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Ectopic expression of Bcl-2 switches over nuclear signalling for cAMP-induced apoptosis to granulocytic differentiation

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Abstract

The IPC-81 myeloid leukaemia cells undergo apoptosis rapidly after cAMP stimulation (6 h) and cell death is prevented by early over-expression of the cAMP-inducible transcription repressor ICER, that blocks cAMP-dependent nuclear signalling. Therefore, the expression of specific genes controlled by CRE-containing promoters is likely to determine cell fate. We now show that cAMP-induced cell death also is abrogated by the over-expression of the anti-apoptotic gene, Bcl-2. Contrary to ICER, Bcl-2 does not affect cAMP-signalling and allows the analysis of cAMP responses in death rescued cells. The Bcl-2 transfected cells treated with 8-CPT-cAMP were growth-arrested and thereafter cells embarked in granulocytic differentiation, with no additional stimulation. Neutrophilic polynuclear granulocytes benefited from a long life span in G0-G1 and remained functional (phagocytosis). This work demonstrates that, using anti-apoptosis regulators, 'death signals' could be exploited to trigger distinct biological responses. Indeed, cAMP signal can trigger several simultaneously developing biological programs, in the same cell, i.e., growth regulation, apoptosis and differentiation. This cell system should prove useful to determine how a tumour cell can be re-programmed for either apoptosis or functional maturation by physiological signals. *Cell Death and Differentiation* (2000) **7,** 1081 – 1089.

Keywords: Bcl-2; apoptosis; cell differentiation; cAMP-signalling; tumour cells; myeloid leukaemia

Abbreviations: cAMP, cyclic adenosine monophosphate; 8-CPT-cAMP, 8-chloro-phenyl-thio-cAMP; CRE, c-AMP responsive element; FACS, fluorescence-activated cell sorting; PKC, Protein kinase C; PKA, c-AMP-dependent protein kinase; RAR, retinoic acid receptor; RXR, retinoid-X-receptor; TUNEL, terminal deoxynucleotide transferase-mediated dUTP-biotin nick-end labelling

Introduction

Cell homeostasis in higher organisms results from a subtle equilibrium between cell proliferation, differentiation and cell death. Programmed cell death (or apoptosis) plays an essential role in the ontogeny and function of normal cells, as well as in the development of tumours.[1–5] Cell death can be read as the ultimate event of the differentiation program by which functionally mature cells are generated. In most tissues, fully differentiated cells function for a limited time and then undergo apoptosis. To avoid tissue ageing and ensure optimal renewal, differentiation and cell death should be tightly coupled programs. This notion is corroborated by analysis of normal haemopoietic cell differentiation where functionally mature cells, like mature polynuclear granulocytes, are short lived and die by apoptosis without additional signalling.[6]

It is well known that regulation of apoptosis occurs through control of pro- or anti-apoptotic genes, among which the genes of the Bcl-2 family are the best described (reviewed in[7–9]). Numerous studies have revealed a key role for Bcl-2 in the development of many tissues, including lymphoid cells, neuronal cells, epithelial cells of glandular tissues (for reviews see[10–13]). Although the mechanism(s) of action of the Bcl-2 protein and its partners remain(s) unclear, their transcriptional and post-transcriptional regulations support the hypothesis that these regulators might constitute molecular targets for several signalling pathways determining cell fate (reviewed in[14]). It is noteworthy that Bcl-2 down-regulation occurs during myeloid leukaemia cell differentiation.[15–18] Bcl-2 and homologous anti-apoptotic proteins not only regulate cell survival, but control cell growth by an action at the cell cycle entry.[19] However, Bcl-2 accomplishes these two distinct functions by genetically dissociated mechanisms.[20] These findings not only suggest a diversification of functions for these proteins, but also that members of the Bcl-2 family may allow one signal to trigger more than one single biological response.

Cyclic-AMP elicits a ubiquitous signalling which plays a central role in diverse responses to hormone for cell proliferation, differentiation and apoptosis (reviewed in[21,22]). The current paradigm is that these distinct

responses reflect either cell type related intrinsic differences, or signal cross-talks. While cAMP stimulates proliferation of some epitheliod cells,[23] it induces differentiation in neuronal cells[24–28] and apoptosis in thymocytes[29] and B cell lymphomas.[30] Alternatively, c-AMP synergises with glucocorticoids and PKC signalling in inducing apoptosis.[28,29,31–33] In the case of human neutrophils several groups have reported that cAMP-elevating agents[34,35] and cAMP analogues[36] inhibited apoptosis. TGF-β and cAMP cooperate to promote apoptosis in B lymphocytes.[37] Cyclic AMP also cooperates with nuclear receptor signalling to trigger cell differentiation. Cyclic-AMP and RARα-dependent signalling cross-talk results in the differentiation of F9 teratocarcinoma[38–41] and granulocytic maturation of various leukaemia cells.[42–46] Recently, a RARα-independent RXR signalling pathway has been evidenced by means of a RXR-PKA cross-talk necessary to induce maturation of retinoid-resistant leukaemia cells.[47] In a specific cellular context, these signallings, taken either independently or combined, can trigger differentiation, proliferation and/or apoptosis. It has been convincingly established that triggering of these biological programs occurs through the transcriptional regulation of primary and secondary sets of genes. However, a pertinacious question that has to be answered is how one defined program is abrogated to allow the execution of another program.

Only a few *in vitro* biological systems offer the proper range of signals and biological responses to decipher the complex networks of molecular events that determine cell fate. The IPC-81 myeloid leukaemia cell line[48] was used to study extensively the pathway for cAMP-induced apoptosis. Cyclic-AMP induces an early G1/S arrest[49] and apoptosis with a complete cell destruction in a few hours.[50–52] This apoptosis process requires PKA-dependent nuclear signalling (CREB phosphorylation) and can be abrogated by constitutive overexpression of the Inducible cAMP Early Repressor (ICER).[53] Although ICER expressing cells were resistant to cAMP-induced cell death, they continued to proliferate with no sign of differentiation. Conceivably, the blockade of gene expression in these cells by constitutive ICER expression should not only block apoptosis-related genes, but also the expression of sets of genes involved in distinct CREB-dependent biological responses. In keeping with this hypothesis, it was of interest to determine whether cAMP could trigger additional biological responses, when only the early apoptotic response to cAMP stimulation was prevented. For this reason, we investigated whether the action of Bcl-2, which occurs downstream cAMP-dependent transcriptional regulation, could block apoptosis and thus allow the analysis of the biological response of death-rescued cells.

In this work, we show that using a physiological agent, a highly tumorigenic leukaemia cell line[54] can be efficiently manipulated, either committed to death or reprogrammed for a normal process of morphological and functional maturation.

Results

Ectopic expression of Bcl-2 uncouples the death machinery from cAMP-dependent nuclear signalling

The MSCVpkg expression vector containing the Bcl-2 coding sequence under the control of the MSCV promoter,[55] was used to produce ectopic retroviral particle in transiently transfected BOSC23 cells.[56] The resulting virus preparations were used to infect both the IPC-81 and IPC-RI$_{D336}$ cells, respectively expressing the wild-type and a mutated regulatory subunit (RI) of the PKA.[57] As experimental control, a similar 'empty vector' has also been used to transfect cells. Several cloned cell lines have been selected for puromycin resistance. To check for the retroviral integration of the Bcl-2 gene into IPC-81 genome and the ectopic expression of its mRNA, a human Bcl-2 cDNA was used in Southern and Northern blots analyses, respectively. A mouse monoclonal antibody directed against human Bcl-2 which cross-reacts with rat Bcl-2 was used for Western blot detection of both rat (endogenous) and human (ectopic) Bcl-2 proteins, comparatively (Figure 1).

Bcl-2 transfected clones showed a positive hybridisation signal for ectopic Bcl-2, after *Eco*RI, *Hind*III and *Bam*HI restriction enzyme digestion of DNA (Figure 1A). 'Empty vector' transfected cells (IPC-e.v.) also resistant to puromycin were modified neither in endogenous Bcl-2 expression, nor in the apoptotic response to cAMP or other agents inducing cell death as okadaic acid, calyculin A, FasL, doxorubicin (not shown). Bcl-2 transfectants (IPC-Bcl-2 and IPC-RI-Bcl-2) show a strong constitutive expression of a 5 kb mRNA (Figure 1B). Western-blot analyses of total cellular extracts indicate (Figure 1C) that Bcl-2 transfected cells also over-expressed a 26 kDa band corresponding to Bcl-2 protein. Thus, these transfected clones showed the presence of the transfected human Bcl-2 gene and the production of mRNAs and protein of expected sizes. These transfected cell clones over-expressing Bcl-2 showed no growth alteration (not shown).

In order to compare IPC-e.v. and IPC-Bcl-2 transfectants for their response to cAMP, we used 8-CPT-cAMP, an analog which binds and activates PKA[50] and induces apoptosis in IPC-81 cells, but not in the IPC-RI$_{D336}$ mutated cell line.[57] Previous experiments on the IPC-81 cell line, showed that a 100 μM concentration of 8-CPT-cAMP induces apoptosis in more than 60% of the cell population after 4 h of incubation and that no cell survived after a 10 h treatment.[50] In order to analyze the features of both IPC-Bcl-2 and IPC-e.v. cells, dose-response assays were performed. Cell viability assays revealed a marked shift in dose-response curves after 17 h of incubation (Figure 2A) showing that IPC-Bcl-2 transfected cells were protected against cAMP-induced cell death. The IC_{50} (35 μM for control cells) is shifted to more than 1000 μM for Bcl-2 transfectants. These results are in accordance with cell morphology (Figure 2B) and with agarose gel DNA fragmentation analysis (Figure 2C). DNA ladder, a typical feature for apoptotic cell death, is observed at 80 μM of 8-CPT-cAMP in control cells after only 5 h of cAMP treatment, whereas Bcl-2-transfected cells showed no

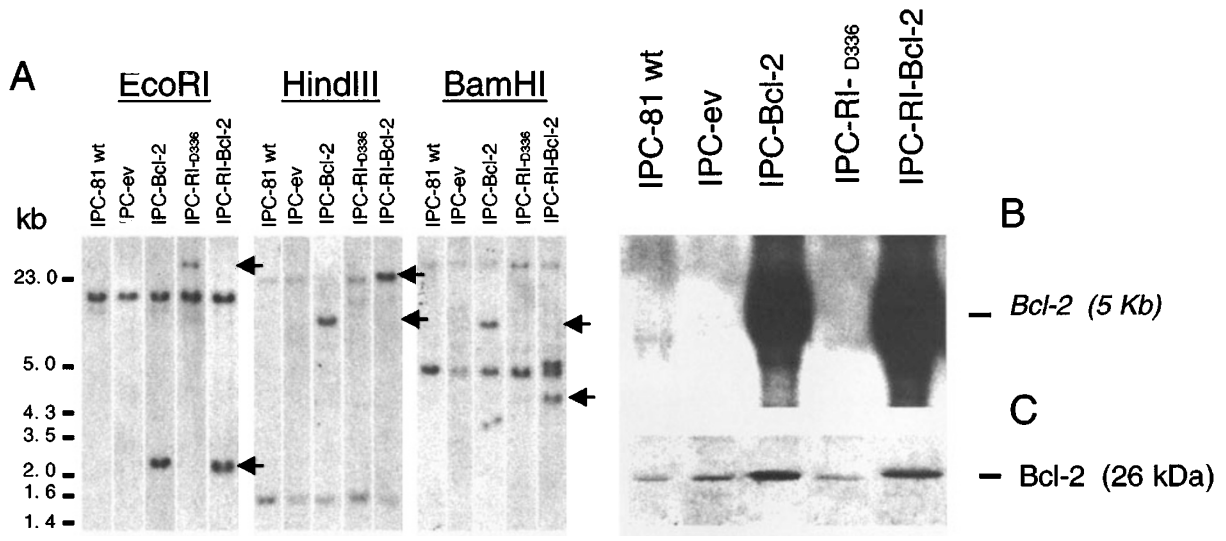

Figure 1 IPC-81 cell transfection and over-expression of Bcl-2 mRNA and protein in IPC-81 and IPC-RI_{D336} cells. (**A**) Southern blot analysis of Bcl-2 transfected IPC-81 cells. *Eco*RI, *Hind*III and *Bam*HI DNA digests from IPC-81, IPC-e.v., IPC-Bcl-2, IPC-RI_{D336} and IPC-RI_{D336}-Bcl-2 cells were hybridised with a human Bcl-2 probe. (**B**) Total mRNA from IPC-81, IPC-RI_{D336} and transfected cells (IPC-e.v.; IPC-Bcl-2; IPC-RI_{D336}-Bcl-2) were analyzed comparatively on Northern blot for Bcl-2 mRNA expression. Note that in IPC-81 cells the endogenous Bcl-2 transcripts are hardly detectable on Northern-blot (endogenous bcl-2 mRNAs sized 7.5 kb, 4.1 kb, 1.9 kb, and therefore are not seen in this figure). (**C**) Total protein extracts from the same cells were analyzed by 15% SDS–PAGE. Bcl-2 proteins were detected by a anti-(rat/human) Bcl-2 mouse monoclonal antibody (see in Materials and Methods)

DNA fragmentation. These experiments demonstrate for the first time that Bcl-2 over-expression protects these cells from PKA dependent apoptosis. To document further the suppression of cAMP-induced apoptosis by Bcl-2, caspase-3 like activation was evaluated after 5 h of treatment in IPC-e.v. and IPC-Bcl-2 cells, comparatively (Figure 2D). Clearly, no enzyme activity in cell extracts could cleave the caspase-3 like colorimetric substrate, Ac-DEVD-pNa. This feature was confirmed at later times during treatment (not shown). Altogether, these data indicated that ectopic over-expression of Bcl-2 in IPC cells, efficiently blocked cAMP-induced apoptosis.

Rapid growth arrest induced by cAMP in Bcl-2 transfected cells

We wanted to determine the fate of cells rescued from death by Bcl-2 over-expression, particularly whether cAMP altered cell growth. Both IPC-e.v., and IPC-Bcl2 were examined for cell cycle and 'TUNEL' analyses during cAMP treatment (Figure 3).

In IPC-e.v. cells used in a control culture, 4 h after addition of 8-CPT-cAMP, a new peak was observed ahead of the G1 peak, on flow cytometry profiles. This additional pre-G1 peak corresponded to the accumulation of apoptotic cells.[58] This peak increased in size after 6 h of cAMP treatment and 'TUNEL' reaction clearly showed a high level of apoptotic cells at the same time (Figure 3A). As expected, these flow cytometry profiles were similar to those obtained with the parental IPC-81 cell line receiving the same treatment (not shown). By contrast, no pre-G1 peak and labelling of DNA fragments could be observed for the Bcl-2-transfected cells, when analyzed after 6 h of cAMP treatments. This confirmed that cAMP-induced apoptosis was blocked in Bcl-2 transfected cells. Furthermore, it was clearly noticed that a decrease of the S-phase peak occurred as early as 6 h of cAMP-treatment (Figure 3A,B) and, remarkably, all cells were growth arrested after 16 h (within a lag-time corresponding to the average doubling-time of IPC-81 cells). By contrast, IPC-$RI_{D336\text{-}Bcl2}$ cells which bear a mutation in the R1 subunit of the PKA[57] showed neither apoptosis nor growth inhibition by cAMP treatment (not shown). These results demonstrate that when Bcl-2 is over-expressed, the early apoptotic response is suppressed and, instead, growth inhibition by cAMP occurs 12–16 h later.

Fate of death-rescued IPC-Bcl-2 cells: recoupling of cAMP-dependent nuclear signalling to the granulocytic maturation program

We wanted to further analyze the fate of Bcl-2 rescued cells. Both viability curves and flow cytometry analyses concurred to support the conclusion that after 16 h of 8-CPT-cAMP treatment, all cells were growth arrested, most of them in G0-G1, a few being blocked in G2-M (Figure 3B). Morphological analysis (Figure 4A–C) carried out in parallel on growth arrested cells showed that after 72 h of cAMP treatment, IPC-Bcl-2 cells were achieving granulocytic maturation (Figure 4B). Granulocytic differentiation of IPC-Bcl-2 cells was then analyzed by flow cytometry, using specific differentiation markers such as the expression of membranous adhesion molecules, the CD11b and CD11c integrins. Figure 4D shows that from 24 to 96 h, Bcl-2 transfected cells treated with cAMP expressed increasing amounts of CD11b/c integrins. Therefore, not only Bcl2 expression rescued cells from death, and allowed growth arrest but, importantly, it allowed myeloid cell maturation to occur. Notice also that cAMP, which did not

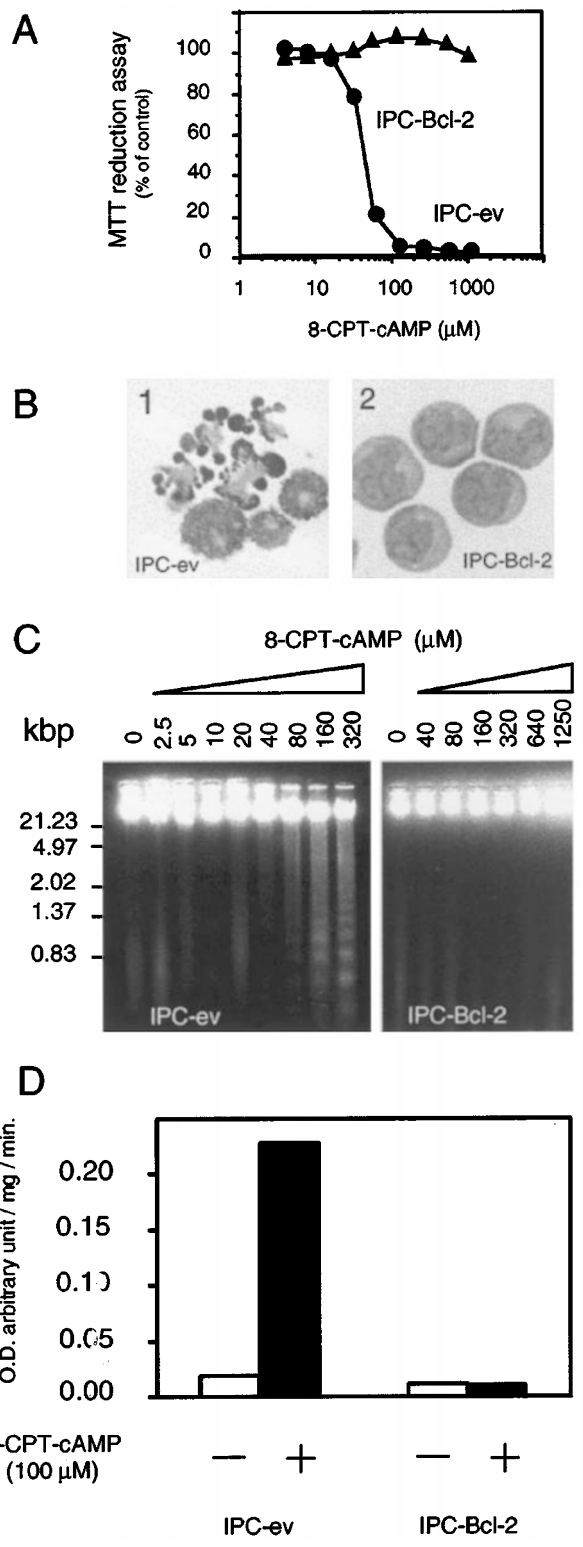

Figure 2 Resistance of IPC-Bcl-2 cells to cAMP-induced apoptosis. (**A**) Cell viability was measured by WST1 assay after 17 h incubation of cultures with 8-CPT-cAMP. Dose-response to 8-CPT-cAMP (**B**). Cell morphology (May-Grünwald-Giemsa staining) after a 5 h 8-CPT-cAMP treatment (100 μM) of IPC-e.v. (B1) and IPC-Bcl-2 (B2) cells. (**C**) DNA fragmentation was evaluated by electrophoretic migration on agarose gels after 5 h incubation with 8-CPT-cAMP; Dose-effect of 8-CPT-cAMP on IPC-e.v. cells (left panel) and IPC-Bcl-2 cells (right panel). (**D**) Differential activation of caspase-3 like protease in IPC-e.v. and IPC-Bcl-2 by cAMP, estimated from the colorimetric measurement of Ac-DEVD-pNa cleavage *in vitro*. All experiments were carried out at least three times. Data shown are from a typical set of analyses

trigger apoptosis in the PKA-mutated IPC-RI_{D336} cells,[57] neither elicited maturation in the Bcl-2 transfected IPC-$RI_{D336\text{-}Bcl2}$ cells (not shown), further supporting the conclusion that PKA signalling triggers the two biological programs.

Bcl-2 over-expression changes normally short-lived cells into long-lived functional polynuclear granulocytic phagocytes

It is well documented that normal granulocytic polynuclear phagocytes show no Bcl-2 expression. They are short-lived and the functional activity of these cells *in vivo* is generally limited to 10 to 15 h.[59] These cells die by apoptosis, and macrophage recognition and phagocytosis occur before any release of their inflammatory content.[6,60] cAMP signalling is also an important regulator for terminal maturation, chemotaxis and the death of these cells.[34–36,61–64] We took advantage of cAMP-induced polynuclear cells to determine whether in leukaemia-derived granulocytes the unphysiological expression of Bcl-2 affected polynuclear phagocyte function and life span. Addition in cell culture medium of *E. Coli* and/or *Saccharomyces cerevisiae* previously killed by fixative treatments allowed the demonstration of an active phagocytosis by the cAMP-induced polynuclear cells, as early as 48 h after cAMP-induced maturation (Figure 4C). Prolonged cultures of the mature phagocytic cells indicated that cell survival of Bcl-2 transfected cells was maintained for up to 2 weeks *in vitro*. However, the function of phagocytes declined with time, then finally cells died.

Discussion

It is generally considered that cellular signalling pathways are univalent (i.e. death signalling, survival signalling, differentiation signalling). When one pathway signals for more than one biological response, the development of multiple responses is commonly understood as the consequence of variations in the intracellular components necessary for signal transduction. This notion could well explain why cAMP generates either growth stimulation, growth arrest, cell differentiation, cell death or inhibition of cell death in various hormone-dependent tissues. Considering that cAMP is a ubiquitous second messenger involved in regulation of gene expression, the involvement of CRE-dependent transcriptional regulation in determining cell fate is suspected.

In a previous work, we showed that in IPC-81 cells, cAMP-induced apoptosis depends on early gene expression, and is blocked by the ectopic expression of the cAMP inducible transcriptional repressor ICER.[53] In this work, we show that, acting downstream the transcriptional regulation by cAMP, Bcl-2 efficiently protected IPC-81 cells from apoptosis. Importantly, although enhanced Bcl-2 expression prevented cAMP from inducing apoptosis, a rapid growth

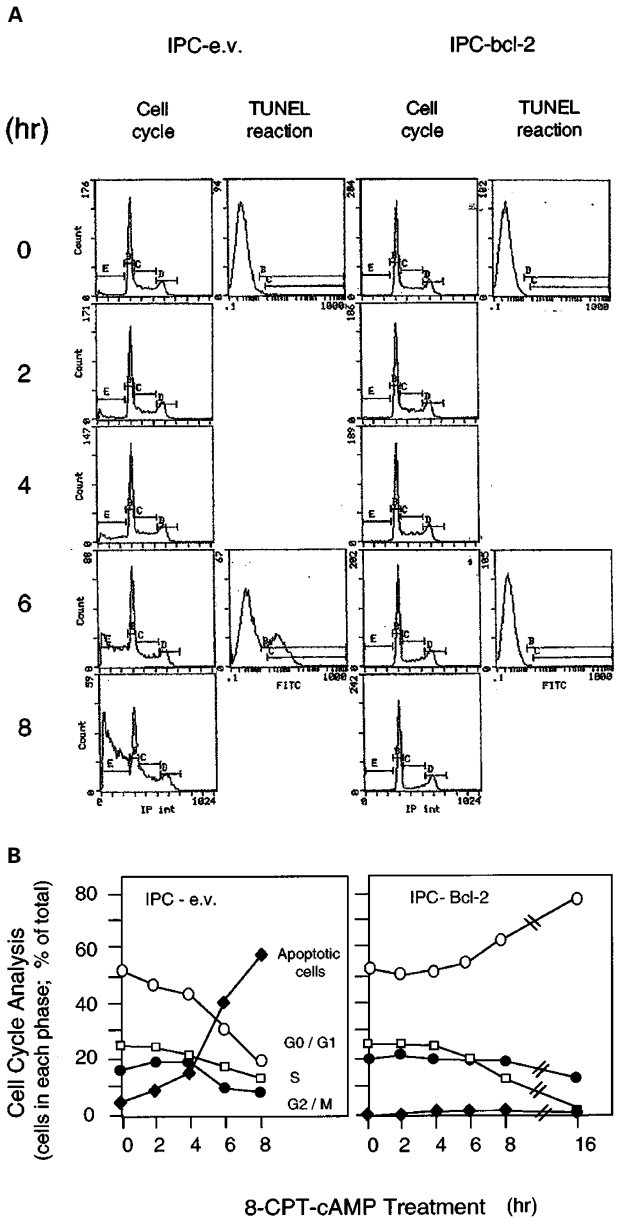

Figure 3 Cell cycle analyses of IPC-e.v. and IPC-Bcl-2 cells by flow cytometry during 8-CPT-cAMP (100 μM) treatment. (**A**) Flow cytometry profiles. Apoptosis was evaluated by TUNEL reaction after 6 h of 8-CPT-cAMP treatment compared to control cultures. (**B**) kinetic of growth arrest *versus* apoptosis in IPC-Bcl-2 and IPC-e.v. cells treated by 8-CPT-cAMP (100 μM). All experiments were carried out at least three times. Data shown are from a typical set of analyses

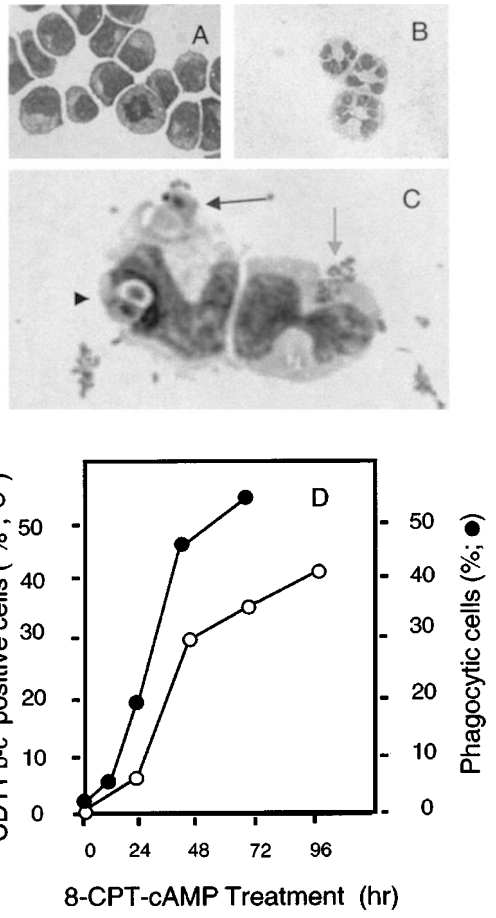

Figure 4 Morphological and functional granulocytic maturation of IPC-Bcl-2 cells in response to 8-CPT-cAMP (100 μM) treatment. May-Grünwald stained cultures, (**A**) control culture; (**B**) 8-CPT-cAMP treated cells (72 h) mature in polymorphonuclear granulocytes; (**C**) 8-CPT-cAMP treated cells (48 h) incubated with fixed *E. coli* and *Saccharomyces cerevisae* for 30 min. Note the typical morphology of mature polynuclear cells. Yeast and bacteria being engulfed by phagocytosis (arrows) and phagosome containing partially digested yeast (arrow head). (**D**) Flow cytometry analyses of CD11b/c integrin expression and yeast phagocytosis, during 8-CPT-cAMP treatment (100 μM). For phagocytosis activity, at least 100 cells were analyzed on each of four to six histological slides for each incubation. Data (positive cells) are expressed as % of the total cell population. All experiments were carried out at least three times. Data shown are from a typical set of analyses

arrest occurred in the death-rescued cells after cAMP triggering, then granulocyte differentiation was observed. Noticeably, in the absence of cAMP treatment, the ectopic expression of Bcl-2 neither modified the cell cycle, nor triggered cell maturation.

Polynuclear neutrophil granulocytes developed in culture after cAMP signalling became fully mature and functional after only 48 h of cAMP stimulation. It is of interest that development of normal granulocytes from myelocytes, during normal haemopoiesis, requires a roughly similar time-course, but that fully mature cells downregulate Bcl-2, are short-lived and die by apoptosis a few hours after cell maturation. In IPC-Bcl-2 leukaemia cells, cAMP acts as a potent intracellular messenger for terminal granulocyte maturation. In these cells, not only does Bcl-2 not affect terminal granulocytic maturation but also it prolongs dramatically the life span of granulocytic phagocytes. Remarkedly, when constitutively expressed, Bcl-2 sequentially protects IPC-81 blast cells from early apoptosis, allows the development of cAMP-induced cell differentiation and increases the survival of terminally mature cells (Figure 5). Considering work reported by other groups[34–36] showing that cAMP inhibited apoptosis of terminally mature human neutrophils, we cannot exclude that cAMP also contributed to a prolonged survival of cells rescued by

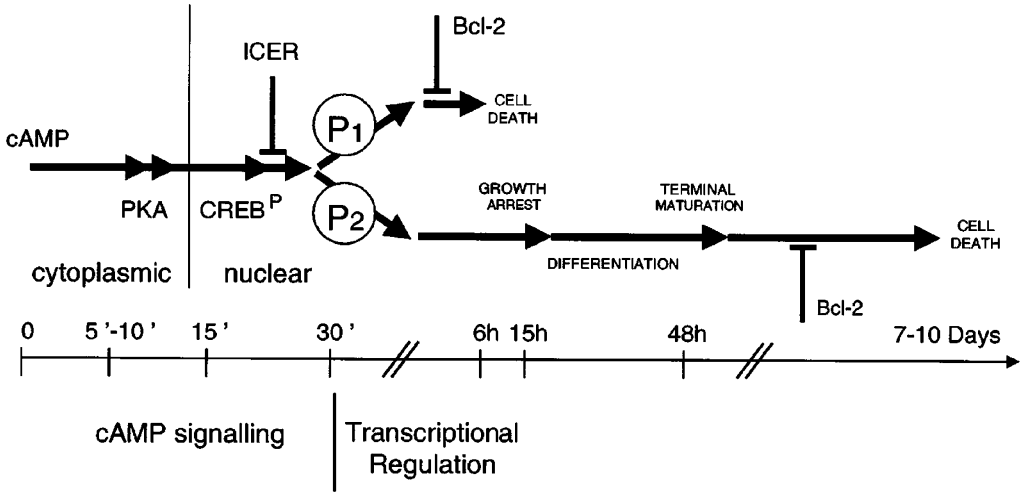

Figure 5 Schematical representation of the cAMP-dependent signalling pathway for cell death and differentiation. A cascade of proximal events (generation of cAMP, activation of PKA, CREB phosphorylation) is shared by death and differentiation signallings and blocked by ICER overexpression.[53] By means of transcriptional gene regulation, cAMP signalling activates distinct sets of genes, each set of genes being necessary to the development of distinct programs, either early cell death of immature blast cells (P1) or myeloid differentiation, terminal maturation, and then death of mature cells (P2). P1 causing early cell death occurs before the development of the distal events of P2 necessary to differentiation. Bcl-2 over-expression represses cAMP-induced cell death without effect on development of P2 program towards cell maturation. Ectopic expression of Bcl-2 in mature cells prolongs their survival as functional phagocytes

Bcl-2 from apoptosis at the immature stage. If this feature was sustained by experimental data, it would mean that, when acting on the same cell type, cAMP can signal for terminal maturation, apoptosis and cell survival, depending upon the stage of cell differentiation. Broadly speaking, the cross-talk between pathways regulating Bcl-2 expression and/or activity and the various signalling for cell differentiation is likely to determine the command of tissue development and remodelling.

Sustained expression of anti-apoptotic regulators like the Bcl-2 family members might be important to the normal development of myelopoiesis. They may provide a second level of regulation for cell survival, after haemopoietic growth factors which are essentially survival factors for haemopoietic progenitors. As a matter of fact, Bcl-2 promotes cell survival in factor-deprived cultures without stimulation of cell proliferation. Bcl-2 rescued cells are most often altered in cell proliferation, at cell cycle entry.[19] However, it is not yet clear whether alteration resulted from a lack of intrinsic capacity to proliferate or from a direct action of Bcl-2 on the cell cycle.[65] Other authors have suggested that Bcl-2 actions on cell survival and growth controls are likely achieved through distinct mechanisms.[20,66] Bcl-2 over-expression suppresses cell death without effect on cell capacity to differentiate.[67] In the case of human neutrophils, ectopic overexpression of Bcl-2 noticeably prolongs cell survival, but interestingly does not block all phenotypic changes preceding or accompanying cell death; indeed Bcl-2 does not prevent ageing neutrophils to be engulfed by macrophages.[68] It has also been reported that death-rescued factor-dependent cells undergo spontaneous differentiation, suggesting intrinsic maturation potential.[69] It is not clear whether this observation can be extended to other cell types (e.g. autonomous tumour cells, like IPC-81 cells), or whether death-rescued cells require appropriate regulatory signal(s) to differentiate. Indeed, this work has shown that in IPC-81 cells, cAMP signalling causing cell death and growth arrest is also required for cell maturation. Because numerous physiological signals known to be important players in haemopoietic differentiation (cAMP, retinoids, glucocorticoids, VD3) also trigger cell death, a sustained Bcl-2 expression (or expression of other members of the Bcl-2 family) could be required for haemopoietic cell development, particularly the full accomplishment of the granulocytic differentiation program. The death program could represent an ancillary program, made available at any time during differentiation, by only modulating the expression of anti-apoptotic regulators.

Finally, this cell model system should prove useful to further investigate to what extent programmed cell death and differentiation share a common signalling pathway and effectors, when activated physiologically. Our knowledge of the molecular network involved in coupling cell death and differentiation programs could benefit to novel therapeutical approaches of tumour treatments.

Materials and Methods

Cell culture and viability assay

The rat promyelocytic leukaemia cell line IPC-81 was previously derived from BN rat myeloid leukaemia (BNML)[48] and cultured as previously described.[50] Note that the IPC-81 cell line is a fully growth-autonomous tumour cell line, and thus it does not require survival factors (CSFs or interleukins) in culture media. Cell morphology was analyzed using the May-Grünwald-Giemsa staining. Cell viability was assessed by morphological integrity of cells under phase-contrast microscopy and by the WST-1 assay (Boehringer Mannheim).

Plasmids, cell transfection, selection of Bcl-2-transfected IPC-81 cells

The MSCVpkgBcl-2 vector was obtained by inserting the complete human Bcl-2 cDNA sequence[10] into the *Eco*RI restriction site of a MSCVpkg vector containing a puromycin resistance gene.[54] The constructs, MSCVpkgBcl-2 and MSCVpkg as control, were used to derive ecotropic retroviral particles in BOSC23 cells.[56] Selection of stable transfected clones was performed by maintaining the culture at a 0.8 μg/ml puromycin concentration during 6 weeks. After amplification of surviving cells, proteins and RNA were analyzed on Western and Northern blots, respectively.

Southern blot and Northern blot analyses

DNA was extracted using standard protocol. After completed digestion with *Eco*RI, *Hind*III and *Bam*HI restriction endonucleases, genomic DNA (10 μg) was electrophoresed, denatured and transferred onto nylon membranes (Hybond-N; Amersham) as described by Maniatis *et al*.[70] and modified by Hillion *et al*.[71] Total RNA was isolated according to the procedure described by Chomczynsky and Sacchi.[72] Twenty μg of total RNA were electrophoresed in a 1.1% agarose/10% formaldehyde gel and then blotted on a nylon membrane.

DNA fragmentation

Cells were pelleted and immediately disrupted in lysis buffer (10 mM Tris-HCl pH 8, 100 mM EDTA, 10 mM EGTA, 0.5% SDS), according to Williams *et al*.[73] DNase-free RNase (SIGMA) was added at 20 μg/ml to the lysates, which were incubated for 2 h at 37°C. The cell lysates were then incubated at 56°C with proteinase K at 100 mg/ml for 1 h. The DNA was extracted with phenol, pelleted with 2 volumes ethanol and 0.1 volume 10 M ammonium acetate, dissolved in TE-buffer (10 mM Tris-HCl pH 8, 1 mM EDTA) and separated on a 1.5% agarose gel (FMC Bioproducts, USA). DNA fragments were visualised on gels after ethidium bromide staining.

Measurement of caspase-3 activity

Caspase-3 like activity was measured using a DEVD cleavage assay modified from Wright *et al*[74] in which DEVD-pNA was used as a colorimetric substrate peptide (Biomol, Tebu, France). Briefly, cells (2×10^6) were pelleted, washed with PBS, pH 7.2, and lysed in 50 mM Tris-HCl, pH 7.5, 0.03% NP-40, 1.0 mM DTT. Lysates were centrifuged at 14 000 r.p.m., for 15 min at 4°C. Total protein determination was done using the Bradford assay. Assays were set up in flat bottom 96-well plates containing 0.2 mM of Ac-DEVD-pNA in a caspase reaction buffer (100 mM HEPES, pH 7.5, 10% sucrose, 0.1% CHAPS, 10 mM DTT) and 0.01 ml of protein extract (20–50 μg) in a total volume of 0.1 ml. Assays were incubated at 37°C. Release of pNa was detected by periodic readings of absorbance at 405 nm taken against a blank containing buffer and peptide alone (i.e., no extract) from 0–5 h to mark the linearity of the enzymatic reaction in time. Enzyme activities were measured as initial velocities and expressed as relative intensity/min/mg total protein within the linear range of the response.

Western blot analysis of protein expression

Cultured cells were washed in PBS and pelleted by centrifugation at $400 \times g$ for 5 min. Pellets of 5×10^5 cells were immediately lysed by adding 100 μl of a boiling Laemmli solution containing β-mercaptoethanol and disrupted with a pestle. Samples were then boiled for 5 min and insoluble material removed by centrifugation at 13 000 $\times g$ for 5 min. Protein extracts (30 μg) were loaded on 15% SDS–polyacrylamide gel, electrophoresed, and blotted onto nitrocellulose membranes (Schleicher & Schuell, Germany). After transfer to the nitrocellulose membrane, proteins were visualised with Ponceau S (Sigma) to confirm equal loading of protein. Membranes were blocked with 5% unfatted milk in PBS, then incubated with a mouse monoclonal antiserum (1/500) raised against human Bcl-2 protein (Dako) in PBS/3% milk for 18 h at 4°C. Membranes were then incubated with horseradish peroxidase-linked protein A for 30 min at 25°C. Each of these steps were followed by three washes for 10 min in PBS/3% milk. Labelling was performed as described in the ECL protocol (Amersham).

Flow cytometry analysis (FACS) of cell surface antigens and cell cycle

The expression of the membrane adhesion molecules CD11b and c integrins was analyzed by direct immuno-fluorescence. After 8-CPT-cAMP (100 μM) incubations, cells were washed in PBS and labelled with an anti-rat CD11b-c FITC mouse monoclonal antibody (Caltag Labs). Cells were then washed twice in PBS and fixed in 1% paraformaldehyde/PBS solution. Cells were analyzed using an FACSCALIBUR (Becton-Dickinson) flow-cytometer. For cell cycle analysis, cells were washed in serum-free medium then fixed in 70% ethanol.

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