Apoptosis Induced by Serum Deprivation of PC12 Cells Is Not Preceded by Growth Arrest and Can Occur at Each Phase of the Cell Cycle

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ABSTRACT

Previous studies have shown that PC12 cells undergo apoptosis (programmed cell death) when deprived of serum. In the present study, we examined the relationship of this death process to the cell cycle. PC12 cell populations synchronized at different, specific phases of the cell cycle exhibit similar kinetics of cell death following deprivation of serum. Flow cytometry analysis was used to examine the levels of apoptotic death in these cell populations in relationship to their progression in the cell cycle during the course of serum deprivation. Such analysis revealed that the cells die during the G1-G2, S, and perhaps G2-M phases and at the G2 to G1 transition.

These results, therefore, suggest that the death of synchronized, serum-deprived PC12 cells occurs throughout the cell cycle and is not dependent on growth arrest. Flow cytometry methodology (acridine orange staining), which determines the RNA content of cells in relationship to their position in the cell cycle, was used to address these questions in nonsynchronized cells. These experiments revealed that, upon serum deprivation, an immediate loss of RNA occurred from cells in G1, S, and G2-M phases. This loss is accompanied by a slower appearance of cells with degraded DNA content. These results show that cells from all phases of the cell cycle are damaged upon serum deprivation and thus suggest that the apoptotic cell death of nonsynchronized PC12 cells may occur from each phase of the cell cycle.

INTRODUCTION

Normal cell death is an important physiological process in which unnecessary cells are eliminated during both development and adult life (1-3). The availability of growth factors can play an important role in controlling normal cell death because most (if not all) cells depend on growth factors for their survival (4, 5). Cells that fail to obtain adequate amounts of their survival factors die. For example, in the nervous system, a massive death of neurons and oligodendrocytes occurs during development, most likely due to the failure of these cells to obtain the specific neurotrophic factors on which they depend (6, 7). In vitro studies have shown that both mitotic and postmitotic differentiated neurons (8, 9) and oligodendrocytes (5) depend on growth factors for their survival. Therefore, the survival effect of growth factors may play an important role in the generation as well as in the differentiation of the nervous system.

One in vitro model system used extensively to study neuronal differentiation is the rat pheochromocytoma cell line, PC12. These cells proliferate in serum-containing medium. In response to NGF, they stop proliferating, differentiate, and acquire a sympathetic, neuron-like phenotype (10, 11). It has recently been demonstrated that proliferating PC12 cells (naïve) depend upon serum, whereas differentiated postmitotic (neuronal) PC12 cells depend upon serum and NGF for their survival. When serum is withdrawn from naïve cells and both serum and NGF from neuronal cells, these cells die and exhibit the characteristic features of the cell death termed apoptosis (programmed cell death; Refs. 12-15). PC12 cells, therefore, can also be used as a model system for studies on the mechanism of growth factor-dependent survival in the nervous system. The advantage offered by the PC12 cells is that they can be used in studies of both mitotic and postmitotic differentiated cells. This is an important feature of the cells, since in the nervous system both mitotic and postmitotic neurons depend on growth factors for their survival. The death of growth factor-deprived PC12 cells can be prevented by some growth factors (e.g., NGF, fibroblast growth factor, and insulin), by the nucleoside inhibitor aminopterin (12, 13), and by exogenous expression of bcl-2 (16). Although some progress has been made in identifying the survival factors and mechanisms which can keep PC12 cells alive, very little is known about the intracellular events involved in the process of apoptosis in these cells. It has recently been suggested that a defective cell cycle control is responsible for inducing apoptosis (17, 18). This hypothesis is based inter alia on the observations that, in some systems, there is a correlation between the cell cycle and apoptosis. For example, in some systems, apoptosis occurs only after cell cycle arrest (19-23), and in other systems, the cells are preferentially susceptible to death at specific phases of the cell cycle (24-26). The purpose of the present study was to determine whether the death of serum-deprived PC12 cells occurs at a specific phase of the cell cycle and whether it depends on growth arrest.

MATERIALS AND METHODS

Cell culture-stock PC12 cells obtained from Dr. Gordon Guroff (NIH Bethesda, MD) were maintained in DMEM high glucose medium supplemented with 8% heat-inactivated horse serum, 8% fetal calf serum, 25 μg/ml penicillin, and 2 mM L-glutamine. For serum-deprivation experiments, confluent stock cultures were washed twice with PBS, detached by 0.5 mM EDTA, and centrifuged. The cells were then resuspended in DMEM and plated in 96 flat-well plates for MTT (Sigma Chemical Co.) assay or in dishes for flow cytometry and DNA fragmentation analyses. For preparation of PC12 cells synchronized in different and specific phases of the cell cycle, subconfluent cultures 6 x 10⁵ cells/140-mm dish were treated with 10 μM thymidine (Sigma). After 24 h, thymidine was removed, and the cells were washed twice with PBS and then grown for additional time periods in drug-free medium. At the times indicated in "Results" (each time point represents a cell population synchronized at a different cell cycle phase), the synchronized cells were detached from dishes and replated in the presence or absence of serum for additional times as indicated. Cell synchronization was assessed at the various times following thymidine release by flow cytometry.

Flow Cytometry Analysis. Three different methods were used for DNA labeling. Nuclei labeling was performed as described previously (27). Briefly, each 200 x g centrifuged cell pellet (10⁶ cells) was resuspended and fixed in 200 μl citrate buffer (250 mM sucrose in 40 mM trisodium citrate) with 5% dimethyl sulfoxide. At this step, samples can be either directly processed or stored at -70°C. Before flow cytometry analysis, cells were incubated at room temperature with 900 μl of stock solution (3.4 mM trisodium citrate, 0.1% NP40, 1.5 mM spermine tetrahydrochloride, and 0.5 mM Tris[base]) containing 30 μg/ml trypsin for 10 min, followed by the addition of 750 μl stock solution containing 0.5 mg/ml trypsin inhibitor with 0.1 mg/ml RNase A. After an additional 10 min of incubation, the cells were stained by the addition of...
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750 μl of solution containing 208 μg/ml PI (Sigma) with 0.58 mg/ml Spermine tetrahydrochloride for 1–3 h at 4°C. PI fluorescence of individual nuclei was then measured using a FACS IV flow cytometer (Becton Dickinson, Mountain View, CA) excited at the 488 nm wavelength and was collected through a 570-nm LP filter. A Consort 40 software computer system was used to analyze the data.

Cell labeling was performed as described previously (28). Briefly, each 200 X g centrifuged cell pellet (10⁶ cells) was resuspended in 1-ml hypotonic fluorochrome solution (50 μg/ml PI in 0.1% sodium citrate plus 0.1% Triton X-100) and stored overnight in the dark at 4°C before the flow cytometric analysis. The PI fluorescence of individual nuclei was measured using a FACS IV flow cytometer as described previously. The mean of the G₁ peak was assigned to channel 100 and, therefore, the events collected between channels 5 and 60 were assigned as apoptotic peaks.

AO staining for the bivariate gating analysis was carried out as described previously (29, 30). Briefly, cells were scraped from dishes with a rubber policeman and centrifuged. The cells were then resuspended at 10⁶ cells/ml in PBS. Aliquots (200 μl) of cell suspensions were mixed for 30 s with 0.4 ml of chilled solution containing 0.08 N HCl, 0.2% Triton X-100, and 0.15 M NaCl, followed by the addition of 1.2 ml of solution containing 10 mM AO in an ice-cold citrate phosphate (0.2 M; pH 6) buffer. The red (630-nm) and green (535-nm) fluorescence of individual cells was measured with an Ortho Cytofluorograf 50 H with an argon laser exciting at 488 nm and 250 mW. Single cells were gated from doublets by measuring the peak width and area of green fluorescence.

DNA Fragmentation Assay. Soluble DNA (27,000 g) was isolated from 10⁷ cells/culture exactly as described previously by Hockenbery et al. (31) except that DNA samples were treated with RNase A (250 μg/ml) before 1/10 of the sample was loaded onto the gel. Agarose gel (1.2%) electrophoresis of DNA was carried out in buffer consisting of 1 μg/ml ethidium bromide, 40 mM Tris HCl (pH 7.9), 4 mM sodiumacetate, and 1 mM EDTA. The DNA was visualized under UV.

Cell Viability Assay. The number of viable cells was determined by the MTT assay (32). MTT was dissolved in PBS at a concentration of 5 mg/ml. From this stock solution, 10 μl/100 μl medium was added to each well, and plates were incubated at 37°C for 4 h. Acid-isopropanol (100 μl of 0.04 N HCl in isopropanol) was then added to the wells and mixed in. After 15 min at room temperature, the plates were read on a Microelisa reader, using a test wavelength of 540 nm and a reference wavelength of 690 nm. Data are presented as the differences between absorbance values at 540 and 690 nm and as means ± SD. The values defined are significant in all cases where Student’s t test yielded P < 0.01.

RESULTS

Serum deprivation has been shown previously to induce the rapid death of proliferating PC12 cells (12) accompanied by the DNA fragmentation characteristic of apoptosis (13, 14).

Fig. 1. DNA histograms from thymidine-synchronized PC12 cells. Cells were treated with 2 mM thymidine for 24 h, and the drug was then removed. At the times indicated in each histogram following thymidine release, nuclei were stained with PI and analyzed by flow cytometry as described in “Materials and Methods.” N.S., nonsynchronized cells. G₀-G₁, S, and G₂-M cell populations are indicated by arrows in the histogram of nonsynchronized cells. The data in insets show the percentage of cells in the different phases of the cell cycle. Results shown are from a representative experiment (of three independent experiments with similar results).
In the present study, we examined whether serum-deprived cells are preferentially susceptible to death at a specific phase of the cell cycle or, alternatively, whether death occurs at all phases of the cell cycle. To address this issue, we prepared PC12 cell populations which had been synchronized at different, specific phases of the cell cycle so that each synchronized population could be deprived of serum and the kinetics of cell death monitored. To generate populations of PC12 cells representing different phases of the cell cycle, PC12 cells were synchronized in early-S phase by treatment with 2 mM thymidine. After 24 h, the drug was removed, and the cells were allowed to progress through the cell cycle. Cell populations obtained at various times following thymidine release corresponded to cells present at different, specific phases of the cell cycle. Cell synchronization was assessed by flow cytometry. As indicated in Fig. 1, exponentially growing PC12 cells were growth arrested at early-S phase after treatment with thymidine. On removal of the drug, the whole population synchronously progressed through the cell cycle. By 12 h, the cells passed through middle-S, late-S, and G2-M, and by 24 h, into the G0-G1 phase. Thirty to thirty-six h following drug removal, some of the cells began to progress from G0-G1 into S and G2-M phases, and the cells exhibited a similar cell cycle distribution as nonsynchronized cells.

The different cell cycle populations were deprived of serum for 6, 12, and 24 h, and the kinetics of their death was measured. Fig. 2 demonstrates that the cell populations corresponding to cells in early-S, middle-S, G2-M, and G0-G1 phases (3, 8, 12, and 24 h following drug release, respectively), as well as nonsynchronized cells, exhibit a similar rate of cell death, regardless of their initial position in the cell cycle when serum was withdrawn. The earliest time point examined in these experiments was 6 h after serum withdrawal, which is the earliest time at which a significant, measurable value of cell death can be monitored by the MTT assay. In order to determine the ability of the different cell populations to undergo apoptosis at earlier times, we examined the appearance of the characteristic internucleosomal DNA fragmentation. This fragmentation is an early event in the death process of serum-deprived PC12 cells and was clearly detectable 4 h after serum deprivation, before the appearance of any morphological signs of cell death (13). Soluble DNA was extracted from the cell populations corresponding to cells in early-S, middle-S, G2-M, and G0-G1 phases (3, 8, 12, and 24 h after drug removal, respectively), as well as from nonsynchronized cells 4 h after serum deprivation. As shown in Fig. 3, a clear pattern of internucleosomal DNA fragmentation is evident in DNA isolated from all these cell populations. No soluble DNA was detected in the samples isolated from nonsynchronized cells (Fig. 3) and from the different cell cycle-specific populations (data not shown) before serum deprivation.

The results depicted in Figs. 2 and 3 demonstrate that the different cell populations exhibited a similar rate of cell death after serum deprivation, regardless of their initial position in the cell cycle. This suggests that death may occur at each phase of the cell cycle. Alternatively, it is possible that death occurs at a specific phase of the cycle, but this cell cycle-specific death cannot be detected, since the cells are progressing in the cell cycle during the course of the experiment and because the cell cycle-specific populations are not completely homogenous. To discriminate between these two possibilities, treated cells were analyzed by flow cytometry.

DNA content histograms obtained by such analysis indicate both the distribution of cells in the cell cycle and the appearance of apoptotic cells (cells with subdiploid DNA content) in each treatment. In addition, this analysis enabled the estimation of the relative levels of the apoptotic cells in the examined cell population. Serum was withdrawn from the different PC12 populations (4, 8, 12, and 24 h following thymidine removal) and from nonsynchronized cells for 4 and 10 h, and the cells were then stained with PI and analyzed by flow cytometry. The results of these experiments are presented in Fig. 4.

It is evident from the experiments depicted in Fig. 4 that cell death can occur directly at the G0-G1 phase, since the G0-G1 cell population did not progress in the cell cycle but remained at the G0-G1 phase during the course of the experiment while cell death still occurred. Cells can also die at the S-phase, as demonstrated in Fig. 4, B and C. Four h after the beginning of the experiments, both early-S (Fig. 4B) and middle-S (Fig. 4C) cell populations did not progress from the S-phase into the G2-M phase (as indicated by the DNA histograms of these cell populations in the presence of serum), although a substantial apoptotic cell population is evident after 4 h of serum deprivation. These apoptotic populations, for the most part, must derive from the majority of cells in this population, i.e., the S-phase cells. Cells may also die at the G2 to G1 transition, as indicated by the failure of the G2-M cells to arrive at G1 (compare Fig. 4, B, C, and D, 4 and 10 h after serum deprivation). The results presented in Fig. 4D indicate that
Fig. 4. DNA histograms from the different cell cycle-specific cell populations following serum deprivation. PC12 cells were synchronized at the different cell cycle phases as described in Figs. 1 and 2. Three h (B), 8 h (C), 12 h (D), and 24 h (E) following thymidine release, the cells were harvested and replated for the indicated times in serum-free medium (—) or in medium supplemented with serum (—). The cells were then stained with PI and analyzed by flow cytometry. DNA histograms from nonsynchronized cells are presented in (A). Ap, G2-G1, S, and G2-M cell populations are indicated by arrows. The data in the insets show the percentage of cells in Ap, G2-G1, S, and G2-M phases, of the different cell populations grown in the presence (+) or absence (-) of serum. Results shown are from a representative experiment (of four independent experiments with similar results).

cell death can also occur at the G2-M phase, since in these experiments the cells that are present are mainly at late-S and G2-M phases during the first 4 h of the experiment. However, in this experiment, it is not possible to distinguish between late-S and G2-M phases, so the possibility that the apoptotic cells are derived from late-S cells and not G2-M cells cannot be excluded. The conclusion that PC12 cells may die at each phase of the cell cycle can be further supported by the results that the different cell cycle populations die at a similar rate after serum deprivation, i.e., a similar level of apoptotic cells is detected in the different cell cycle populations as well as in nonsynchronized cells 4 and 10 h after serum deprivation (about 20 and 40% of the total cell populations, respectively; Fig. 4). The results presented in Fig. 4 also reveal that the cells can die without being growth arrested previously. Accordingly, cell death can occur in cell populations which are progressing anywhere in the cell cycle (Fig. 4, B, C, and D).

The experiments described in Fig. 2–4 strongly suggest that the death of synchronized, serum-deprived PC12 cells is not restricted to one particular point in the cell cycle. To assess whether serum-deprived, non-synchronized PC12 cells also die throughout the cell cycle, we applied the flow cytometric methodology which simultaneously stains cellular DNA and RNA (AO staining; Refs. 29 and 30). This method allowed us to examine the effect of serum deprivation on the RNA content of cells in relation to their position.
Fig. 5. Bivariate (DNA/RNA) counter plots of exponentially growing PC12 cells following serum deprivation. PC12 cells were transferred to serum-free medium for the indicated times. The cells were then stained with AO and analyzed by flow cytometry as described in “Materials and Methods.” Based on DNA values (green fluorescence), cells were classified in G₁, S, and G₂-M phases (as shown by the horizontal lines), whereas G₀ and G₁ cells were distinguished based on RNA values (red fluorescence). G₀ cell population is indicated by the arrow. Apoptotic cells (Ap) have lower DNA staining and consist of two populations: Ap-high (Ap-H), which exhibit high RNA values, and Ap-low (Ap-L), which exhibit low RNA values. The data in insets show the percentage of cells in each phase of the cell cycle and univariate DNA frequency distribution histograms in which the Ap, G₀-G₁, S, and G₂-M cell populations are indicated. Control, exponentially growing cells. Results shown are from a representative experiment (of two independent experiments with similar results).

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in the cell cycle. As shown in Fig. 5, serum deprivation results in a rapid reduction of the RNA content of most cells in the S and G₂-M phases and many of G₁ cells as well. This reduction in the RNA content may indicate that these cells have initiated the process of apoptosis and, therefore, suggest that serum-deprived, nonsynchronized cells may initiate the apoptotic process in all phases of the cell cycle. In addition to the rapid effect on the RNA content of the cells, there is a slow appearance of cells with subdiploid DNA content (apoptotic cells) in both the high RNA fraction (Ap-high population) and low RNA fraction (Ap-low population).

DISCUSSION

PC12 cells can be induced to differentiate and acquire the phenotype of nondividing neurons (10, 11). Both naive and neuronal PC12 cells depend on growth factor for their survival. Upon withdrawal of serum from naive cells or both serum and NGF from neuronal PC12 cells, these cells die and exhibit the DNA fragmentation characteristic of apoptosis (12–15). These features of PC12 cells resemble the dependence of mitotic and postmitotic neurons on growth factors in many aspects and thus make these cells a useful model system for studying the mechanisms of apoptosis induced in mitotic and postmitotic neurons after growth factor deprivation. The present study concentrates on the death of mitotic PC12 cells after serum deprivation. We asked whether this death is preceded by growth arrest and whether it occurs at a specific phase of the cell cycle. Our results demonstrate that the death of serum-deprived PC12 cells is not restricted to one particular point in the cell cycle and that it is not preceded by growth arrest. The rationales for these conclusions are: (a) PC12 cell populations synchronized at specific and different phases of the cell cycle, as well as nonsynchronized cells, exhibited similar kinetics of cell death after serum deprivation. Cell death in these experiments was indicated by three independent parameters, loss of viability (Fig. 2), DNA fragmentation (Fig. 3), and the appearance of the apoptotic cell population in DNA histograms (Fig. 4); (b) the fact that serum-deprived cells can die at some point between the late-S-G₂ phase and the next G₁ phase is shown in Fig. 4 by the failure of cycling cells to arrive at the G₀-G₁ phase; (c) the fact that serum-deprived cells can die within the S and G₂-M phases is shown in Fig. 5 by the abrupt loss of RNA from these cells; and (d) the fact that serum-deprived cells die within G₀-G₁ or when trying to enter or leave G₀-G₁ is shown in Fig. 4 by the appearance of cell death during the time periods in which the G₀-G₁ cell population did not progress in the cell cycle. Similarly, Fig. 5 shows that there is a cell death excess not accounted for by cells in the S or G₂-M phases, which is represented by an apoptotic fraction that is either due to cell death within G₁ or death in cells trying to enter or leave the G₁ phase. At this time, we cannot distinguish between the latter two possibilities.

Examination of the RNA content of cells in relation to their position in the cell cycle (Refs. 29 and 30; AO staining) indicates that the death of serum-deprived exponentially growing cells may occur by at least two pathways. One involves rapid RNA loss, which affects most of S and G₂-M cells and many of G₁ cells. This RNA loss may reflect RNA degradation and shuts off total RNA synthesis as shown previously to occur in some apoptotic systems (33, 34) or stripping of the cytoplasm. The exact mechanism which leads to the RNA loss is not known,
although it is clear that it succeeds in killing the cells. The second pathway involves DNA degradation, which results in the appearance of two cell populations, Ap-high and Ap-low. The Ap-high cell population may derive from each of G1, S, or G2-M phases which contain high RNA levels; however, since the vast majority of these cells are G1 cells, the Ap-high cells are most probably derived mainly from G1 cells. The origin of Ap-low is not clear; it may derive from any of the cell populations with low RNA content or from the Ap-high cells.

Serum withdrawal from some cell types like fibroblasts causes growth arrest in the G0 state (35). This may also be the case in PC12 cells, because flow cytometry analysis (Fig. 5) suggests that G0 cells (cells with G1 DNA content and low RNA level) accumulate after serum deprivation. It is not clear from the present study whether G0 cells can also undergo apoptosis; however, since these cells accumulate during the course of serum deprivation, and since at a later time following serum deprivation essentially all cells die, it seems likely that G0 cells can also undergo apoptosis.

Apoptosis in many systems is often preceded by growth arrest, mainly but not only in the G0-G1 phase (19–23). The data presented here demonstrate that, although serum deprivation may drive the cells into the G0 state, death may occur also at the G1, S, and G2-M phases, i.e., the apoptotic death of PC12 cells does not require the cells to be first growth arrested at the G0 stage. In summary, upon serum deprivation, cell death occurs at all phases of the cell cycle. Cells that escaped from death continue to progress in the cell cycle. These cells can either die in the next phase of the cell cycle or can continue their progression in the cell cycle until they are arrested at the G0-G1 state and then die.

Previous studies showed that apoptosis induced by some antitumor agents also occurs in all phases of the cell cycle (36, 37). Our studies extend these conclusions to a different system in which apoptosis is induced by growth factor deprivation. The importance of the system used in the present study is that it represents apoptosis that is likely to occur in normal cell death.

The present study examined the relationship between apoptosis and the cell cycle in proliferating PC12 cells. It is interesting to compare these studies to nondenervating neuronal PC12 cells. It has recently been reported that the death of growth factor-deprived neuronal PC12 cells is accompanied by a burst of DNA synthesis (38) and activation of P34\(^{\text{cdc2}}\) kinase (39). The interpretation of these results implied that the postmitotic neuronal PC12 cells die because of aborted attempts to reenter the cell cycle. These results may, therefore, suggest that apoptosis is induced differently in growth factor-deprived PC12 and neuronal PC12 cells. However, the observation that every growth factor or agent which protects naive PC12 cells from serum deprivation also protects neuronal PC12 from serum and NGF deprivation suggests that the death process of these two cell systems is very similar. Clearly, further work is needed to address the issue of how apoptosis is induced in growth factor-deprived naive and neuronal PC12 cells.

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