ELSEVIER

EXPERIMENTAL HEMATOLOGY

Experimental Hematology 34 (2006) 1420–1428

Activation of cyclic AMP pathway prevents CD34$^+$ cell apoptosis

Soledad Negrotto[a], Natalia Pacienza[a], Lina Paola D'Atri[a], Roberto Gabriel Pozner[a], Elisa Malaver[a], Oscar Torres[a], Maria Angela Lazzari[a], Ricardo Martín Gómez[b], and Mirta Schattner[a]

[a]*Hematological Research Institute, National Academy of Medicine, National Research Council (CONICET), Buenos Aires, Argentina;*
[b]*Biochemistry and Molecular Biology Institute, Department of Biological Sciences, National University of La Plata, CONICET, Buenos Aires, Argentina*

(Received 20 January 2006; revised 3 May 2006; accepted 25 May 2006)

Objective. **Although cAMP is involved in a number of physiologic functions, its role in hematopoietic cell fate decision remains poorly understood. We have recently demonstrated that in CD34$^+$-derived megakaryocytes, cAMP-related agents prevent apoptosis. In this study we addressed the question of whether cAMP also regulates survival of their precursors, CD34$^+$ cells.**

Methods. **Apoptosis was evaluated by fluorescence microscopy, and detection of hypodiploid or annexin V$^+$ cells by flow cytometry. Mitochondrial membrane potential and *bcl*-xL or caspase-3 expression were assessed by flow cytometry. Colony-forming units were studied by clonogenic assays in methylcellulose.**

Results. **We found that two different cAMP analogs such as Dibutiril-cAMP and sp-5,6-DCl-BIMPS (BIMPS) promoted survival of human umbilical cord–derived CD34$^+$ cells by suppressing apoptosis induced by either nitric oxide (NO) or serum deprivation. Involvement of PKA and PI3K pathway was demonstrated by the ability of their specific inhibitors Rp-cAMP and Wortmannin or LY294002 respectively to reverse the antiapoptotic effect of BIMPS. Treatment of CD34$^+$ cell with BIMPS not only restrained the *bcl*-xL downregulation but also suppressed the loss of mitochondrial membrane potential and caspase-3 activation induced by serum starvation. While thrombopoietin (TPO), granulocyte colony-stimulating factor (G-CSF) or stem cell factor (SCF) were not able to increase cAMP levels, the antiapoptotic activity exerted by these growth factors was blocked by inhibition of the adenylate cyclase and synergized by BIMPS. Cyclic AMP analogs suppressed the decreased colony formation in cells exposed to NO or serum deprivation.**

Conclusion. **Altogether, our results strongly suggest that cAMP appears to be not only a key pathway controlling CD34$^+$ survival, but also a mediator of the TPO-, G-CSF- and SCF-mediated cytoprotection.**

Hematopoiesis, a complex process in which hematopoietic stem cells (HSCs) proliferate, differentiate, and generate a large number of lineage-committed blood cells, is closely dependent on stromal cells and their secreted cytokines and chemokines. An adjusted balance between self-renewal and differentiation is necessary to maintain an adequate number of hematopoietic progenitors and mature blood cells [1].

Programmed cellular death or apoptosis occurs under physiologic conditions such as embryogenesis, tissue homeostasis, and immune processes. In hematopoiesis, apoptosis has been implicated in the elimination of differentiated terminal cells, including monocytes, neutrophils, and eosinophils [2]. However, apoptosis of hematopoietic cells can also be induced under pathologic conditions such as x-irradiation, infection, or chemotherapy [3]. Hematopoietic cells require appropriate factors for their survival, including interleukin-3 (IL-3), thrombopoietin (TPO), Flt3 ligand, granulocyte colony-stimulating factor

Offprint requests to: Mirta Schattner, Ph.D., Pacheco de Melo 3081, 1425 Buenos Aires, Argentina; E-mail: mschattner@hematologia.anm.edu.ar

0301-472X/06 $–see front matter.
doi: 10.1016/j.exphem.2006.05.017

(G-CSF), stem cell factor (SCF), IL-6, or stromal-derived factor-1 (SDF-1) [4–7]. Otherwise, these cells not only cease proliferation but also undergo a rapid apoptotic death. On the other hand, tumor necrosis factor-α (TNF-α), transforming growth factor β (TGF-β), interferon γ (IFN-γ), and the ligands of Fas/TNF-α family enhance apoptosis [8]. Regarding HSCs, expression of antiapoptotic members of the IAPs and *bcl*-2 family, caspase inhibition, and activation of the PI3K axis are some of the signaling pathways that have been shown to participate in the preservation of HSC lifespan [9–11].

In $CD34^+$-derived megakaryocytes, we have recently demonstrated that prostacyclin and cAMP-related agents prevent nitric oxide (NO)-induced apoptosis through inhibition of both cGMP raises and caspase-3 activation [12].

Considering that the role of cyclic nucleotides in HSC survival has received little attention, in this study we addressed the question of whether cAMP is also a mediator of $CD34^+$ cell survival. Our results show that two different cAMP analogs effectively inhibited human $CD34^+$ programmed cellular death by activating PKA and PI3K pathways. Prevention of mitochondrial transmembrane potential dissipation, *bcl*-xL downregulation and caspase-3 activation were downstream signaling pathways involved in cAMP antiapoptotic activity. Moreover, cAMP rescue from apoptosis renders functional $CD34^+$ cells and it also appears to be a critical mediator of the cytoprotective action exerted by growth factors such as TPO, G-CSF, and SCF.

Materials and methods

Materials

N^6,2′-O-dibutyryladenosine-3′,5′-cyclic monophosphate sodium salt (Dib-cAMP), Rp-Adenosine-3′,5′-cyclic monophosphorothioate (Rp-cAMP), 9-(Tetrahydro-2-furyl)adenine (SQ22536), Sp-5,6-dichloro-1-β-D-ribofuranosylbenzimidazole-3′,5′-monophosphorothioate (BIMPS), Wortmannin, 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), and 2′-amino-3′-methoxyflavone (PD98059) were from Biomol International L.P. (Plymouth Meeting, PA, USA). 1-propanamine,3-(2-hydroxy-2-nitroso-1-propylhydrazino) (PAPA/NO) was from Cayman Chemical (Ann Arbor, MI, USA). TPO was from Peprotech (Veracruz, Mexico). SCF and G-CSF were purchased from Sigma Chemical Co. (St. Louis, MO, USA). PAPA/NO was dissolved in NaOH (0.01 M) and TPO in Tris (10 mM, pH 8.0).

Stock solutions of Wortmannin, LY294002, and PD98059 were prepared in dimethylsulfoxide (DMSO) and dilutions in Iscove's modified Dulbecco medium (IMDM). The final DMSO concentration never exceeded 0.01% v/v. All other drugs were dissolved in MilliQ water and further dilutions of all reagents were carried out in IMDM.

Isolation of $CD34^+$ cells

Umbilical cord blood was collected during normal full-term deliveries with informed consent of the mother and used within 24 hours. After collection, samples were diluted one-third in phosphate-buffered saline (PBS) and centrifuged to remove platelets. Low-density mononuclear cells were prepared by centrifugation of the remaining blood over a Ficoll Hypaque (1.077 g/cm^3) gradient. Cells collected from the interface were washed and resuspended in PBS containing EDTA (2 mM) and human albumin (0.5% w/v). $CD34^+$ cells were purified using a magnetic cell sorting system (Miltenyi Biotec, Bergisch Gladbach, Germany) in accordance with the manufacturer's recommendations. The purity of the $CD34^+$-enriched population was determined by immunolabeling with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies against CD34 (Clone 581, Beckman Coulter, Miami, FL, USA) that reacted with an epitope other than the antibody used for separation. After two Mini-MACS column separations, the purity of cell suspension was determined by flow cytometry and ranged typically from 95 to 99% for $CD34^+$.

Apoptosis induction

Apoptosis was induced by addition of the NO donor, PAPA/NO, or serum deprivation (SD) and percentage of apoptotic cells was determined 24 or 48 hours later, respectively. Experimental medium was IMDM supplemented with antibiotics and 5% human serum, except in SD assays.

Quantitation of $CD34^+$ cell apoptosis by fluorescence microscopy

Cells (5×10^5/mL) were labeled with a mixture of the fluorescent DNA-binding dyes acridine orange (100 μg/mL) to determine the percentage of cells that had undergone apoptosis and ethidium bromide (100 μg/mL) to differentiate between viable and nonviable cells. With this method, nonapoptotic cell nuclei show "structure" variations in fluorescence intensity that reflect the distribution of euchromatin and heterochromatin. In contrast, apoptotic nuclei exhibit highly condensed chromatin that is uniformly stained by acridine orange. In order to assess the percentage of cells showing morphologic features of apoptosis, at least 300 cells were scored in each experiment.

Detection of hypodiploid $CD34^+$ cells by propidium iodide staining and flow cytometry

$CD34^+$ population displaying a hypodiploid DNA peak was determined as previously [12]. Briefly, cells were fixed in 0.5% paraformaldehyde. DNA was then stained by the incubation of the cells for 20 minutes at 4°C with 400 μL of a solution containing propidium iodide (PI) (5 μg/mL in 0.1% sodium citrate) and 0.1% Triton X-100 for cell permeabilization. This was followed by RNA digestion. The red fluorescence of PI in individual nuclei was measured using a FACScan flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA).

Annexin V binding assay

Phosphatidylserine expression was detected by annexin V binding and PI staining using a commercial kit (Sigma). Briefly, 24 hours after different treatments, HSCs (1.5×10^5) were washed and incubated for 30 minutes with annexin V–FITC at 37°C. Cells were then resuspended in 400 μL of binding buffer containing PI and immediately analyzed by flow cytometry.

Analysis of caspase-3 activation and bcl-xL expression by flow cytometry

Cells (1×10^6/mL) were fixed and permeabilized using Fix & Perm kit (Beckman Coulter) following the manufacturer's instructions. After labeling with a polyclonal antibody specific for the

activated form of caspase-3 (Asp175, Cell Signaling Technology, Inc. Beverly, MA, USA) for 2 hours at room temperature, cells were washed and incubated with secondary FITC-conjugated swine anti-rabbit immunoglobulins (Sigma Chemical Co.) for 30 minutes at room temperature. As nonspecific binding control, anti-cleaved caspase-3 antibody was replaced by a similar concentration of rabbit IgG. For *bcl*-xL detection, cells were incubated for 30 minutes with FITC-conjugated anti-human *bcl*-xL monoclonal antibody. Percentage of activated caspase-3- or *bcl*-xL-expressing cells was analyzed by flow cytometry.

Mitochondrial membrane potential ($\Delta\varphi_m$)

$\Delta\varphi_m$ was examined using Mitochondrial Permeability Detection Kit (MTt-E-φ) according to manufacturer instructions (Biomol International LP). Briefly, 2×10^5 cells were stained with MTt-E-φ reagent and incubated at 37°C for 15 minutes. After three washings, cells were then suspended in PBS and analyzed immediately. The structure of MTt-E-φ allows it to penetrate healthy mitochondria. Once inside, it aggregates and fluoresces red. When the mitochondrial $\Delta\varphi_m$ collapses in apoptotic cells, the dye no longer accumulates in the mitochondria and it is distributed throughout the cells. When dispersed in this manner, the reagent assumes a monomeric form which fluoresces green [13]. Fluorescence was detected by flow cytometry and results were expressed as the ratio between the means of red/green fluorescence. At least 10,000 events were collected for sample.

Clonogenic progenitor assays

Clonogenic colony assays were performed as previously described [14]. Briefly, $CD34^+$ cells (5×10^2/100 μL) in IMDM were exposed to PAPA/NO or serum deprivation for 18 hours in the absence or presence of BIMPS or Dib-cAMP. After confirmation that cell number did not change by any treatment, cells were cultured into 1 mL of MethoCult H4434 supplemented with SCF (50 ng/mL), GM-CSF (10 ng/mL), IL-3 (10 ng/mL), and erythropoietin (3 U/mL) and plated in 35-mm-diameter plates (Stem Cell Technology, Vancouver, BC, Canada). Colony-forming units (CFU) (granulocyte-monocyte CFU [CFU-GM], erythroid burst-forming units [BFU-E], and granulocyte-erythroid-macrophage-megakaryocyte CFU [CFU-GEMM]) were identified by morphology and counted under an inverted microscope at 10 to 12 days postplating.

Statistical analysis

Data are expressed as mean ± SEM and were analyzed by one-way analysis of variance (ANOVA) followed by the Newman-Keuls procedure to determine significant differences between groups. p values lower than 0.05 were considered statistically significant.

Results

Cyclic AMP protects $CD34^+$ cell apoptosis

We have previously demonstrated that cAMP protects NO-induced human megakaryocyte apoptosis [12]. In the present study we examined whether a similar effect was exerted on their precursors, $CD34^+$ cells. In a first set of experiments, HSCs were subjected to NO or SD in the presence or absence of Dib-cAMP, a cAMP-permeable analog. After 24 or 48 hours of incubation respectively, apoptosis was revealed by fluorescence microscopy using the fluorescent DNA-binding dyes acridine orange and ethidium bromide. Table 1 shows that Dib-cAMP significantly prevented NO- and SD-mediated apoptosis. As it has been reported that butyrate released from Dib-cAMP molecule may trigger cellular responses that often interfere with second messenger pathways [15], we analyzed the specificity of action of cAMP using another cAMP analog such as BIMPS. Treatment of $CD34^+$ cells with BIMPS significantly inhibited NO- and SD-mediated apoptosis (Table 1). Since cellular death triggered by serum withdrawal was more pronounced than that induced by NO, in the rest of experiments we used serum starvation as the apoptotic stimulus. Under this condition, the antiapoptotic effect of Dib-cAMP or BIMPS was concentration dependent with an $IC_{50} = 10 \pm 2$ μM and 2.7 ± 0.5 μM respectively (Fig. 1).

During the course of apoptosis, phosphatidylserine, a negatively charged phospholipid, becomes exposed at

Table 1. Cyclic AMP prevents NO- and SD-induced apoptosis

NO-induced apoptosis		SD-induced apoptosis	
Treatments	Apoptosis (%)	Treatments	Apoptosis (%)
Control	6 ± 3	Control	10 ± 1
NO	18 ± 2*	SD	46 ± 5*
NO + Dib-cAMP	10 ± 1#	SD + Dib-cAMP	22 ± 2#
NO + BIMPS	8 ± 1#	SD + BIMPS	19 ± 2#

$CD34^+$ cells deprived of serum were treated with Dib-cAMP (20 μM) or BIMPS (10 μM). In NO-induced apoptosis, both analogs were added 1 minute before PAPA/NO (100 μM). Cell death was evaluated 48 and 24 hours later respectively by fluorescent microscopy. Values are expressed as means ± standard error of 5 independent experiments. *$p < 0.05$ vs control, #$p < 0.05$ vs NO or SD.

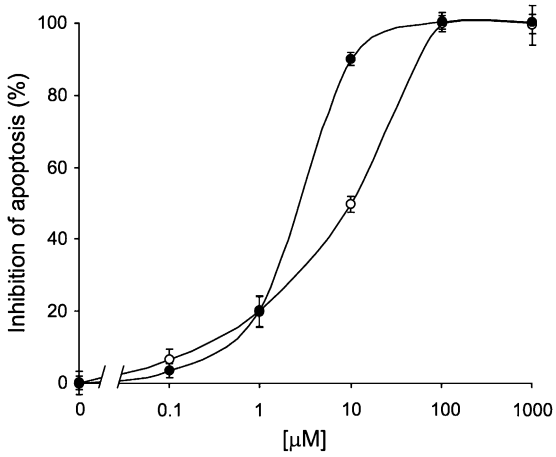

Figure 1. Concentration response curve of cAMP antiapoptotic activity. Cells were treated with BIMPS (solid circles) or Dib-cAMP (open circles) and apoptosis was evaluated 48 hours later by fluorescence microscopy (n = 4, in duplicate).

the cell surface [16]. The ability of cAMP to prevent apoptosis was then analyzed by flow cytometry using FITC-labeled annexin V, which specifically binds to phosphatidylserine. In agreement with the results obtained by fluorescence microscopy (Fig. 2A), we found that while SD increase the number of apoptotic cells (percentage of annexin V^+/PI^- cells), cells pretreated with BIMPS showed a marked decrease in the percentage of apoptotic cells (C = 4.1% ± 0.9%; SD = 27.7% ± 2.1%*; SD+BIMPS = 6.9% ± 1.0%$^{\#}$ of annexin V^+/PI^- cells, n=4, *$p < 0.05$ vs C, $^{\#}p < 0.05$ vs SD) (Fig. 2B). BIMPs protection was also observed when apoptosis was analyzed by detection of hypodiploid nuclei in permeabilized cells stained with PI (C = 12% ± 3%; SD = 50% ± 4%*; SD+BIMPS = 21% ± 3%$^{\#}$ of apoptosis, n=4, *$p < 0.05$ vs C, $^{\#}p < 0.05$ vs SD) (Fig. 2C).

Role of PKA on cAMP-mediated cytoprotection

PKA constitutes an ubiquitous enzyme present in all eukaryotic cells responsible for the mediation of most biological effects of cAMP [17]. To distinguish the role of this kinase, Rp-cAMP (a potent inhibitor of PKA) was applied to block PKA activity in $CD34^+$ cells. Preincubation with Rp-cAMP (50 μM) almost completely suppressed the antiapoptotic effect mediated by BIMPS (5 μM) (C = 11% ± 2%; SD = 43% ± 0.5%*; SD+Rp-cAMP = 42% ± 1%, SD+BIMPS = 28% ± 2%$^{\#}$, and SD+Rp-cAMP+BIMPS = 41% ± 2%$^{\&}$ of apoptosis, n=4, *$p < 0.05$ vs C, $^{\#}p < 0.05$ vs SD, $^{\&}p < 0.05$ vs BIMPS) (Fig. 3).

Cyclic AMP antiapoptotic action is mediated by PI3K-dependent signaling pathway

Activation of both the PI3K/PKB and MAPK pathways plays an important role in proliferation and survival of many cell types including hematopoietic cells [18,19]. We investigated whether cAMP could prevent $CD34^+$ cell apoptosis through these signaling pathways by using Wortmannin or LY294002 and PD098059 as PI3K- and MAPK-specific inhibitors respectively. Figure 4 shows that none of these drugs were able to modify cell survival per se at

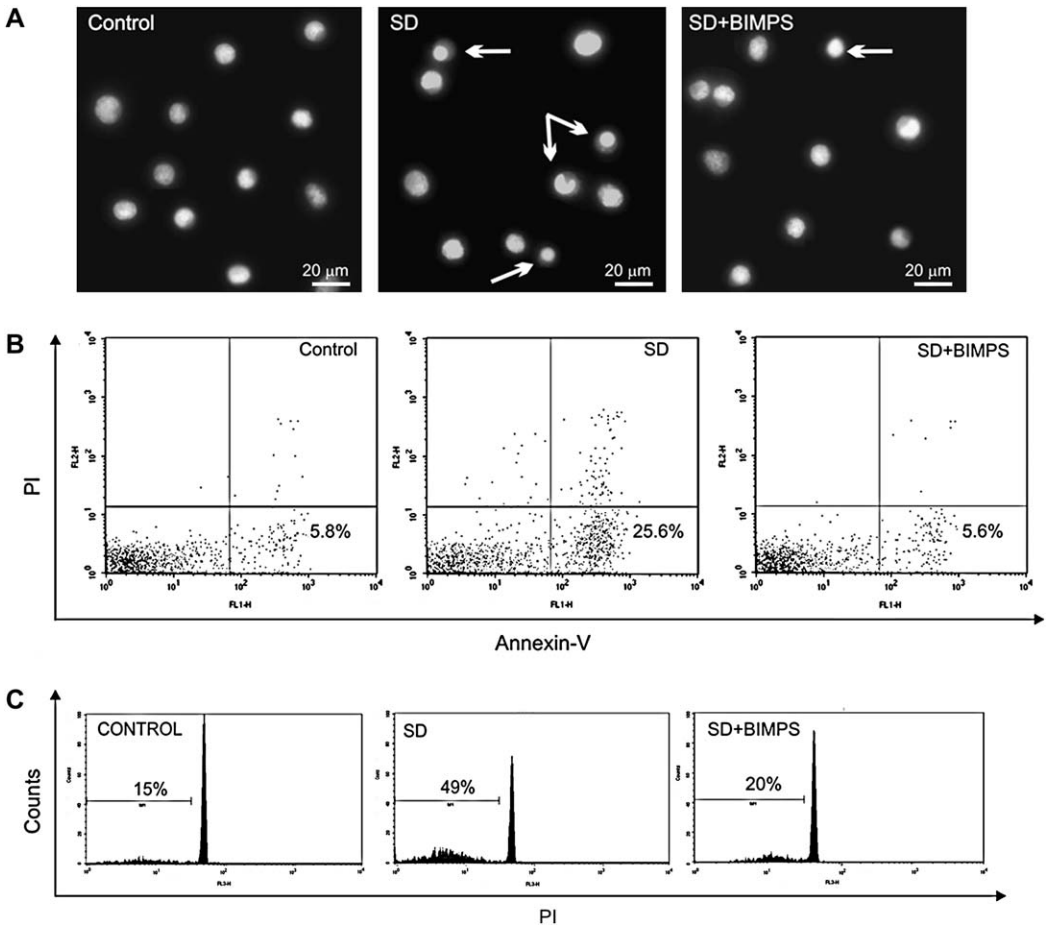

Figure 2. Cyclic AMP suppresses HSC apoptosis induced by SD. **(A)** $CD34^+$ cells were treated with BIMPS (10 μM) and apoptosis was evaluated by fluorescence microscopy 48 hours later. Arrows indicate apoptotic cells (original magnification 400×). **(B)** Cells were treated with BIMPS (10 μM) and 24 hours later the percentage of annexin V^+ cells was determined by flow cytometry. **(C)** Cells were treated with BIMPS (10 μM) and apoptosis was measured by PI staining and flow cytometry. The percentage of hypodiploid cells is indicated on each panel. One representative experiment is shown for each assay (n = 5).

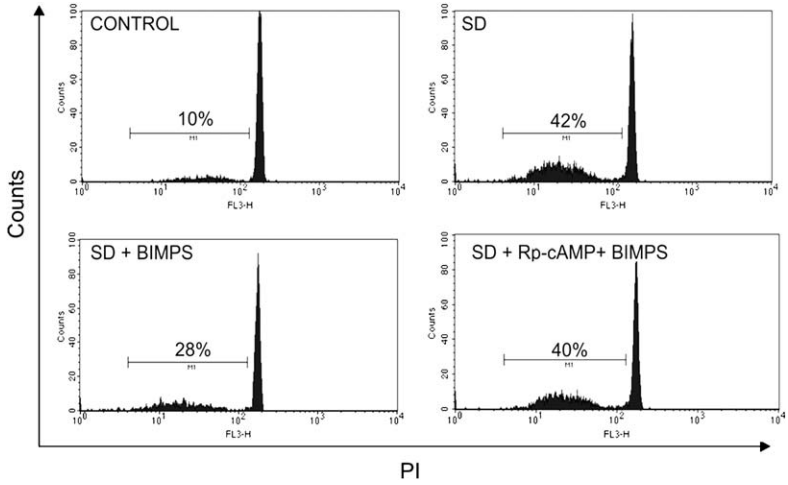

Figure 3. Role of PKA in cAMP-mediated cytoprotection. $CD34^+$ cells were preincubated for 30 minutes with Rp-cAMP (50 μM) before BIMPS (5 μM) addition. After 48 hours, apoptosis was evaluated by detection of hypodiploid nuclei by flow cytometry. One representative experiment from 4 independent experiments performed in duplicate is shown.

the concentrations employed. While both PI3K inhibitors completely suppressed BIMPS-mediated cytoprotective action, the MAPK inhibitor had no effect. The activity of PD098059 was confirmed by the observation that it significantly reduced TPO-induced $CD34^+$ proliferation (data not shown).

bcl-xL, a mediator of cAMP survival pathway

Most primitive $CD34^+$ cells express *bcl*-xL, which appears to be essential for the survival of the HSC population [20]. Thus, we next examined *bcl*-xL expression and its regulation by cAMP using flow cytometry. Fluorescence histograms showed that serum-starved cells exhibited a marked reduction in *bcl*-xL expression (C=90% ± 1%, SD=66% ± 3%* of positive cells, n=3) that was completely inhibited by BIMPS pretreatment (BIMPS+SD=94% ± 2%#, *$p < 0.05$ vs C, #$p < 0.05$ vs SD, n=3) (Fig. 5).

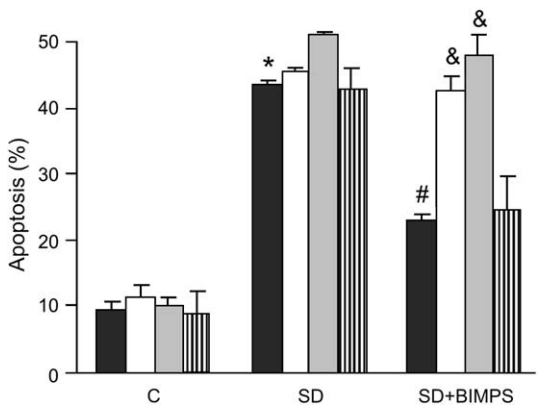

Figure 4. Role of PI3K in cAMP antiapoptotic activity. Cells were preincubated for 30 minutes in the absence (black bars) or presence of Wortmmanin (100 ng/mL) (white bars), LY294002 (10 μM) (grey bars), or PD098059 (50 μM) (striped bars) before BIMPS (5 μM) addition. Apoptosis was evaluated by detection of hypodiploid nuclei by flow cytometry (n = 4 in duplicate). *$p < 0.05$ vs control, #$p < 0.05$ vs SD, &$p < 0.05$ vs BIMPS alone.

Caspase-3 inhibition is involved in cAMP antiapoptotic activity

Members of the caspase family are considered the executer proteins of programmed cellular death. Since caspase-3 is activated when $CD34^+$ cells are deprived of growth factors [9], we evaluated its expression in our experimental model. Figure 5 shows that expression of caspase-3, in freshly isolated $CD34^+$ cells (C=12% ± 1% of positive cells), was increased up to 36% ± 3% ($p < 0.05$, n=3) after 5 hours of serum starvation. Addition of BIMPS significantly reduced caspase-3 expression levels to 20% ± 2% ($p < 0.05$ vs SD, n=3).

Cyclic AMP inhibited SD-induced $\Delta\varphi_m$ reduction

Cytochrome-c release is associated with opening of permeability transition pore (PTP) of mitochondrial membrane [21]. Once cytochrome-c is released, it triggers caspase activation and cell apoptosis. Reduction of $\Delta\varphi_m$ could be an indicator of PTP opening [22]. While apoptotic $CD34^+$ cells showed a sharp decrease in $\Delta\varphi_m$, reflected by a lower red/green fluorescence ratio, BIMPS pretreatment abrogated this effect (Fig. 6).

Role of cAMP in $CD34^+$ cell survival mediated by TPO, G-CSF, and SCF

It is well known that some growth factors such us TPO, SCF, and G-CSF not only promote cell proliferation but also have antiapoptotic activity [4,5]. Having demonstrated that cAMP is a survival pathway in HSCs, we next examined whether this second messenger was involved in the antiapoptotic activity of different cytokines. In agreement

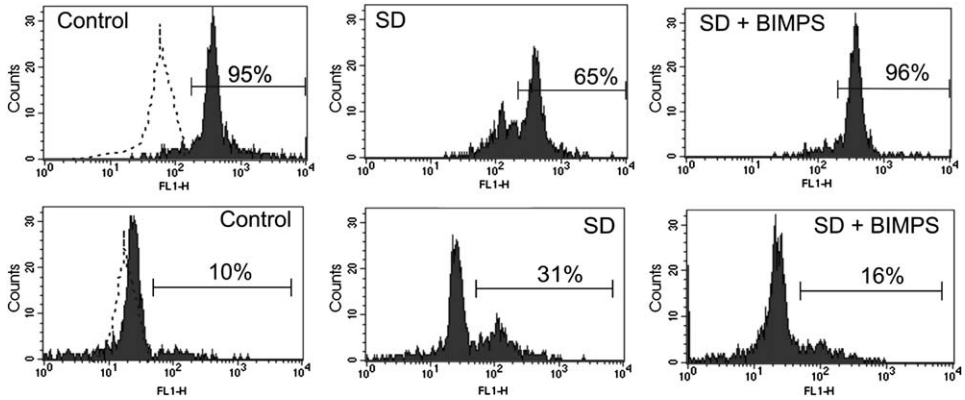

Figure 5. Cyclic AMP suppresses SD-induced *bcl*-xL downregulation and caspase-3 activation. $CD34^+$ cells were treated with BIMPS (10 μM). *bcl*-xL expression (upper picture) and caspase-3 activation (lower picture) were evaluated by flow cytometry 48 and 5 hours later respectively. One representative experiment is shown for each assay (n = 3 in duplicate).

with previous data [4,5], TPO, G-CSF, and SCF decreased the number of apoptotic cells induced by serum withdrawal (Fig. 7). Pretreatment of $CD34^+$ cells with SQ22536, an adenylate cyclase inhibitor, markedly decreased the cytokine-mediated effects (Fig. 7). In addition, when cells were treated simultaneously with threshold TPO, G-CSF, or SCF concentrations and BIMPS, they synergistically prevented SD-induced cell death (Table 2). Interestingly, TPO, G-CSF, and SCF failed to increase cAMP levels (data not shown).

Role of cAMP in colony-forming activity of $CD34^+$ cells

To evaluate the effects of cAMP on a functional activity of HSCs, clonogenic progenitor assays were performed in the presence or absence of BIMPS or Dib-cAMP. We found that the total number of colonies formed by $CD34^+$ cells subjected to NO or SD was almost twofold and fivefold lower respectively than control samples (Fig. 8). Although BIMPS or Dib-cAMP failed to modify colony-forming activity of $CD34^+$ cells compared to control samples, both analogs significantly inhibited the decrease in colony formation by HSC exposed to NO or SD (Fig. 8). In all experiments, no changes were observed either in the percentage of different types (Fig. 8) or in the size of the colonies formed (data not shown).

Discussion

Our results show that cAMP is a survival pathway in $CD34^+$ cells. The inhibitory effect of cAMP toward $CD34^+$ cell apoptosis was observed in both NO- and SD-induced cellular death. This observation makes a specific interaction of cAMP with any of these transduction

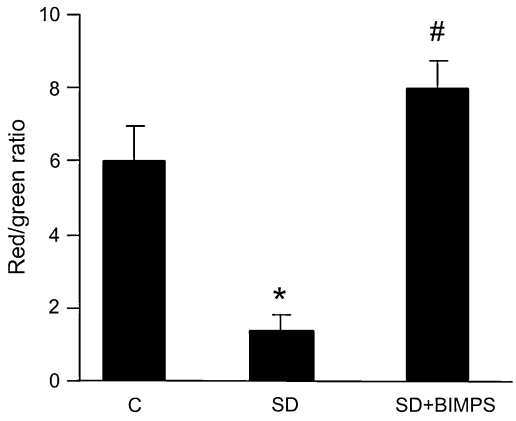

Figure 6. Cyclic AMP inhibited SD-induced $\Delta\varphi_m$ reduction. $CD34^+$ cells were treated with BIMPS (10 μM) for 18 hours. After labeling with MTt-E-φ reagent for 15 minutes, cells were analyzed by flow cytometry. Results are expressed as the ratio between the means of red/green fluorescence intensity (n = 4 in duplicate). $^*p < 0.05$ vs control, $^{\#}p < 0.05$ vs SD.

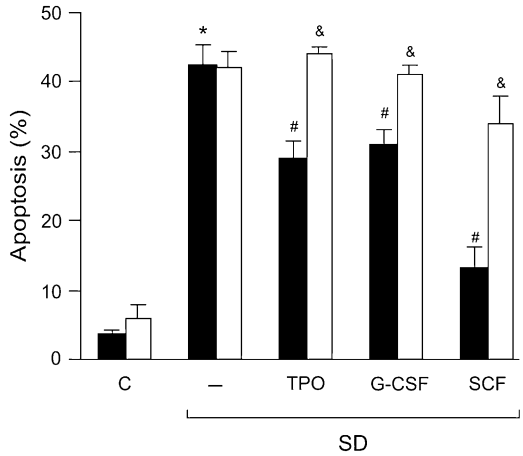

Figure 7. Role of cAMP in TPO-, G-CSF-, or SCF-mediated cytoprotection. Cells were incubated for 5 minutes without (black bars) or with (white bars) SQ22536 (0.5 mM) before TPO (20 ng/mL), G-CSF (20 ng/mL), or SCF (20 ng/mL) addition. Apoptosis was evaluated by fluorescence microscopy 48 hours later (n = 5 in duplicate). $^*p < 0.05$ vs control, $^{\#}p < 0.05$ vs SD, $^{\&}p < 0.05$ vs cytokines without SQ22536.

Table 2. Cyclic AMP potentiates the TPO, G-CSF, and SCF antiapoptotic effect

Treatments	Apoptosis (%)	
	− BIMPS	+ BIMPS
Control	3 ± 1	2 ± 1
SD	47 ± 3*	38 ± 3
TPO + SD	40 ± 1	25 ± 2§
G-CSF + SD	38 ± 2	22 ± 2§
SCF + SD	35 ± 6	18 ± 3§

$CD34^+$ cells were incubated with TPO (2 ng/mL), G-CSF (2 ng/mL), or SCF (1 ng/mL) in the absence or presence of BIMPS (2 μM). Apoptosis was determined 48 hours later by detection of hypodiploid cells. Values are expressed as means ± standard error of 3 independent experiments performed in duplicate. *$p < 0.05$ vs control, §$p < 0.05$ vs BIMPS, TPO, G-CSF, or SCF alone.

pathways unlikely, and rather supports a general inhibitory effect of cAMP toward $CD34^+$ cell apoptotic pathways.

In our experiments, inhibition of PKA completely suppressed the effect of cAMP, pointing out that this kinase is a *downstream* effector of the cAMP pathway. In addition, the participation of the PI3K axis in the cAMP rescue of $CD34^+$ from apoptosis was confirmed by observing that two specific inhibitors of PI3K completely suppressed the cAMP antiapoptotic effect. In contrast, failure of PD098059 to inhibit the cAMP effect suggests that MAPK do not appear to be involved. These results are in agreement with previous studies on TPO antiapoptotic pathways [7,23].

Activation of PKA and PKB results in phosphorylation of different proteins that regulate expression of antiapoptotic and proapoptotic members of the *bcl*-2 family proteins

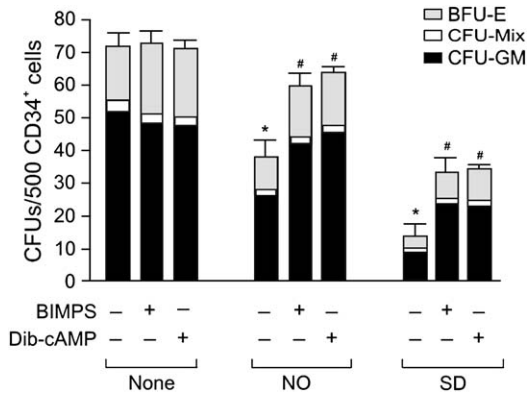

Figure 8. Cyclic AMP inhibits the decrease in colony-forming activity of HSCs induced by NO and SD. $CD34^+$ cells (5×10^2/100 μL) were deprived of serum or exposed to NO in the absence or presence of Dib-cAMP (20 μM) or BIMPS (10 μM) for 24 hours and then plated into methylcellulose semisolid medium. In NO-induced apoptosis, both analogs were added 1 minute before PAPA/NO (100 μM). After 12 days of culture, total number and types of colonies per dish was determined by light microscopy. Values are expressed as means ± standard error of 3 independent experiments performed in duplicate. *$p < 0.05$ vs control, #$p < 0.05$ vs NO or SD.

[24–27]. We found that serum starvation induced downregulation of antiapoptotic *bcl*-xL protein. The observation that treatment of cells with a cAMP analog completely reversed the changes of *bcl*-xL levels revealed that expression of this protein is another step of the apoptotic program at which cAMP acts to prevent cellular death. The relative levels and competing dimerization between *bcl*-2 family members can indirectly prevent the activation of executioner caspases by controlling cytochrome-c efflux from mitochondria. In particular, Bax interacts with mitochondrial membrane allowing cytochrome-c release, whereas *bcl*-2 and *bcl*-xL act in the opposite way, interfering with the activation of Bax [28]. Our results demonstrate that inhibition of both loss of mitochondrial potential membrane and caspase-3 activation triggered during the apoptosis process are also involved in the antiapoptotic cAMP signaling pathway.

Several reports have shown that some growth factors promote not only proliferation, but also cell survival through activation of antiapoptotic signaling pathways [7,29]. Regarding $CD34^+$ cells, it has been demonstrated that TPO, IL-3, G-CSF, IL-6, Flt3 ligand, and SCF support cell survival and prevent programmed cellular death [4,5,7]. Our findings, showing that the cytoprotective action of TPO, G-CSF, and SCF was completely suppressed in the presence of an adenylate cyclase inhibitor and increased by a cAMP analog, indicate that cAMP is a key mediator in the antiapoptotic activity of these growth factors. Interestingly, none of these growth factors were capable of increasing intracellular cAMP levels; however, it is still conceivable that TPO, G-CSF, or SCF do need basal cAMP levels to upregulate antiapoptotic genes.

During preparation of this manuscript, Goichberg et al. [30] reported that sustained elevation of cAMP upregulates the expression of the chemokine receptor CXCR4 on human $CD34^+$ cells. The functional significance of this enhanced CXCR4 expression was demonstrated by an increased ability of human $CD34^+$ cells to transmigrate and adhere to bone marrow stroma and augmented homing potential. They also observed that cAMP elevation and subsequent PKCζ activation had a beneficial effect on $CD34^+$ cell survival. Our present data confirms and further extends the findings of Goichberg et al. demonstrating that PKA and PKB as well as regulation of antiapoptotic proteins and caspase activation are involved in the cAMP-mediated prevention of $CD34^+$ cell apoptosis. In the study of Goichberg et al. [30] they also observed that sustained elevation of cAMP decreased $CD34^+$ proliferation through inhibition of ERK activation. Using colony-forming assays, we observed that although treatment with cAMP did not modify the number or type of colonies formed by $CD34^+$ cells, it was capable to abrogate the decrease in colony formation mediated by NO or SD, indicating that cAMP rescue from apoptosis renders functional $CD34^+$ cells. Taken together with these observations, it could be speculated that

cAMP may help to maintain the survival and the restrictive proliferative capacity of the quiescent HSC in the bone marrow but allows functional activity of these cells when exposed to appropriate signaling.

Early investigations have shown that activation of the adenylate cyclase plays an important role in hematopoietic progenitor proliferation. There is a general consensus that while cAMP raises inhibit granulocyte [31–34] and B-lymphocyte [35] progenitor proliferation, they enhance erythroid colony growth [33,36], and controversial data exist regarding megakaryoblast proliferation [33,37]. The fact that we did not observe any of these cAMP effects on progenitor proliferation could be related to the different experimental conditions employed in each study (e.g., type and origin of cells, cAMP source, pretreatment time of cells, previous plating, and growth factors).

In conclusion, the results presented in this study identify cAMP as a protective factor for $CD34^+$ cells and may promote a future use of cAMP-related drugs in supportive care of $CD34^+$ cell transplantation.

Acknowledgments

This work was supported by grants from the National Agency of Scientific and Technological Support (PICT 14353).

References

1. Attar EC, Scadden DT. Regulation of hematopoietic stem cell growth. Leukemia. 2004;18:1760–1768.
2. Koury MJ. Programmed cell death (apoptosis) in hematopoiesis. Exp Hematol. 1992;20:391–394.
3. Allen PD, Bustin SA, Newland AC. The role of apoptosis (programmed cell death) in haemopoiesis and the immune system. Blood Rev. 1993;7:63–73.
4. Borge OJ, Ramsfjell V, Cui L, Jacobsen SE. Ability of early acting cytokines to directly promote survival and suppress apoptosis of human primitive $CD34^+CD38^-$ bone marrow cells with multilineage potential at the single-cell level: key role of thrombopoietin. Blood. 1997; 90:2282–2292.
5. Philpott NJ, Prue RL, Marsh JC, Gordon-Smith EC, Gibson FM. G-CSF-mobilized CD34 peripheral blood stem cells are significantly less apoptotic than unstimulated peripheral blood CD34 cells: role of G-CSF as survival factor. Br J Haematol. 1997;97:146–152.
6. Lataillade JJ, Clay D, Bourin P, et al. Stromal cell–derived factor 1 regulates primitive hematopoiesis by suppressing apoptosis and by promoting G(0)/G(1) transition in $CD34^+$ cells: evidence for an autocrine/paracrine mechanism. Blood. 2002;99:1117–1129.
7. Majka M, Ratajczak J, Villaire G, et al. Thrombopoietin, but not cytokines binding to gp130 protein-coupled receptors, activates MAPKp42/44, AKT, and STAT proteins in normal human $CD34^+$ cells, megakaryocytes, and platelets. Exp Hematol. 2002;30:751–760.
8. Maciejewski J, Selleri C, Anderson S, Young NS. Fas antigen expression on $CD34^+$ human marrow cells is induced by interferon gamma and tumor necrosis factor alpha and potentiates cytokine-mediated hematopoietic suppression in vitro. Blood. 1995;85:3183–3190.
9. Wang LS, Liu HJ, Xia ZB, Broxmeyer HE, Lu L. Expression and activation of caspase-3/CPP32 in $CD34^+$ cord blood cells is linked to apoptosis after growth factor withdrawal. Exp Hematol. 2000;28:907–915.
10. Weber-Nordt RM, Henschler R, Schott E, Wehinger J, Behringer D, Mertelsmann R, Finke J. Interleukin-10 increases Bcl-2 expression and survival in primary human $CD34^+$ hematopoietic progenitor cells. Blood. 1996;88:2549–2558.
11. Fukuda S, Pelus LM. Regulation of the inhibitor-of-apoptosis family member survivin in normal cord blood and bone marrow $CD34^+$ cells by hematopoietic growth factors: implication of survivin expression in normal hematopoiesis. Blood. 2001;98:2091–2100.
12. Pozner RG, Negrotto S, D'Atri LP, et al. Prostacyclin prevents nitric oxide–induced megakaryocyte apoptosis. Br J Pharmacol. 2005;145: 283–292.
13. Cossarizza A, Baccarani-Contri M, Kalashnikova G, Franceschi C. A new method for the cytofluorimetric analysis of mitochondrial membrane potential using the J-aggregate forming lipophilic cation 5, 5′, 6,6′-tetrachloro-1, 1′, 3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1). Biochem Biophys Res Commun. 1993;197:40–45.
14. Bernstein ID, Andrews RG, Zsebo KM. Recombinant human stem cell factor enhances the formation of colonies by $CD34^+$ and $CD34^+lin^-$ cells, and the generation of colony-forming cell progeny from $CD34^+lin^-$ cells cultured with interleukin-3, granulocyte colony-stimulating factor, or granulocyte-macrophage colony-stimulating factor. Blood. 1991;77:2316–2321.
15. Schwede F, Maronde E, Genieser H, Jastorff B. Cyclic nucleotide analogs as biochemical tools and prospective drugs. Pharmacol Ther. 2000;87:199–226.
16. Anthony RS, McKelvie ND, Cunningham AJ, Craig JI, Rogers SY, Parker AC. Flow cytometry using annexin V can detect early apoptosis in peripheral blood stem cell harvests from patients with leukaemia and lymphoma. Bone Marrow Transplant. 1998;21: 441–446.
17. Walsh DA. Role of the cAMP-dependent protein kinase as the transducer of cAMP action. Biochem Pharmacol. 1978;27:1801–1804.
18. Zhang W, Liu HT. MAPK signal pathways in the regulation of cell proliferation in mammalian cells. Cell Res. 2002;12:9–18.
19. Cantrell DA. Phosphoinositide 3–kinase signalling pathways. J Cell Sci. 2001;114:1439–1445.
20. Park JR, Bernstein ID, Hockenbery DM. Primitive human hematopoietic precursors express Bcl-x but not Bcl-2. Blood. 1995;86: 868–876.
21. Green DR, Reed JC. Mitochondria and apoptosis. Science. 1998;281: 1309–1312.
22. Kowaltowski AJ, Castilho RF, Vercesi AE. Mitochondrial permeability transition and oxidative stress. FEBS Lett. 2001;495:12–15.
23. Kirito K, Watanabe T, Sawada K, Endo H, Ozawa K, Komatsu N. Thrombopoietin regulates Bcl-xL gene expression through Stat5 and phosphatidylinositol 3–kinase activation pathways. J Biol Chem. 2002; 277:8329–8337.
24. Harada H, Becknell B, Wilm M, et al. Phosphorylation and inactivation of BAD by mitochondria-anchored protein kinase A. Mol Cell. 1999;3:413–422.
25. Srivastava RK, Srivastava AR, Korsmeyer SJ, Nesterova M, Cho-Chung YS, Longo DL. Involvement of microtubules in the regulation of Bcl2 phosphorylation and apoptosis through cyclic AMP-dependent protein kinase. Mol Cell Biol. 1998;18:3509–3517.
26. Seo JH, Rah JC, Choi SH, et al. Alpha-synuclein regulates neuronal survival via Bcl-2 family expression and PI3/Akt kinase pathway. FASEB J. 2002;16:1826–1828.
27. Nimmanapalli R, O'Bryan E, Kuhn D, Yamaguchi H, Wang HG, Bhalla KN. Regulation of 17-AAG-induced apoptosis: role of Bcl-2, Bcl-XL, and Bax downstream of 17-AAG-mediated down-regulation of Akt, Raf-1, and Src kinases. Blood. 2003;102:269–275.
28. Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. Science. 1998;281:1322–1326.
29. Gottlieb RA, Giesing HA, Zhu JY, Engler RL, Babior BM. Cell acidification in apoptosis: granulocyte colony-stimulating factor delays

programmed cell death in neutrophils by up-regulating the vacuolar H(+)-ATPase. Proc Natl Acad Sci U S A. 1995;92:5965–5968.
30. Goichberg P, Kalinkovich A, Borodovsky N, et al. cAMP-induced PKCζ activation increases functional CXCR4 expression on human $CD34^+$ hematopoietic progenitors. Blood. 2006;107:870–879.
31. Taetle R, Mendelsohn J. Modulation of normal and abnormal myeloid progenitor proliferation by cyclic nucleotides and PGE1. Blood Cells. 1980;6:701–718.
32. Lee AW. Synergistic activation of mitogen-activated protein kinase by cyclic AMP and myeloid growth factors opposes cyclic AMP's growth-inhibitory effects. Blood. 1999;93:537–553.
33. Long MW, Heffner CH, Gragowski LL. Cholera toxin and phorbol diesters synergistically modulate murine hematopoietic progenitor cell proliferation. Exp Hematol. 1988;16:195–200.
34. Gualtieri RJ, Berne RM, McGrath HE, Huster WJ, Quesenberry PJ. Effect of adenine nucleotides on granulopoiesis and lithium-induced granulocytosis in long-term bone marrow cultures. Exp Hematol. 1986;14:689–695.
35. Shimozato T, Kincade PW. Prostaglandin E(2) and stem cell factor can deliver opposing signals to B lymphocyte precursors. Cell Immunol. 1999;198:21–29.
36. Rossi GB, Migliaccio AR, Migliaccio G, et al. In vitro interactions of PGE and cAMP with murine and human erythroid precursors. Blood. 1980;56:74–79.
37. Mantel C, Aronica S, Luo Z, et al. Macrophage inflammatory protein-1 alpha enhances growth factor–stimulated phosphatidylcholine metabolism and increases cAMP levels in the human growth factor–dependent cell line M07e, events associated with growth suppression. J Immunol. 1995;154:2342–2350.