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Programmed cell death and Bcl-2 protection in the absence of a nucleus

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The molecular basis of programmed cell death (PCD) is unknown. An important clue is provided by the Bcl-2 protein, which can protect many cell types from PCD, although it is not known where or how it acts. Nuclear condensation, DNA fragmentation and a requirement for new RNA and protein synthesis are often considered hallmarks of PCD. We show here, however, that anucleate cytoplasts can undergo PCD and that Bcl-2 and extracellular survival signals can protect them, indicating that, in some cases at least, the nucleus is not required for PCD or for Bcl-2 or survival factor protection. We propose that PCD, like the cell cycle, is orchestrated by a cytoplasmic regulator that has multiple intracellular targets.

Key words: apoptosis/*bcl-2*/cell death/cytoplast/staurosporine

Introduction

Normal cell death is a fundamental feature of animal cells. It occurs in most developing tissues and continues in many tissues throughout life (Glucksmann, 1951). Despite its importance as a mechanism for eliminating unwanted cells (Ellis *et al.*, 1991), it remains a mystery how the cells die. It is often called programmed cell death (PCD) because it is thought that the cells activate an intrinsic death program and kill themselves.

Genetic studies of normal cell death in the nematode *Caenorhabditis elegans* provide the best evidence that PCD involves a cell-intrinsic death program (Ellis *et al.*, 1991). Two genes, *ced-3* and *ced-4*, have been shown to be required for the 131 cell deaths that normally occur during the development of the hermaphrodite: both genes must act in the cells (or their precursors) that die, and if either gene is inactivated by mutation, none of the 131 cells die. Both genes have been cloned and sequenced; *ced-3* encodes a cysteine protease (Yuan *et al.*, 1993), while *ced-4* encodes a novel protein (Yuan and Horvitz, 1992).

Because the molecular nature of PCD in vertebrates is not known, there is no single characteristic that unambiguously defines a vertebrate cell death as PCD. The majority of the deaths, however, share common morphological features, collectively referred to as apoptosis (Kerr *et al.*, 1972; Wyllie *et al.*, 1980). One of the earliest and most distinctive features is the condensation and fragmentation of the nucleus (pyknosis). Frequently, the nuclear DNA is degraded, producing a 'ladder' of oligonucleosomal-sized double-stranded DNA fragments visible by gel electrophoresis (Williams *et al.*, 1974; Skalka *et al.*, 1976; Wyllie, 1980); in many cells, larger DNA fragments and single-stranded nicks are produced (Ucker *et al.*, 1992; Tomei *et al.*, 1993). Other changes include rounding up of the cell, blebbing of the plasma membrane, cytoplasmic condensation, loss of mitochondrial function, and, in many cases, the breaking up of the dead cell into fragments called apoptotic bodies (Wyllie *et al.*, 1980). Perhaps the most fundamental feature of PCD, however, is that the dead cells or fragments are rapidly phagocytosed by neighboring cells or macrophages, before there is any leakage of cytoplasmic contents and without an inflammatory response. These features distinguish apoptosis from cell necrosis, in which cells dying as a result of acute injury swell and lyse, spilling their contents into the extracellular space, eliciting an inflammatory response (Kerr *et al.*, 1972).

A potential breakthrough in understanding the molecular mechanism of PCD in vertebrate cells has come from studies of the mammalian gene *bcl-2*, which was first described as an oncogene in a common form of human B cell lymphoma (Tsujimoto *et al.*, 1985). Overexpressed *bcl-2* can protect various cell types, both *in vitro* and *in vivo*, from PCD, at least temporarily (Vaux *et al.*, 1988; McDonnell *et al.*, 1989; Nuñez *et al.*, 1990; Sentman *et al.*, 1991; Strasser *et al.*, 1991; Garcia *et al.*, 1992; Allsopp *et al.*, 1993), whereas *bcl-2*-deficient mice show abnormalities consistent with loss of a death repressor in specific cells (Veis *et al.*, 1993). It has recently been found that *bcl-2* shares sequence similarity with the *C.elegans* gene *ced-9* (Hengartner and Horvitz, 1994), which normally acts to suppress *ced-3*- and *ced-4*-dependent PCD in the worm. Remarkably, the human *bcl-2* gene can function in *C.elegans* to suppress PCD (Vaux *et al.*, 1992; Hengartner and Horvitz, 1994). These findings indicate that *ced-9* and *bcl-2* are functionally as well as structurally homologous and suggest that both PCD and its regulation have been highly conserved in evolution from worms to humans, confirming that PCD is a fundamental feature of animal cells and that a normal function of *bcl-2* is to suppress PCD. Recently, a number of *bcl-2*-related genes have been identified, including *bax* (Oltvai *et al.*, 1993), *bcl-x* (Boise *et al.*, 1993), *A1* (Lin *et al.*, 1993) and *mcl1* (Kozopas *et al.*, 1993) in vertebrate cells and *E1B(19k)* in adenovirus (Rao *et al.*, 1992; E.White, personal communication), LMW5-HL in African swine fever virus (Neilan *et al.*, 1993), and BHRF1 in Epstein–Barr virus (Henderson *et al.*, 1993).

Although it is generally agreed that the Bcl-2 protein is intracellular and membrane-bound (Tsujimoto *et al.*, 1987; Chen-Levy *et al.*, 1989; Hockenbery *et al.*, 1990; Alnemri *et al.*, 1992), its subcellular location has been controversial. Some studies found it to be mainly in the inner mitochondrial membrane (Hockenbery *et al.*, 1990), while others found

it mainly associated with the endoplasmic reticulum, nuclear envelope and outer mitochondrial membrane (Chen-Levy *et al.*, 1989; Alnemri *et al.*, 1992; Monaghan *et al.*, 1992; Jacobson *et al.*, 1993). We recently showed that *bcl-2* can protect cells from PCD even when the cells lack mitochondrial DNA and therefore do not have a functional respiratory chain (Jacobson *et al.*, 1993), suggesting that neither PCD nor Bcl-2 protection depends on mitochondrial respiration. Hockenbery *et al.* (1993) recently reported that Bcl-2 has no effect on oxidative phosphorylation or intracellular peroxide formation, although it inhibited the lipid peroxidation seen in dexamethasone-treated T cell hybridoma cells; similarly, Kane *et al.* (1993) reported that Bcl-2 inhibited the generation of reactive oxygen species in a neural cell line treated with glutathione synthesis inhibitors.

In the present report we examine the role of the nucleus in PCD and in Bcl-2-mediated and survival-factor-mediated protection against PCD. There are many reasons for thinking that the nucleus might be important in PCD. Chromatin condensation and DNA degradation occur early in the process, and it has been suggested that the activation of an endonuclease and the resulting DNA degradation may be the primary events in PCD (Wyllie, 1980; Umansky, 1982; Duke *et al.*, 1983; Cohen and Duke, 1984; McConkey *et al.*, 1989; Orrenius *et al.*, 1989; Alnemri and Litwack, 1990; Batistatou and Greene, 1991; Gaido and Cidlowski, 1991; Ojcius *et al.*, 1991; Deckwerth and Johnson, 1993), a suggestion supported by the findings that endonuclease inhibitors such as aurintricarboxylic acid (ATA) and Zn^{2+} can often inhibit PCD (Duke *et al.*, 1983; Cohen and Duke, 1984; McConkey *et al.*, 1989; Shi *et al.*, 1990; Batistatou and Greene, 1991; Crompton, 1991; Ojcius *et al.*, 1991; Vukmanovic and Zamoyska, 1991; Mesner *et al.*, 1992). In addition, RNA synthesis is sometimes required for PCD (Tata, 1966; Lockshin, 1969; Stanisic *et al.*, 1978; Cohen and Duke, 1984; Wyllie *et al.*, 1984; Sellins and Cohen, 1987; Martin *et al.*, 1988; Kizaki *et al.*, 1989; Ucker *et al.*, 1989; Oppenheim *et al.*, 1990; Schwartz *et al.*, 1990; Scott and Davies, 1990; Shi *et al.*, 1990) and in some cases new species of mRNAs can be detected just prior to PCD (reviewed in Freeman *et al.*, 1993), although it has yet to be shown that any of these mRNAs encode proteins that are part of the effector machinery of PCD. Moreover, a number of proteins that function in the nucleus have been found to regulate PCD, including members of the nuclear receptor superfamily (Tata, 1966; Wyllie, 1980; Cohen and Duke, 1984; Kyprianou and Isaacs, 1988), c-Myc (Askew *et al.*, 1991; Evan *et al.*, 1992; Shi *et al.*, 1992), c-Fos (Colotta *et al.*, 1992), and p53 (Yonish-Rouach *et al.*, 1991; Shaw *et al.*, 1992; Ryan *et al.*, 1993). Finally, treatments that damage nuclear DNA can induce PCD by a mechanism that depends on p53 (Clarke *et al.*, 1993; Lowe *et al.*, 1993).

We show here that, in the case of two cell lines, anucleate cytoplasts and their nucleated parent cells both die with the characteristic morphological features of apoptosis when deprived of survival factors or treated with high concentrations of the protein kinase inhibitor staurosporine. The cells and cytoplasts die with the same kinetics and sequence of intracellular changes and both can be transiently protected from dying by Bcl-2, and, in the case of the one cell line tested, by extracellular survival factors. These findings suggest that, for some cells at least, the nucleus is required neither for PCD nor for Bcl-2-mediated or survival-factor-mediated protection against PCD. We hypothesize that, like the cell cycle, PCD may be orchestrated by a cytoplasmic regulator.

Results

Staurosporine induces PCD in GM701 cells

We previously showed that high (micromolar) concentrations of staurosporine induce nuclear pyknosis and fragmentation in an SV40-transformed human fibroblast cell line called GM701, and that the overexpression of *bcl-2* in these cells protects them against the lethal effects of this drug (Jacobson *et al.*, 1993). We have now further characterized the death induced by staurosporine to see if it has other features of PCD. We first examined the kinetics of staurosporine-induced death in GM701 cells. As shown in Figure 1A,

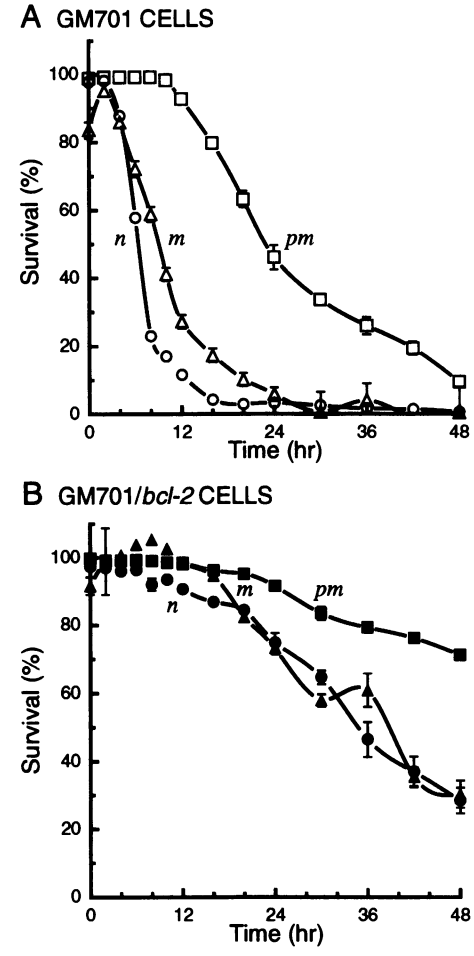

Fig. 1. Staurosporine-induced death of GM701 (**A**) and GM701/*bcl-2* (**B**) cells. About 6000 cells were plated per well in 96-well plates in DMEM-10%FCS, and the next day they were treated with staurosporine (time = 0) and then assayed at various times afterwards. Nuclear morphology (n) was assessed with Hoechst 33342 dye. Plasma membrane integrity (pm) was assessed simultaneously in unfixed cells with ethidium homodimer. Mitochondrial function (m) was assessed in separate wells using the bulk cell photometric MTT assay (see Materials and methods). Each point represents the mean ± SEM of five cultures (in this and the following figures, where error bars cannot be seen it is because they are smaller than the diameter of the central symbol). At least 200 cells were counted per culture. In control cultures, where staurosporine was omitted, <2% of the cells were dead at 48 h when assessed by Hoechst dye or ethidium homodimer staining.

staurosporine induced a rapid decline in mitochondrial function, as assessed by the ability of cells to convert the tetrazolium dye MTT to a dark-blue reaction product (Mosmann, 1983). This loss of mitochondrial function did not recover when staurosporine was washed out (data not shown), and it paralleled the appearance of pyknotic nuclei in staurosporine-treated GM701 cells. Both nuclear pyknosis and loss of mitochondrial function occurred >12 h before plasma membrane integrity was lost, as assessed by the uptake of the membrane-impermeant DNA dye ethidium homodimer (Figure 1A). As expected, GM701 cells that had been infected with a retroviral vector containing a human *bcl-2* cDNA (GM701/*bcl-2* cells), and therefore overexpressed the Bcl-2 protein, were transiently protected against the effects of staurosporine and showed markedly delayed appearance of all of these signs of cell death (Figure 1B).

When viewed by time-lapse video microscopy, GM701 cells changed their shape within minutes of staurosporine treatment: they became stellate, with long, thin processes. This immediate morphological response occurred indistinguishably in both GM701 and GM701/*bcl-2* cells. After 2−10 h, GM701 cells rapidly shrank, became phase-bright, and eventually stopped all movement (Figure 2A); the time course of appearance of these changes (Figure 2B) was similar to the time course of appearance of pyknosis and of the loss of MTT activity in other experiments (compare Figures 1A and 2B). GM701/*bcl-2* cells showed none of these changes until 24 h or so later (not shown).

When viewed by electron microscopy after only 1 h of staurosporine treatment, both GM701 and GM701/*bcl-2* cells showed similar ultrastructural changes, including nuclear membrane convolution, slight mitochondrial swelling and enlargement of nucleoli with dispersion of nucleolar granules (not shown). Within 4 h of staurosporine treatment, however, GM701 cells (Figure 3C), but not GM701/*bcl-2* cells (not shown), showed changes that are characteristic of apoptosis, including chromatin margination, nuclear fragmentation, the appearance of vacuoles in the cytoplasm, and cytoplasmic condensation, without further mitochondrial swelling. Staurosporine-treated GM701/*bcl-2* cells eventually showed all of the same changes, but with a delay of >24 h (not shown), consistent with the delayed time-course of pyknosis, loss of MTT activity, and loss of plasma membrane integrity observed by light microscopy (see Figure 1).

Thus, staurosporine-induced cell death in GM701 cells occurs with the morphological features and sequence of intracellular changes characteristic of apoptosis and is inhibited by overexpression of *bcl-2*, suggesting that it is a suitable model of PCD.

Staurosporine induces PCD in anucleate GM701 cytoplasts and Bcl-2 is protective

To determine whether the nucleus is required for cells to undergo PCD, we studied the effects of staurosporine on

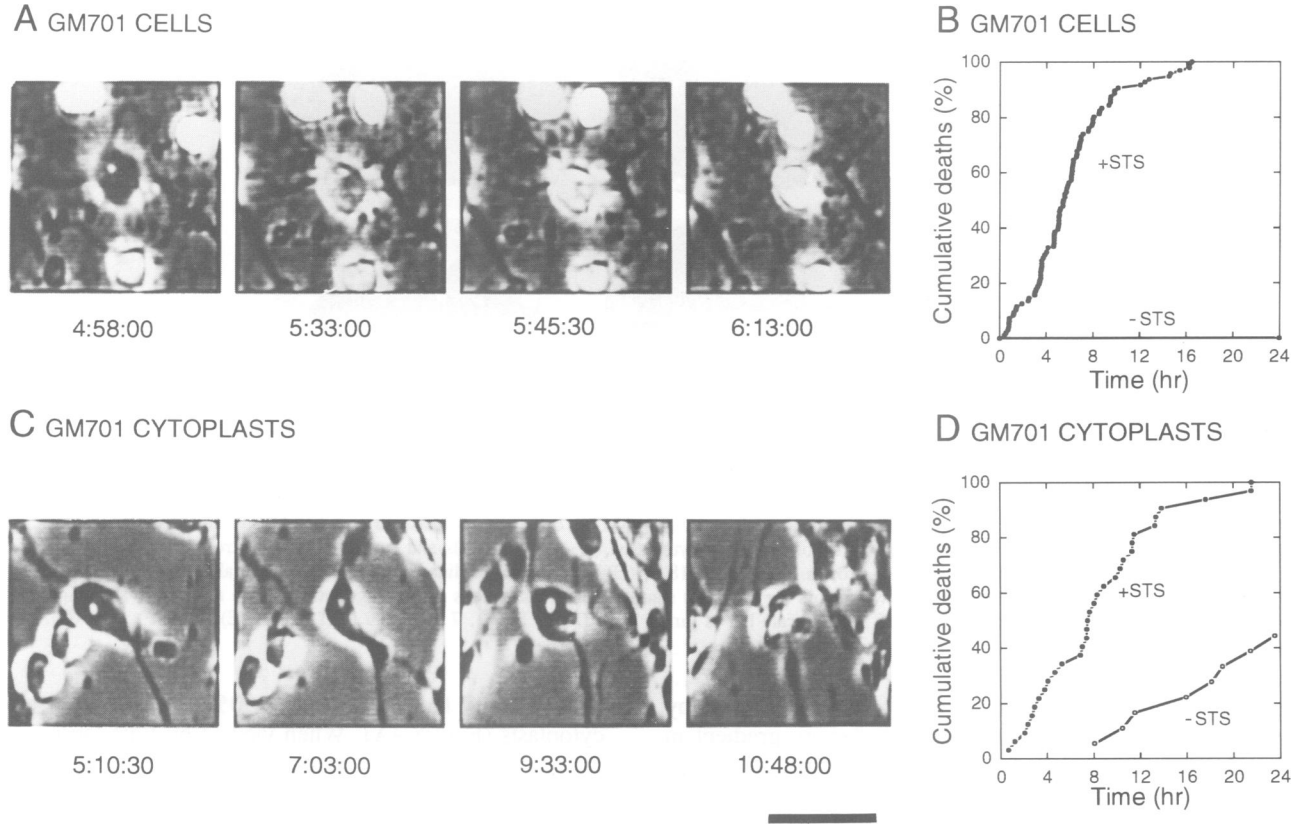

Fig. 2. Time-lapse video micrographs of staurosporine-treated GM701 cells and cytoplasts. **(A)** GM701 cell after the start of staurosporine treatment; times are indicated in h:min:s. **(B)** Time-course of death (assessed by time-lapse video microscopy in a single microscopic field) of staurosporine-treated GM701 cells, with staurosporine added at time = 0. No cell death was seen in untreated cultures when cells in a single microscopic field were followed for 24 h. **(C)** GM701 cytoplast after the start of staurosporine treatment. **(D)** Time-course of death of control (open symbols) and staurosporine-treated (closed symbols) GM701 cytoplasts. The enucleation efficiency of the cytoplast preparations was >95%. The total number of cells assessed in (B) was 96 with staurosporine (+STS), while in (D) 32 cytoplasts were assessed with staurosporine and 18 without (−STS); cells or cytoplasts that migrated out of the field, or were obscured by other cytoplasts or free nuclei, were not counted. Scale bar = 50 μm.

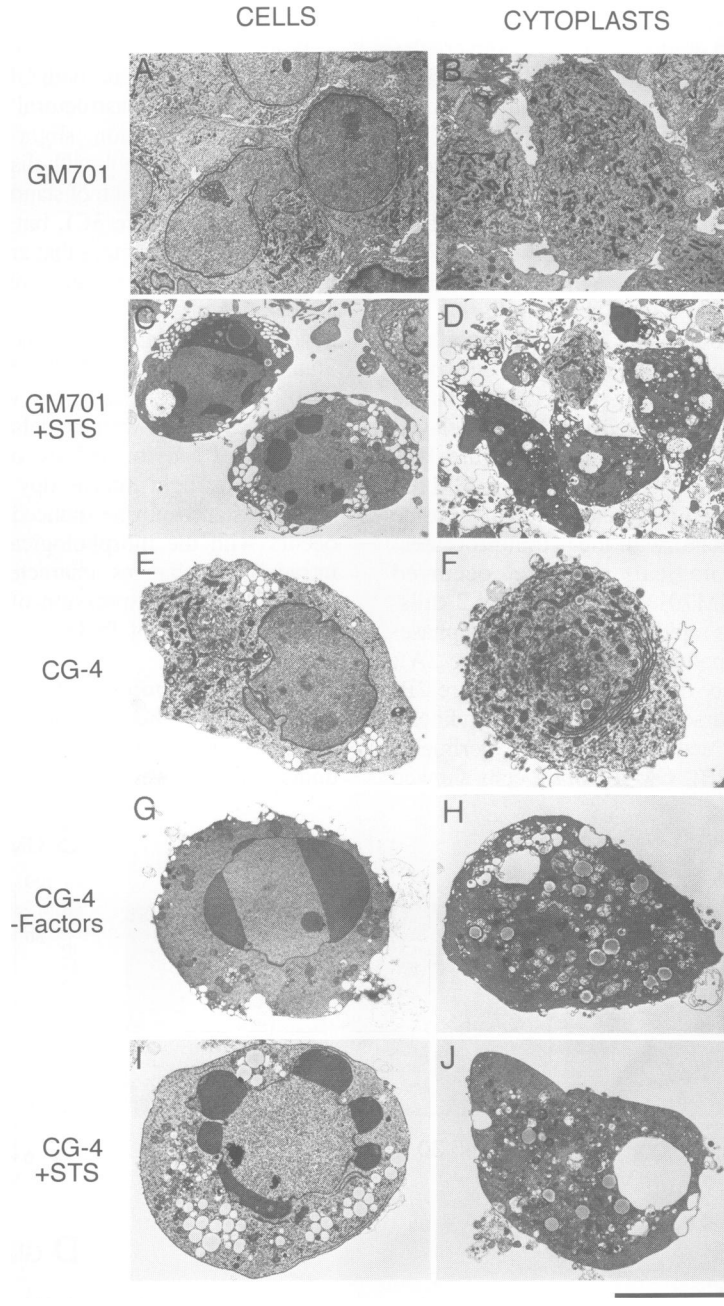

Fig. 3. Electron micrographs of cells (**left**) and cytoplasts (**right**). (**A–D**) GM701 cells and cytoplasts after 8 h: (A) control cells, (B) control cytoplasts, (C) staurosporine-treated cells, (D) staurosporine-treated cytoplasts. (**E–J**) CG-4 cells and cytoplasts: (E) control cells in CG-4 growth medium for 6 h, (F) control cytoplasts in CG-4 growth medium for 18 h, (G) cells in B-S medium without added survival factors for 18 h, (H) cytoplasts in B-S medium without added survival factors for 18 h, (I) staurosporine-treated cells in CG-4 growth medium at 6 h, (J) staurosporine-treated cytoplasts in CG-4 growth medium at 6 h. The scale bar is (μm): 11 in (A), 9 in (B), 7 in (C), 9 in (D), 8 in (E), 6 in (F), 8 in (G), 5 in (H), 7 in (I), and 6 in (J).

enucleated GM701 cells. Nuclei were removed by centrifuging the cells through a Ficoll density gradient in the presence of cytochalasin B (see Materials and methods). More than 90% of the live cells lacked nuclei in these preparations. These cytoplasts adhered to the plastic tissue-culture plate, flattened out, extended processes (Figure 2C) and retained normal mitochondrial function (as assessed by the MTT assay) for approximately 24 h after plating (Figure 4A). Addition of 1 μM staurosporine caused a loss of mitochondrial function with a very similar time-course to that seen with nucleated GM701 cells, so that by 16 h MTT activity had decreased by 90% relative to untreated cytoplasts (Figure 4A). When viewed by time-lapse video microscopy, GM701 cytoplasts underwent the same morphological changes (Figure 2C), with similar kinetics (Figure 2D), as intact cells in response to staurosporine treatment. Staurosporine-treated cytoplasts also underwent the same ultrastructural changes as intact cells, including shrinkage, vacuolation and, at later stages, increased electron density (Figure 3D). Most untreated cytoplasts remained unchanged and appeared similar to untreated nucleated cells except for the absence of a nucleus (Figure 3A and B),

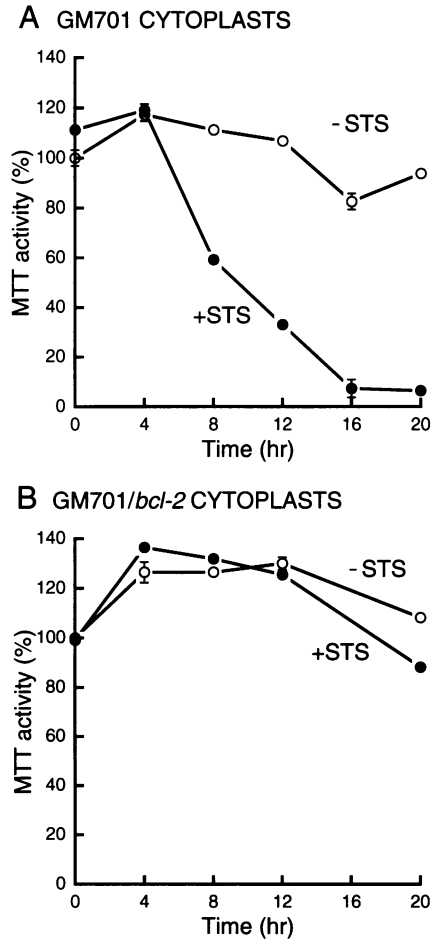

Fig. 4. Effect of staurosporine on MTT activity in GM701 (**A**) and GM701/*bcl-2* (**B**) cytoplasts. About 60 000 cytoplasts were plated per well in 96-well tissue-culture plates in DMEM-10% FCS and either were left untreated (controls, open symbols) or were treated with 1 μM staurosporine (closed symbols) after 5.5 h (time = 0). MTT activity was assessed at various times thereafter using the bulk cell photometric assay and the results were normalized to the activity in untreated cells at time = 0. Enucleation efficiencies were >97%, as determined by fixing the cells and cytoplasts (~6 h after plating) in 4% paraformaldehyde followed by acid-alcohol, staining them with propidium iodide, and counting the proportion of adherent cells that lacked a propidium iodide-stained nucleus. The results represent the means ± SEM of quadruplicate (A) or triplicate (B) cultures.

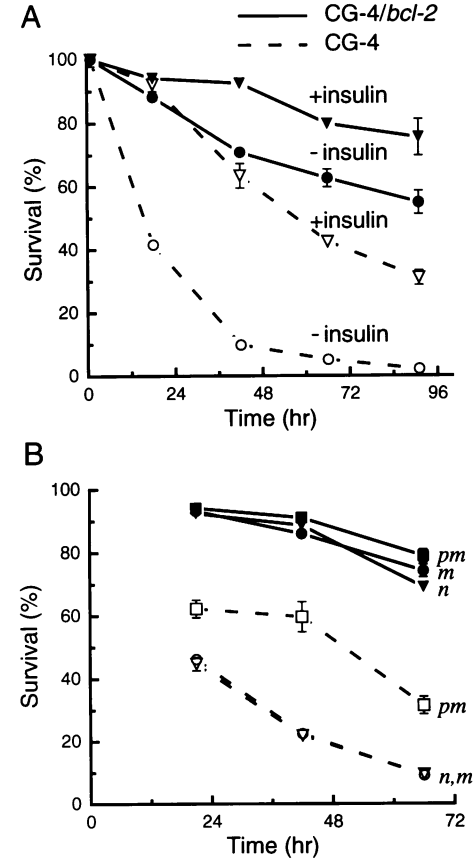

Fig. 5. Survival of CG-4 and CG-4/*bcl-2* cells in the presence or absence of survival factors. (**A**) CG-4 cells (dashed lines) and CG-4/*bcl-2* cells (solid lines) were cultured in serum-free B-S medium, with (triangles) or without (circles) insulin (5 μg/ml); survival was assessed by the microscopic MTT assay. (**B**) Survival of CG-4 cells (dashed lines) and CG-4/*bcl-2* cells (solid lines) in serum-free B-S medium without insulin, assayed by nuclear morphology after Hoechst 33342 staining (n), together with either the microscopic MTT assay (m) or the ethidium homodimer assay for plasma membrane integrity (pm) in the same cells. The results were not significantly different when nuclear morphology was assessed together with MTT or with ethidium homodimer staining. In both (A) and (B) the % survival was determined as the proportion of live cells [live/(live + dead)]. Each point represents the mean ± SEM of three (A) or five (B) cultures, normalized to survival after 1 (A) or 21 (B) h in CG-4 growth medium. At least 100 cells were counted per culture.

although occasional apoptotic cytoplasts were observed. Thus staurosporine can induce all of the cytoplasmic features of apoptosis in enucleated cells.

As was the case with intact GM701/*bcl-2* cells, GM701/*bcl-2* cytoplasts were relatively resistant to staurosporine, as assessed by the MTT assay (Figure 4B), time-lapse video microscopy and electron microscopy (not shown). Thus the protective effect of Bcl-2 does not require a nucleus.

Growth-factor withdrawal or staurosporine treatment induces PCD in CG-4 cells

We previously showed that purified oligodendrocyte precursor cells isolated from the developing rat optic nerve depend on signals from other cells to survive in serum- and growth-factor-free cultures, and that signalling molecules such as insulin-like growth factor-1 (IGF-1) and platelet-derived growth factor (PDGF) promote the survival of these cells *in vitro* (Barres *et al.*, 1992). Without such survival factors, the cells die by PCD. The rat oligodendrocyte precursor cell line CG-4 (Louis *et al.*, 1992) also requires survival factors to live *in vitro* (Barres *et al.*, 1993; Louis *et al.*, 1993) and, like their primary cell counterparts, high levels of insulin (which activate IGF-1 receptors) promoted their survival in serum-free culture (Figure 5A); in the absence of survival factors, CG-4 cells died within 18–48 h with the characteristic morphology of apoptosis (Figure 3G). Nuclear pyknosis and loss of mitochondrial function preceded loss of plasma membrane integrity (Figure 5B) and seemed to occur at around the same time since >98% of the pyknotic cells were MTT-negative, and >99% of the MTT-negative cells were pyknotic at all times (not shown). CG-4 cells that had been infected with a retroviral vector containing a human *bcl-2* cDNA (CG-4/*bcl-2* cells) were transiently protected from dying in medium lacking survival factors (Figure 5A and B). Similarly, when primary oligodendrocyte precursors from rat brain were infected with

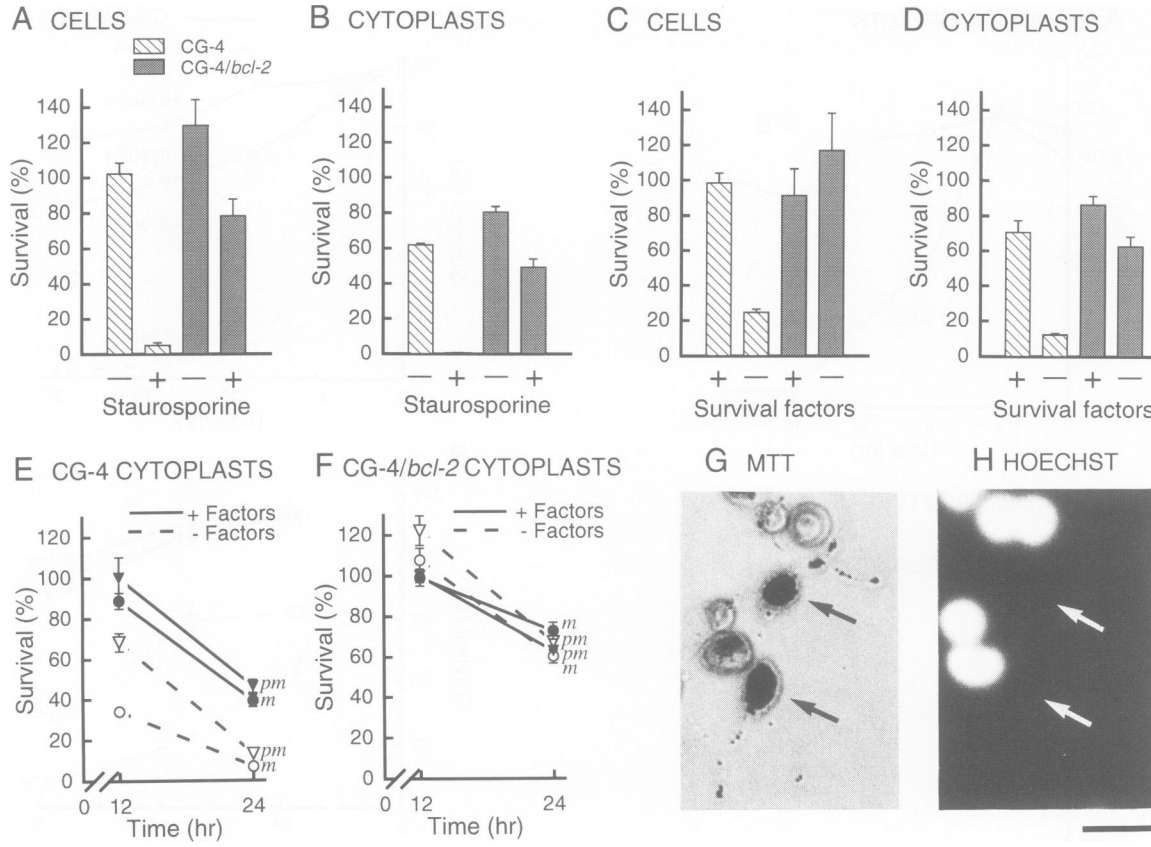

Fig. 6. Death of CG-4 and CG-4/*bcl-2* cells and cytoplasts induced by treatment with staurosporine for 22 h (**A** and **B**) or the withdrawal of survival factors for 24 h (**C**–**F**). Cells were cultured in CG-4 growth medium, except in (C–F) where they were cultured in B-S medium alone, without insulin. In (A–D), survival was determined by counting the total number of live cells or cytoplasts per high-power field in separate wells by the microscopic MTT assay in the presence of Hoechst 33342 (to distinguish cytoplasts from cells and free nuclei). In (E) and (F), survival was determined as in (A–D) by the microscopic MTT assay (m), or by assessing plasma membrane integrity by staining with calcein-AM (pm), both in the presence of Hoechst 33342 to distinguish cytoplasts from cells and free nuclei. Results were normalized to the number of live cells or cytoplasts per high-power field after 12 h in CG-4 growth medium without staurosporine. Time 0 was either the time of plating (for the survival factor withdrawal experiments in (C–F), or the time of staurosporine addition (~1.5 h after plating). Each point represents either the mean ± SEM of three experiments, in each of which at least four fields were counted in each of five cultures (A and B), or the mean ± SEM of five cultures from one experiment (C–F). (G) and (H) show the appearance of stained cells. Two live CG-4 cytoplasts (arrows) stained with MTT are shown in (G), while four free nuclei stained with Hoechst 33342 are shown in (H). Cytoplasts were plated in CG-4 growth medium, stained simultaneously with MTT and Hoechst 33342 3 h later, and then photographed in an Olympus IMT-2 inverted fluorescence microscope using a 40× objective after a 1 h incubation. The density of MTT staining was typically similar in live CG-4 cytoplasts and intact cells, indicating that the healthy cytoplasts metabolized MTT to a similar extent to their nucleated parent cells. Scale bar = 20 μm.

the same retroviral vector (Babe Puro/*bcl-2*), then purified by immunopanning (Barres *et al.*, 1992; Collarini *et al.*, 1992), *bcl-2* promoted the survival of these cells when they were cultured in serum-free medium lacking added survival factors or, at longer times, in medium containing high concentrations of insulin as the only survival factor (not shown).

CG-4 cells, like GM701 cells, died when treated with 1 μM staurosporine (Figure 6A). The dead cells lost MTT activity and displayed the typical morphological and ultrastructural features of apoptosis, including nuclear pyknosis, marginated chromatin, and cytoplasmic condensation and vacuolation (Figure 3I). As expected, CG-4/*bcl-2* cells were transiently protected from the lethal effects of staurosporine (Figure 6A).

Bcl-2 and extracellular survival signals protect anucleate CG-4 cytoplasts from PCD

CG-4 cytoplasts died with the characteristic features of PCD when treated with staurosporine, and Bcl-2 transiently protected them, just as with CG-4 cells. Death was assessed by electron microscopy (Figure 3J) and by the MTT assay (Figure 6B).

CG-4 cytoplasts also died with the features of PCD when deprived of survival factors, and Bcl-2 was able to protect them. They underwent the same ultrastructural changes as CG-4 cells, including shrinkage, vacuolation, and, at later stages, increased electron density (Figure 3H). When assayed by the MTT assay, only 10% of the CG-4 cytoplasts were MTT-positive after 24 h in the absence of added survival factors, whereas 70% were MTT-positive at this time in the presence of survival factors (Figure 6D), similar to the pattern observed with nucleated cells (Figure 6C). By contrast, there was comparatively little difference in the number of CG-4/*bcl-2* cytoplasts that were MTT-positive after 24 h in the presence or absence of survival factors (Figure 6D), as was the case for CG-4/*bcl-2* cells (Figure 6C). Similar results were obtained when CG-4 cytoplast survival was assayed by plasma membrane integrity: fewer cytoplasts survived in the absence of survival

factors than in the presence of survival factors (Figure 6E), and CG-4/*bcl-2* cytoplasts survived better than CG-4 cytoplasts when survival factors were withdrawn (Figure 6F). As with CG-4 cells, loss of mitochondrial function in CG-4 cytoplasts preceded loss of plasma membrane integrity (Figure 6E).

Protein synthesis inhibitors do not block PCD in cells or cytoplasts

The findings that cytoplasts could undergo PCD when either treated with staurosporine or deprived of survival factors suggested that RNA synthesis was not required for PCD; the possibility remained, however, that new protein synthesis (from stable cytoplasmic mRNAs) might be required. We therefore tested whether the protein synthesis inhibitors cycloheximide or anisomycin, at concentrations that inhibited the incorporation of ^{35}S-labelled methionine and cysteine into cells by >95% (not shown), could prevent PCD in intact and enucleated cells. As shown in Figure 7, the inhibitors did not prevent the loss of mitochondrial function (assessed by the MTT assay) induced in either GM701 cells and cytoplasts by staurosporine treatment (Figure 7A and B) or in CG-4 cells deprived of survival factors (Figure 7C), nor did they prevent Bcl-2 from protecting these cells and cytoplasts (Figure 7A–C). The inhibitors on their own, even in the presence of survival factors, killed all of the CG-4 cytoplasts (Figure 7D), which died with the morphological features of PCD (not shown). Interestingly, Bcl-2 protected CG-4 cytoplasts from PCD induced by the inhibitors (Figure 6D). Thus, in GM701 and CG-4 cells and cytoplasts at least, PCD and Bcl-2 protection do not require the synthesis of new proteins.

Discussion

In the present study we show that enucleated cells (cytoplasts) can die with the same kinetics and cytoplasmic changes as their nucleated parent cells when treated so as to induce PCD. This is the case for the two very different cell lines we studied—a transformed human fibroblast line (GM701), a non-transformed rat oligodendrocyte precursor line (CG-4)—and for two PCD-inducing conditions—treatment with 1 μM staurosporine and withdrawal of survival factors. We also show for both the cells and cytoplasts that overexpression of the human Bcl-2 protein delays PCD and that protein synthesis is required for neither PCD nor Bcl-2 protection. Moreover, we show that extracellular survival factors can protect against PCD in the absence of a nucleus, suggesting that they can operate, in part at least, independently of the nucleus. In addition, we show that Bcl-2 protects normal oligodendrocyte precursor cells from PCD, adding to the growing list of cell types that are protected by Bcl-2.

Staurosporine-induced cell death as a model of PCD

Four lines of evidence suggest that the death induced by staurosporine treatment of GM701 cells and CG-4 cells is PCD. (i) The cells display the morphological features of apoptosis, including nuclear condensation and fragmentation, cell shrinkage and cytoplasmic condensation and vacuolation. (ii) The sequence of changes in the treated cells is characteristic of PCD *in vitro*: nuclear condensation occurs early, then mitochondrial dehydrogenase activity (as assessed

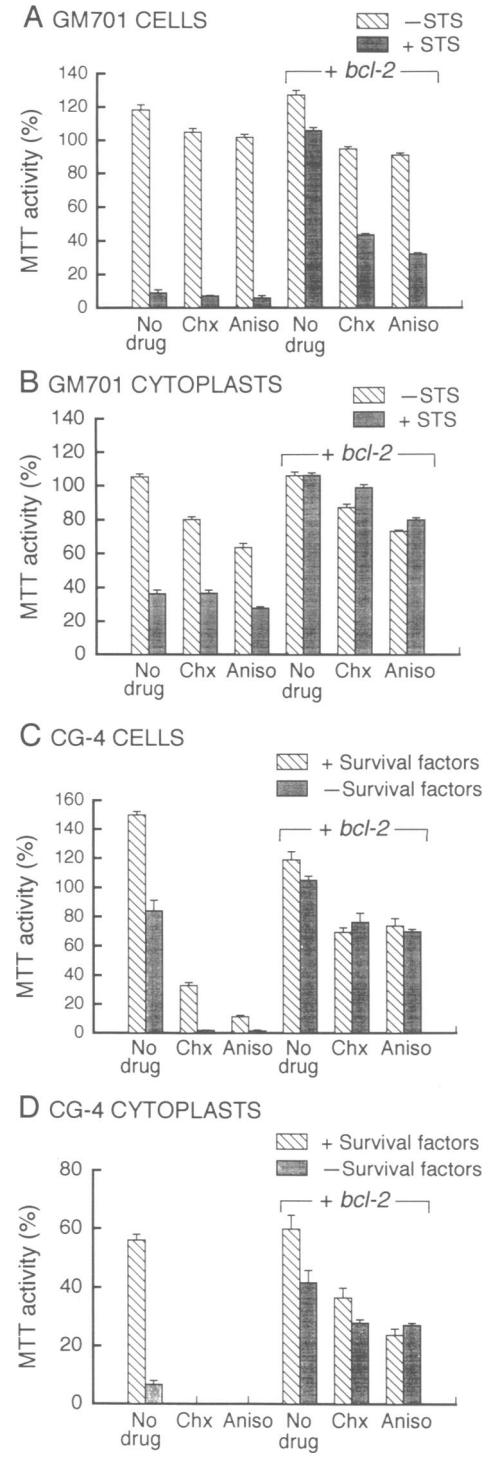

Fig. 7. Effect of protein synthesis inhibitors on the death of cells and cytoplasts induced by staurosporine or survival factor withdrawal. Survival was assessed, in the presence or absence of protein synthesis inhibitors, by the MTT assay, using either the bulk cell photometric method (A and B) or the microscopic method (C and D). In (A) and (B) GM701 cells and cytoplasts were cultured in DMEM-10%FCS and treated with staurosporine for 22 h. In (C) and (D) CG-4 cells and cytoplasts were assessed 24 h after plating, in either CG-4 growth medium (with survival factors) or B-S medium (without survival factors). Each point is the mean ± SEM of five cultures. Results were normalized to survival after 12 h in DMEM-10%FCS or CG-4 growth medium in the absence of drugs. The enucleation efficiencies were >90% for GM701 and GM701/*bcl-2* cytoplast preparations, and >65% for CG-4 and CG-4/*bcl-2* cytoplast preparations. Chx = cycloheximide (10 μg/ml); Aniso = anisomycin (3 μg/ml).

by the MTT assay) shuts down, and finally, many hours later, plasma membrane integrity (as assessed by permeability to ethidium homodimer) is lost. The finding that the loss of plasma membrane integrity occurs late demonstrates that the nuclear and cytoplasmic changes are not secondary to cell necrosis (Sheridan *et al.*, 1981); *in vivo*, cells that undergo PCD would be phagocytosed before the plasma membrane breaks down (Wyllie *et al.*, 1980; Kerr *et al.*, 1987). (iii) Overexpression of *bcl-2* delays these changes and cell death by at least a day or so. (iv) Both ATA (0.5 mM) and Zn^{2+} (2 mM), which have been reported to inhibit PCD in other systems (Duke *et al.*, 1983; Cohen and Duke, 1984; McConkey *et al.*, 1989; Shi *et al.*, 1990; Batistatou and Greene, 1991; Crompton, 1991; Ojcius *et al.*, 1991; Vukmanovic and Zamoyska, 1991; Mesner *et al.*, 1992), also inhibit staurosporine-induced cell death in GM701 cells and CG-4 cells (unpublished observations). Bertrand *et al.* (1993) have also reported that staurosporine induces PCD in various cell lines and have suggested that it may activate a final common pathway to PCD. Some additional changes occur in staurosporine-treated cells that are not characteristic of PCD, including an almost immediate change in cell morphology and motility, slight mitochondrial swelling and convolution of the nuclear envelope. These, however, precede the PCD-like changes by hours and occur with an indistinguishable time course in Bcl-2-protected cells.

Recognition of PCD in cytoplasts

Although the *cell* deaths that we have studied are typical of PCD, the conclusions of this paper rely largely on the evidence that the *cytoplast* deaths are also examples of PCD. Because there is no definitive marker of PCD in vertebrate cells, this evidence is necessarily indirect. First, the cytoplasmic changes—mainly shrinkage, condensation, vacuolation and cessation of movement—are the same in cytoplasts and their nucleated parents when assessed by electron microscopy and by time-lapse video recording. Second, the sequence and time-course of the loss of mitochondrial dehydrogenase activity and of plasma membrane integrity are the same in cytoplasts and cells, with the mitochondrial change preceding the plasma membrane change by at least 8–24 h. Third, at least for CG-4 cells, both cells and cytoplasts plated in survival-factor-free medium can be protected from rapid death by the addition of survival factors to the medium. Finally, overexpression of the Bcl-2 protein delays all of these changes in both cytoplasts and cells. Taken together, these findings make it highly likely that the cytoplasts and cells are dying by the same mechanism—PCD.

PCD in the absence of nuclear events or protein synthesis

As cytoplasts can die with the characteristics of PCD, we conclude that, in some cells at least, PCD does not depend on nuclear events. Thus, although nuclear condensation, nuclear fragmentation and DNA degradation have been considered hallmarks of PCD, they are unlikely to be essential for the process.

Previous studies have suggested that DNA degradation may not be required for PCD. In *C.elegans*, for example, at least one nuclease responsible for degrading the DNA in cells that undergo PCD operates in the phagocytic cells that ingest the dead cells rather than in the dead cells themselves; consequently, PCD occurs normally in mutants that lack the nuclease, although the nucleus remains intact after phagocytosis (Hedgecock *et al.*, 1983; Ellis and Horvitz, 1986). Similarly, cytotoxic T cell-mediated cell death does not require the endonuclease normally responsible for degrading nuclear DNA in the target cells, as cell death occurs with normal kinetics and morphological features in endonuclease-deficient target cells (Ucker *et al.*, 1992). Moreover, although the endonuclease inhibitors ATA and Zn^{2+} inhibit PCD in many systems, it is not clear that they do so by inhibiting nucleases (Bina-stein and Tritton, 1976): these agents inhibit staurosporine-induced PCD in GM701 and CG-4 cells (unpublished observations), for example, even though staurosporine can induce GM701 and CG-4 cytoplasts to undergo PCD.

How can our finding that cytoplasts can undergo PCD, even when protein synthesis is inhibited, be reconciled with the many previous observations that inhibitors of RNA or protein synthesis can sometimes suppress PCD and that PCD is often preceded by the synthesis of new species of mRNAs? We favor the possibility that RNA and protein synthesis are required in these cases for the regulation of the death program rather than for the synthesis of components of the program itself. There are many examples where inhibitors of RNA or protein synthesis fail to inhibit PCD, or even induce PCD, indicating that macromolecular synthesis cannot be a general requirement for PCD (see Wyllie *et al.*, 1980; reviewed in Martin, 1993). In some cases such inhibitors block PCD induced by one stimulus but fail to block PCD induced by a different stimulus in the same cell type (Duke *et al.*, 1983; Cotter *et al.*, 1992), consistent with the view that while macromolecular synthesis may be required to activate PCD, it is not required for the process itself. Corticosteroid-induced PCD in thymocytes, for example, requires both RNA and protein synthesis, as corticosteroids activate an intracellular signalling cascade that depends on transcription and translation; thymocytes, however, can be induced to undergo PCD by prolonged treatment with RNA or protein synthesis inhibitors (Waring *et al.*, 1990; Nieto *et al.*, 1992; M.D.Jacobson, unpublished observations), indicating that macromolecular synthesis is not required for PCD in these cells. If RNA synthesis is not a general requirement for PCD then searches for 'death genes' by subtractive hybridization are unlikely to succeed (see, for example, Harrigan *et al.*, 1989; Schwartz *et al.*, 1990; Briehl and Miesfeld, 1991; Owens *et al.*, 1991); instead, such searches should identify genes encoding proteins involved in activating PCD or in degrading the dead cell.

The regulation of PCD, like the regulation of cell proliferation, is likely to be complex, with some intracellular proteins like c-Myc tending to activate PCD (Askew *et al.*, 1991; Evan *et al.*, 1992; Shi *et al.*, 1992) and others like Bcl-2 tending to suppress it. Moreover, some Bcl-2-related proteins such as Bax and the short form of Bcl-x may act to antagonize the protective effects of Bcl-2 and the long form of Bcl-x, respectively. The effects of inhibitors of RNA or protein synthesis in a particular cell would be expected to depend on the specific combination of intracellular PCD activators and suppressors, the half-lives of these proteins and those of putative PCD effector proteins (see below), and the half-lives of the mRNAs encoding all of these proteins. It is not surprising, therefore, that these drugs have different

effects on different cells, as well as on the same cells in different circumstances.

Constitutive expression of the machinery for PCD

The findings that PCD can occur without protein synthesis in many types of mammalian cells suggests that the putative proteins that mediate PCD are constitutively expressed in many, and perhaps all, mammalian cells. Cytotoxic T lymphocytes, for example, can kill most types of mammalian cells that become infected with an intracellular microbe such as a virus; the killed cells show the typical features of PCD and the killing is not prevented by inhibitors of protein synthesis (Duke *et al.*, 1983). Since many viruses shut off host cell protein synthesis early in infection (Fields, 1985), it makes sense for this type of defense system to operate independently of host cell protein synthesis. We have found that 1 μM staurosporine can induce PCD in a variety of cell types, including a number of cell lines (Jacobson *et al.*, 1993), lens epithelial cells (Ishizaki *et al.*, 1993), retinal cells (Jacobson *et al.*, 1993), oligodendrocytes and their precursor cells (unpublished observations), chondrocytes (Y.Ishizaki, J.Burne and M.Raff, submitted) and thymocytes (unpublished observations); and in all of the cases tested, protein synthesis is not required.

We previously proposed that most, and perhaps all, mammalian cells other than blastomeres are programmed to kill themselves and require continuous signalling from other cells to avoid PCD (Raff, 1992). We can now extend the proposal and suggest that the protein components of the death program are constitutively expressed by all of these cells but are normally kept suppressed by extracellular survival signals. Our finding that cytoplast death can be inhibited by survival factors suggests that this suppression can, in part at least, operate independently of the nucleus. In some circumstances, the suppression can be overridden by specific extracellular signals that activate PCD—such as corticosteroids acting on thymocytes (Wyllie, 1980), or thyroid hormone acting on cells in the tadpole tail at metamorphosis (Tata, 1966).

A cytoplasmic PCD control system

Like mitosis, PCD can affect many organelles. Typically, the cytoskeleton reorganizes so that the cell rounds up, the plasma membrane blebs, the nuclear envelope breaks down, the nucleus condenses, some mitochondrial functions shut down and the cell surface becomes marked for phagocytosis (Wyllie *et al.*, 1980). It is not clear whether this characteristic series of events implies a directly coupled sequence in which each step triggers the next, or whether, like the cell cycle (Kirschner, 1992), these events are controlled independently by a cytoplasmic control system (Figure 8A). Our findings are most compatible with a cytoplasmic control system that orchestrates the various events of PCD. Since cells without mitochondrial respiration (Jacobson *et al.*, 1993) and cells without a nucleus seem to undergo PCD normally, it is unlikely that either changes in mitochondrial respiration or changes in the nucleus are required steps in a coupled sequence of events constituting PCD. Our findings exclude the possibility, for example, that the breakdown of the nuclear envelope releases a signal that induces one or more later essential steps in PCD, at least for the cells we have tested. Vukmanovic and Zamoyska

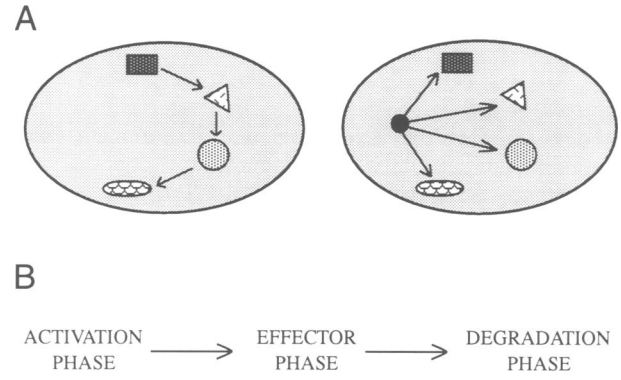

Fig. 8. Hypothetical models of PCD. (**A**) On the left, a sequential series of events mediates PCD, with each event dependent on the preceding one. On the right, the various events of PCD are activated in parallel by a central control system. (**B**) Three phases of PCD.

(1991) previously provided evidence that DNA degradation and loss of mitochondrial function can occur independently in PCD.

It may be useful to consider PCD as occurring in three successive, but possibly overlapping, phases (Figure 8B): (i) an activation phase, in which the cytoplasmic control system is activated (or derepressed), (ii) an effector phase, in which the activated control system acts on multiple target organelles (including the nucleus) in the cell, and (iii) a degradation phase, in which the dead or dying cell is broken down, beginning in the cell itself and ending in a phagocytic cell. In this scheme, exogenous inhibitors of RNA and protein synthesis (as discussed above), as well as endogenous gene-regulatory proteins such as c-Myc, c-Fos, and p53, are suggested to influence the activation phase of PCD rather than the effector phase.

There is increasing evidence that the control mechanisms that regulate the cell cycle and those that regulate PCD share some components. Depending on the conditions, for example, c-Myc can activate either cell proliferation or PCD (Evan *et al.*, 1992; Shi *et al.*, 1992) and p53 can either arrest the cell cycle (Michalovitz *et al.*, 1990; Kastan *et al.*, 1991) or activate PCD (Yonish-Rouach *et al.*, 1991; Shaw *et al.*, 1992; Clarke *et al.*, 1993; Lowe *et al.*, 1993; Ryan *et al.*, 1993). Moreover, it has been pointed out that PCD and mitosis have a number of features in common, including plasma membrane blebbing, nuclear envelope breakdown, chromatin condensation, and cytoskeletal changes that cause the cell to round up, suggesting that mitosis and PCD may share some common mechanisms (Ucker, 1991; Colombel *et al.*, 1992; Rubin *et al.*, 1993).

It has even been suggested that PCD can be viewed as an abortive or premature mitosis (Ucker, 1991; Colombel *et al.*, 1992; Rubin *et al.*, 1993). The induction during PCD of cdc2 kinase activity during PCD (Brooks *et al.*, 1993; Shi *et al.*, 1994) is consistent with this suggestion and raises the possibility that the effector mechanism may involve a cyclin-dependent kinase (Rubin *et al.*, 1993; Shi *et al.*, 1994). Sharing of components between the cell cycle and the death program could help explain why it has been so difficult to identify proteins that are specifically involved in the effector phase of PCD in vertebrate cells. It remains to be seen whether the Ced-3 and Ced-4 proteins, which are specifically required for PCD in *C.elegans* (Ellis and

Horvitz, 1986), operate in the effector or activation phase of PCD.

The role of Bcl-2

Although it is clear that Bcl-2 can inhibit PCD in many types of mammalian cells, it is not known how it does so. Our finding that Bcl-2 can delay PCD in cytoplasts indicates that the protective function of Bcl-2 does not require the nucleus [or presumably the nuclear envelope, where a substantial proportion of the Bcl-2 seems to be located (Chen-Levy *et al.*, 1989; Alnemri *et al.*, 1992; Monaghan *et al.*, 1992; Jacobson *et al.*, 1993)]. We find that Bcl-2 also protects cells and cytoplasts in which protein synthesis has been inhibited and can protect cells and cytoplasts from death induced by protein synthesis inhibitors, suggesting that the protective function of Bcl-2 does not require new protein synthesis. These findings are not inconsistent with the recent proposal that Bcl-2 functions in an antioxidant pathway (Hockenbery *et al.*, 1993; Veis *et al.*, 1993). The simplest possibility is that Bcl-2 suppresses the activity of the cytoplasmic PCD control system. Whether this turns out to be the case or not, the search for proteins that interact with Bcl-2 and Bcl-2-like proteins is likely to be a rewarding strategy for eventually identifying the components of the death program in mammalian cells (Oltvai *et al.*, 1993).

Materials and methods

Cell culture and reagents

Ethidium homodimer and calcein-AM were purchased from Molecular Probes, Inc. (Eugene, OR). Cell culture media, antibiotics and fetal calf serum (FCS) were from Gibco. All other reagents and chemicals were from Sigma Chemical Corp.

B-S medium consisted of Dulbecco's modified Eagle's medium (DMEM) supplemented with transferrin, crystalline grade bovine serum albumin (BSA, Sigma A-4161), putrescine, progesterone, thyroxine, tri-iodothyronine, and selenium (modified from Bottenstien and Sato, 1979), as previously described (Lillien and Raff, 1990). The DMEM used in all experiments contained 4.5 g/l glucose and 4 mM L-glutamine, and was supplemented with 1 mM sodium pyruvate. GM701 cells were provided by M.King, and GM701/*bcl-2* cells were generated as previously described (Jacobson *et al.*, 1993); both cell lines were maintained in DMEM containing 10% FCS (DMEM-10%FCS). CG-4 cells were provided by J.-C.Louis and were grown on poly-D-lysine (PDL)-coated 9 cm Falcon plastic tissue-culture dishes in serum-free B-S medium supplemented with 30% B104 cell conditioned medium, insulin (10 μg/ml), and antibiotics (CG-4 growth medium, modified from Louis *et al.*, 1992). For survival assays, CG-4 cells or cytoplasts were plated in 100 μl of B-S medium (with or without added survival factors) or in CG-4 growth medium; GM701 cells and cytoplasts were plated in DMEM-10%FCS.

Assays of cell survival and death

MTT assay. Mitochondrial function was assayed by the ability of cells or cytoplasts to convert soluble MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) into an insoluble dark-blue formazan reaction product (Mosmann, 1983): 10 μl of MTT solution (5 mg/ml in PBS, sterilized by filtration) was added to cells or cytoplasts growing in 100 μl of medium. In the microscopic MTT assay, individual cells or cytoplasts were counted in an Olympus IMT-2 inverted fluorescence microscope after ~1 h at 37°C, using the dye Hoechst 33342 to distinguish cytoplasts from cells and nucleoplasts, as described below. In the bulk cell photometric MTT assay, the bulk conversion of MTT in a culture well was measured photometrically as previously described (Tada *et al.*, 1986): after 4 h at 37°C, the reaction was stopped by adding 0.1 ml of 10% SDS in 0.01 M HCl; after a further overnight incubation at 37°C, the absorbance (570−630 nm) was measured using a Vmax microplate reader (Molecular Dynamics) and % survival was defined as [(experimental − blank)/(control − blank)] × 100, where the blank was the value obtained in wells containing medium and MTT without cells.

Vital nuclear stain. The membrane-permeant bisbenzimide dye Hoechst 33342 was used to stain nuclei in both live and dead cells. A concentrated stock (2 mg/ml) was made up in water and sterilized by filtration. When used alone the stock was diluted 1:50 in sterile phosphate-buffered saline (PBS) and 10 μl was added directly to cells or cytoplasts growing in 100 μl medium, giving a final concentration of 4 μg/ml. When combined with MTT, ethidium homodimer, or calcein-AM (see below), the concentrated stock of Hoechst 33342 was diluted to the appropriate concentration in the working solution containing the other dye. When used alone or with ethidium homodimer, cells were incubated at 37°C for 15−30 min before counting; when combined with MTT, cells were incubated at 37°C as described above. Cells were viewed in an inverted fluorescence microscope.

Plasma membrane permeability. The membrane-impermeant DNA dye ethidium homodimer was used to identify dead cells in which plasma membrane integrity was disrupted. The membrane-permeant dye calcein-AM was used to label live cells; it is metabolized by cytoplasmic esterases to yield the membrane-impermeant fluorescent dye calcein, which is retained only in cells with an intact plasma membrane. The concentrated dyes were prepared according to the manufacturer's (Molecular Probes, Inc.) instructions, and were added directly to unwashed cells in culture to final concentrations of 4 μM for ethidium homodimer and 2.5 μM for calcein-AM.

Retrovirus construction and infection of CG-4 cells

The 1.3 kb *Eco*RI fragment from pSV2-bcl2α, which contains a cDNA encoding the human Bcl-2α protein (Reed *et al.*, 1988), was inserted into the *Eco*RI site of the retroviral vector pBabe Puro (Morgenstern and Land, 1990), to create the plasmid pBabe Puro/*bcl-2*. Murine retrovirus-producing lines were made by transiently transfecting the helper line GP+E/86 (Markowitz *et al.*, 1988), and then using the virus-containing supernatant to infect tunicamycin-treated GP+E/86 cells, as described by Morgenstern and Land (1991). Producer lines were selected in 2.5 μg/ml puromycin, subcloned, and selected for high titre virus production. CG-4 cells were infected with retrovirus and then selected in 1 μg/ml puromycin; this line (CG-4/*bcl-2*) was not subcloned. Bcl-2 expression was confirmed by immunofluorescence staining, using a monoclonal anti-human Bcl-2 antibody (Pezzella *et al.*, 1990), as previously described (Jacobson *et al.*, 1993).

Preparation of cytoplasts

GM701, GM701/*bcl-2*, CG-4 and CG-4/*bcl-2* cells were enucleated as previously described by Wigler and Weinstein (1975) and Gudas *et al.* (1986), except for minor modifications. Cells were removed from tissue-culture plates by incubation with 0.5 mM EDTA in PBS (for GM701 cells) or with 0.25% trypsin−0.02% EDTA (for CG-4 cells). After washing in minimum Eagle's medium for spinner culture (S-MEM; Gibco) containing 10% FCS, cells were resuspended by vigorous trituration with a 1 ml Pipetman (Gilson) in S-MEM containing 25 μM HEPES, pH 7.4, 5% FCS, 2 mM L-glutamine, 1 mM pyruvate and 21 μM (10 μg/ml) cytochalasin B (S-MEM/CB); DNase I (0.004%) was added to the CG-4 cell suspensions to reduce clumping. Each cell suspension was adjusted to a final concentration of $5 \times 10^6 - 2 \times 10^7$/ml and incubated at 37°C for 45 min before being layered onto a previously prepared discontinuous Ficoll density gradient, consisting of 2 ml of 25%, 2 ml of 17%, 0.5 ml of 16%, 0.5 ml of 15%, and 2 ml of 12.5% Ficoll, all in S-MEM/CB. Gradients were pre-equilibrated for 6−12 h at 37°C in an atmosphere of humidified 5% CO_2. Three milliliters of the cytochalasin-treated cell suspension was applied to the gradient and then overlaid with S-MEM/CB. The gradients were centrifuged for 60 min in a pre-warmed Beckman SW41 rotor at 25 000 r.p.m. at 33°C. Cytoplasts were collected from the central interface between the 15% and 17% Ficoll layers, diluted into DMEM-10%FCS, centrifuged, resuspended in DMEM-10%FCS (for GM701 cytoplasts) or CG-4 growth medium, and plated. For survival factor withdrawal experiments with CG-4 cytoplasts, the cytoplast fractions were diluted into L15 medium containing 0.5% crystalline-grade BSA, washed twice in this medium, and resuspended in B-S medium for plating. Enucleation efficiency was determined 12 h after plating by counting the proportion of MTT-positive cells that did not contain a Hoechst 33342-stained nucleus (except as indicated in Figure 4). More than 90% of the cytochalasin-treated GM701 and GM701/*bcl-2* cells did not contain a nucleus, whereas 60−90% of the CG-4 and CG-4/*bcl-2* cells did not contain a nucleus. For experiments using the bulk cell photometric MTT assay the exact enucleation efficiencies are given in the figure legends.

Time-lapse video microscopy

Cells and cytoplasts were plated in sealed, PDL-coated Nunc SlideFlasks, which were examined on a Zeiss inverted microscope using phase-contrast optics and a 10× objective. Digital images were recorded every 150 s using

an Avcam 405 video camera attatched to a Dell 310 computer with a Matrox video graphics board, all controlled by Cigal image analysis software (©J.Voyvodic). The images were then transferred to videotape using a Panasonic time-lapse video recorder. Still pictures were photographed from the digital images. Cumulative deaths were counted from the video images using a Mitsubishi video tape player at the ICRF image analysis facility.

Electron microscopy

GM701 and GM701/*bcl-2* cells and cytoplasts were fixed in 2.5% glutaraldehyde, while CG-4 and CG-4/*bcl-2* cells and cytoplasts were fixed in 4% paraformaldehyde plus 0.5% glutaraldehyde, both in phosphate buffer pH 7.4 (PB) for 2–4 h at room temperature. The cells and cytoplasts were then postfixed in 1% osmium tetroxide for 1 h at 4°C, stained in tannic acid and uranyl acetate, dehydrated through graded ethanol, and embedded in Epon 812 resin. Thin sections were cut parallel to the substratum, stained with uranyl acetate and lead citrate, and examined with a Jeol 100 CXII electron microscope at 80 kV.

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