Okadaic acid-induced apoptosis of HL60 leukemia cells is preceded by destabilization of bcl-2 mRNA and downregulation of bcl-2 protein

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Abstract We have studied the actions of the protein phosphatase inhibitor okadaic acid (OA) on the expression of bcl-2 in HL60 human leukemia cells. OA induced downregulation of bcl-2 mRNA and protein prior to the induction of apoptosis. Downregulation of bcl-2 mRNA levels did not result from actions of OA on the bcl-2 upstream negative response element. Nuclear run-off analyses confirmed that OA did not affect bcl-2 gene transcription. However, OA caused a rapid increase in the rate of degradation of bcl-2 mRNA. Therefore, OA induces downregulation of bcl-2 expression via destabilization of its transcript. The constitutive action of an OA-sensitive protein phosphatase may therefore maintain HL60 cell survival by blocking bcl-2 mRNA degradation.

Key words: Apoptosis; Bcl-2; Messenger RNA; Leukemia; Phosphoprotein phosphatase; Okadaic acid

1. Introduction

The sensitivity of cells to the induction of apoptosis is determined by the relative levels of anti-apoptotic proteins, including bcl-2 and bcl-X_L and pro-apoptotic gene products including bax [1]. Recent studies have shown that these proteins regulate the release of cytochrome c from mitochondria [2–4] and the consequent activation of caspases, the cysteine proteases which initiate cell commitment to apoptosis [5].

High bcl-2 expression by malignant cells is an obstacle to the treatment of some cancer patients. For example, high bcl-2 expression by the malignant cells of acute myeloid leukemia patients is associated with a poor response to chemotherapy [6]. Therefore, the mechanisms which regulate bcl-2 expression are important determinants of the sensitivity of some tumor cells to cytotoxic regimes. Transcription of the bcl-2 gene is initiated at two distinct promoter sites, P1 and P2. A 1.3 kb negative regulatory element (NRE), located between P1 and P2, potently downregulates transcription from P1 and from heterologous promoters [7]. The biochemical mechanisms which regulate P1, P2 and the NRE are poorly understood.

Okadaic acid (OA) inhibits protein phosphatase 2A (PP2A) at low nanomolar concentrations and protein phosphatases 1 and 2B (PP1 and 2B) at higher levels [8]. OA induces apoptosis in a variety of tumor cell lines [9–12]. Here we show that induction of apoptosis following OA treatment of HL60 cells was preceded by the downregulation of bcl-2 mRNA and protein levels via destabilization of bcl-2 transcripts.

2. Materials and methods

2.1. Cell culture and quantitation of apoptosis

HL60 acute promyelocytic leukemia cells were maintained as described [13]. Apoptosis was quantitated by the terminal transferase-mediated fluoresceinated dUTP nick end-labelling (TUNEL) method using the Apoptosis Detection System (Promega, Southampton, UK). In some experiments, apoptosis was quantified by FACScan analysis of cells containing a sub-diploid DNA content [14].

2.2. Competitive reverse transcriptase polymerase chain reaction (RT PCR) procedures

Relative levels of actin and bcl-2 transcripts were quantitated by competitive reverse transcriptase polymerase chain reaction (RT PCR) [15] using primers and internal standard mimic templates as detailed elsewhere [16]. PCR products were quantitated by Southern blot hybridization [16] and densitometric scanning.

2.3. Western blotting

Protein extraction and Western blot analysis were as described [13].

2.4. Construction and electroporation of luciferase reporter plasmids

The control plasmid pCMV-GL2 (Fig. 3) was derived by blunt-end cloning of the *Nhe*I-*Nsi*I CMV promoter fragment of pBKCMV (Stratagene, UK) into the *Sma*I site located upstream of the luciferase gene of the pGL2 reporter plasmid (Promega, UK). pCMV-GL2-bcl2 (Fig. 3) was constructed by sub-cloning a bcl-2 upstream genomic sequence into pGL2. Details of cloning procedures are available on request. Ten million HL60 cells were suspended in 0.4 ml OptiMEM (Life Technologies, Paisley, UK) and transfected with 10 μg plasmid at 960 μF and 300 V using a Gene Pulser (Bio-Rad, UK). Expression from reporter constructs was quantitated using the Luciferase Assay System (Promega, UK).

2.5. Run-off transcription assays

Run-off assays were carried out as described [17]. Control experiments in which α-amanitin (Sigma, Poole, UK) was included in the run-off reactions abolished hybridization to both bcl-2 and actin targets (not shown).

2.6. Estimation of mRNA stability

HL60 cells were treated with 1 μg ml^{-1} actinomycin D in the presence or absence of 20 nM OA. RNA was isolated from 2×10^6 cell aliquots at 1 h intervals [18]. As a control for recovery, 10^9 copies of the synthetic mouse TEL RNA transcript [19] were added to each 0.5 ml of RNA extraction solution. Relative bcl-2 and actin transcript levels were determined by competitive RT PCR [16].

3. Results

3.1. OA-induced apoptosis of HL60 cells is preceded by downregulation of bcl-2 protein and mRNA

Following the addition of 20 nM OA to HL60 cultures, a small increase in TUNEL-positive apoptotic cells was evident at 24 h. However, substantial induction of apoptosis was not detected until 48 h. Concentrations of OA above 10 nM induced apoptosis of HL60 cells in a dose-dependent manner (Fig. 1B). Expression of the structural protein actin was es-

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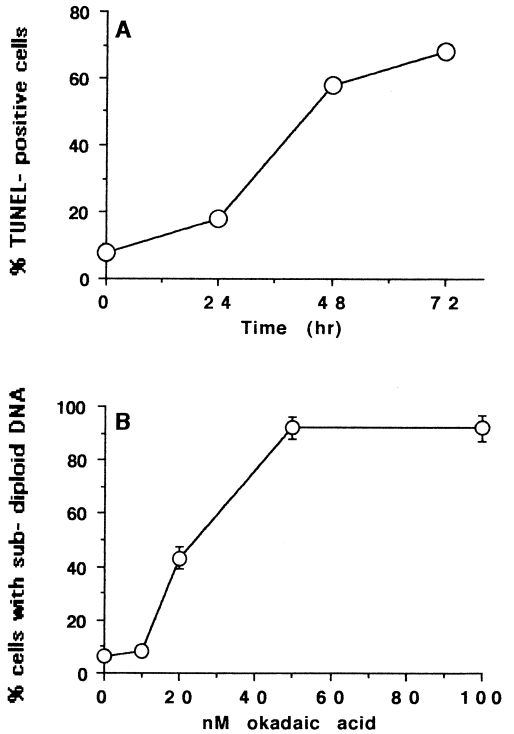

Fig. 1. Induction of apoptosis in OA-treated HL60 cells. A: The percentage of apoptotic cells was determined by TUNEL assay. B: HL60 cells were incubated for 48 h with increasing concentrations of OA. Apoptotic cells were quantitated by FACScan analysis.

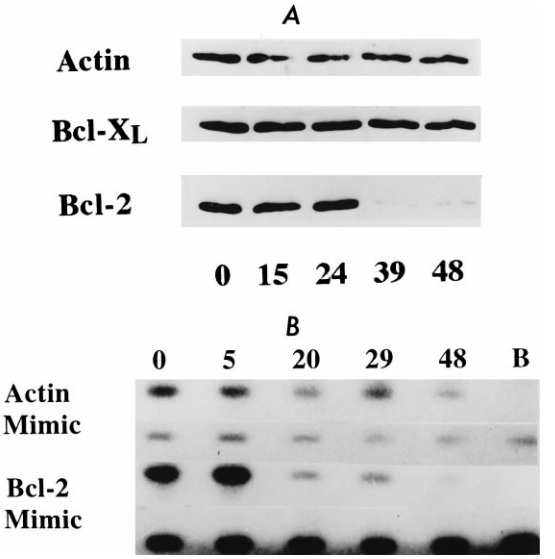

Fig. 2. A: Western blot analysis for actin, bcl-X_L and bcl-2. Hours of incubation with 20 nM OA are indicated under the lanes. B: Competitive RT PCR determination of actin and bcl-2 mRNA in OA-treated HL60 cells. Hours of incubation with OA are indicated above the lanes. Lane B: Reverse transcriptase reagent blank (RNA omitted).

sentially unchanged up to 48 h after addition of 20 nM OA. Bcl-X_L expression was also unaffected. By contrast, a marked downregulation of bcl-2 protein occurred between 24 and 39 h (Fig. 2A).

Bcl-2 cDNA probes cross-hybridize to ribosomal RNA on Northern blots [20]. We therefore used a competitive RT PCR procedure [15,16] for semi-quantitative analysis of bcl-2 and actin transcripts. Bcl-2 mRNA levels were markedly decreased between 5 and 24 h following OA addition, whereas actin transcripts were substantially more stable (Fig. 2B).

3.2. *Effects of OA on the activity of the bcl-2 NRE*

The plasmid pCMV-GL2, which contains the CMV promoter inserted immediately 5′ of the luciferase gene, gave a strong signal on transfection into HL60 cells (Fig. 3A). Luciferase expression by this plasmid was unaffected by OA (Fig. 3A). Insertion of the bcl-2 NRE between the CMV promoter and the luciferase reporter in plasmid pCMV-GL2-bcl2 resulted in a strong inhibition of luciferase expression compared to pCMV-GL2 (compare control transfections in Fig. 3A and B). Therefore, the bcl-2 NRE is functional in HL60 cells.

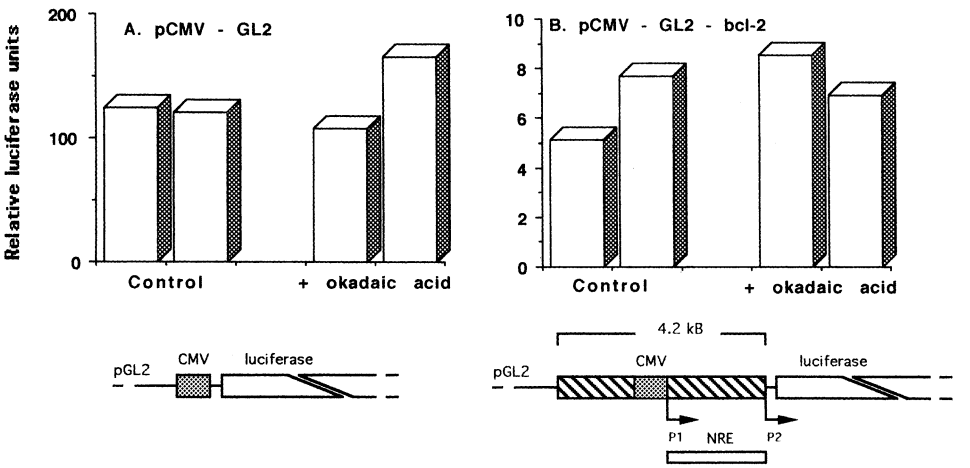

Fig. 3. Actions of OA on luciferase reporter activity in HL60 cells. HL60 cells were electroporated with 10 μg pCMV-GL2 (A) or pCMV-GLC (B). Structures of the plasmids are shown. Bcl-2 upstream sequences are in cross-hatch. Each pool of transfected cells was immediately divided into four aliquots. Two aliquots were incubated in the presence of 20 nM OA for 24 h prior to extraction and assay for luciferase activity while the remaining two aliquots were left untreated. Luciferase activities were normalized with respect to protein content to allow for small variations in extraction efficiency. Note different scales in A and B.

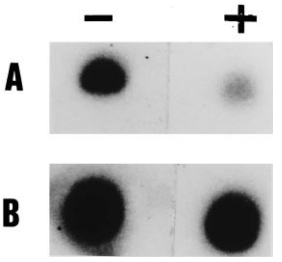

Fig. 4. Run-off analysis of actin and bcl-2 gene transcription. Equal counts min^{-1} of radiolabelled run-off transcripts from control (−) or 24 h OA-treated (+) HL60 cells were hybridized to 5 μg dot-blotted actin (A) or bcl-2 (B) cDNA targets. Run-off transcripts did not hybridize to empty vector (not shown).

However, OA did not further reduce luciferase expression by pCMV-GL2-bcl2 (Fig. 3B), indicating that the downregulation of bcl-2 transcripts induced by this agent (Fig. 2B) was not mediated via an action on the NRE. Similar results were obtained in an independent experiment.

3.3. Run-off analysis of bcl-2 transcripts

Following 24 h OA treatment, the level of actin-specific run-off transcription had declined, whereas bcl-2 transcription was only marginally affected (Fig. 4). Scanning densitometry of two separate run-off hybridizations showed an OA-induced decline in actin transcription of 80% and 69%. Bcl-2 run-off transcription was augmented by 12% in OA-treated cells in the first experiment and decreased by 16% in the second. Therefore, the marked decline in steady-state bcl-2 transcripts observed 20 h following OA addition was not attributable to an effect of the inhibitor on bcl-2 transcription. The maintenance of actin mRNA levels in OA-treated cells (Fig. 3B) despite the decrease in transcription rate (Fig. 4) may be attributable to the relative stability of these transcripts [21].

3.4. Stability of bcl-2 and actin transcripts in OA-treated HL60 cells

Finally, we estimated the stability of bcl-2 transcripts in HL60 cells following blockade of transcription by actinomycin D. Incubation was terminated at 3 h because actinomycin D itself induced caspase activation at 5 h and morphologically observable apoptosis at 6 h (not shown). Comparable RNA recovery and reverse transcription of all samples was monitored by RT PCR analysis of a synthetic TEL RNA transcript which was added at the time of cell lysis (Fig. 5A). Efficiency of individual RT reactions as well as loading and Southern blot transfer of products were controlled by the use of internal PCR controls [15,16].

In the absence of added OA, bcl-2 and actin transcripts were essentially stable following actinomycin D addition (Figs. 5B and 6). By contrast, bcl-2 mRNA was rapidly degraded in HL60 cells incubated in the presence of both actinomycin D and OA (Figs. 5B and 6). The stability of actin transcripts was not decreased by OA addition, suggesting that the effect of OA on bcl-2 mRNA stability was not the result of a general effect on mRNA stability. The OA-induced decrease in stability of bcl-2 transcripts was seen in two additional experiments.

4. Discussion

Here we have shown that OA-induced apoptosis of HL60 cells was preceded by downregulation of bcl-2 protein. Therefore, the decrease in bcl-2 levels may contribute to OA-induced cell killing. By contrast, levels of bcl-X_L protein were not downregulated.

Bcl-2 mRNA levels declined dramatically by 20 h after addition of OA to HL60 cells, suggesting that OA-induced bcl-2 downregulation was the result of decreased transcript levels. Reporter transfection studies showed that bcl-2 mRNA downregulation was not modulated via the upstream NRE of the bcl-2 gene. Run-off analyses confirmed that transcription of the bcl-2 gene was unaffected by the inhibitor. However, OA treatment resulted in the rapid destabilization of bcl-2 mRNA. It is unlikely that this destabilization was the consequence of apoptosis, since the decrease in bcl-2 mRNA half-life was observed within 3 h of OA addition, whereas apoptotic changes were not evident until 24 h.

In contrast to the relatively slow induction of apoptosis by 20 nM OA, micromolar levels of this inhibitor induced apoptosis within 2 h [10]. Apoptosis induction was associated with enhanced serine phosphorylation of bcl-2, which inhibits its ability to neutralize the cytotoxic actions of bax [22]. This action of OA may be mediated by inhibition of protein phosphatase 2B [10]. The slow induction of apoptosis by 20 nM OA reported here may be attributable to a different mechanism involving bcl-2 downregulation resulting from the inhibition of PP2A or PP1.

In conclusion, the data here suggest that the constitutive activity of an OA-sensitive protein phosphatase contributes to the survival of HL60 cells by maintaining stability of bcl-2 mRNA. Earlier observations that ectopic expression of bcl-2

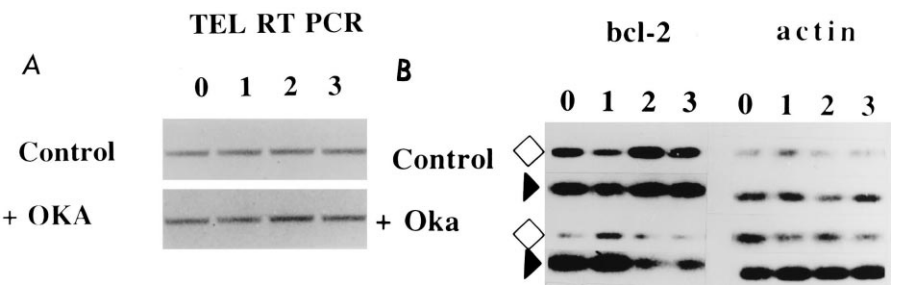

Fig. 5. Estimation of degradation rate of bcl-2 transcripts. HL60 cells were treated with 1 μg ml^{-1} of actinomycin D in the presence or absence of 20 nM OA. RNA was extracted at intervals using a lysis solution containing a synthetic TEL gene transcript. A: Each sample was analyzed by RT PCR to assess recovery of the TEL transcript. B: RT PCR analysis for actin and bcl-2 mRNA. Open diamond, mimic band; closed triangle, bcl-2 band; closed diamond, actin band. The time (hours) after actinomycin D addition is shown above each lane.

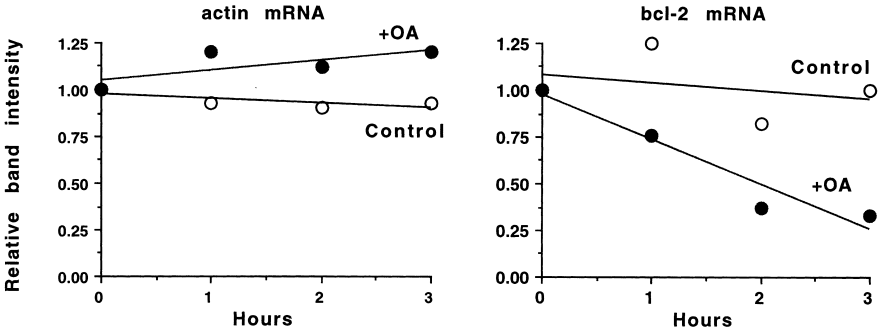

Fig. 6. Rate of decay of actin and bcl-2 transcripts in HL60 cells treated with actinomycin D. The densitometric intensities of the actin and bcl-2 bands shown in Fig. 5B were normalized with respect to the appropriate mimic band and plotted against the time (hours) after actinomycin addition. The band/mimic ratio at time zero was set at unity.

inhibited OA-induced apoptosis in K562 cells [12] is compatible with this hypothesis.

Aberrant regulation of PP2A may play a role in leukemogenesis. The SET gene, which is translocated to the Nup214 gene in some cases of myeloid leukemia [23] encodes an inhibitor of PP2A [24]. It is therefore possible that impaired regulation of PP2A may contribute to leukemogenesis in cells expressing the chimeric SET/Nup214 oncoprotein. Therapeutic modulation of the activity of PP2A-dependent pathways may therefore provide a means of enhancing the sensitivity of some tumor cells to the induction of apoptosis by cytotoxic drugs.

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