Internucleosomal DNA Cleavage and Neuronal Cell Survival/Death

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Abstract. Serum-free PC12 cell cultures have been used to study the mechanisms of neuronal death after neurotrophic factor deprivation. We previously reported that PC12 cells undergo "apoptotic" internucleosomal DNA cleavage after withdrawal of trophic support. Here, we have used a sensitive method to detect PC12 cell DNA fragmentation within three hrs of serum removal and have exploited this assay to examine several aspects regarding the mechanisms of neuronal survival/death. Major advantages of this assay are that it permits acute experiments to be performed well before other manifest signs of cell death and under conditions that cannot be applied chronically. We find that this apopotic DNA fragmentation is distinct from the random DNA degradation that occurs during necrotic death. Major observations include the following: (*a*) There is a good correlation between the ability of trophic substances to promote PC12 cell survival and to inhibit early DNA fragmentation. (*b*) Phorbol ester, an activator of PKC, acutely suppresses DNA fragmentation, but does not promote long-term survival or inhibition of endonuclease activity when applied chronically due to its downregulation of PKC. (*c*) Cells undergoing apoptosis within 3 h of serum withdrawal have a "commitment point" of only 1.0–1.5 h beyond which they can no longer be rescued by NGF. (*d*) Aurin, a non-carboxylic analog of the endonuclease inhibitor ATA, also inhibits DNA fragmentation and promotes short-term survival of PC12 cells. (*e*) Macromolecular synthesis is not required for DNA fragmentation or for NGF to prevent this event. (*f*) Extracellular Ca^{2+} is not required for internucleosomal DNA cleavage caused by serum withdrawal or for suppression of this by NGF. (*g*) DNA fragmentation can also be detected in cultures of rat sympathetic neurons as early as 10 h after removal of NGF. As in PC12 cell cultures, this precedes morphological signs of cell death.

CELL death in the nervous system is a prominent feature of normal development. Overabundant production of vertebrate neurons is followed by naturally occurring neuronal death after projection of axons to their targets (Hamburger and Oppenheim, 1982; Oppenheim, 1991). It is believed that neurotrophic factors produced in limited amounts by target tissues regulate the survival of neurons during this developmental phase (Purves, 1986; Barde, 1989; Oppenheim, 1989). Moreover, neurons appear to depend on trophic support even after this critical period (Hendry and Campbell, 1976; Otto et al., 1987; Rich et al., 1987). The best characterized interaction is between NGF and sympathetic neurons. The significance of NGF for the survival of these neurons has been demonstrated in vivo, both during development and in the adult (Levi-Montalcini and Booker, 1960; Gorin and Johnson, 1979, 1980), and in vitro (Levi-Montalcini and Angeletti, 1963; Martin et al., 1988). The mechanisms whereby NGF promotes survival are not yet clear, nor is it known how neurons die, though progress has been made on both issues.

The rat pheochromocytoma PC12 cell line has been successfully used for studying the survival promoting actions of NGF and the mechanisms of neuronal cell death (Rukenstein et al., 1991; Batistatou and Greene, 1991). When cultured in medium without serum or NGF, both naive and NGF-pretreated (i.e., neuronally differentiated) PC12 cells die. Moreover under these conditions, the cells can be rescued by NGF (Greene, 1978). Therefore, in serum-free medium PC12 cells resemble sympathetic neurons in their requirement for NGF. Furthermore, analysis of DNA from serum-free cultures of both naive or neuronally differentiated PC12 cells and of NGF-deprived sympathetic neurons reveals a pattern of internucleosomal DNA fragmentation characteristic of a type of cell death designated as apoptosis (Batistatou and Greene, 1991; Edwards et al., 1991). In serum-deprived PC12 cultures, this fragmentation is detectable well before morphologic signs of cell degeneration. DNA fragmentation in PC12 cultures is prevented by NGF. In addition ATA, a nuclease inhibitor that prevents that DNA fragmentation, rescues both PC12 cells and sympathetic neurons from death caused by withdrawal of trophic support (Batistatou and Greene, 1991).

In the present study we introduce a modified Southern blot technique that permits robust detection of PC12 cell DNA cleavage within 3 h of serum removal. This assay has the advantage that it permits acute experiments to be carried out

well before other manifest signs of cell death and under culture conditions not amenable to chronic application. We have exploited this sensitive assay to investigate a number of issues concerning the mechanisms of neuronal survival/death. For example, our findings support nontranscriptional mechanisms both for induction of apoptotic DNA fragmentation and for the prevention thereof by NGF.

Materials and Methods

Materials

Mouse NGF was prepared from adult male submaxillary glands as previously described (Mobley et al., 1976) and was used at a concentration of 100 ng/ml. Insulin, 8-(4-chlorophenylthio)cAMP (CPT-cAMP), 12-*o*-tetradecanoylphorbol-13-acetate (TPA),[1] cycloheximide, camptothecin, uridine, 5-fluoro-2'-deoxyuridine, aurin (rosolic acid) and the triammonium salt of aurintricarboxylic acid were purchased from Sigma Chemical Co. (St. Louis, MO). EGF was generously provided by Dr. J. Schlessinger (New York University Medical Center, NY). Guinea pig complement and Ca^{2+}-free MEM were from GIBCO. Affinity-purified anti-NGF polyclonal rabbit antibodies were generously provided by Fidia Research Laboratories (Abano Terme, Italy).

Cell Culture

PC12 cells were cultured as previously described (Greene and Tischler, 1976, 1982) on collagen-coated dishes in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum and 5% FBS (complete medium). For the experiments in serum-free medium, cells were washed extensively with RPMI 1640 medium as described previously (Rukenstein et al., 1991; Batistatou and Greene, 1991) and plated in the presence of various agents. For experiments with elevated K^+, the medium was maintained at an iso-osmotic level by mixing appropriate volumes of 160 mM KCl and RPMI 1640 medium.

Primary cultures of dissociated sympathetic neurons were prepared from the superior cervical ganglia of postnatal day two rats (Lee et al., 1980*b*). Cells were plated at a density of ~1 ganglion per well, in 24-well plastic culture dishes precoated with collagen. Cultures were maintained in RPMI 1640 culture medium supplemented with 10% FBS, 3 mg/ml glucose (to a final 5 mg/ml) and 100 ng/ml NGF. Uridine (10 μM) and fluorodeoxyuridine (10 μM) were added on days 1–3 to eliminate non-neuronal cells. After 3–5 d, the cultures were washed three times with RPMI 1640 medium containing 10% FBS and an additional 3 mg/ml glucose and then maintained in the same medium (0.5 ml/well) in the presence of either 100 ng/ml NGF or anti-NGF polyclonal rabbit antibodies at a dilution of 1:250.

Extraction of DNA and Southern Blot Analysis

PC12 cells (3–10 × 10^6 per condition; equal amount of cells per condition in each experiment) were washed and plated in uncoated 100-mm plastic culture dishes in RPMI 1640 medium with or without additives. After incubation for the indicated times at 37°C, the cells were triturated off the dishes, centrifuged at 800 *g* for 5 min and the pellet was washed with ice-cold PBS. Soluble DNA was extracted by the method of Hockenbery et al. (1990) and resuspended in TE buffer (10 mM Tris, 1 mM EDTA). Subsequently the samples were incubated with 50 mg/ml DNase-free RNase (Boehringer Mannheim Corp., Indianapolis, IN) at 37°C for 30 min. All the soluble DNA recovered per condition (total soluble DNA/culture) was subjected to electrophoresis on a 1.2% agarose gel and blotted onto Gene Screen Plus Membrane (Dupont/NEN, Boston, MA). Blots were probed with total genomic PC12 cell DNA digested with Sau 3A (Boehringer Mannheim), a modification of the method described by Edwards et al. (1991). ^{32}P-labeled probe was prepared by the random priming reaction (Sambrook et al., 1989) and hybridization and washings were performed according to the manufacturer's protocol (Dupont/NEN).

The same procedure was followed for isolation and analysis of soluble DNA from superior cervical ganglionic neurons. At various times, after addition of anti-NGF antibodies, the cultures were washed once with ice-cold PBS and the neurons (three ganglia per condition) were harvested directly in lysing solution. All the soluble DNA recovered per condition was analyzed as described above.

Cell Counts

PC12 cells were washed, resuspended in RPMI 1640 medium and plated at a density of 8–12 × 10^4 per well in collagen-coated 24-well plastic culture dishes (0.5 ml of medium/well) in the presence of various agents. At various times the culture medium was removed and replaced with 0.25 ml of a solution that lyses the cell membrane but leaves the nuclei intact (Soto and Sonnenschein, 1985). The nuclei were counted in a hemacytometer. Counts were performed on triplicate wells in every experiment.

Complement-mediated Cytotoxicity

PC12 cells were washed and incubated in medium containing three parts of RPMI 1640 medium with 15% serum and one part of guinea pig serum (as a source of complement), in the presence of heat-inactivated antiserum prepared against PC12 cells, used at a dilution 1:100. This antiserum possesses potent cytotoxic activity against PC12 cells (Lee et al., 1980*a*).

Results

Apoptotic Internucleosomal Cleavage and Necrotic Random Degradation of PC12 Cell DNA

Previous studies showed that PC12 cells die when cultured in serum-free RPMI 1640 medium, and in so doing exhibit a pattern of DNA fragmentation characteristic of apoptosis (Greene, 1978; Batistatou and Greene, 1991). This fragmentation was apparent before any morphological signs of cell death. In such experiments, soluble PC12 cell DNA was analyzed by agarose gel electrophoresis and visualized under UV light after ethidium bromide staining. Using this procedure, DNA fragmentation was barely detectable 3 h after serum deprivation (Batistatou and Greene, 1991). To increase the sensitivity for detection of fragmented DNA, we carried out Southern blot analysis of soluble DNA from serum-deprived PC12 cells using radioactive probes derived from PC12 cell total genomic DNA. As shown in Fig. 1 (lane 2) a clear pattern of internucleosomal DNA cleavage (i.e., discrete fragments differing in size from one another by multiples of ~180 bps) is prominent as early as 3 h after serum deprivation. This pattern is detectable even at 2 h after withdrawal of serum or when as few as 10^6 PC12 cells are used per condition (data not shown).

In addition to the apoptotic mode of cell death, with its characteristic DNA fragmentation, the other major type of cell death is necrosis (for review see Wyllie et al., 1980; Duvall and Wyllie, 1986). In many cell types, necrosis occurs as a result of lethal stimuli such as complement attack or exposure to toxins, and is accompanied by random DNA degradation. We asked whether internucleosomal DNA cleavage, like that observed after growth factor or serum withdrawal, is the only mode of DNA fragmentation in PC12 cells, and thus non-specific. To examine this, we exposed PC12 cells to complement-mediated cytotoxicity. Cells were incubated in complete growth medium in the presence of an anti-PC12 cell antiserum and guinea pig serum (as a source of complement). Degeneration of most cells, as seen by phase-contrast microscopy, was apparent by 30 min. Soluble DNA was extracted from the cultures at various times and analyzed as described above. In addition, soluble DNA from

1. *Abbreviations used in this paper*: ATA, aurintricarboxylic acid; PKC, protein kinase C; TPA, 12-*o*-tetradecanoylphorbol-13-acetate.

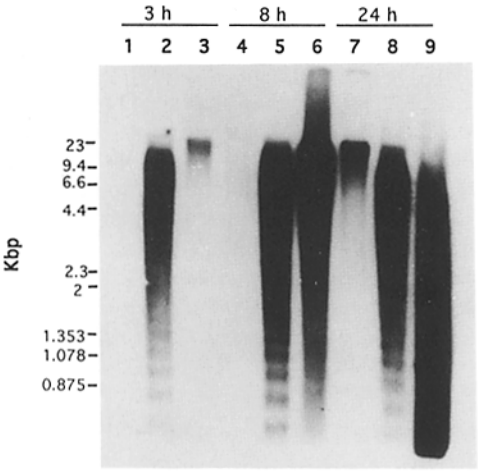

Figure 1. Complement-mediated lysis of PC12 cells leads to random DNA degradation whereas withdrawal of trophic support causes internucleosomal DNA fragmentation. PC12 cells were washed and cultured in RPMI 1640 medium containing 15% serum (lanes *1*, *4*, and *7*), in RPMI 1640 medium with no additives (lanes *2*, *5*, and *8*), or in medium containing three parts of RPMI 1640 medium with 15% serum, one part guinea pig serum, and antiserum prepared against PC12 cells, at a dilution 1:100 (lanes *3*, *6*, and *9*). After the indicated times soluble DNA was extracted from the cultures, separated on a 1.2% agarose gel, blotted onto Gene Screen Plus membrane and analyzed as described in Materials and Methods. Comparable results were obtained in two independent experiments.

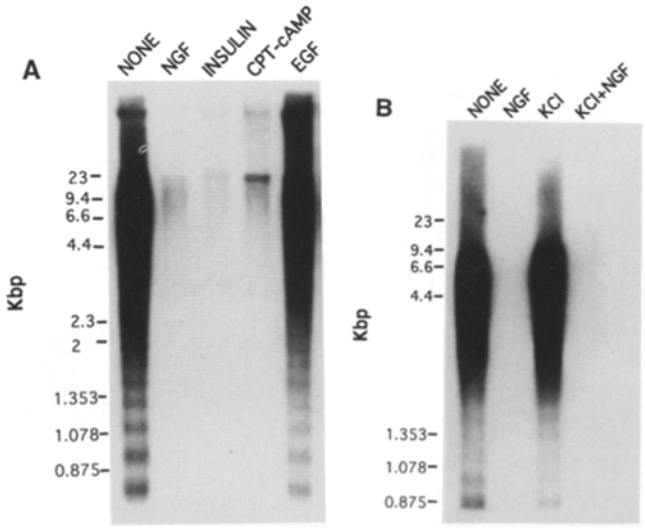

Figure 2. Survival-promoting agents inhibit internucleosomal DNA fragmentation. (*A*) PC12 cells were washed and cultured in serum-free RPMI 1640 medium, in the presence no additives (*NONE*), 100 ng/ml NGF, 3 μM insulin, 100 μM CPT-cAMP, or 5 ng/ml EGF for 3 h. Soluble DNA was extracted from the cultures and analyzed as described in Materials and Methods. Comparable results were obtained in two independent experiments. (*B*) PC12 cells were washed and cultured in RPMI 1640 medium with no additives (*NONE*), in RPMI 1640 medium with 100 ng/ml NGF, or in medium containing three volumes RPMI 1640 and one volume of 160 mM KCl with or without 100 ng/ml NGF (KCl + NGF and KCl respectively) for 3 h. Soluble DNA was subsequently extracted from the cultures and analyzed as described. Comparable results were obtained in two independent experiments.

parallel cultures of cells incubated in complete growth medium or in serum-free RPMI 1640 medium alone was isolated and analyzed. As shown in Fig. 1 (lanes *3*, *6*, and *9*) in the case of complement-mediated lysis, the soluble DNA fraction contains a continuous spectrum of sizes, indicative of random degradation. Interestingly, such DNA degradation is a relatively late event, and is undetectable at 3 h (Fig. 1, lane *3*), a time when cellular degeneration was prominent. This contrasts with the soluble DNA from serum-deprived PC12 cells which resolves as a "DNA ladder," early after serum withdrawal (Fig. 1, lane *2*).

Survival-promoting Agents Prevent PC12 Cell DNA Fragmentation

The above findings indicate that early internucleosomal DNA cleavage is a specific event occurring in serum- and growth factor-free cultures of PC12 cells that are destined to die. Using our sensitive assay we asked whether agents known to promote PC12 cell survival in serum-free medium also inhibit early DNA fragmentation. As shown in Fig. 2 *A*, besides NGF, insulin and 8-(4-chlorophenylthio)cAMP (CPT-cAMP) at concentrations that rescue the cells from death (Rukenstein et al., 1991), also inhibit formation of the DNA ladder. Conversely, agents that do not promote PC12 cell survival, including EGF or 40 mM KCl, fail to block internucleosomal DNA cleavage (Fig. 2, *A* and *B*). It has been reported that EGF and elevated K^+ slightly delay cell death in serum-free medium (Rukenstein et al., 1991). This slight effect is reflected in our assay, with appearance of a somewhat less prominent DNA ladder in the treated cultures as compared with the untreated serum-free controls.

Effects of TPA on DNA Fragmentation

Phorbol esters are potent activators of protein kinase C (PKC) and support survival of chick peripheral neurons in culture (Montz et al., 1985; Wakade et al., 1988). However, they do not promote survival of PC12 cells in serum-free medium or of rat sympathetic neurons cultured in the absence of NGF (Rukenstein et al., 1991; Rydel and Greene, 1988). To analyze this apparent discrepancy, we exploited our early DNA fragmentation assay to examine the effects of the phorbol ester TPA. As shown in Fig. 3, 100 nM TPA added to serum-free PC12 cell cultures is able to significantly inhibit the DNA ladder that is apparent 3 h after serum withdrawal. 50 nM–10 μM TPA are equally effective in blocking the internucleosomal DNA cleavage (data not shown). However, unlike the case of NGF, this inhibition is transient. By 7.5 h, the extent of DNA fragmentation in serum-free cultures treated with 100 nM TPA is comparable with that in control serum-free cultures with no additives. This delayed appearance of DNA ladder is not due to a possible toxic effect of TPA since addition of 100 nM TPA in NGF-treated cultures does not affect the ability of NGF to inhibit DNA fragmentation at this time (data not shown). One possible interpretation for this transient effect of TPA is that this agent initially activates PKC, and thereby leads

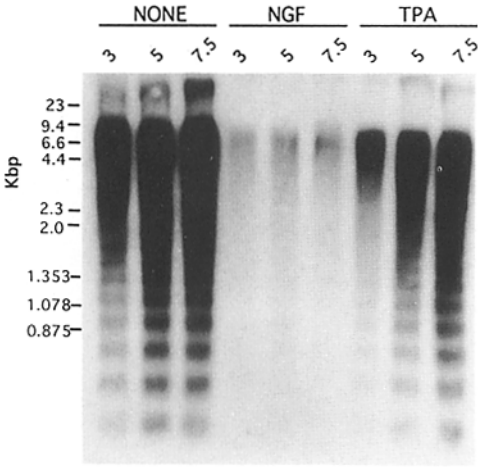

Figure 3. Effects of TPA on DNA fragmentation. PC12 cells were washed and cultured in RPMI 1640 medium with no additives (*NONE*), 100 ng/ml NGF or 100 nM TPA for 3, 5, or 7.5 h, as indicated. Subsequently soluble DNA was isolated from the cultures and analyzed. Comparable results were obtained in two independent experiments.

to inhibition of the DNA fragmentation, but after a more prolonged time of treatment it causes PKC downregulation, and consequently becomes ineffective. To test this hypothesis, PC12 cells were preincubated with 1 μM TPA in serum-containing medium for 3 days. This treatment has been shown to cause significant downregulation of PC12 cell PKC activity (Matthies et al., 1987). Subsequently, the cells were washed and plated in serum-free medium in the presence of various agents. As shown in Fig. 4 *B*, in contrast to its effects in control non-pretreated cultures (Fig. 4 *A*), 100 nM TPA is not able to inhibit early DNA fragmentation in cultures that were subjected to downregulation of PKC. It has been previously reported that PKC downregulation by pretreatment with TPA does not compromise the survival promoting actions of NGF or CPT-cAMP (Rukenstein et al., 1991). As shown in Fig. 4 *B* downregulation of PKC also does not affect the ability of these agents to inhibit internucleosomal DNA fragmentation. The same phenomenon is observed with aurintricarboxylic acid (ATA), a general nuclease inhibitor that is able to promote long-term survival of PC12 cells in serum-free medium (Batistatou and Greene, 1991).

Temporal Aspects of the Effects of NGF and ATA on PC12 Cell DNA Fragmentation

Previous studies have shown that addition of NGF to PC12 cell cultures at the time of change-over to serum-free conditions rescues the cells from death and inhibits internucleosomal DNA cleavage (Greene, 1978; Batistatou and Greene, 1991). The present sensitive assay system permitted us to ask the time that cells could be deprived of trophic support before becoming irreversibly committed to DNA fragmentation. Presumably, after this commitment point NGF will no longer be able to inhibit the DNA ladder. The experimental paradigm used was as follows: PC12 cells were washed and plated in serum-free medium, and NGF was added to the cultures at various times. 3 h after the switch to serum-free conditions soluble DNA was isolated and analyzed. Fig. 5 *A* shows that if NGF is added to the cultures within the first 1.0–1.5 h after withdrawal of serum (and thus is present for the last 1.5–2.0 h of incubation in serum-free medium), the DNA fragmentation does not appear. However, after 1.5 h, addition of NGF can no longer prevent formation of the DNA ladder that is present after 3 h of serum deprivation.

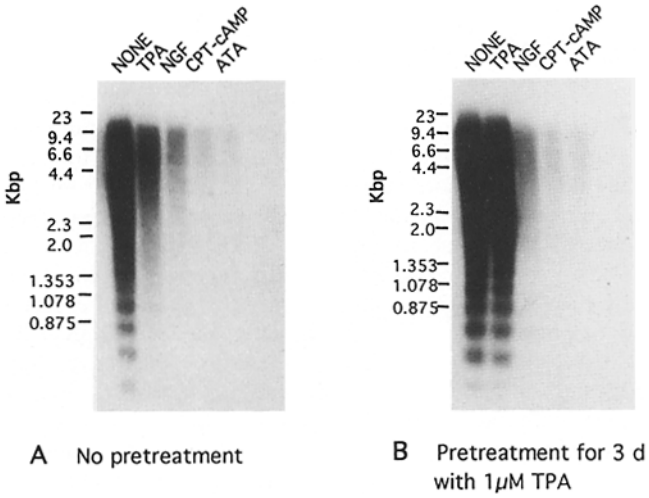

Figure 4. Effects of TPA pretreatment on the ability of various agents to inhibit internucleosomal DNA cleavage. Replicate PC12 cell cultures were maintained in complete medium in the presence (*B*) or absence (*A*) of 1 μM TPA for 3 d, then washed and cultured in RPMI 1640 medium containing no additives (*NONE*), 100 nM TPA, 100 ng/ml NGF, 100 μM CPT-cAMP, or 100 μM ATA, for 3 h. Soluble DNA was subsequently isolated from the cultures and analyzed as described in Materials and Methods. Comparable results were obtained in two independent experiments.

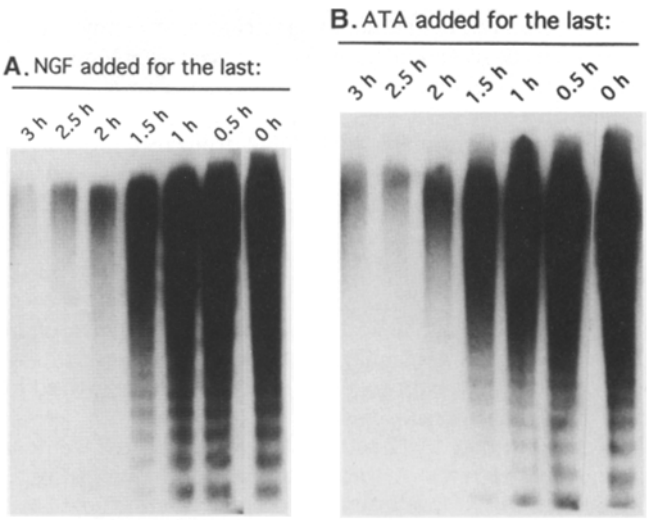

Figure 5. Effects of delayed NGF and ATA addition on DNA fragmentation. PC12 cells were washed and plated in serum-free RPMI 1640 medium. 100 ng/ml NGF (*A*) or 100 μM ATA (*B*) were added to the cultures either immediately after plating (*3 h*), at various intermediate times (*0, 5, 1, 1.5, 2 or 2.5 h* after plating) or not at all (*0 h*). 3 h after the switch to serum-free conditions, soluble DNA was isolated from the cultures and analyzed. Comparable results were obtained in two independent experiments.

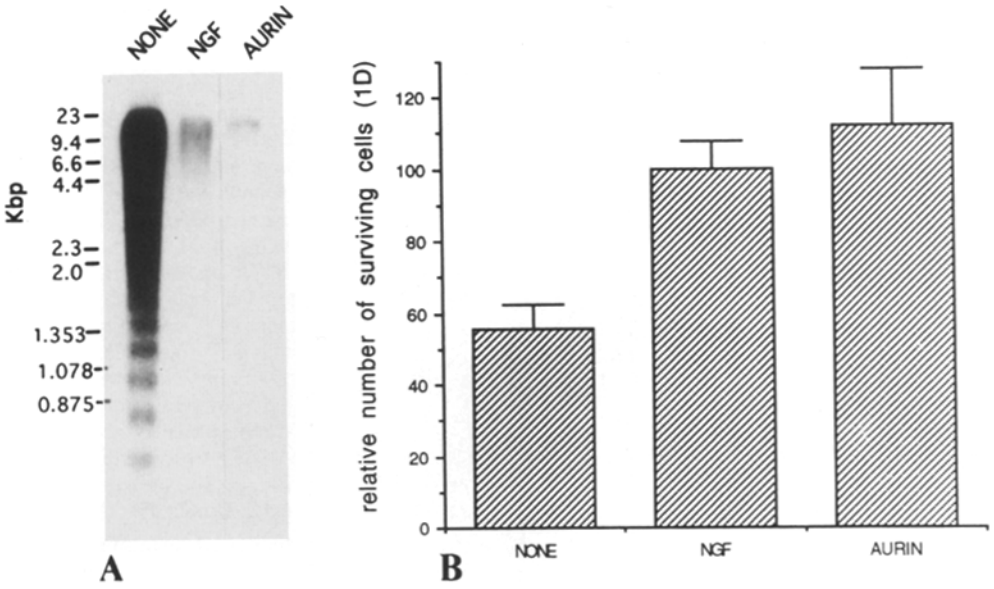

Figure 6. Effects of aurin on DNA fragmentation and short-term survival of PC12 cells in serum-free medium. PC12 cells were washed and plated in serum-free RPMI 1640 medium with no additives (*NONE*), 100 ng/ml NGF or 25 μM aurin. (*A*) After 3 h in culture, soluble DNA was extracted from the cells and analyzed. Comparable results were obtained in two independent experiments. (*B*) After 24 h in culture viable cells were counted as described in Materials and Methods. Cell numbers are expressed relative to those in NGF-treated cultures (designated as 100). Error bars represent SEM (n = 3).

ATA, like NGF, inhibits DNA fragmentation and prevents death of PC12 cells in serum-free medium (Batistatou and Greene, 1991). Since ATA is a nuclease inhibitor it has been suggested that it acts by inhibiting the endogenous endonuclease activity which leads to cell death. Because NGF and ATA presumably act at different mechanistic points to prevent cell death, we compared the times at which they are able to prevent formation of the DNA ladder in PC12 cultures deprived of serum. Fig. 5 *B* shows that ATA is able to inhibit the DNA fragmentation even if added 1.0–1.5 h after serum withdrawal. Interestingly, the commitment point—the time of culture in serum-free medium after which ATA can no longer inhibit the internucleosomal DNA cleavage that is present at 3 h—is similar to that of NGF (about 1.5 h).

Aurin Inhibits DNA Fragmentation and Promotes Survival of PC12 Cells

Past studies have shown that ATA, in addition to inhibiting nucleases, can bind to and inhibit the activity of at least several other proteins (Bina-Stein and Tritton, 1976). Such general binding activity of ATA could be due to its anionic charge. Moreover ATA binds to and is an indicator for Al^{3+} (Smith et al., 1949). Therefore, it might be argued that the survival promoting effects of ATA are due to either its anionic properties, chelation of ions, or binding of metals necessary for cell death to occur. To examine these possibilities we used aurin, a structural analog of ATA that lacks the three carboxylic groups, and thus the anionic charge. As shown in Fig. 6 *A*, 25 μM aurin, when added to serum-free cultures inhibits DNA fragmentation.

Since aurin mimics the effect of ATA on DNA fragmentation we examined whether, like ATA, it is able to promote survival of PC12 cells in serum-free medium. The data in Fig. 6 *B* show that 25 μM aurin added to serum-free cultures is able to prevent the death of PC12 cells that occurs by 24 h of serum deprivation. In three independent experiments the concentration of aurin necessary to promote maximal survival of the cells was 20–30 μM. However, aurin was not able to promote optimal long-term survival of PC12 cells. After 2–3 d the number of surviving cells in serum-free cultures containing aurin decreased significantly, though it was still higher than that in control cultures in serum-free medium alone (data not shown).

Role of RNA and Protein Synthesis in Prevention of Internucleosomal DNA Cleavage

Our past studies indicated that inhibition of protein or RNA synthesis does not rescue PC12 cells from death caused by serum deprivation and that NGF prevents serum-free PC12 cell death even when macromolecular synthesis is blocked (Rukenstein et al., 1991). Interpretation of these experiments is subject to the caveats that suppression of synthesis itself may lead to cell degeneration and that NGF may indirectly protect cells from such death. Because DNA fragmentation can be detected early and before clear toxic effects of synthesis inhibitors are apparent, we assessed this parameter in serum-deprived PC12 cell cultures that were treated with or without NGF and with or without inhibitors of macromolecular synthesis. We used 20 μM camptothecin, previously shown to inhibit PC12 cell RNA synthesis by 85% (Burstein and Greene, 1978), and cycloheximide, at 10 μg/ml, a level that blocks protein synthesis by 90% (Greenberg et al., 1986). A portion of the cultures was preincubated with the inhibitors for 1.0–1.5 h in serum-containing medium to assure adequate entry into the cells and inhibition of synthesis. These and non-treated controls were then washed and plated in serum-free medium with the appropriate combinations of inhibitors and NGF. After 3 h, soluble DNA was extracted and analyzed. As shown by the data in Fig. 7, the presence of the inhibitors did not prevent the internucleosomal DNA cleavage apparent after serum withdrawal. Moreover, blockade of synthesis did not interfere with the ability of NGF to inhibit DNA fragmentation.

Role of Extracellular Calcium in DNA Fragmentation

Influx of extracellular calcium appears to play a critical role in certain types of cell death, such as the neuronal degenera-

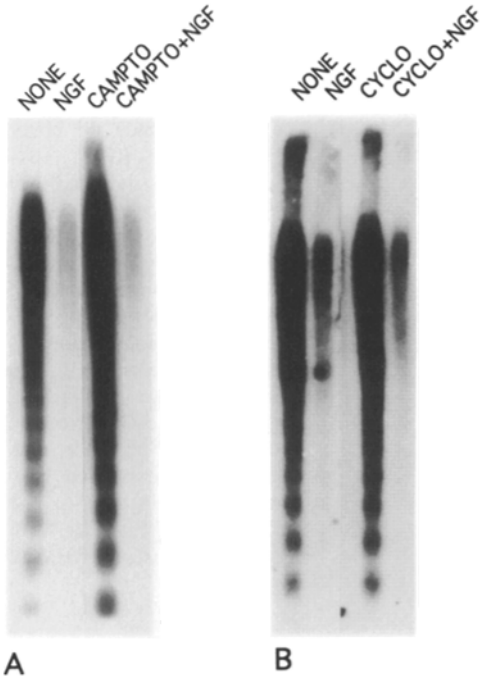

Figure 7. Effects of macromolecular synthesis inhibitors on internucleosomal DNA cleavage. (*A*) Replicate cultures of PC12 cells were maintained in complete medium in the presence or absence of 20 μM camptothecin for 1.2 h. Subsequently the cells were washed and plated in serum-free RPMI 1640 medium in the presence of no additives (*NONE*) or 100 ng/ml NGF (for cells not pretreated with camptothecin) or in the presence of 20 μM camptothecin with or without 100 ng/ml NGF (*CAMPTO + NGF* and *CAMPTO*, respectively, for cells pretreated with camptothecin). Equal numbers of cells were plated for each condition (about 5×10^6 cells per condition). After 3 h, soluble DNA was extracted from the cultures and analyzed. Comparable results were obtained in three independent experiments. (*B*) Replicate cultures of PC12 cells were maintained in complete medium in the presence or absence of 10 μg/ml cycloheximide for 1.2 h. Subsequently the cells were washed and plated in RPMI 1640 medium in the presence of no additives (*NONE*) or 100 ng/ml NGF (for cells not pretreated with cycloheximide) or in the presence of 10 μg/ml cycloheximide with or without 100 ng/ml NGF (*CYCLO + NGF* and *CYCLO*, respectively, for cells pretreated with cycloheximide). Equal numbers of cells were plated for each condition ($\sim 5 \times 10^6$ cells/condition). After 3 h, soluble DNA was extracted from the cultures and analyzed. Comparable results were obtained in three independent experiments.

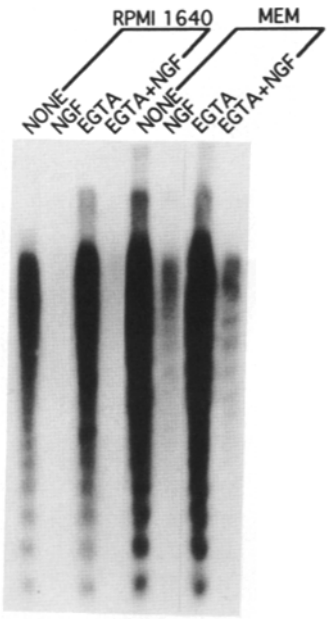

Figure 8. Role of extracellular Ca^{2+} on DNA fragmentation. PC12 cells were extensively washed with RPMI 1640 medium containing 1 mM EGTA. Subsequently cells were cultured in RPMI 1640 medium containing no additives (*NONE*), 100 ng/ml NGF, 1 mM EGTA, or 1 mM EGTA and 100 ng/ml NGF for 3 h. In a parallel experiment PC12 cells were washed with Ca^{2+}-free MEM medium containing 1 mM EGTA, and cultured in Ca^{2+}-free MEM containing no additives (*NONE*), 100 ng/ml NGF, 1 mM EGTA, or 1 mM EGTA and 100 ng/ml NGF for 3 h. Soluble DNA was subsequently extracted from all cultures and analyzed. Comparable results were obtained in two independent experiments.

tion due to toxicity of excitatory amino acids (for review see Choi, 1988; Choi et al., 1990) or after glucose deprivation (Cheng and Mattson, 1991, 1992). We sought to determine the role of extracellular Ca^{2+} in the response of PC12 cells to growth factor deprivation. In preliminary experiments we observed that prolonged culture in calcium-free medium was toxic for PC12 cells (as judged by the appearance of small phase-dark cells and debris after 1–3 d of incubation in serum-free medium with added NGF). However, incubation of PC12 cells in calcium-free medium for only 3 h did not appear to be detrimental. We therefore investigated the role of extracellular Ca^{2+} in DNA fragmentation apparent 3 h after serum withdrawal. To this end we washed and incubated PC12 cells in serum-free RPMI 1640 medium (containing 0.4 mM Ca^{2+}) in the presence of 1 mM EGTA. As shown in Fig. 8 the absence of extracellular calcium does not inhibit the internucleosomal DNA cleavage apparent 3 h after serum deprivation. Comparable results were obtained when the RPMI 1640 medium was adjusted to contain 2–20 mM of EGTA. Moreover, the same effects were observed when cells were washed and incubated in Ca^{2+}-free Minimum Essential Medium with or without 1 mM EGTA (Fig. 8).

We further examined whether influx of extracellular Ca^{2+} is also necessary for NGF's ability to inhibit the DNA fragmentation. The data in Fig. 8 show that despite the absence of extracellular calcium, NGF is able to inhibit internucleosomal DNA cleavage.

Early Apoptotic DNA Fragmentation in NGF-deprived Neurons

A past study has shown that sympathetic neurons deprived of NGF display apoptotic internucleosomal DNA fragmentation (Edwards et al., 1991). Since ATA, a nuclease inhibitor, is able to promote their long-term survival in the absence of NGF, it has been hypothesized that an endogenous endonuclease activity leads to DNA fragmentation and neuronal cell death after growth factor deprivation (Batistatou and Greene, 1991). We investigated the time of appearance of internucleosomal DNA cleavage in NGF-deprived cultures of sympathetic neurons, reasoning that if it is one of the first events of cell death, then DNA fragmentation should appear early after growth factor removal. Sympathetic neurons were isolated from superior cervical ganglia of 2-d-old rats and cultured for 3–5 d. Subsequently the cultures were washed and maintained in the presence of either NGF or anti-NGF antibodies. The first morphological changes in NGF-deprived cultures appeared after 24–36 h; some cell bodies were smaller and phase-dark and debris appeared in the culture medium. It should be noted that some debris were

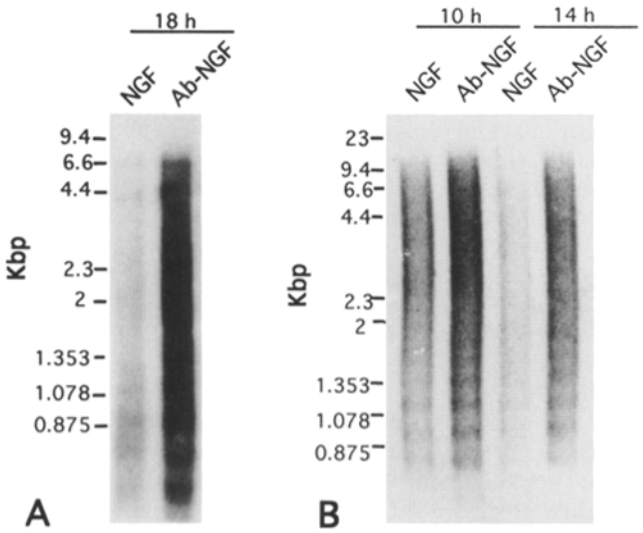

Figure 9. NGF-deprived neurons display DNA fragmentation. Newborn rat sympathetic neurons were cultured for 4 d in the presence of NGF as described in Materials and Methods. Subsequently cultures were washed and maintained in the presence of either 100 ng/ml NGF or anti-NGF polyclonal antibodies (*Ab-NGF*). (*A*) 18 h after addition of the antibodies soluble DNA was extracted from the cultures and analyzed. (*B*) In an independent experiment 10 or 14 h after addition of the antibodies soluble DNA was extracted from the cultures and analyzed.

also apparent in the NGF-treated cultures. This is consistent with reports that the number of sympathetic neurons gradually decreases during the first week in culture even in the presence of NGF (Chun and Patterson, 1977). At various times soluble DNA in both NGF-deprived and control cultures was isolated and analyzed. As seen in Fig. 9 a background DNA fragmentation is present in NGF-treated control cultures at all times, probably reflecting the small amount of dying sympathetic neurons. However, as early as 10 h after addition of anti-NGF antibody there is a clear pattern of internucleosomal cleavage that is more prominent than in control cultures and that becomes more pronounced at later times. In three independent experiments enhanced DNA fragmentation was apparent in anti-NGF-treated cultures before any morphological signs of cell death were seen by phase-contrast microscopy.

Discussion

This study has explored several issues on the subject of neuronal cell survival/death. We have primarily exploited the appearance of DNA fragmentation in cultures of PC12 cells deprived of serum and NGF as a means to investigate neuronal cell death. To significantly increase the sensitivity for detection of fragmented DNA, based on modification of methods described by Edwards et al. (1991), we used Southern blot analysis with random probes derived from PC12 cell genomic DNA. This technique permits detection of a clear DNA fragmentation pattern in soluble DNA of PC12 cells deprived of serum for only 3 h. Thus, it has allowed us to probe the survival/death mechanisms with agents or treatments that would themselves cause cell death or injury if applied for longer times. This early appearance of internucleosomal DNA cleavage is evidently predictive for the later-occurring PC12 cell death that takes place after withdrawal of trophic support. Thus, agents that rescue PC12 cells from serum-free death, including NGF, insulin, CPT-cAMP, and ATA, also inhibit the early cleavage of DNA. In contrast, agents that do not rescue the cells, including EGF or elevated K^+, do not suppress DNA fragmentation.

Recently, Mesner et al. (1992) confirmed our observations (Batistatou and Greene, 1991) that ATA prevents death of PC12 cells and sympathetic neurons after withdrawal of trophic support. However, these workers also reported that death of PC12 cells (naive or NGF-treated) and cultured superior cervical ganglionic neurons after trophic factor withdrawal is not accompanied by DNA fragmentation and therefore must occur by a mechanism other than apoptosis. This contrasts with our findings with PC12 cells (Batistatou and Greene, 1991; and present data) as well as those of Edwards et al. (1991), Deckwerth and Johnson (Deckwerth, T. L., and E. M. Johnson. 1992. *Soc. Neurosci. Abstr.* 30.2) and the present study with sympathetic neurons. One possible explanation for this apparent discrepancy could be methodological sensitivity. Although Mesner et al. (1992) used radioactive DNA probes to detect DNA fragmentation, the small quantity (5 μg per condition) of total genomic DNA analyzed may have been limiting. These authors claimed to have ruled out this possibility by quantitative comparison of genomic DNA from trophic factor deprived PC12 cells or sympathetic neurons with that of thymocytes undergoing glucocorticoid-induced cell death. It should be noted however, that such thymocyte cell death is a more rapid and synchronized event (Wyllie, 1980) than in the cases of PC12 cells and sympathetic neurons (Rukenstein et al., 1991; Martin et al., 1988). Therefore, a significantly higher percentage of treated thymocytes are likely to undergo DNA fragmentation at any given time in comparison with PC12 cells and sympathetic neurons which exhibit a more protracted time course of degeneration (Greene, 1978; Martin et al., 1988). Hence, quantitative comparison between these populations, namely PC12 cells, sympathetic neurons and thymocytes should be interpreted with caution. We previously reported that although we were able to detect the "DNA ladder" in preparations of total genomic DNA from dying PC12 cells, the sensitivity of detection was greatly enhanced by analysis of soluble DNA. This is because elimination of high molecular weight DNA permits significantly larger amounts of relevant material to be analyzed by agarose gel electrophoresis. The present PC12 cell studies use naive PC12 cells deprived of serum. In addition to these, Mesner et al. (1992) also used PC12 cells primed with NGF in the presence of serum and then deprived simultaneously of serum and NGF. However, in contrast to Mesner et al. (1992), we have observed internucleosomal DNA fragmentation in NGF-primed cultures deprived of both serum and NGF (Batistatou and Greene, 1991), as well as in cultures primed with NGF under serum-free conditions and then deprived of the factor (Farinelli, S. E., and L. A. Greene, unpublished).

Apoptotic Versus Necrotic Death of PC12 Cells

To test whether the internucleosomal DNA cleavage indicating apoptotic death is specific for PC12 cell degeneration af-

ter withdrawal of trophic support, we used complement attack to induce necrotic death of PC12 cells. Necrosis is a form of cell death that is distinct from apoptosis and is characterized by early dilatation of cytoplasmic organelles, later followed by rupture of cell membranes and nuclear disruption (Wyllie et al., 1980; Duvall and Wyllie, 1986). As reported for other cell types, necrotic PC12 cell death was accompanied by random rather than internucleosomal DNA degradation. Thus, the appearance of a DNA ladder after serum or growth factor withdrawal is a rather specific event. Moreover, necrotic DNA degradation was a late phenomenon that was detected several hours after cellular degeneration was evident. This late onset of random DNA degradation after cell lysis is in concordance with the proposed mechanism of necrotic death, in which chromatin digestion by nucleases and proteases is the consequence rather than the cause of cell death (Duvall and Wyllie, 1986). In contrast, the early internucleosomal DNA fragmentation that occurs after serum or NGF withdrawal appears before overt morphological signs of cell degeneration and could thus play a causative role in cell death.

ATA and Aurin

One observation that supports a causative role for internucleosomal DNA cleavage in cell death is that ATA, a nuclease inhibitor, prevents the death of PC12 cells and sympathetic neurons after withdrawal of serum and NGF, respectively (Batistatou and Greene, 1991). In a previous study we found that ATA did not affect a number of examined responses to NGF, suggesting that it has rather specific actions on the cells (Batistatou and Greene, 1991). Moreover, it is unlikely that ATA directly activates membrane tyrosine kinase receptors since, in contrast to growth factors, it does not induce detectable changes in tyrosine phosphorylation of cellular proteins in serum-free cultures (Batistatou, A., unpublished data). However, ATA has a number of known activities in addition to nuclease inhibition, and it might be argued that one or more of these is responsible for its survival-promoting actions. Such activities include binding to a variety of cellular proteins and chelating metal ions such as Al^{3+} (Bina-Stein and Tritton, 1976; Smith et al., 1949). To investigate such possibilities, we tested the actions of aurin, an analog of ATA that lacks tricarboxylic acid groups and anionic charge and that consequently would be less likely to undergo charge-based chelation of metal ions and non-specific binding to proteins. We found that aurin promotes excellent short-term serum-free survival of PC12 cells. However, it did not promote effective long-term survival. The reasons for this are unclear, but may include instability or toxic side effects. To avoid such problems of long-term drug exposure, we used our short-term assay of early DNA fragmentation. This clearly showed that aurin, like ATA, effectively inhibits early DNA fragmentation.

Temporal Aspects of Commitment to DNA Fragmentation

Our past and present findings reveal that DNA fragmentation can be detected in PC12 cell cultures within 2–3 h after withdrawal of trophic support. The levels of such fragmentation continue to increase for several hours, at least in part because the onset of this phenomenon is not synchronized in the population. This is supported by the observations that cell death in this system is prolonged over a period of several days (Greene, 1978). Moreover, it is likely that fragmentation of DNA is a rapid event and therefore that the DNA ladder we observe at any given time represents material in rapid transit between intact high molecular weight DNA and fragments too small to register in our assay. Thus, the DNA fragments we detect at 3 h of serum withdrawal are presumably derived from a small subpopulation of cells. Irrespective of the basis for the absence of synchrony in the cultures, we took advantage of the early subpopulation of vulnerable cells to address the issue of the time at which PC12 cells become committed to DNA fragmentation after withdrawal of trophic support. This was achieved by progressively delaying the time of addition of NGF. We found that for those cells that undergo fragmentation by 3 h of withdrawal, commitment to this event occurs after ~1.5 h. Our findings suggest that neuronal cells without trophic support can rapidly become vulnerable to irreversible processes such as DNA fragmentation and thus that there is only a very narrow time window during which they can be rescued by trophic factors. In agreement with this, during development of the nervous system, cell death and selection of those neurons that will survive in any given population takes place over a relative short time period (Harris-Flanagan, 1969; Horsburgh and Sefton, 1987; Provis, 1987).

PKC and Inhibition of DNA Fragmentation

Although agents that activate a variety of cellular protein kinases are capable of supporting PC12 cell and neuronal survival (Rukenstein et al., 1991), the role of PKC in this regard has been unclear. Evidence in PC12 cell cultures has suggested that activation of PKC is not required for the survival-promoting actions of NGF and other agents (Rukenstein et al., 1991) and our present experiments indicate that NGF, CPT-cAMP, or ATA do not require activated PKC to inhibit internucleosomal DNA fragmentation. With regard to a direct role for PKC activation in survival, past work established that phorbol esters (activators of PKC) do not support the serum-free survival of PC12 cells or of rat sympathetic neurons deprived of NGF (Rukenstein et al., 1991; Rydel and Greene, 1988). In contrast, the phorbol ester 12,13 dibutyrate (PDB) has been shown to maintain cultured chick sympathetic neurons (Wakade et al., 1988). Resolution of this apparent discrepancy appears to lie in the different behavior of PKC in the two systems. In chick sympathetic neurons, 2 d of exposure to PDB leads to an increase in PKC activity as compared with that in cultures maintained with NGF (Wakade et al., 1988). However, for both PC12 cells and rat sympathetic neurons, continuous treatment with phorbol esters causes initial activation followed by profound downregulation of PKC (Matthies et al., 1987). To be able to examine a survival-related parameter before PKC downregulation, we studied PC12 cell DNA fragmentation. Our experiments revealed that TPA significantly inhibited DNA cleavage after initial application (3 h), but not at later times of treatment (5 or 7.5 h). In addition, long-term pretreatment with TPA abolished the early effect of phorbol ester on DNA fragmentation. These observations suggest that activation of PKC can inhibit DNA cleavage and presumably support neuronal survival, but that prolonged exposure to phorbol ester in the rat, but not the chick, leads to PKC depletion and loss of survival-promoting activity. An alternative interpretation

consistent with our data is that in the rat, PKC delays, but does not prevent cell death.

Role of Macromolecular Synthesis

We previously reported that inhibition of RNA and protein synthesis fails to rescue PC12 cells from death after 1 d in serum-free medium (Rukenstein et al., 1991). One interpretation of our observations is that macromolecular synthesis is not required for PC12 cell death. An alternative explanation is that serum-deprived PC12 cells cannot sustain survival without continuous macromolecular synthesis and are therefore eventually killed by exposure to appropriate inhibitors. To distinguish between these possibilities, we exploited our assay for early detection of internucleosomal DNA cleavage after serum withdrawal. We found that such fragmentation occurred even after inhibition of macromolecular synthesis. We therefore conclude that macromolecular synthesis is not required for induction of endonuclease activity responsible for DNA fragmentation and suggest that de novo protein synthesis is not necessary for PC12 cell death caused by withdrawal of trophic support.

Our observations with PC12 cells contrast with the case of sympathetic neurons in which death caused by withdrawal of NGF is considerably delayed by suppression of macromolecular synthesis (Martin et al., 1988). The latter findings have been interpreted to suggest that neuronal death is due to de novo synthesis of "death proteins" whose expression is otherwise suppressed by neurotrophic agents such as NGF (Martin et al., 1988). However, we have hypothesized that such an effect in neurons might be due to causes other than suppression of death-inducing genes (Rukenstein et al., 1991; Batistatou and Greene, 1991). For example, it has been suggested that inhibitors of macromolecular synthesis prevent apoptotic cell death because they lead to depletion of rapidly turning over, constitutively expressed proteins (such as endonucleases) rather than to inhibition of newly expressed death genes (McConkey et al., 1990; Gaido and Cidlowski, 1991). An alternative suggestion that would also be consistent with the differential sensitivity of PC12 cells and sympathetic neurons to synthesis inhibitors is that trophic-factor-deprived cells die because they unsuccessfully attempt to enter the cell cycle (Colombel et al., 1992). Since neurons are postmitotic, their attempt to re-initiate DNA synthesis would presumably require synthesis of cell cycle proteins; in contrast, naive PC12 cells are both transformed and capable of mitosis and therefore might not require de novo synthesis to initiate replication. Consistent with this are the reports (Rukenstein et al., 1991; Mesner et al., 1992; Pittman, R. N., A. J. DiBenedetto, and S. Wang. 1992. *Soc. Neurosci. Abstr.* 247.10) that NGF-pretreated PC12 cells can be at least partially rescued from cell death in serum-free medium by RNA and protein synthesis inhibitors after NGF withdrawal. In such cultures, NGF treatment induces PC12 cells to enter a postmitotic state similar to that of sympathetic neurons (Greene, 1978). Thus, from the viewpoint of this model, NGF-untreated PC12 cells would differ from their NGF-pretreated counterparts and from sympathetic neurons principally in that they already possess proteins that permit them to attempt entry into the cell cycle. Once sympathetic neurons and NGF-pretreated PC12 cells synthesize such proteins, cell death in all cases may be predicted to occur by a similar mechanism.

Our past work indicated that NGF and other agents promote survival of serum-deprived PC12 cells even when macromolecular synthesis is blocked (Rukenstein et al., 1991). This led to the proposal that NGF maintains survival by a mechanism that is independent of synthesis. Such a post-translational mechanism is supported by the report that NGF maintains sympathetic neurons in the presence of protein synthesis inhibitors, even under conditions in which such inhibitors themselves cannot prevent death (Edwards et al., 1991). However, such experiments are open to the alternate possibility that NGF prevents or delays death caused by the inhibitors themselves. Here again, the assay of early DNA ladder formation proved valuable because it could be applied well before any evident signs of cell degeneration caused by cessation of synthesis. Our present observation that inhibition of macromolecular synthesis does not interfere with the ability of NGF to block internucleosomal DNA cleavage thus significantly supports a posttranslational mechanism for NGF action on DNA fragmentation and cell survival. We have previously suggested that a protein kinase/phosphorylation-driven pathway would be an attractive candidate in this regard (Rukenstein et al., 1991; Batistatou and Greene, 1991).

The Role of Extracellular Ca^{2+}

Extracellular calcium is a possible regulator of neuronal survival/death. Influx of extracellular Ca^{2+} appears to play a necessary role in the neurotoxic effects of excitatory neurotransmitters (Choi, 1988; Choi et al., 1990). Moreover, it has been reported that trophic factors including NGF can protect neurons from such neurotoxicity (Cheng and Mattson, 1991, 1992). We therefore asked whether extracellular Ca^{2+} plays a role in the response of PC12 cells to growth factor deprivation or in the ability of NGF to maintain survival. Attempts to investigate these issues were hampered by the finding that long-term removal of calcium from serum-deprived PC12 cell cultures is itself toxic. We therefore turned to assessment of early DNA fragmentation. Our findings indicate that neither the presence nor influx of extracellular Ca^{2+} are required for the induction of DNA fragmentation, and therefore probably cell death, caused by withdrawal of trophic support. In addition, we found that the capacity of NGF to inhibit DNA fragmentation does not depend on the presence of extracellular Ca^{2+}. These results complement previous observations that removal of extracellular Ca^{2+} neither prevents death of sympathetic neurons caused by NGF withdrawal nor compromises NGF's ability to maintain survival (Koike et al., 1989). Further studies will be necessary to determine the potential role of intracellular Ca^{2+} in regulation of cell death/survival by NGF and other trophic factors.

DNA Fragmentation in NGF-deprived Sympathetic Neurons

It was previously reported that sympathetic neurons cultured in the absence of NGF display apoptotic DNA fragmentation 18 h after NGF withdrawal (Edwards et al., 1991). We confirmed this observation and extended our studies to even earlier times to show that enhanced levels of internucleosomal DNA fragmentation are detectable at 10 h after NGF deprivation. This is well before overt morphological signs of deterioration. These observations, together with our previ-

ous finding that ATA promotes long-term survival of NGF-deprived sympathetic neurons, are consistent with the possibility that NGF promotes survival by suppressing the action of a constitutively-expressed endonuclease activity. The assay for early DNA fragmentation used here should facilitate the elaboration and testing of this hypothesis.

In summary our findings indicate that early internucleosomal DNA fragmentation is specifically associated with PC12 cell death after serum and NGF deprivation and is a valuable marker for investigating the mechanisms of cell survival/death. Moreover, its appearance in cultures of sympathetic neurons early after NGF withdrawal suggests that similar mechanisms may be responsible for cell survival/death in both systems.

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