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Neither Macromolecular Synthesis nor Myc Is Required for Cell Death via the Mechanism That Can Be Controlled by Bcl-2

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Expression of c-*myc* and macromolecular synthesis have been associated with physiological cell death. We have studied their requirement for the death of factor (interleukin-3)-dependent cells (FDC-P1) bearing an inducible *bcl-2* expression construct. FDC-P1 cells expressing *bcl-2* turned off expression of c-*myc* when deprived of interleukin-3 but remained viable as long as *bcl-2* was maintained. A subsequent decline in Bcl-2 allowed the cells to undergo apoptosis directly from G_0, in the absence of detectable c-*myc* expression. Thus c-*myc* expression may lead to apoptosis in some cases but is not directly involved in the mechanism of physiological cell death that can be controlled by Bcl-2. The macromolecular synthesis inhibitors actinomycin D and cycloheximide triggered rapid cell death of FDC-P1 cells in the presence of interleukin-3, but the cells could be protected by Bcl-2. Thus, the cell death machinery can exist in a quiescent state and can be activated by mechanisms that do not require synthesis of RNA or protein.

Cell death is the outcome of specific cellular processes that have evolved to eliminate unwanted cells and is required for normal development and homeostasis (8, 24, 32). The human *bcl-2* gene (2, 30) encodes a protein which has been shown to block physiological cell death in a variety of mammalian cell types, including neurons, myeloid cells, and lymphocytes (12, 33). The mechanism of cell death that Bcl-2 can control is well conserved, as Bcl-2 is able to block the death of cells in *Caenorhabditis elegans* that are normally mediated by *ced-3* and *ced-4* (7, 35). Furthermore, Bcl-2 can prevent death of cells due to a wide variety of agents, including many drugs, heat shock, growth factor deprivation, and viral infection (15, 27, 29).

The ability of macromolecular synthesis inhibitors such as actinomycin D and cycloheximide to prevent cell death in some circumstances has been used as evidence that the cell actively participates in its own demise (18, 19). However, there are numerous reports of cells failing to be protected by actinomycin D or cycloheximide (for example, see reference 16) or actually undergoing apoptosis in response to them (4, 37). We wished to test whether cell death mediated by the mechanism that can be blocked by Bcl-2 required synthesis of RNA or protein.

Recent reports have associated expression of the c-*myc* oncogene with apoptosis. For example, activation of T-cell hybridomas by antigen or antibodies to CD3 led to apoptosis, but death could be blocked by antisense oligodeoxynucleotides to c-*myc* (25). Rat-1 fibroblasts expressing c-*myc* constitutively were unable to arrest in low serum and underwent apoptosis (9). Chinese hamster ovary cells that overexpressed c-*myc* also died by apoptosis (1). As Myc resides in the nucleus and is able to bind DNA, the association of c-*myc* expression with cell death supports the idea that cell death can be regulated by transcription. Furthermore, three groups reported that apoptosis associated with c-*myc* expression could be blocked by expression of the *bcl-2* gene, showing that in these cases the cell death mechanism triggered by Myc was the one that can be regulated by Bcl-2 (1, 10, 36). We wished to investigate further the requirement for c-*myc* expression during apoptosis and to study the role of transcription and translation in cell death in general.

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MATERIALS AND METHODS

Cell lines. FDC-P1 cells (5) and their derivatives were maintained in RPMI containing 10% fetal calf serum and interleukin-3 provided by medium conditioned by the Wehi3B cell line (14). Cells were maintained at 37°C in a humidified atmosphere of 7% CO_2 in air. Cell counts were performed by using a hemocytometer, and viable cells were distinguished by exclusion of eosin or trypan blue. Where indicated, cells were treated for 24 h with either actinomycin D (2.5 μg/ml) or cycloheximide (2.5 μg/ml).

Constructs. The pMT*bcl*-2TKNeo construct was made by inserting a fragment encoding the entire human *bcl-2* coding region (2) and simian virus 40 poly(A) addition signals into a vector bearing the sheep metallothionein promoter (22) and a thymidine kinase promoter-*neo* cassette to allow selection of transformed cells. FDC-P1 cells were transformed by electroporation with linearized plasmid pMT*bcl*-2TKNeo, and G418-resistant colonies were picked from soft agar cultures containing growth factor and G418 (400 μg/ml). Individual clones were tested for inducible expression of human Bcl-2 by culture in the presence or absence of 100 μM $ZnCl_2$, followed by immunofluorescence using a monoclonal antibody specific for human Bcl-2 (23) (Dako, Glostrup, Denmark). The MPZen*bcl*-2SVNeo and MPZenSVNeo retroviruses were constructed and introduced into FDC-P1 cells by transfection as described previously (34).

Northern (RNA) analysis. FDC-P1 were cells digested with proteinase K, and poly(A) RNA was isolated by adherence to oligo(dT)-cellulose. Two micrograms of mRNA was loaded onto a 1% agarose–formaldehyde gel. The gel was

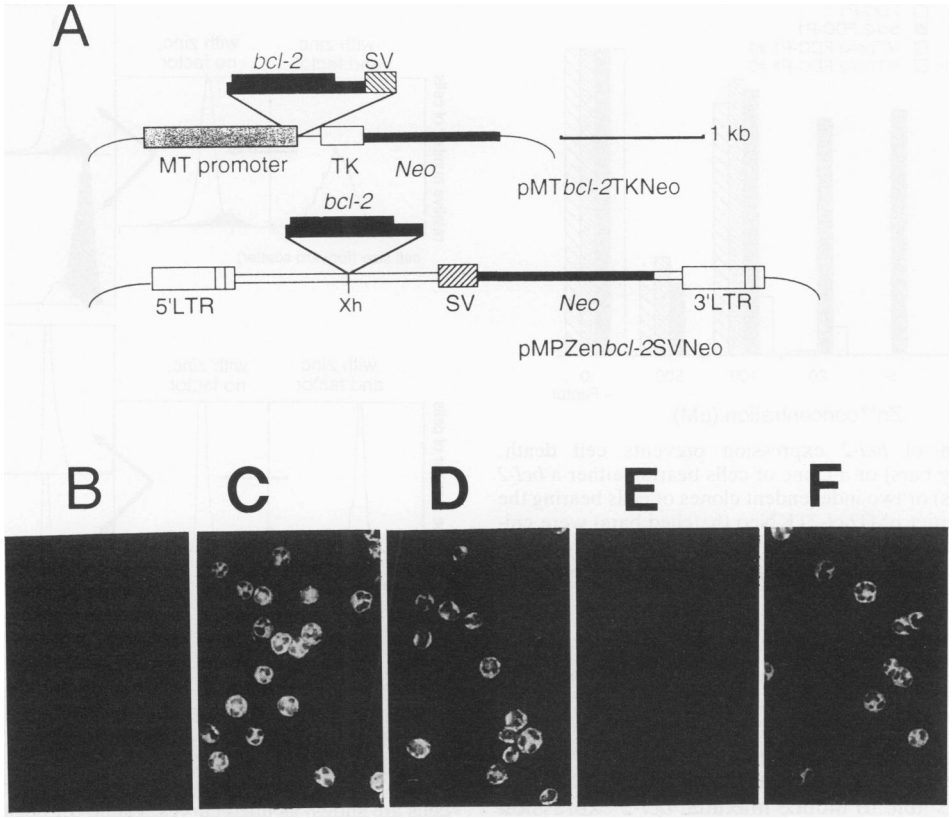

FIG. 1. Expression of human *bcl-2* in FDC-P1 cells. (A) pMT*bcl-2*TKNeo and pMPZen*bcl-2*SVNeo constructs. pMT*bcl-2*TKNeo was constructed by inserting a fragment bearing the human *bcl-2* cDNA and simian virus 40 (SV)poly(A) sites into a vector bearing the sheep metallothionein (MT) promoter (22) and a neomycin resistance gene (*Neo*) driven by the thymidine kinase (TK) promoter. The pMPZen*bcl-2*SVNeo construct allows expression of human *bcl-2* from a myeloproliferative sarcoma virus long terminal repeat (LTR) and was used for the production of helper-free retrovirus as described previously (34). Regions encoding *bcl-2* and *neo* genes are filled; sequences derived from simian virus 40 are hatched; the sheep metallothionein promoter is shaded. (B to F) FDC-P1 cells bearing ZenSVNeo (B), Zen*bcl-2*SVNeo (C), and MT*bcl-2*TKNeo (D to F) constructs were stained with a monoclonal antibody to human Bcl-2. Panel D shows MT*bcl-2*TKNeo-bearing cells treated with 100 μM Zn^{2+} for 24 h; the cells in panel E were not treated with zinc. Panel F depicts a mixture of 12 Zn^{2+}-treated and 5 untreated cells. All exposures were for the same time.

blotted onto a nitrocellulose filter to allow hybridization to [α-^{32}P]dATP-labeled *bcl-2*, *c-myc*, and β-actin probes.

Cell cytometry. Flow cytometry was used to distinguish live and dead cells by propidium iodide (PI) fluorescence. Forward light scatter was used as an index of cell size. Cells that failed to stain with 1 μg of PI per ml were sorted into a solution containing 1% Triton X, 10 μg of PI per ml, and 500 μg of RNase A per ml to allow quantitation of the DNA content of viable cells during a second round of fluorescence-activated cell sorting (FACStar II; Becton Dickinson) analysis (28).

DNA electrophoresis. DNA was extracted by dissolving the cells in 6 M guanidine hydrochloride–0.3 M sodium acetate and precipitating the nucleic acids with 95% ethanol. The pellet was dissolved in 10 mM Tris–1 mM EDTA and treated with RNase prior to electrophoresis in a 2% agarose gel. To visualize the DNA, the gel was stained with 1 μg of ethidium bromide per ml.

RESULTS

Cell lines expressing *bcl-2*. For these experiments, we used FDC-P1 cells, a mouse myeloid cell line that depends upon interleukin-3 or granulocyte-macrophage colony-stimulating factor for growth but undergoes apoptosis when deprived of growth factor (34, 38). These cells were transformed with a retrovirus (MPZen*bcl-2*SVNeo) that directs constitutive expression of the human *bcl-2* gene (34) (Fig. 1A). For inducible expression of *bcl-2*, the FDC-P1 cells were transformed with a vector (pMT*bcl-2*TKNeo) bearing the human *bcl-2* gene under control of the sheep metallothionein promoter (22). This construct allows *bcl-2* expression in the presence of zinc. Inducible expression of *bcl-2* in Zn^{2+}-treated cells was confirmed by staining with antibodies specific for the human Bcl-2 protein (23) (Fig. 1B to F). When Zn^{2+} was withdrawn, *bcl-2* expression decreased, and after 3 days the protein was no longer above the level seen when untreated cells were examined by fluorescence microscopy (data not shown).

In the presence of 100 μM Zn^{2+}, clones of FDC-P1 cells bearing the MT*bcl-2*TKNeo construct survived in the absence of growth factor (Fig. 2), as did cells bearing the Zen*bcl-2*SVNeo provirus. When factor is removed, cells expressing *bcl-2* leave the cell cycle and enter G_0, but they do not die (see Fig. 3). Bcl-2 was required for survival in the absence of growth factor, as wild-type FDC-P1 cells, as well as MT*bcl-2*.FDC-P1 cells not treated with Zn^{2+}, died when growth factor was withdrawn.

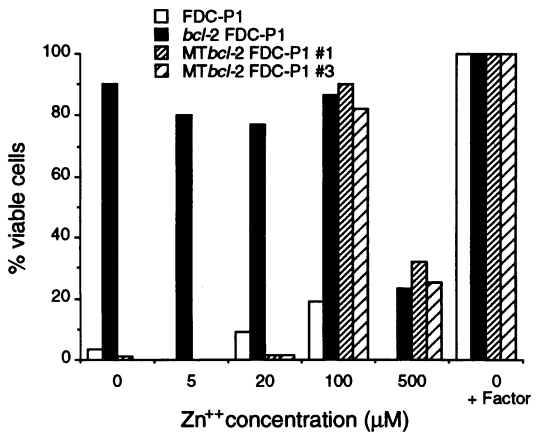

FIG. 2. Induction of *bcl-2* expression prevents cell death. FDC-P1 cells (empty bars) or a clone of cells bearing either a *bcl-2* retrovirus (filled bars) or two independent clones of cells bearing the zinc-inducible construct pMT*bcl-2*TKNeo (hatched bars) were cultured for 2 days in the absence of growth factor in various concentrations of Zn^{2+}. Cell viability was then determined by dye exclusion.

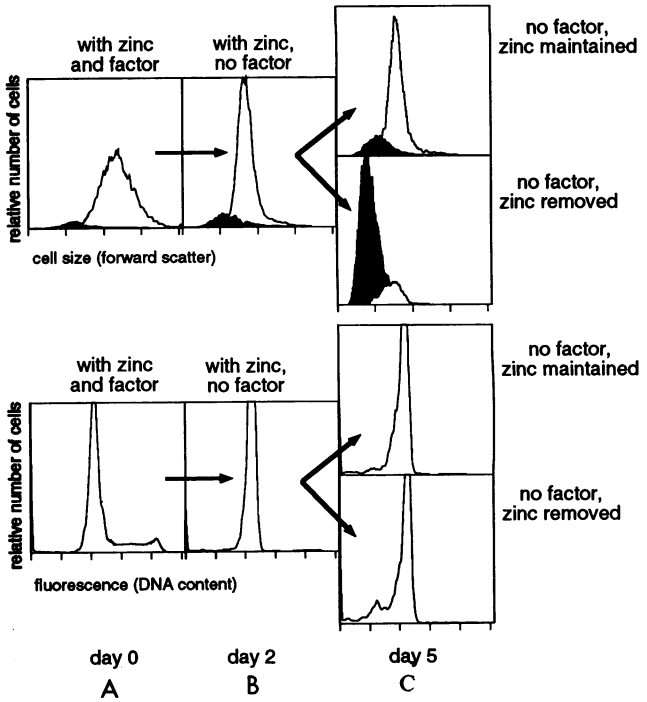

FIG. 3. Maintenance of *bcl-2* is required to prevent apoptosis, and cells can undergo apoptosis directly from G_0. Cell viability and size (upper graphs) and DNA content of sorted viable cells (lower graphs) were determined by flow cytometry. Nonviable (PI-positive) cells are shown as filled curves; viable (PI-negative) cells are shown as unfilled curves. MT*bcl-2*.FDC-P1 cells were cultured with growth factor and 100 μM Zn^{2+} (A; the cells are large and cycling) or deprived of factor for 2 days in the presence of Zn^{2+} (B; the cells have shrunk and entered G_0). Cells cultured with Zn^{2+} but no factor for 2 days were washed and cultured a further 3 days either in the presence or in the absence of Zn^{2+} (C). At this time, most of the cells removed from Zn^{2+}-containing medium had died (filled curves), whereas cells maintained in Zn^{2+} remained alive (unfilled curves).

Zinc ions have the ability to block DNase function (21), but it is likely that Zn^{2+} was able to protect the cells by induction of *bcl-2* rather than by direct inhibition of the endonuclease responsible for DNA cleavage for two reasons. First, the concentration of Zn^{2+} needed to protect the cells (100 μM) was able to induce maximal *bcl-2* expression but was less than the concentrations (typically >1 mM) reportedly necessary to inhibit the endonuclease responsible for fragmenting the DNA (11, 21). Second, the levels of Zn^{2+} that protected the MT*bcl-2*.FDC-P1 cells did not protect the parental FDC-P1 line (Fig. 2).

Apoptosis of cells in G_0. Initially, the cells were propagated in medium containing growth factor and 100 μM Zn^{2+} to induce expression of *bcl-2* (Fig. 3A). To arrest the cells, they were washed and returned to medium containing zinc but no growth factor. Two days after growth factor was withdrawn, the volume of the cells had decreased markedly and the cells had left the cell cycle to enter G_0 (Fig. 3B). These cells remained alive, however, as readdition of growth factor allowed them to proliferate once more (data not shown). The arrested MT*bcl-2*.FDC-P1 cells were then washed again and split into two aliquots for culture either in the presence or in the absence of 100 μM Zn^{2+}. Cells returned to medium containing Zn^{2+}, which consequently continued to express *bcl-2* (see Fig. 4), remained viable. In contrast, the factor-deprived cells transferred to medium without Zn^{2+} died over the following 3 days as the levels of Bcl-2 declined.

As shown in Fig. 4A, in a typical experiment, less than 2% of MT*bcl-2*.FDC-P1 cells could survive in the absence of factor for 3 days after zinc was withdrawn, but 80% of the cells survived when maintained in zinc. Significantly, the dying cells exhibited all of the characteristic signs of apoptosis, including DNA degradation (Fig. 4B).

Expression of c-*myc* in cells undergoing apoptosis. Northern analysis was performed to monitor the levels of c-*myc* expressed by FDC-P1 cells (Fig. 5). Cells provided growth factor (lanes 1 to 3) expressed high levels of c-*myc*. When growth factor was removed, however, c-*myc* expression became undetectable (lanes 4 to 10). In the presence of 100 μM Zn^{2+}, MT*bcl-2*.FDC-P1 cells expressed *bcl-2* message (lanes 5 to 7), but when zinc was removed, *bcl-2* expression was extinguished (lanes 8 to 10). Fluorescence microscopy showed that human Bcl-2 protein gradually declined in abundance in the 3 days following loss of *bcl-2* message (data not shown). Significantly, no c-*myc* was detected in cells undergoing apoptosis (lanes 8 to 10). Since these cells had not synthesized detectable levels of c-*myc* message for 5 days, and the half-life of c-Myc protein is less than 60 min (17), it is unlikely any c-Myc protein remained. Thus, although we did not measure c-Myc protein directly, we conclude that the mechanism of cell death that can be blocked by Bcl-2 does not require c-Myc. To confirm that c-*myc* was not involved in the death of FDC-P1 cells deprived of growth factor, we treated cells with antisense oligonucleotides to c-*myc* kindly provided by Doug Green (1, 25). We found that antisense oligonucleotides at concentrations that protected a T-cell hybridoma from apoptosis due to treatment with anti-CD3 offered no protection to factor-deprived FDC-P1 cells (data not shown).

Effect of macromolecular synthesis inhibitors on the induction of cell death. To test the requirement for macromolecular synthesis in cell death, clones of FDC-P1 cells were treated with actinomycin D or cycloheximide, either in the presence or in the absence of growth factor. Cells were cultured for 24 h in combinations of 2.5 μg of actinomycin D

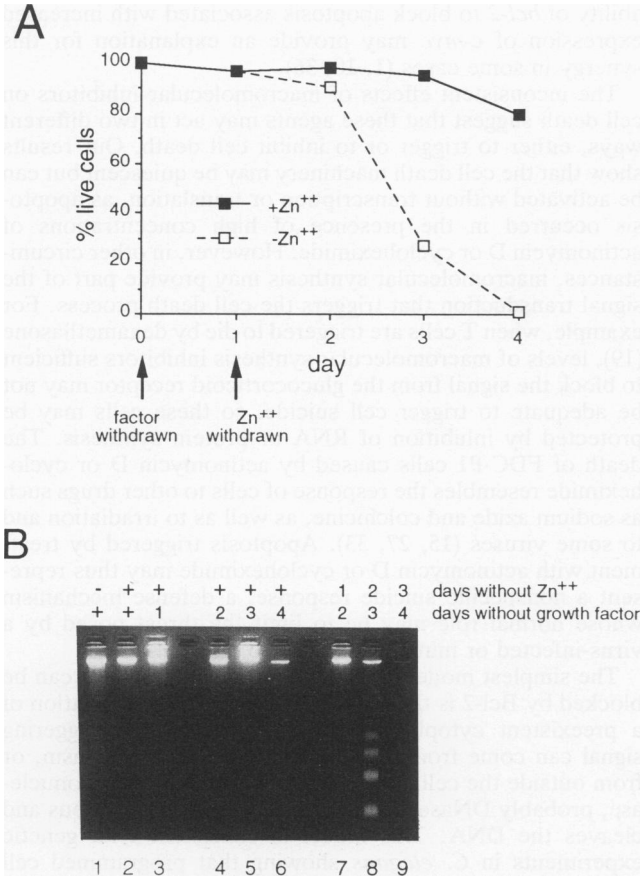

FIG. 4. Cells die as Bcl-2 declines. (A) Growth factor was withdrawn from Zn^{2+}-treated MT*bcl-2*.FDC-P1 cells, and they were cultured for another day in the presence of Zn^{2+}. The cells were then washed and either returned to Zn^{2+}-containing medium (■) or placed in medium without Zn^{2+} (□). Cell viability was determined by dye exclusion. (B) Aliquots of cells from the experiment depicted in panel A were harvested, and their DNA was subjected to electrophoresis. Lanes: 1, DNA from cells cultured with growth factor but no Zn^{2+}; 2, cells cultured with Zn^{2+} and factor; 3 to 6, cells cultured with Zn^{2+} but deprived of factor for 1 to 4 days; 7, cells deprived of factor for 2 days and Zn^{2+} for 1 day; 8, cells deprived of factor for 3 days and Zn^{2+} for 2 days; 9, cells deprived of factor for 4 days and deprived of Zn^{2+} for 3 days. Each lane was loaded with DNA from a similar number of cells, although the fraction of viable cells in each sample varied. The reduced intensity of the bands in lane 9 is due to ongoing degradation of the DNA following its cleavage at internucleosomal regions.

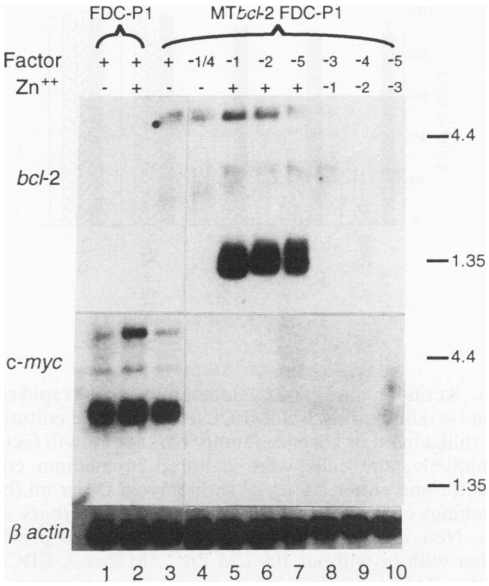

FIG. 5. Expression of c-*myc* is not required for apoptosis to occur. Cells were cultured in the presence or absence of Zn^{2+} and growth factor, and their mRNA was isolated. Lanes 1 and 2 show FDC-P1 cells cultured with growth factor either with (lane 1) or without Zn^{2+} (lane 2). Lanes 3 to 10 show mRNA from MT*bcl-2*.FDC-P1 cells either in the presence of growth factor (lane 3) or when deprived of growth factor from 6 h to 5 days (lanes 4 to 10). Lanes 5 to 7 show mRNA from cells treated with Zn^{2+} to induce *bcl-2* expression; lanes 8 to 10 show mRNA from cells cultured for 1 to 3 days following withdrawal of zinc. Equal amounts of mRNA were loaded into each lane, but considerably more cells were required to yield the mRNA for lanes 9 and 10, as many of these cells were not viable. The Northern blot was hybridized to probes for human *bcl-2*, mouse c-*myc*, and β-actin, as indicated.

per ml, 2.5 μg of cycloheximide per ml, growth factor, and 100 μM Zn^{2+}, and cell viability was determined by dye exclusion.

In the presence of growth factor, both actinomycin D and cycloheximide triggered rapid cell death, and the dying cells exhibited the characteristics of apoptosis. Cell death must have occurred via the Bcl-2-dependent pathway, as expression of *bcl-2*, either constitutively from a retroviral long terminal repeat or when induced by zinc, prevented rapid death of the cells (Fig. 6). The fact that cell death occurred in the presence of growth factor suggests that growth factor does not promote cell survival by inducing *bcl-2* or a Bcl-2-like activity. This conclusion is supported by observations that exogenous Bcl-2, but not growth factor, will protect FDC-P1 cells against apoptosis due to azide or colchicine (data not shown). In the absence of growth factor, we saw no protection from cell death by actinomycin D or cycloheximide, although we repeated the experiment using a range of concentrations of drug (data not shown). These results indicate that the cell death mechanism that can be controlled by Bcl-2 can exist in a quiescent state in a cell, but its activation will culminate in cell suicide unless it is blocked by Bcl-2. Growth factor does not promote FDC-P1 cell viability through induction of *bcl-2* but allows cell survival by stopping default activation of the cell death machinery. As cells cultured with factor are still susceptible to suicide induced by actinomycin D and cycloheximide, as well as azide and colchicine (33), survival signals from the growth factor receptor cannot prevent activation of the cell death machinery by alternative routes.

DISCUSSION

These experiments demonstrate that although synthesis of RNA and protein and expression of c-*myc* have been associated with physiological cell death, none of these processes are required for FDC-P1 cells to die by the mechanism that can be inhibited by Bcl-2. As the mammalian gene *bcl-2* appears to act in the same manner as the *C. elegans* gene *ced-9* (35), which functions in the nematode by inhibiting the activity of *ced-3* or *ced-4* (13), Bcl-2 is likely to prevent the death of mammalian cells by blocking the activity of the mammalian homolog of *ced-3* or *ced-4*. Thus, it is reasonable to propose from these experiments that the cell death

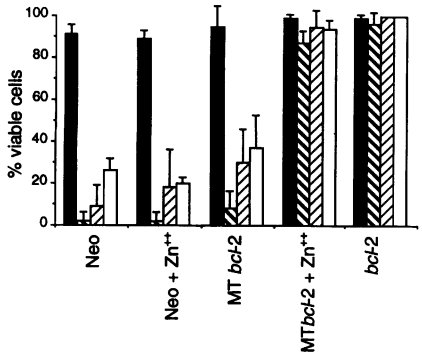

FIG. 6. Actinomycin D and cycloheximide cause rapid cell death which can be inhibited by Bcl-2. FDC-P1 cells were cultured in the presence (filled bars) or absence (empty bars) of growth factor for 24 h. Alternatively, the cells were cultured in medium containing growth factor and either 2.5 μg of actinomycin D per ml (bars with thick hatching) or 2.5 μg of cycloheximide per ml (bars with thin hatching). Neo, FDC-P1 cells bearing a ZenSVNeo provirus cultured either with or without 100 μM Zn^{2+}; MT*bcl-2*, FDC-P1 cells bearing the Zn^{2+}-inducible *bcl-2* expression construct cultured either with or without 100 μM Zn^{2+}; *bcl-2*, FDC-P1 cells bearing a Zen*bcl-2*SVNeo virus that were cultured without Zn^{2+}. Results show mean ± 3 standard errors of the mean cell viability for triplicate cultures.

mechanism, presumably involving the homologs of *ced-3* and *ced-4*, can exist in an inactive state in mammalian cells but can be activated to kill the cell without the need for RNA or protein synthesis or c-*myc* expression. The ability of physiological cell death to occur in the absence of transcription or Myc is consistent with cell death being a cytoplasmic rather than a nuclear process. This idea is consistent with the localization of Bcl-2 to cytoplasmic membranes (20) and observations that nuclear DNA degradation is not essential for apoptosis (31).

These results do not preclude a role for c-*myc* in triggering apoptosis in some circumstances. Although our results indicate that c-*myc* is not required for apoptosis, in some cases it may act upstream of the gene products that carry out cell death. An increase in expression of c-*myc* may lead to apoptosis is two ways. First, unphysiological disturbances of many kinds, such as viral infection and exposure to cytotoxins, can be recognized by cells, which trigger cell suicide mechanisms (15, 32). Inappropriate expression of c-*myc* may similarly be detected by the cell, which then kills itself. Second, Myc may provide part of a physiological signal transduction pathway that culminates in activation of a cell death mechanism. Although treatment of FDC-P1 cells with interleukin-3, which is required for cell survival, results in an increase in c-*myc* expression, it is conceivable that in other circumstances c-*myc* may activate the cell death process, just as c-*myc* expression is seen during the differentiation of some cell types (6) but prevents the differentiation of others (3). The results presented here show that c-*myc* expression is not a required part of the death mechanism itself, as G_0-arrested FDC-P1 cells that do not express c-*myc* could still undergo apoptosis.

The ability of *bcl-2* and c-*myc* to synergize in oncogenesis was first demonstrated experimentally by infecting bone marrow from Eμ-*myc* transgenic mice with *bcl-2*-bearing retroviruses (34) and was confirmed by experiments that crossed Eμ-*myc* and Eμ–*bcl-2* transgenic mice (26). The ability of *bcl-2* to block apoptosis associated with increased expression of c-*myc* may provide an explanation for this synergy in some cases (1, 10, 36).

The inconsistent effects of macromolecular inhibitors on cell death suggest that these agents may act in two different ways, either to trigger or to inhibit cell death. Our results show that the cell death machinery may be quiescent but can be activated without transcription or translation, as apoptosis occurred in the presence of high concentrations of actinomycin D or cycloheximide. However, in other circumstances, macromolecular synthesis may provide part of the signal transduction that triggers the cell death process. For example, when T cells are triggered to die by dexamethasone (19), levels of macromolecular synthesis inhibitors sufficient to block the signal from the glucocorticoid receptor may not be adequate to trigger cell suicide, so these cells may be protected by inhibition of RNA or protein synthesis. The death of FDC-P1 cells caused by actinomycin D or cycloheximide resembles the response of cells to other drugs such as sodium azide and colchicine, as well as to irradiation and to some viruses (15, 27, 33). Apoptosis triggered by treatment with actinomycin D or cycloheximide may thus represent a nonspecific suicide response, a defense mechanism whose normal role may be to limit the threat posed by a virus-infected or mutagenized cell to its neighbors.

The simplest model for the cell death process that can be blocked by Bcl-2 is that cell death results from activation of a preexistent cytoplasmic mechanism, but the triggering signal can come from the nucleus, from the cytoplasm, or from outside the cell. After death of the cell, an endonuclease, probably DNase I (21), gains access to the nucleus and cleaves the DNA. This model is consistent with genetic experiments in *C. elegans* showing that programmed cell death still occurs in *nuc-1* mutants, but loss of endonuclease function prevents degradation of the DNA in the dead cell's nucleus (7). In this model, the endonuclease does not participate in killing the cell; nevertheless, it may play an important role by degrading viral or mutated DNA that may threaten other cells of the host.

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