washed twice with 500 μl of PBS. IP antibody-IP matrix complex was incubated with the lysate at 4°C on a rotator overnight. The matrix containing the immunoprecipitated sample was then pelleted and washed three times with RIPA buffer. Samples were boiled and stored at −80°C.

Western Blotting Analysis

Total cell lysates (20 μg) or immunoprecipitated p-CREB^{Ser133} were electrophoresed and transferred to nitrocellulose membranes. After blocking in 5% non-fat milk, 10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween-20, nitrocellulose membranes were probed with mouse CREB and pCREB^{Ser133} (Cell Signalling Technology Inc., Beverly, MA), mouse PARP monoclonal antibodies (Calbiochem), rabbit p300 polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and developed with specific enzyme conjugated horseradish peroxidase (Calbiochem, Darmstadt, Germany). Sample normalization was done normalized incubating membranes with mouse β actin monoclonal antibody. Bands were detected with ECL detection system (Amersham Intl, UK).

Caspase-3 Activity Assay

Caspase-3 activity was measured with a colorimetric assay using a peptide-based substrate Ac-DEVD-pNA according to the manufacturer's instructions (Calbiochem, Darmstadt, Germany). Cells (25×10^3) were collected, washed in PBS and lysed on ice for 10 min in cell lysis buffer provided in the kit. Cell extracts were then incubated in the presence of colorimetric caspase substrate at 37°C, and color changes were followed for 2 h at 10 min intervals with a 96-well microtest plate spectrophotometer Anthos 2010 (Anthos Labtec Instruments, Salzburg, Austria). To determine whether color changes were specific for caspase-3 activity, a peptide-based caspase-3 inhibitor (Ac-DEVD-CHO) was added to the incubation mix.

p38 MAP Kinase ELISA Assay

p38 MAP kinase activity was quantified by means of fast activated cell-based ELISA (FACE) kit (Active Motif, CA). Briefly, cells were seeded in poly-L-Lysine pre-treated wells and immediately fixed with 8% formaldehyde. Cells were then incubated with a primary antibody that recognizes either phosphorylated or total p38 and subsequently with a secondary HRP-conjugated antibody. The color, developed with a developing solution provided in the kit, was detected with a 96-well microtest plate spectrophotometer Anthos 2010 (Anthos Labtec Instruments, Salzburg, Austria). The relative number of cells in each well was then determined using the provided Crystal Violet solution.

Imaging and Statistics

Densitometric values of protein bands, expressed as Integrated Optical Intensity (IOI), were estimated in a CHEMIDOC XRS System by the QuantiOne 1-D analysis software (BIORAD, Richmond, CA). Values obtained were normalized with reference to densitometric values of internal beta actin. Data were analyzed using the two tailed, two sample *t*-test. Results were expressed as mean ± SD. Values of $P < 0.05$ were considered significant.

RESULTS

Effects of Hemin on K562 Erythroleukemia Cell Differentiation

Phenotypic analysis performed with flow cytometry on K562 erythroleukemia cells, induced to differentiate with 30 μM hemin, disclosed an high percentage (47.5%) of surface CD71/Gly erythroid differentiation markers positive cells after 4 days of treatment. After treatment for 6 days, considered the time of terminal differentiation, this percentage was further increased to 66.3%, and was reduced in the presence of SB203580, p38 MAPK specific inhibitor (Fig. 1) [Choi et al., 2003]. The parallel evaluation of cell cycle in flow cytometry (not shown) displayed percentages of dead cells in the range between 23 and 30%, respectively after 4 and 6 days of treatment, against the 5% of dead cells in

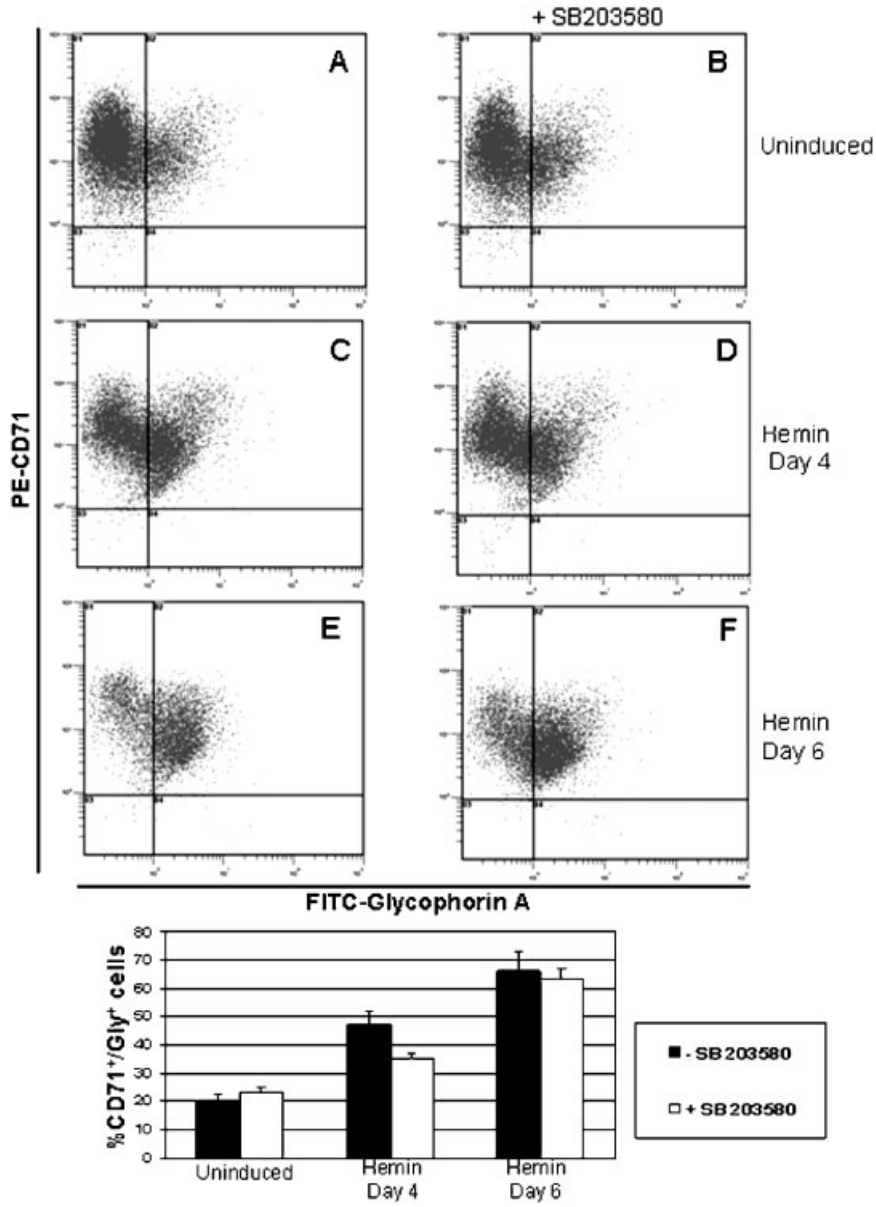

Fig. 1. Flow cytometry analysis of the expression of CD71 and Glycophorin A surface erythroid differentiation markers in K562 cells induced to differentiation with 30 μM hemin up to 6 days. When required, cells were co-incubated with 20 μM SB203580 for 1 h. Mean ± SD of three different consistent experiments is reported in the graph. **A**: Uninduced; **B**: Uninduced plus SB203580 (1 h); **C**: Hemin (4 days); **D**: Hemin (4 days) plus SB203580 (1 h); **E**: Hemin (6 days); **F**: Hemin (6 days) plus SB203580 (1 h). Hemin treated (4 and 6 days) samples vs. uninduced. $^*P < 0.05$ Hemin treated (4 and 6 days) samples + SB203580 vs. hemin treated (4 and 6 days) samples − SB203580. $^*P < 0.05$.

uninduced samples. In the presence of SB203580, this percentage lowered to 19 and 22%, respectively.

CREB Expression and Activation in Hemin-Induced K562 Differentiation

Since CREB is a nuclear transcription factor involved in c-AMP signaling in a number of experimental models [Sassone-Corsi, 1995; Xing et al., 1996; Andreatta et al., 2004], we evaluated the possible role of this protein in the occurrence of K562 differentiation. Western blotting analysis of total homogenates showed that both CREB expression and phosphorylation increased early (1 h) after hemin induction and reached the highest level at 4 days of treatment, which is the time when all biochemical events would be completed. It was interesting to note that both CREB expression

and phosphorylation were down-modulated in the presence of SB203580 (20 μM) at all experimental points (Fig. 2A). To ascertain whether the increased phosphorylation corresponded to an increased activation of this transcription factor, pCREB was co-immunoprecipitated with p300, coactivator protein which mediates transcriptional activation of CREB [Shaywitz and Greenberg, 1999; Blobel, 2000]. Interestingly, pCREB/p300 immune complex was detected only 1 h after hemin treatment (Fig. 2B), when pCREB was already translocated into the nucleus (Fig. 3). Both these events are further evidences of CREB activation, as elsewhere already reported [Mayr et al., 2001]. We hypothesize that the formation of CREB/CBP complex, 1 h after hemin treatment, could be responsible for switching on CREB activation, which determines later changes in the nuclear morphology (Fig. 3, insets). In fact, from 3 h onwards, pCREB positive nuclei displayed the hallmarks of apoptotic nuclei, showing a typical chromatin condensation at earlier time intervals (3 h) and a higher incidence of micronuclei formation after 4 and 6 days of hemin induction. Lastly, SB203580 was able to down-modulate CREB phosphorylation only when this protein was located at cytoplasm level (Fig. 3).

SB203580 Reduces K562 Erythroleukemia Cells Differentiation

The involvement of p38 MAP kinase pathway was confirmed by ELISA assay of the enzyme, which was activated 1 h after hemin treatment, stable after 4 days and reduced after 6 days of hemin treatment (Fig. 4). It is worth noting that at all experimental time points, in the presence of SB203580, both CREB phosphorylation and p38 MAP kinase activity were downregulated (Figs. 2 and 4) as well as the percentage of differentiated cells (Fig. 1D,F).

Relationship Between K 562 Differentiation and Apoptotic Machinery

Finally by means of diaminobenzidine staining, which detects hemoglobin accumulation, and May Grunwald Giemsa staining, we confirmed the relationship between pCREB positive reaction in apoptotic nuclei and the pattern of terminally differentiated cells. In fact, cells highly positive for diaminobenzidine staining disclose apoptotic nuclei, which, in turn, are positive for pCREB immune labeling (Figs. 3 and 5). In the same experimental conditions caspase-3 activity started to increase after 4 days and further after 6 days of hemin treatment and, again, was reduced in the presence of SB203580 (Fig. 6). As expected,

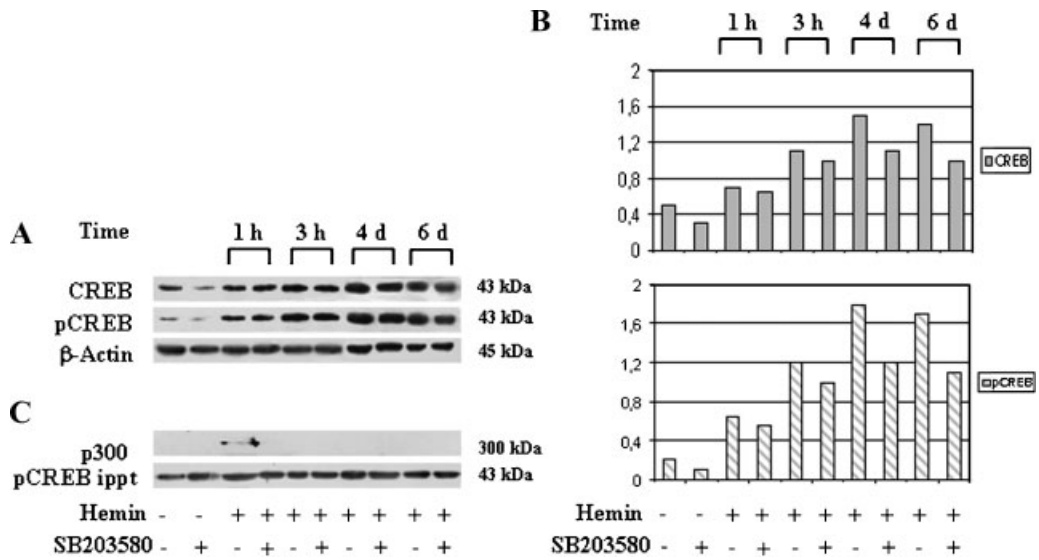

Fig. 2. Effects of hemin (30 μM) and SB203580 (20 μM) on CREB expression and Ser^{133} phosphorylation (**A**). As shown, samples were normalized by incubating membranes in the presence of mouse β actin monoclonal antibody. **B**: Densitometric analysis of CREB and pCREB expression. **C**: Co-immunoprecipitation of pCREB and p300. Immunoprecipitated p-CREB was probed against rabbit p300 polyclonal antibody and reprobed against mouse pCREB monoclonal antibody. Note that pCREB/p300 immune complex appeared 1 h after hemin treatment. The most representative out of three different consistent experiments is shown.

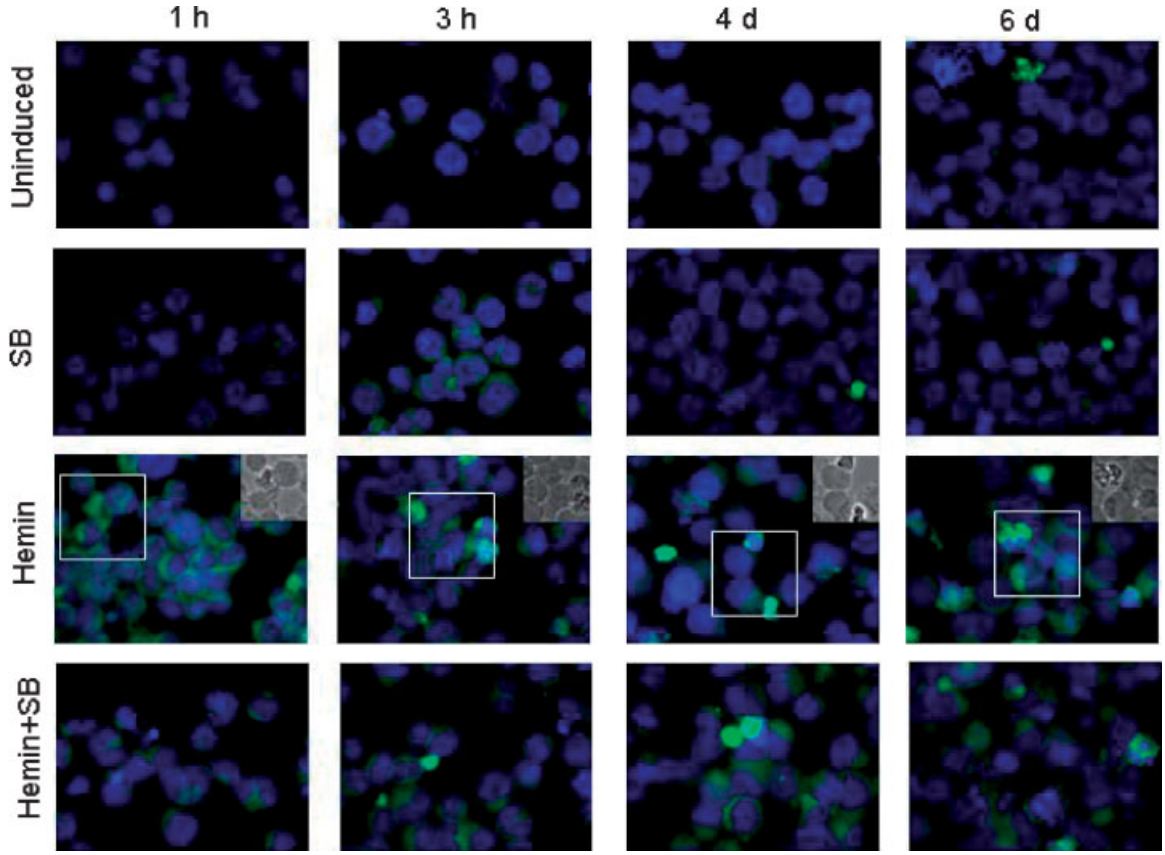

Fig. 3. Immunofluorescence analysis of pCREB localization in K562 cells induced to differentiation with 30 μM hemin up to 6 days. Green fluorescence of FITC (Fluorescein-Isothiocyanate) refers to pCREB labeling; blue fluorescence of DAPI (4–6 diamino-2-phenyl-indol) indicates counterstained nuclei. Note that pCREB nuclear positivity is already evident after 1 h of hemin treatment. As displayed in the insets, acquired by phase contrast light microscopy, pCREB positive nuclei resemble the features of apoptotic nuclei. Magnification: 40×.

PARP cleaved fragment appeared after 4 days and was still detectable, although to a lower extent, 6 days after induction of differentiation (Fig. 7).

DISCUSSION

The process of cell differentiation consists of two phases known as commitment and

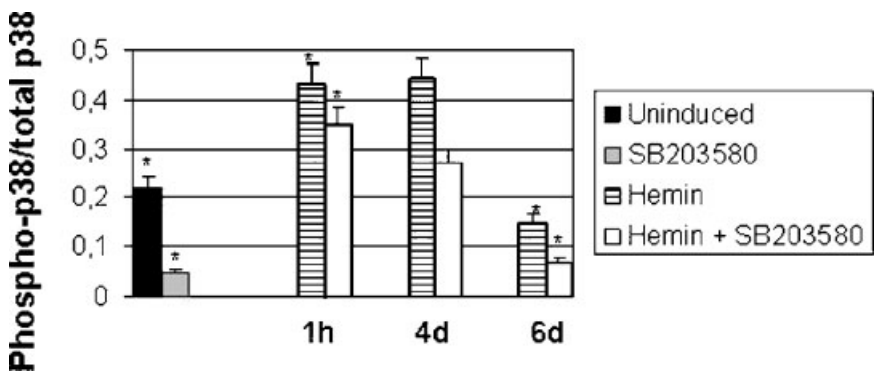

Fig. 4. Effects of hemin (30 μM) and SB203580 (20 μM) on p38 MAP kinase activation. Phosphorylated and total p38, determined at 450 nm wavelength, were assayed in triplicate using the phospho-p38 and the total p38 antibodies from the FACE p38 ELISA kit. The mean ratio (±SD) of phospho-specific p38 to total p38 indicates the effect of the stimulus administered. Hemin treated (1 h and 6 days) ± SB203580 samples vs. uninduced samples: $^{*}P < 0.05$.

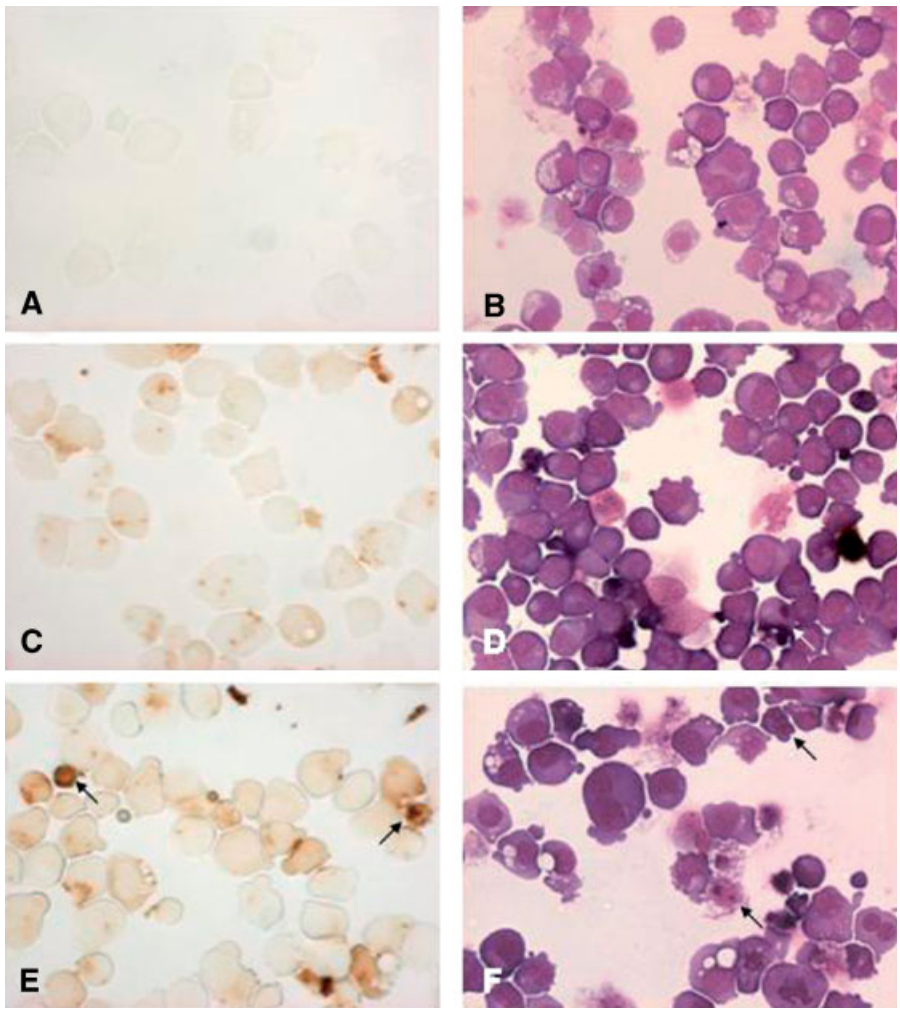

Fig. 5. Diaminobenzidine staining of K562 erythroleukemia cells induced to differentiation with 30 μM hemin up to 6 days (**A**–**C**–**E**). May Grunwald Giemsa staining of the same cells (**B**–**D**–**F**). Arrows indicate that marked Diaminobenzidine highly positive cells (E) disclose apoptotic features (F) after 6 days of hemin treatment. Magnification: 40×. A,B: uninduced, C,D: 4 days hemin treatment, E,F: 6 days hemin treatment.

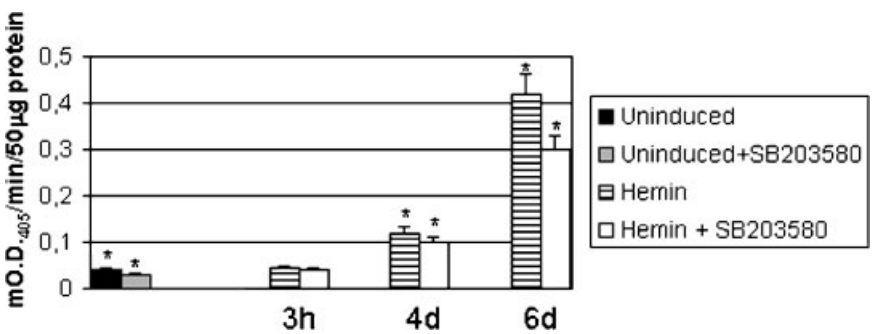

Fig. 6. Effects of hemin (30 μM) and SB203580 (20 μM) treatment on caspase-3 activity. Analysis of caspase-3 activity was performed in triplicate with a colorimetric assay using peptide-based substrate AC-DEVD-pNA. Results are expressed as m.O.D./min 50 μg protein. Mean (±SD) of three different consistent experiments. Hemin treated (4 and 6 days) ± SB 203580 samples vs. uninduced samples: $^{*}P < 0.05$.

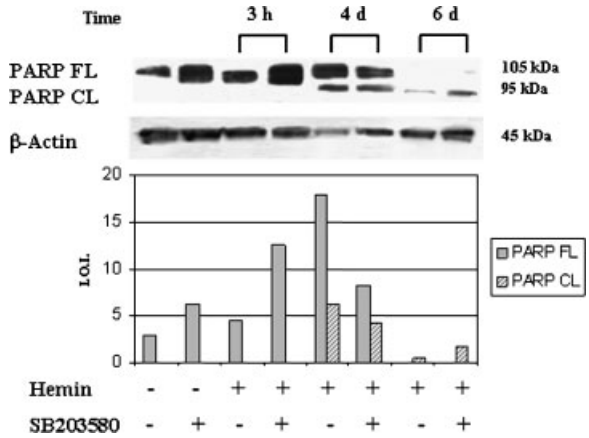

Fig. 7. Effects of hemin (30 μM) and SB 203580 (20 μM) treatment on PARP full length and cleaved fragment expression. As shown, samples were normalized by incubating membranes in the presence of mouse β actin monoclonal antibody. The most representative out of three different consistent experiments is shown.

expression of differentiation. Even though the commitment phase seems to be intrinsically determined [Suda et al., 1984], extrinsic factors can commit the cell towards a particular lineage. During cell division leukemia cells can undergo the process of self-renewal without commitment, or can be committed to differentiate into different cell types [Herrera et al., 1998]. The commitment of erythroid progenitors is related to an alteration in the cell cycle that includes prolongation of G1 and possibly requires DNA synthesis [Marks et al., 1977]. The second phase of differentiation is characterized by the reduction in cell proliferation and by the appearance of hemoglobin and erythroid structural proteins, including those of the membrane skeleton (spectrin, ankyrin, band 3) [Fibach et al., 1977; Cataldi et al., 2000]. Terminal erythroid differentiation involves nuclear and chromatin condensation, loss of organelles and, ultimately, enucleation, which are morphological changes similar to those occurring during apoptosis as a consequence of caspase-3 activation [Bessis, 1973; Zamai et al., 2004].

The commitment and expression of a differentiated phenotype, including the accumulation of mRNA for specific proteins, represent a multistep process that involves a number of regulatory elements. On the basis of recent evidences concerning the role exerted "in vivo" by CREB in hematopoiesis and in acute myeloid leukemia [Shankar et al., 2005a,b], we investigated the role of the CREB pathway in the molecular and morphological changes occurring during "in vitro" hemin-induced differentiation of human K562 neoplastic cells. Our results show that CREB activation occurs after 1 h of hemin treatment, persists until 4 days and slightly lowers after 6 days (Fig. 2), when the majority of the cells (66.3%) express phenotypic (Fig. 1) or cytoplasm (Fig. 5) markers of differentiation. The finding of a reduced rate of CREB activation along with a reduced percentage of differentiated cells upon SB203580 treatment prompted us to hypothesize a role for p38 MAP kinase in mediating CREB phosphorylation, thus indicating that the commitment of K562 cells might depend on the early upregulation of p38 MAP kinase/CREB pathway, which persists up to 4 days when the expression of phenotypic markers occurs.

In parallel, caspase-3 enzyme activity increases after 4 days and reaches a particularly high level at 6 days, when terminal differentiation is in progress (Figs. 1 and 5) and the highest number of dead cells is achieved (30%). Although we cannot exclude the presence of residual caspase-3 activity in the fraction of dead cells (30%), we hypothesize that this enzyme is involved in the erythroid differentiation within the still viable subpopulation, as elsewhere already reported [Secchiero et al., 2002]. This interpretation is supported by previous reports related to erythroblast differentiation [Zermati et al., 2001; Zamai et al., 2004]. The late increase in caspase-3 activity and PARP cleavage, with respect to p38 MAP kinase/CREB pathway activation may account for the nuclear structural changes associated with erythroblast maturation, by influencing gene expression during the last steps of differentiation.

All in all, our observations suggest that the biphasic process of K562 cell differentiation is switched on by the early activation of a cell signaling network driven by p38 MAP kinase pathway, which leads to the downstream activation of CREB. CREB phosphorylation is possibly required, together with caspase-3 enzyme activation, in determining the nuclear structural changes typical of erythroblast maturation, which could influence, in turn, the final steps of differentiation and prepare the process of enucleation.

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