Aurintricarboxylic Acid Rescues PC12 Cells and Sympathetic Neurons from Cell Death Caused by Nerve Growth Factor Deprivation: Correlation with Suppression of Endonuclease Activity

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Abstract. Past studies have shown that serum-free cultures of PC12 cells are a useful model system for studying the neuronal cell death which occurs after neurotrophic factor deprivation. In this experimental paradigm, nerve growth factor (NGF) rescues the cells from death. It is reported here that serum-deprived PC12 cells manifest an endonuclease activity that leads to internucleosomal cleavage of their cellular DNA. This activity is detected within 3 h of serum withdrawal and several hours before any morphological sign of cell degeneration or death. NGF and serum, which promote survival of the cells, inhibit the DNA fragmentation. Aurintricarboxylic acid (ATA), a general inhibitor of nucleases in vitro, suppresses the endonuclease activity and promotes long-term survival of PC12 cells in serum-free cultures. This effect appears to be independent of macromolecular synthesis. In addition, ATA promotes long-term survival of cultured sympathetic neurons after NGF withdrawal. ATA neither promotes nor maintains neurite outgrowth. It is hypothesized that the activation of an endogenous endonuclease could be responsible for neuronal cell death after neurotrophic factor deprivation and that growth factors could promote survival by leading to inhibition of constitutively present endonucleases.

NATURALLY occurring neuronal death is a well established phenomenon that occurs during development of the vertebrate nervous system. About 20–80% of embryonic postmitotic neurons in a given population ultimately die (Oppenheim, 1991). It is believed that the survival of neurons is, at least in part, mediated by neurotrophic factors that are released in limited supplies by target tissues and that those neurons that acquire sufficient amounts of such factors survive (Barde, 1988; Oppenheim, 1989).

The presently best characterized neurotrophic factor is nerve growth factor (NGF)[1]. Sympathetic neurons are among the major neuronal populations affected by NGF. The significance of NGF for their survival has been demonstrated both in vivo and in vitro. Administration of exogenous NGF to prenatal rats decreases the naturally occurring death of sympathetic neurons (see Levi-Montalcini and Angeletti, 1968, for review). Moreover, exposure of rat fetuses to maternal anti-NGF antibodies or of newborn animals to injected anti-NGF antiserum leads to degeneration of sympathetic ganglia (Levi-Montalcini and Booker, 1960; Levi-Montalcini and Angeletti, 1966; Gorin and Johnson, 1979). Sympathetic neurons cultured from neonatal rodents also require NGF for survival (Levi-Montalcini and Angeletti, 1963; Martin et al., 1988).

The mechanism by which NGF promotes survival and prevents death of its responsive neurons is largely unknown. The morphology of degenerating superior cervical ganglionic neurons during naturally occurring death or after anti-NGF treatment is illuminating. In vivo the earliest degenerative changes are observed in the nuclear compartment with nucleolar alterations and chromatin clumping (Levi-Montalcini et al., 1969; Wright et al., 1983). These changes are similar to the "nuclear" type of degeneration described in other developing neuronal populations (Chu-Wang and Oppenheim, 1978; Pilar and Landmesser, 1976). In vitro, both "nuclear"- and "cytoplasmic"-like types of degeneration are observed when sympathetic neurons are deprived of NGF (Martin et al., 1988).

The ultrastructural changes that take place during the "nuclear" type of degeneration resemble the morphological changes observed in cells undergoing a mode of degeneration termed "apoptosis" (Wyllie et al., 1980). In vivo, apoptosis characteristically affects scattered single cells and has been observed in many instances of cell death, e.g., during embryonic development and metamorphosis or as part of normal tissue turnover (Duvall and Wyllie, 1986). Apoptosis also occurs in vitro. One of the best characterized examples is the immature thymocyte, which dies when exposed to glucocorticoid hormones, calcium ionophores, or antibodies to the CD3-T-cell receptor complex (McConkey et al., 1990b). As first described in glucocorticoid-induced thymocyte cell

1. *Abbreviations used in this paper*: ATA, aurintricarboxylic acid; NGF, nerve growth factor; ODC, ornithine decarboxylase.

death, the chromatin of apoptotic cells is degraded into discrete fragments of oligonucleosome length (Wyllie, 1980). The correlation of apoptosis with internucleosomal DNA cleavage has led to the suggestion that such cell death is due to induction or activation of endonuclease activity.

Much of the present information on NGF-dependent neuronal survival has been obtained through studies on primary cultures of sympathetic neurons. An alternative is the rat pheochromocytoma PC12 cell line (Greene and Tischler, 1976). When exposed to NGF, PC12 cells acquire a sympathetic neuron-like phenotype. Moreover, NGF rescues PC12 cells from death in serum-free medium (Greene, 1978). Previous studies have utilized PC12 cells cultured in serum-free medium to study the mechanisms by which neurotrophic factors prevent neuronal cell death and have indicated that these cells may be a useful model for this purpose (Rukenstein et al., 1991).

In the present study, we investigated the mechanisms of neuronal death after growth factor deprivation. We found that DNA fragmentation characteristic of apoptotic cell death rapidly appears when PC12 cells are challenged with serum-free medium and that this fragmentation is prevented by NGF. In addition, a general nuclease inhibitor, aurintricarboxylic acid (ATA), that blocks the DNA fragmentation, also rescues the cells from death. Moreover, ATA is able to promote the survival of NGF-deprived sympathetic neurons in culture.

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 50 ng/ml. Actinomycin D, anisomycin, camptothecin, and the triammonium salt of ATA ("aluminon", Lot No 118F0449) were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

PC12 cells were cultured as previously described on collagen-coated dishes in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum and 5% FBS (Greene and Tischler, 1976). For the experiments in serum-free medium, cells were washed three to four times with RPMI 1640 medium while attached to the collagen-coated culture dish. Then the cells were detached by trituration and washed in RPMI 1640 medium by three to four cycles of centrifugation-resuspension. For the experiments on promotion of cell survival, washed cells were resuspended in RPMI 1640 medium and plated at a density of 8–12 $\times$ 10^4 per well in uncoated 24-well plastic culture dishes. Under these conditions the cells grew mainly in suspension. To refeed, but to avoid loss of floating cells, fresh medium (0.25 ml) was added to the cultures every 3–4 d. For experiments in which PC12 cells were induced to morphologically differentiate in the presence of NGF, the cells were plated in 24-well plastic culture dishes, whose bottom surfaces were precoated with rat tail collagen (Greene and Tischler, 1982) or matrigel (dilution 1:50, Collaborative Research Inc.). For the rest of the experiments, cells were grown in 35- and 100-mm collagen-coated culture dishes at a density of 1-2 $\times$ 10^6 and 10–15 $\times$ 10^6 cells per dish, respectively.

Primary cultures of dissociated sympathetic neurons were prepared from the superior cervical ganglia of postnatal-day-2 or -3 rats (Lee et al., 1980). Cells were plated at a density of ~0.25 ganglion per well, in 24-well plastic culture dishes precoated with collagen. Cultures were grown in RPMI 1640 culture medium supplemented with 10% heat-inactivated horse serum and 50 ng/ml NGF. Cytosine arabinoside (10 μM) was added to the cultures from days 1-3 to kill dividing non neuronal cells. After 3–6 d, the cultures were washed extensively (5×) with RPMI 1640 medium containing 1% horse serum and the cells were then maintained in the same medium (0.5 ml/well) in the presence of either no additives, NGF, or ATA. The lowered serum concentration was used to avoid possible diminution of free ATA levels by interaction with serum proteins (Lindenbaum and Schubert, 1956).

Extraction and Electrophoretic Analysis of DNA

PC12 cell soluble DNA was extracted by the method of Hockenbery et al. (1990). The nucleic acid concentration was determined by UV absorbance at 260 nm. The same amount of nucleic acid from each sample (10–30 μg, depending on the experiment) was subjected to electrophoresis on a 1.2% agarose gel. Subsequently, the gel was incubated at 37°C with RNase A (20 μg/ml) and then stained with ethidium bromide. In some experiments, the samples were pretreated with RNase A (the latter was adjusted to 0.25 mg/ml final concentration in the extraction buffer, and the samples were incubated for 2 h at 37°C, which led to complete digestion of the RNA as seen by agarose gel electrophoresis) before determination of DNA concentration and electrophoresis of equal amounts of DNA per sample.

Total PC12 cell DNA was prepared as follows. About 10^8 cells per sample were incubated in a solution containing 0.1 M NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA (pH 8), 1% SDS, and proteinase K (adjusted to a final concentration of 200 μg/ml) for 30 min at 37°C. The samples were further incubated on a rotating wheel overnight at room temperature. After extraction with an equal volume of phenol, RNase (DNase-free) was added to the samples to a final concentration of 20 μg/ml, and the solution was incubated at 37°C for 60 min. Samples were extracted sequentially with equal volumes of phenol, phenol-chloroform, and chloroform, and the DNA was precipitated with 0.3 M sodium acetate and 2.2 vol of ethanol. The DNA was resuspended in 10 mM Tris-HCl and 1 mM EDTA and its concentration was determined by UV absorbance at 260 nm. 60 μg of DNA per sample were subjected to electrophoresis on a 1.2% agarose gel. As might be anticipated, the sensitivity with which endonuclease-fragmented DNA was detected was considerably greater with preparations of total soluble DNA that with total cellular DNA. For this reason, unless otherwise indicated, experiments employed the former preparation.

Cell Counts

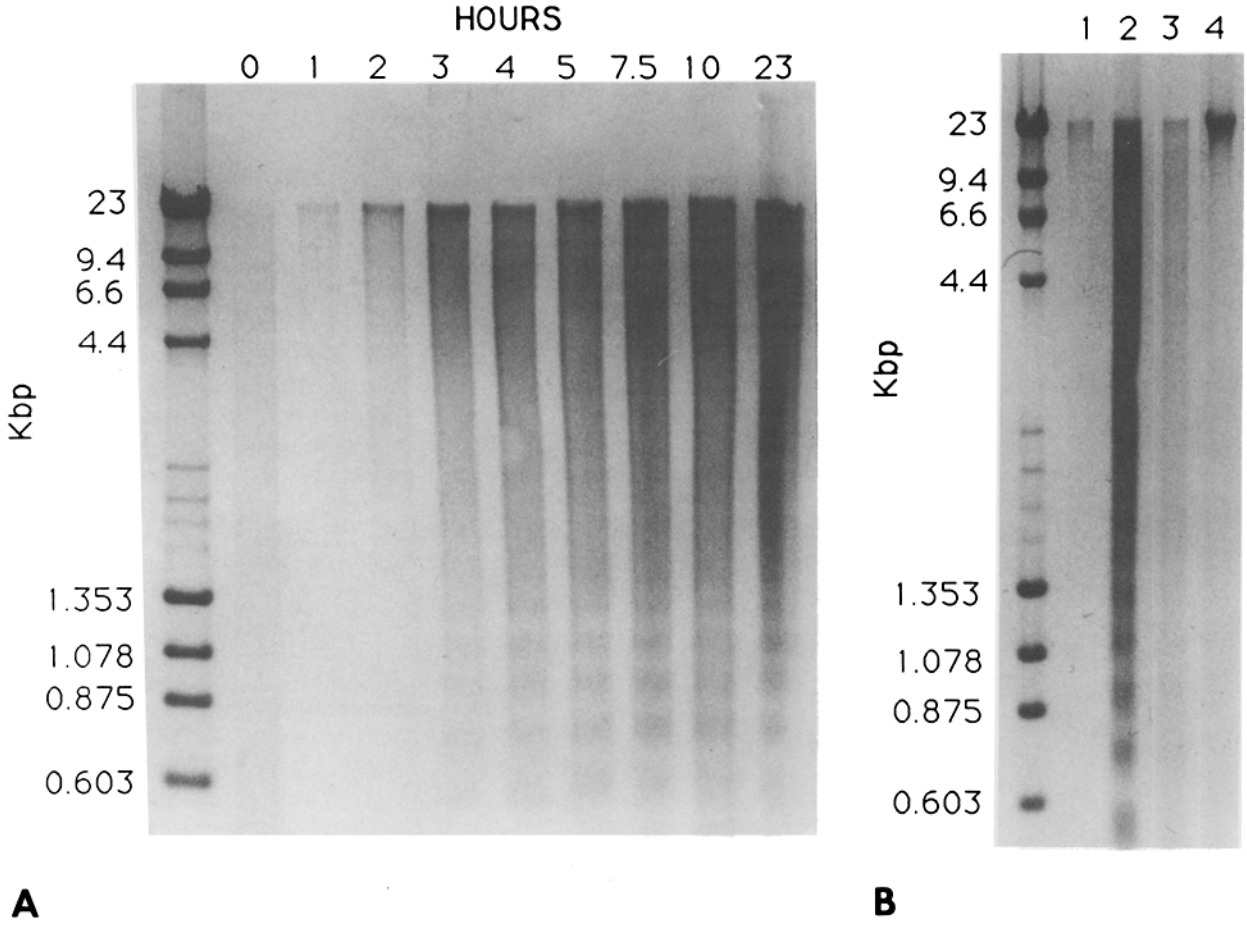
PC12 cells were removed from the wells and centrifuged (500 *g*, 5 min). The supernatant containing cell debris was discarded and the cell pellet was resuspended in 0.25 ml of a solution that lyses the cell membrane but leaves the nuclei intact (Soto and Sonnenschein, 1985). The nuclei were counted in a hemacytometer. Counts were performed on triplicate wells and are presented as means $\pm$ SEM. The results are presented relative to the cell number initially plated per well (designated as 100). For sympathetic neurons, neuronal cell number was determined by strip counts (Greene et al., 1990). The neuronal cell bodies, as seen by phase-contrast microscopy, are round and phase-bright and thus easily distinguishable in the cultures. Cells in triplicate wells were scored and the counts are presented as means $\pm$ SEM. The results are presented relative to the cell number in NGF-treated cultures (designated as 100).

Northern Blot Analysis of c-fos mRNA

PC12 cells were washed as described previously and grown in 100-mm collagen-coated dishes in RPMI 1640 culture medium in the presence of various concentrations of ATA. After 18–22 h or 7 d, NGF (50 ng/ml) was added for 30 min or 4 h and, subsequently, the cells were used for isolation of total cellular RNA, by a single step acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). 15 μg of RNA per sample were separated on 1% agarose-formaldehyde gels and transferred to nitrocellulose membranes (Maniatis et al., 1982). The blots were hybridized with a ^{32}P-labeled Pst-1 fragment of plasmid p-fos-1 (Curran et al., 1982), washed, and subjected to autoradiography. Subsequently, the blots were stripped and hybridized with a probe to glyceraldehyde-3-phosphate dehydrogenase (Piechaczyk et al., 1984), a gene whose message is not rapidly regulated by NGF. The quantitative results are presented as the ratio of the c-*fos* signal (as determined by densitometry performed on autoradiograms) to the GAPDH signal.

Measurement of Ornithine Decarboxylase (ODC) Activity

PC12 cells were washed and cultured on collagen-coated 35-mm dishes in



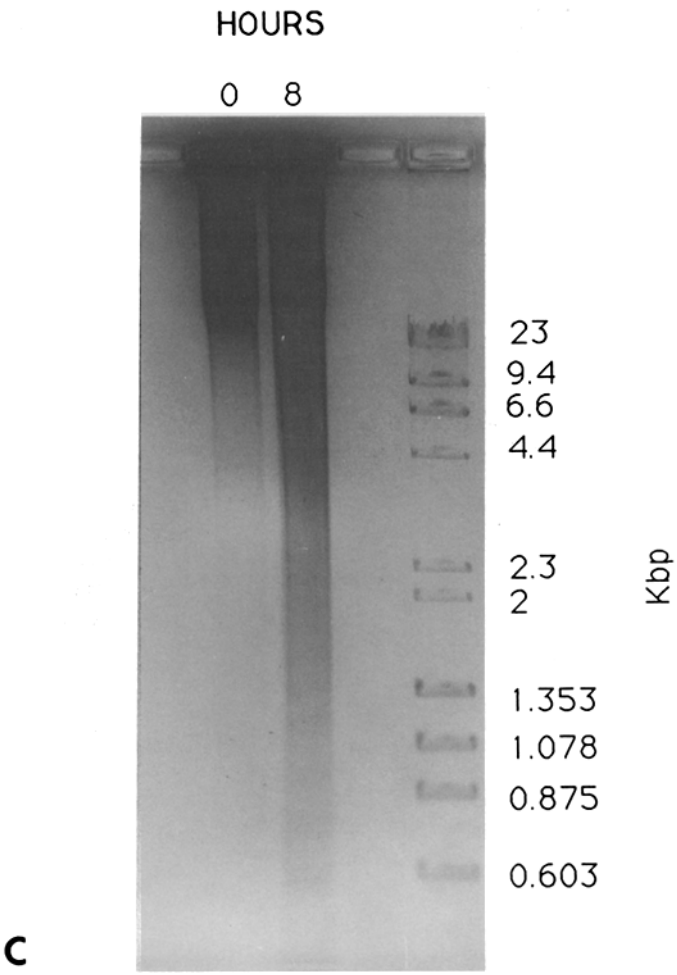
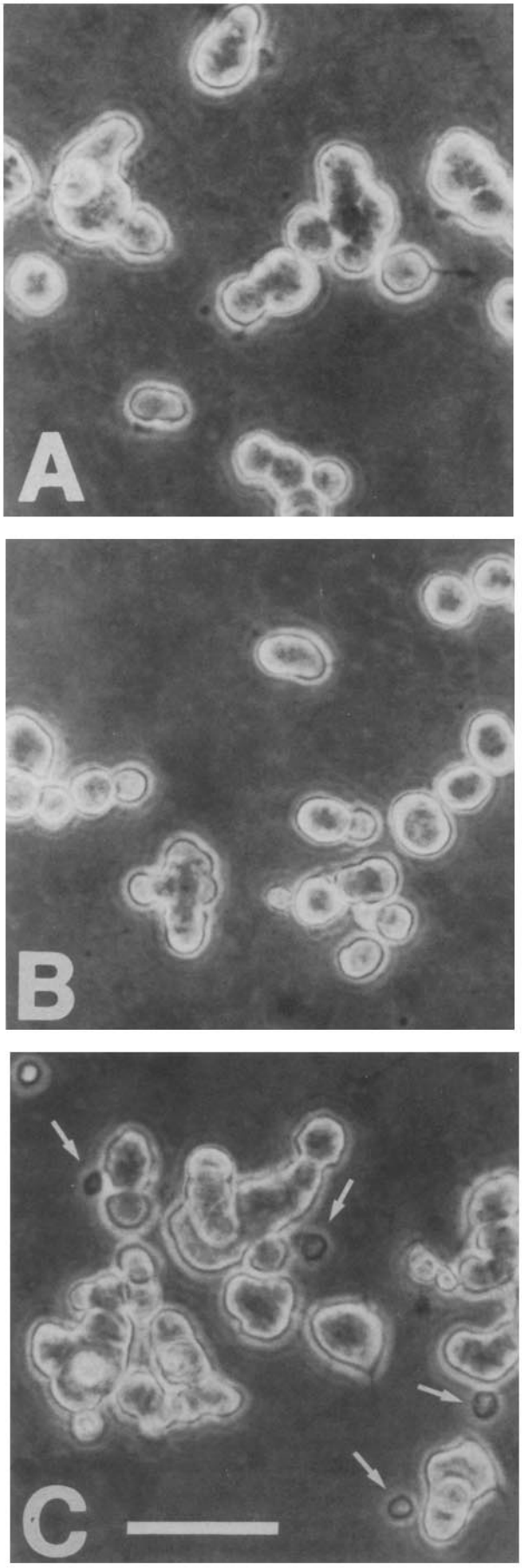


Figure 1. (*A*) Agarose gel electrophoresis of soluble DNA extracted from PC12 cells washed and cultured for 0, 1, 2, 3, 4, 5, 7.5, 10, or 23 h with RPMI 1640 medium without serum. 20 μg of nucleic acid per sample was subjected to electrophoresis on a 1.2% agarose gel. After RNase treatment the gel was stained with ethidium bromide and the DNA was visualized under UV light and photographed. The negative image of the stained gel is shown. (*B*) Prevention of internucleosomal cleavage of DNA by NGF and ATA. PC12 cells were washed and cultured with RPMI 1640 medium in the presence of either serum (lane *1*), no additives (lane *2*), 50 ng/ml NGF (lane *3*), or 100 μM ATA (lane *4*) for 6.5 h. PC12 cell-soluble DNA was subsequently extracted and analyzed by agarose gel electrophoresis as above. (*C*) Agarose gel electrophoresis of total cellular DNA from PC12 cells washed and cultured for 0 or 8 h in RPMI 1640 medium. Total PC12 cell DNA was extracted as described under Materials and Methods. 60 μg of DNA per sample were subjected to electrophoresis on a 1.2% agarose gel.



RPMI 1640 medium in the presence or absence of 100 μM ATA. After 18–22 h, NGF (50 ng/ml) was added for 6 h. The cultures were then washed with ice-cold-buffered saline and frozen on dry ice until assay. ODC activity was measured as previously described (Greene and McGuire, 1978). [1-^{14}C] L-ornithine (0.1 μCi/μl, ICN Radiochemicals Inc., Irvine, CA) was used at 0.1 μCi per assay. Assays were performed using 100–250 μg of protein/sample.

Results

Serum and NGF Deprivation Induce Internucleosomal Cleavage of PC12 Cell DNA

To obtain insight regarding the mechanism by which growth factor deprivation leads to cell death, PC12 cell DNA was isolated at various times after serum deprivation and then analyzed by agarose gel electrophoresis. As shown in Fig. 1 *A*, in the case of serum-deprived, but not of serum-treated PC12 cells, the soluble DNA fraction contains fragments whose molecular weights are multiples of ~180 bp, the length of a nucleosome subunit. Comparable observations were made with samples of soluble DNA that were treated with RNase before quantification and analysis (data not shown) as well as with total cellular DNA (Fig. 1 *C*). The "ladder-like" pattern of DNA degradation is evident as early as 3 h after serum deprivation. However, at this time there is no overt morphological evidence of cell death as seen by phase-contrast microscopy (Fig. 2 *B*). Membrane fragments and debris that reflect cell death appear in the culture medium only after 6–7 h of serum deprivation (Fig. 2 *C*) and become prominent by 24 h, when >50% of the cells are dead and disintegrating. In the above experiments we used PC12 cells that were not previously exposed to NGF. Past work established that process-bearing, NGF-pretreated ("primed") PC12 cells also die when deprived of NGF and serum and can be rescued by NGF (Rukenstein et al., 1991). The same DNA fragmentation is apparent in primed PC12 cells switched to serum free medium without NGF (data not shown).

NGF Prevents PC12 Cell DNA Fragmentation

Previous studies have shown that addition of NGF to serum-free medium rescues PC12 cells from death (Greene, 1978). To determine whether in addition to preventing death, NGF also inhibits the fragmentation of DNA, PC12 cells were incubated for ~6 h in RPMI 1640 medium in the presence of either serum, NGF, or no additives. As shown in Fig. 1 *B*, NGF, as well as serum, prevents the internucleosomal cleavage of PC12 cell DNA.

ATA Prevents PC12 Cell DNA Fragmentation

Past work has shown the ladder-like pattern of DNA degradation in several cell types undergoing cell death, and the induction of endogenous endonuclease activity has been correlated with apoptosis (Wyllie, 1980; McConkey et al.,

Figure 2. Phase-contrast micrographs of PC12 cells washed and maintained in RPMI 1640 medium in: (*A*) the presence of serum for 24 h; (*B*) the absence of serum for 3 h; (*C*) the absence of serum for 10 h. Arrows show degenerating cells. Bar, 50 μm.

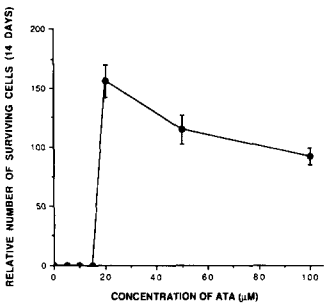


Figure 3. Dose-response relationship for the effects of ATA on 2-wk survival of PC12 cells in serum-free medium. Cells were washed and plated in RPMI 1640 medium in the presence of various concentrations of ATA. After 2 wk, viable cells were counted as described in Materials and Methods. The number of surviving cells is presented relative to the number of cells initially plated (8.8×10^4; designated as 100). Error bars represent SEM ($n = 3$).

1989; Cohen and Duke, 1984). The triphenylmethane dye ATA, which is a general inhibitor of nucleases in vitro (Hallick et al., 1977), has been shown to inhibit glucocorticoid-induced thymocyte DNA fragmentation and cell death (McConkey et al., 1989). To test whether ATA would inhibit fragmentation of PC12 cell DNA after serum deprivation, cultures were incubated for ~6 h in RPMI 1640 medium with the presence of either serum, 100 μM ATA, or no additives. As shown in Fig. 1 *B*, ATA effectively prevents the fragmentation of PC12 cell DNA.

ATA Promotes Long-term Serum-free Survival of PC12 Cells

Since ATA mimics the effects of serum and NGF on DNA fragmentation, we sought to determine whether, like trophic factors, it can prevent the death of PC12 cells in serum-free medium. As shown in Figs. 3 and 4, ATA promotes the long-term (2 week) survival of PC12 cells in serum-free medium at concentrations between 20–100 μM. The minimum effective concentration varies between 10–20 μM in different experiments. There is a sharp concentration dependence, which may be due to the need for achievement of threshold levels. We noticed that ATA-treated PC12 cells lose their adherence to the collagen substratum after several days. Increased concentrations of collagen led to better cell attachment, but somewhat higher concentrations of ATA were needed to maintain survival in serum-free medium. This suggests that ATA binds to the collagen substratum and that this affects its free concentration. For these reasons most experiments were performed with cells grown in suspension on noncoated plastic culture dishes.

Fig. 5 shows a time course for cell survival versus time. The ATA-promoted survival of PC12 cells was consistently accompanied by an increase in cell number. There was no evidence for cell death in the cultures during the first week (i.e., there was no debris, as observed in serum-free cultures maintained with no additives).

In the above experiments we used PC12 cells that were not previously exposed to NGF. As shown in Fig. 4, ATA, unlike NGF, does not cause morphological differentiation of PC12 cells. ATA was also able to rescue "primed" cells from serum-free death, but, in contrast to NGF, did not cause regeneration of neurites from passaged cells (data not shown).

Role of RNA and Protein Synthesis in the Effect of ATA on PC12 Cell Survival in Serum-free Medium

To determine whether RNA or protein synthesis are required for ATA to promote survival of PC12 cells in serum-free medium, we used actinomycin-D and camptothecin at concentrations previously shown to inhibit PC12 cell RNA-synthesis by 95% and by 85%, respectively (Burstein and Greene, 1978), and anisomycin at a concentration that inhibits PC12 cell protein synthesis by >99% (Greenberg et al., 1986). PC12 cells were preincubated in serum-free medium in the presence of either of the inhibitors for 1.5 h and then ATA was added to the cultures. After 16–20 h, viable PC12 cells were counted as described under Materials and Methods. As shown in Fig. 6, in the presence of ATA and macromolecular synthesis inhibitors the numbers of surviving cells are ~3–10-fold higher than those in RPMI 1640 with the inhibitors alone. Thus, ATA is capable of promoting PC12 cell survival in serum-free medium, even in the absence of macromolecular synthesis. The partial degree of cell death that occurred in presence of ATA appears due to toxic effects of the inhibitors at the concentrations used to effectively block synthesis (Rukenstein et al., 1991).

Effects of ATA on NGF-induced Responses

Previous in vitro studies have shown that in addition to being a general inhibitor of nucleases, ATA can inhibit other enzymes as well (Bina-Stein and Tritton, 1976). Moreover, it has been shown that in cell lysates ATA can interfere with protein synthesis (Blumenthal and Landers, 1973; Kulkarni et al., 1987; Mathews, 1971). As a partial test to whether ATA has generalized nonspecific effects on PC12 cells, we assessed its ability to affect several NGF-induced responses. Among the early transcription-dependent responses of PC12 cells to NGF is the induction of c-*fos* mRNA (Greenberg et al., 1985). As shown in Fig. 7, ATA pretreatment in serum-free medium does not abolish the increase of c-*fos* mRNA that occurs when NGF is added to the cultures. Moreover, ATA does not interfere with subsequent down regulation of c-*fos* mRNA since the c-*fos* message is undetectable 4 h after NGF addition to the ATA-treated cultures (data not shown). Comparable results were obtained when cells were pretreated for 7 d with ATA in serum-free medium. Although direct quantitative comparison with ATA-free controls could not be carried out due to the cell death that occurs without the drug, the levels of c-*fos* mRNA induced by NGF in serum-free medium plus ATA are similar to those induced in serum-containing medium (Greenberg et al., 1985).

Another effect of NGF on PC12 cells is the transcription-dependent induction of ODC activity (Greene and McGuire, 1978). Fig. 8 shows that overnight pretreatment with ATA does not abolish the induction of ODC activity by NGF. The level of ODC activity stimulated by NGF in the presence of ATA compares favorably with those achieved with NGF in serum-containing cultures (Greene and McGuire, 1978).

NGF induces morphological differentiation of PC12 cells in the absence as well as presence of serum (Greene, 1978). Previous studies have shown that NGF-induced neurite outgrowth is a complex process that requires both transcription-dependent and -independent actions of NGF (Greene, 1984). To test whether ATA affects NGF-induced neurite outgrowth, PC12 cells were plated in serum-free medium on

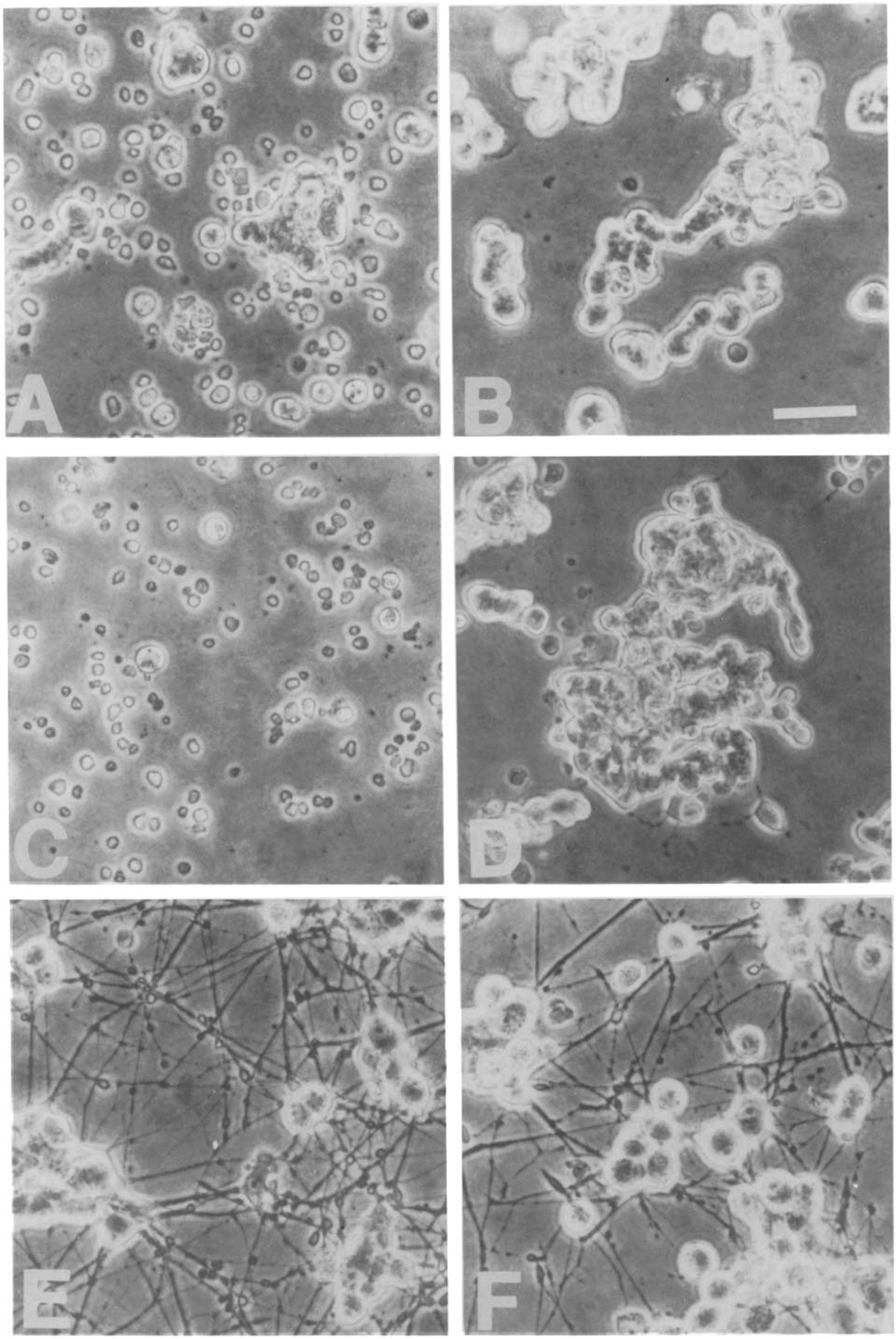


Figure 4. Phase-contrast micrographs of PC12 cells washed and maintained on collagen-coated (*A–D*) or on matrigel-coated dishes (*E* and *F*). Cells were grown in RPMI 1640 medium with: (*A*) no additives for 3 d; (*B*) 100 μM ATA for 3 d; (*C*) no additives for 7 d; (*D*) 30 μM ATA for 7 d; (*E*) 50 ng/ml NGF for 7 d; (*F*) 50 ng/ml NGF and 20 μM ATA for 7 d. Bar, 50 μm.

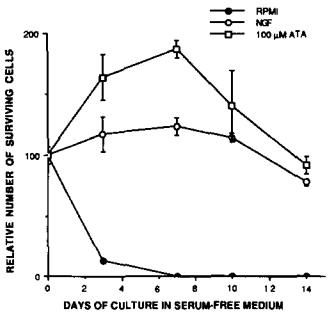


Figure 5. Effect of ATA on long-term survival of PC12 cells in serum-free medium. Cells were washed and plated in RPMI 1640 medium in the presence of either no additives, 50 ng/ml NGF, or 100 μM ATA. The cultures were fed every 3–4 d by addition of 0.25 ml of the same medium. Viable cells, in triplicate wells, were counted every 3–4 d as described in Materials and Methods. The numbers of surviving cells are presented relative to the number of cells initially plated (8.8×10^4; designated as 100). Error bars represent SEM ($n = 3$).

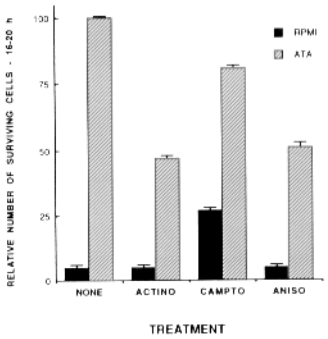


Figure 6. Effects of RNA and protein synthesis inhibitors on ATA-induced survival of PC12 cells in serum-free medium. Cells were washed and plated in RPMI 1640 medium and pretreated with no additives (*NONE*), 10 μM actinomycin D (*ACTINO*), 20 μM camptothecin (*CAMPTO*), or 100 μM anisomycin (*ANISO*). After 1.5 h, 30 μM ATA was added to the cultures where indicated. Surviving cells were counted after 16–20 h. The numbers of surviving cells are expressed relative to the number of cells present with ATA alone. Error bars represent SEM ($n = 3$). Comparable results were obtained in two independent experiments.

collagen- or matrigel-coated dishes in the presence of NGF and ATA. As illustrated in Fig. 4 *F*, ATA does not inhibit NGF-promoted neurite outgrowth. Under these conditions, ATA without NGF promotes survival, but does not stimulate the formation of neurites.

ATA Promotes Survival of Cultured Rat Neonatal Sympathetic Neurons

Sympathetic neurons require NGF for survival when cultured in vitro irrespective of the presence of serum (Levi-Montalcini and Angeletti, 1963; Martin et al., 1988). Since the PC12 cell line has many properties in common with sympathetic neurons, we tested whether ATA could promote survival of sympathetic neurons in the absence of NGF. Neonatal (P2-3) rat superior cervical ganglionic neurons were cultured for 3 d in the presence of NGF, then washed extensively to remove NGF and maintained in the presence of 99% RPMI 1640, 1% horse serum, and various concentrations of ATA. As shown at Fig. 9, cell counts performed 2 d later reveal that ATA promotes NGF-free survival of cultured neurons. The numbers of surviving neurons were 60–80% of those rescued by NGF. The effective ATA concentrations were somewhat higher than those observed with PC12 cells, but this could reflect the presence of the collagen coating on the bottom of the culture dishes and the presence of serum, both of which could affect the free concentration of the drug. By 3 d of treatment, although the ATA-exposed neurons were still clearly alive (Fig. 10 *C*), they began to form large clumps and accurate quantitation of cell numbers was no longer possible. However, under the same conditions ATA was able to promote NGF-free survival of sympathetic neurons for at least 2 wk (Fig. 10, *E* and *F*). As seen with

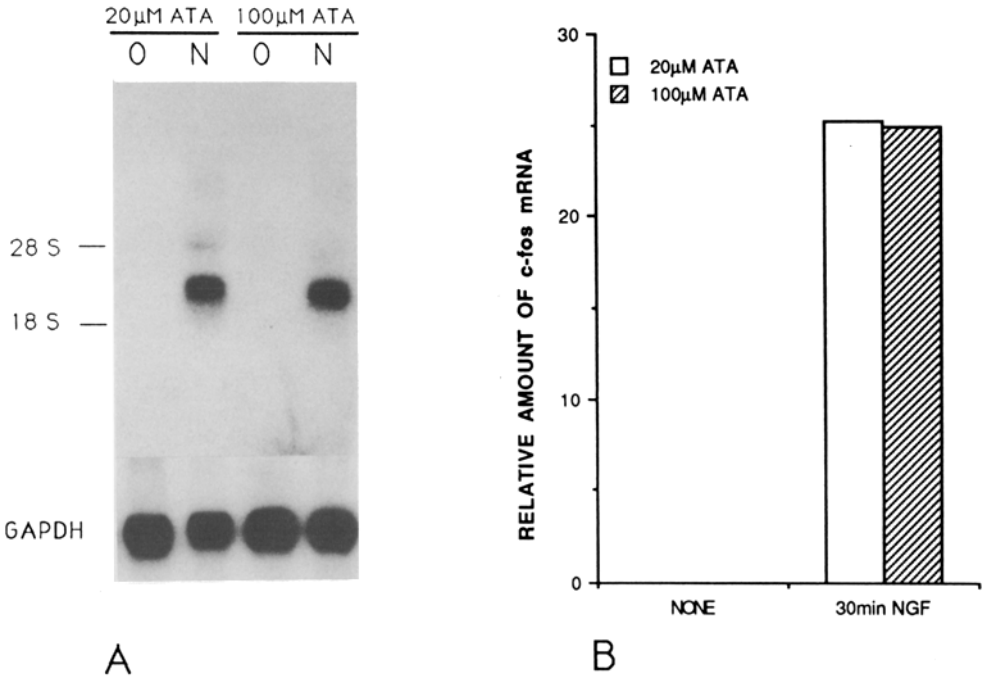


Figure 7. Effect of ATA on the NGF-induced increase of c-*fos* mRNA. (*A*) Cells were washed and grown on 100-mm collagen-coated dishes in RPMI 1640 medium in the presence of 20 or 100 μM ATA. After 16 h of preincubation, NGF was added to the indicated cultures for 30 min and the cells were used for isolation of total RNA. 15 μg of total RNA per sample were separated on a 1% agarose-formaldehyde gel and transferred to a nitrocellulose membrane. The Northern blot analysis of c-*fos* and GAPDH mRNA was performed as described under Materials and Methods. (*B*) The results are presented as the ratio of the c-*fos* signal (as determined by quantitative densitometry performed on autoradiograms) versus the nonregulated GAPDH signal. It should be noted that in control cultures in which the cells were grown in RPMI 1640 medium without additives, many cells were dying by 16 h. The responses of these cells to added NGF were not similar to those evoked from healthy cells and thus are not presented here.

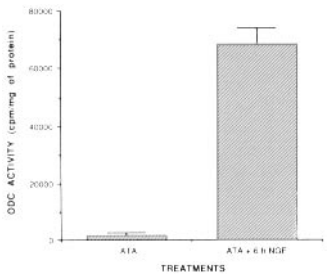


Figure 8. Effect of ATA on NGF-induced ODC activity. PC12 cells were washed and plated on collagen-coated 35-mm culture dishes in RPMI 1640 medium in the presence of 100 μM ATA. After 16 h of preincubation, NGF was added to the indicated cultures for 6 h. Error bars represent SEM (n = 3 replicate cultures). In control cultures without ATA, as noted in the legend of Fig. 7, the cells were dying at the time that NGF was added, thus, the results are not presented here.

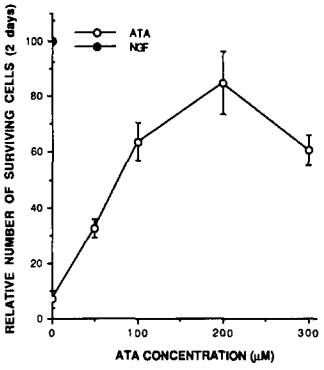


Figure 9. Dose-response relationship for the effects of ATA on 2-d survival of sympathetic neurons cultured in the absence of NGF. Primary cultures of superior cervical ganglionic neurons from postnatal-day-2 rats were established as described in Materials and Methods. After 3 d the cultures were washed and maintained in RPMI 1640 medium containing 1% horse serum (0.5 ml/well) in the presence of no additives, NGF, or ATA. After 2 additional days the living neurons were counted and the resultant values are presented as means ± SEM (n = 3 independent cultures). The results are presented relative to the cell number in NGF-treated cultures (designated as 100).

neuronally differentiated PC12 cells, ATA did not maintain the neurite network and the processes disintegrated, while the cell bodies remained phase-bright and viable (Fig. 10). This loss of neurites, as well as decreased adhesion, may contribute to the formation of the large neuronal clumps in the ATA-treated cultures. Readdition of NGF to such cultures resulted in the reappearance of healthy neurites (data not shown).

Discussion

The purpose of this study has been to explore the mechanisms by which neurotrophic factors promote survival and prevent cell death. Primarily, we have used serum-free cultures of PC12 cells as a model system. Previous studies have indicated that this system may be useful for understanding the neuronal cell death that occurs after growth factor deprivation (Rukenstein et al., 1991).

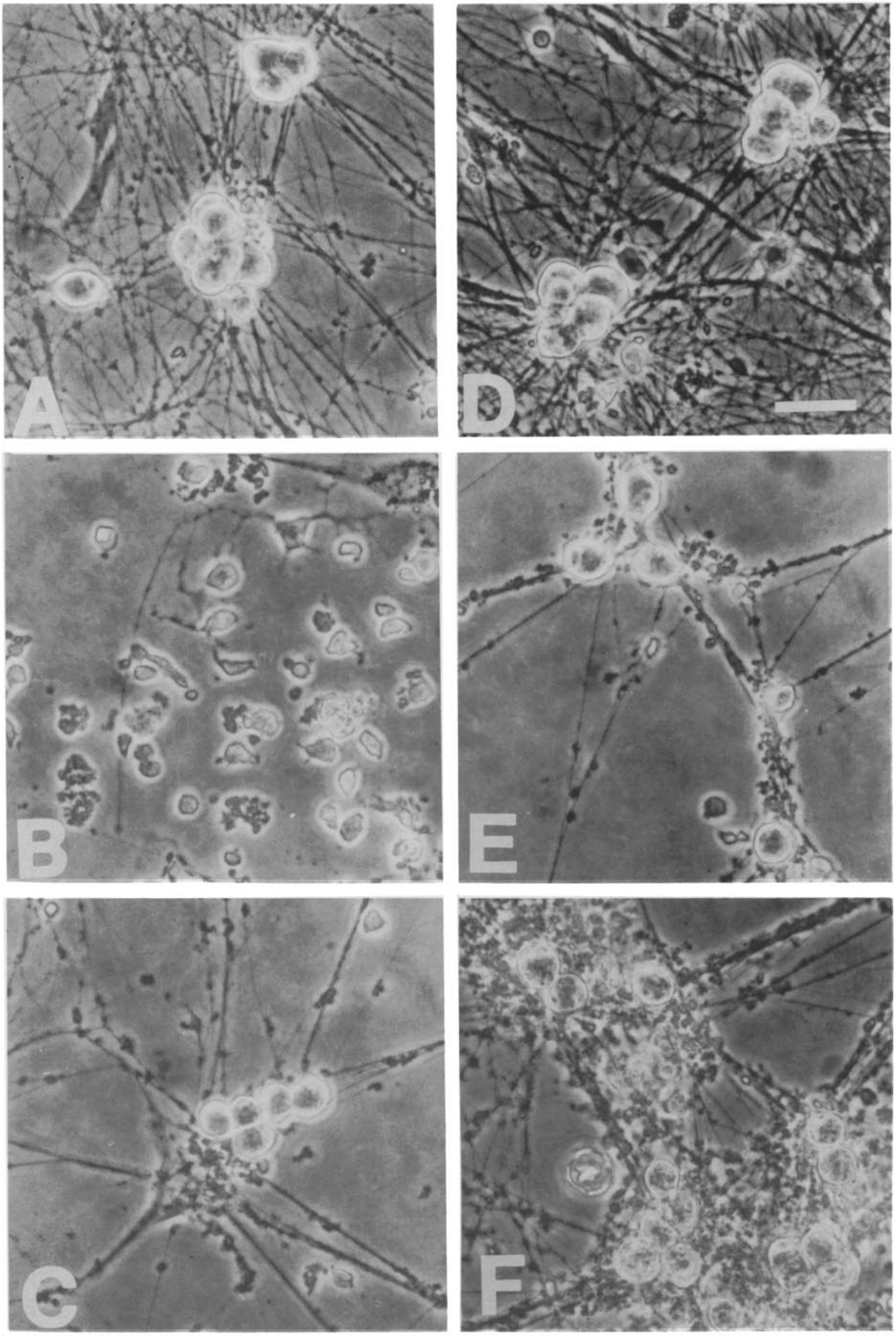
Past work has shown that a common morphological motif during naturally occurring neuronal death resembles a form of cell death called "apoptosis" (Wyllie et al., 1980). The characteristic ultrastructural changes of cells undergoing apoptosis (dense chromatin aggregates, fragmentation of the nucleolus, conservation of mitochondria, and sparing of the membranes at early stages) have also been described in SCG neurons in vivo, during naturally occurring death or after anti-NGF treatment (Levi-Montalcini et al., 1969; Wright et al., 1983). In vitro, after NGF deprivation by treatment with polyclonal anti-NGF antiserum, a percentage of superior cervical ganglionic neurons also show this type of cell death (Martin et al., 1988). The mechanism of apoptotic cell death has not been fully elucidated, but closely associated with this phenomenon is a characteristic pattern of DNA fragmentation that appears to be due to activation of endogenous endonuclease activity (Wyllie, 1980; McConkey et al., 1989; Cohen and Duke, 1984). The low molecular weight DNA of apoptotic cells consists of fragments whose molecular weights are multiples of about 180 base-pairs (the length of a nucleosome). It is reported here that PC12 cells cultured in serum-free medium without additives exhibit the characteristic pattern of DNA-fragmentation associated with apoptosis. Moreover, this pattern appears as early as 3 h after serum deprivation, before any overt morphological signs of cell death. NGF and serum which rescue the cells, also inhibit the DNA fragmentation.

Several points indicate that the observation of fragmented DNA only in unsupplemented, serum-free cultures is unlikely to be an artifact caused by analysis of unequal amounts of DNA. The same results were obtained with preparations of soluble DNA irrespective of RNase pretreatment, as well as with preparations of total cellular DNA. Furthermore, at early times of serum deprivation (0–10 h) when there was little or no cell death, the recovery of total soluble DNA/culture, and therefore per cell, was nearly the same (both with and without RNase pretreatment). Thus, electrophoretic analysis in such cases was essentially normalized for cell number as well as for amount of DNA.

The conclusion drawn from these data is that an endonuclease activity is correlated with PC12 cell death after serum withdrawal and growth factor deprivation. The question arises, however, whether this endonuclease activity causes cell death or whether it is merely a consequence of the process of cell degeneration. If the former is true then inhibition of the responsible endonuclease should promote survival of PC12 cells in serum-free medium. No specific inhibitor for the endonuclease has been described. However, in glucocorticoid- and Ca^{2+}-ionophore–induced thymocyte apoptosis, aurintricarboxylic acid, a general in vitro inhibitor of nucleases (Hallick et al., 1977), has been reported to suppress endogenous endonuclease activity and to promote cell survival (McConkey et al., 1989). We found here that ATA inhibited the fragmentation of PC12 cell DNA when added to serum-free cultures and promoted cell survival in a manner independent of macromolecular synthesis.

In vitro studies with ATA have shown that in addition to inhibiting nucleases (Hallick et al., 1977), this compound

Figure 10. Phase-contrast micrographs of sympathetic neurons washed and maintained in RPMI 1640 culture medium containing 1% horse serum and (*A*) 50 ng/ml NGF for 3 d; (*B*) no additives for 3 d; (*C*) 100 μM ATA for 3 d; (*D*) 50 ng/ml NGF for 7 d; (*E*) 50 μM ATA for 7 d; (*F*) 200 μM ATA for 14 d.


A
D
B
E
C
F

can suppress at least several additional enzymatic activities (Bina-Stein and Tritton, 1976) and can interfere with protein synthesis in cell-free systems (Blumenthal and Landers, 1973; Kulkarni et al., 1987; Mathews, 1971). This raises the possibility that ATA prevents cell death by mechanisms other than inhibiting nucleases. While this cannot be ruled out at present, we did not observe generalized inhibitory effects of ATA on PC12 cells as judged by several criteria. This compound permitted a degree of cell replication and did not appear to interfere with several different NGF responses, i.e., the transient regulation of c-*fos* mRNA; the induction of ODC activity, a transcription- and translation-requiring action (Greene and McGuire, 1978, Greenberg et al., 1985); and the promotion of neurite outgrowth (a complex action with both transcription-dependent and -independent actions of NGF). Moreover, although ATA mimicked the effect of NGF on survival, it did not promote neuronal differentiation. The attractive possibility, therefore, remains that ATA promotes PC12 cell survival by inhibiting an endonuclease activity which in turn leads to cell death. The observations that NGF and serum inhibit the DNA fragmentation are also consistent with the possibility that other growth factors prevent cell death by inhibiting an endogenous endonuclease activity.

Previous studies have shown that macromolecular synthesis is not required for NGF-promoted survival of serum-deprived PC12 cells (Rukenstein et al., 1991). Moreover, macromolecular synthesis inhibitors do not prolong survival of PC12 cells in serum-free medium (Rukenstein et al., 1991). It is thus possible that NGF inhibits the fragmentation of PC12 cell DNA by leading to posttranslational modification, and thus inactivation of an endogenous endonuclease. Alternatively, NGF may affect the chromatin and render it less susceptible to endonucleases. Phosphorylation could be the key step for these actions of NGF. Past studies have documented the ability of NGF to affect phosphorylation of intracellular substrates, including several nuclear proteins (Halegoua and Patrick, 1980; Yu et al., 1980).

We observed that ATA also promotes long-term survival of sympathetic neurons in NGF-deprived cultures. This is consistent with the possibility that the same mechanism is responsible for death in both cell systems. Although the issue of cell numbers available for experimentation has thus far precluded our carrying out parallel studies with neurons, it is possible that the same type of endonuclease is activated in NGF-deprived sympathetic neurons as in PC12 cells. It has been reported that inhibition of macromolecular synthesis prolongs NGF-free survival of cultured sympathetic neurons (Martin et al., 1988). One interpretation that has been offered for this observation is that cell death requires *de novo* synthesis of specific "death" proteins required for cell suicide. An alternative explanation that has been suggested for apoptosis in thymocytes (McConkey et al., 1990a), which could also pertain to neurons, is that cell death is due to a constitutively expressed, but trophic-factor-regulated, endonuclease that rapidly turns over and is hence subject to rapid depletion when macromolecular synthesis is blocked.

With both PC12 cells and sympathetic neurons, ATA promotes survival but is unable to initiate or maintain neurite outgrowth. This supports prior studies with these two systems that indicated a distinction between the final pathways by which NGF prevents cell death and stimulates neurite outgrowth (Greene, 1978; Greene et al., 1990; Rukenstein et al., 1991).

In summary, we have shown that an endonuclease activity that results in internucleosomal cleavage of DNA is correlated with PC12 cell death after NGF and serum deprivation. ATA inhibits the DNA fragmentation and promotes long-term survival of the cells. In addition it rescues sympathetic neurons from death after NGF deprivation. These findings provide the first evidence for an "apoptotic" mechanism of cell death in neuronal cells.

We thank Dr. A. Rukenstein for aid with cell culture.

This work was supported by grants from the National Institutes of Health–National Institute of Neurological Diseases and Stroke. A. Batistatou was supported in part by a Frederic E. G. Valergakis Graduate Research Grant.

Received for publication 8 April 1991 and in revised form 2 July 1991.

References

Barde, Y. A. 1988. What, if anything, is a neurotrophic factor? *Trends Neurosci.* 11:343–351.

Bina-Stein, M., and T. R. Tritton. 1976. Aurintricarboxylic acid is a nonspecific enzyme inhibitor. *Mol. Pharmacol.* 12:191–193.

Blumenthal, T., and T. A. Landers. 1973. The inhibition of nucleic acid-binding proteins by aurintricarboxylic acid. *Biochem. Biophys. Res. Commun.* 55:680–688.

Burstein, D. E., and L. A. Greene. 1978. Evidence for both RNA-synthesis-dependent and -independent pathways in stimulation of neurite outgrowth by nerve growth factor. *Proc. Natl. Acad. Sci. USA.* 76:6059–6063.

Chomczynski, P., and N. Sacchi. 1987. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156–159.

Chu-Wang, I. W., and R. W. Oppenheim. 1978. Cell death of motoneurons in the chick embryo spinal cord. *J. Comp. Neurol.* 177:33–58.

Cohen, J. J., and R. C. Duke. 1984. Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. *J. Immunol.* 132:38–42.

Curran, T., G. Peters, C. VanBeveren, N. M. Teich, and I. M. Verma. 1982. FBJ murine osteosarcoma virus: identification and molecular cloning of biologically active proviral DNA. *J. Virol.* 44:674–682.

Duvall, E., and A. H. Wyllie. 1986. Death and the cell. *Immunol. Today.* 7:115–119.

Gorin, P. D., and E. M. Johnson, Jr. 1979. Experimental autoimmune model of nerve growth factor deprivation: effects on developing peripheral sympathetic and sensory neurons. *Proc. Natl. Acad. Sci. USA.* 76:5382–5386.

Greenberg, M. E., L. A. Greene, and E. B. Ziff. 1985. Nerve growth factor- and epidermal growth factor-induced rapid transient changes in protooncogene transcription in PC12 cells. *J. Biol. Chem.* 260:14101–14110.

Greenberg, M. E., A. L. Hermanowski, and E. B. Ziff. 1986. Effect of protein synthesis inhibitors on growth factor activation of c-*fos*, c-*myc* and actin gene transcription. *Mol. Cell. Biol.* 6:1050–1057.

Greene, L. A. 1978. Nerve growth factor prevents the death and stimulates neuronal differentiation of clonal PC12 pheochromocytoma cells in serum-free medium. *J. Cell Biol.* 78:747–755.

Greene, L. A. 1984. The importance of both early and delayed responses in the biological actions of nerve growth factor. *Trends Neurosci.* 7:91–94.

Greene, L. A., and J. C. McGuire. 1978. Induction of ornithine decarboxylase by nerve growth factor dissociated from effects on survival and neurite outgrowth. *Nature (Lond.).* 276:191–194.

Greene, L. A., and A. S. Tischler. 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. USA.* 73:2424–2428.

Greene, L. A., and A. S. Tischler. 1982. PC12 pheochromocytoma cells in neurobiological research. *Adv. Cell. Neurobiol.* 3:373–414.

Greene, L. A., C. Volonté, and A. Chalazonitis. 1990. Purine analogues inhibit nerve growth factor-promoted neurite outgrowth by sympathetic and sensory neurons. *J. Neurosci.* 10:1479–1485.

Halegoua, S., and J. Patrick. 1980. Nerve growth factor mediates phosphorylation of specific proteins. *Cell.* 22:571–581.

Hallick, R. B., B. K. Chelm, P. W. Gray, and E. M. Orozco, Jr. 1977. Use of aurintricarboxylic acid as an inhibitor of nucleases during nucleic acid isolation. *Nucleic Acids Res.* 4:3055–3064.

Hockenbery, D., G. Nunez, C. Milliman, R. D. Schreiber, and S. J. Korsmeyer. 1990. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature (Lond.).* 348:334–336.

Kulkarni, G. R., G. R. Kantharaj, C. Fluellen, B. G. Niranjan, and N. G. Avadhani. 1987. Stimulation of transcription and translation by aurintricarboxylic acid in mitochondrial lysates from Ehrlich ascites cells. *Biochem. Biophys. Res. Commun.* 145:1149–1157.

Lee, V. M., M. L. Shelanski, and L. A. Greene. 1980. Characterization of antisera raised against cultured rat sympathetic neurons. *Neuroscience.* 5:2239–2245.

Levi-Montalcini, R., and P. U. Angeletti. 1963. Essential role of the nerve growth factor on the survival and maintenance of dissociated sensory and sympathetic embryonic nerve cells in vitro. *Dev. Biol.* 7:653–659.

Levi-Montalcini, R., and P. U. Angeletti. 1966. Immunosympathectomy. *Pharmacol. Rev.* 18:619–628.

Levi-Montalcini, R., and P. U. Angeletti. 1968. Nerve growth factor. *Physiol. Rev.* 48:534–569.

Levi-Montalcini, R., and B. Booker. 1960. Destruction of sympathetic ganglia in mammals by an antiserum to nerve growth factor protein. *Proc. Natl. Acad. Sci. USA.* 46:384–391.

Levi-Montalcini, R., F. Caramia, and P. U. Angeletti. 1969. Alterations in the fine structure of nucleoli in sympathetic neurons following NGF-antiserum treatment. *Brain Res.* 12:54–73.

Lindenbaum, A., and J. Schubert. 1956. Binding of organic anions by serum albumin. *J. Phys. Chem.* 60:1663–1665.

Maniatis, T. M., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 545 pp.

Martin, D. P., R. E. Schmidt, P. S. DiStefano, O. H. Lowry, J. G. Carter, and E. M. Johnson, Jr. 1988. Inhibitors of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation. *J. Cell Biol.* 106:829–844.

Mathews, M. B. 1971. Mammalian chain initiation: the effect of aurintricarboxylic acid. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 15:201–204.

McConkey, D. J., P. Hartzell, P. Nicotera, and S. Orrenius. 1989. Calcium-activated DNA fragmentation kills immature thymocytes. *FASEB J.* 3:1843–1849.

McConkey, D. J., P. Hartzell, and S. Orrenius. 1990*a*. Rapid turnover of endogenous endonuclease activity in thymocytes: effects of inhibitors of macromolecular synthesis. *Arch. Biochem. Biophys.* 278:284–287.

McConkey, D. J., S. Orrenius, and M. Jondal. 1990*b*. Cellular signalling in programmed cell death (apoptosis). *Immunol. Today.* 11:120–121.

Mobley, W. C., A. Schenker, and E. M. Shooter. 1976. Characterization and isolation of proteolytically modified nerve growth factor. *Biochemistry.* 15:5543–5551.

Oppenheim, R. W. 1989. The neurotrophic theory and naturally occurring motoneuron death. *Trends Neurosci.* 12:252–255.

Oppenheim, R. W. 1991. Cell death during development of the nervous system. *Annu. Rev. Neurosci.* 14:453–501.

Piechaczyk, M., J. M. Blanchard, L. Marty, C. Dani, F. Panabieres, S. E. Sabouty, P. H. Fort, and P. H. Jeanteur. 1984. Post-transcriptional regulation of glyceraldehyde-3-phosphate dehydrogenase gene expression in rat tissues. *Nucleic Acids Res.* 12:6951–6963.

Pilar, G., and L. Landmesser. 1976. Ultrastructural differences during embryonic cell death in normal and peripherally deprived ciliary ganglia. *J. Cell Biol.* 68:339–356.

Rukenstein, A., R. E. Rydel, and L. A. Greene. 1991. Multiple agents rescue PC12 cells from serum-free cell death by translation- and transcription-independent mechanisms. *J. Neurosci.* 11:2552–2563.

Soto, A. M., and C. Sonnenschein. 1985. The role of estrogens on the proliferation of human breast tumor cells (MCF-7). *J. Steroid Biochem.* 23:87–94.

Wright, L. L., T. J. Cunningham, and A. J. Smolen. 1983. Developmental neuron death in the rat superior cervical sympathetic ganglion: cell counts and ultrastructure. *J. Neurocytol.* 12:727–738.

Wyllie, A. H. 1980. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature (Lond.).* 284:555–556.

Wyllie, A. H., J. F. R. Kerr, and A. R. Currie. 1980. Cell death: the significance of apoptosis. *Int. Rev. Cytol.* 68:251–306.

Yu, M. W., N. W. Tolson, and G. Guroff. 1980. Increased phosphorylation of specific nuclear proteins in superior cervical ganglia and PC12 cells in response to nerve growth factor. *J. Biol. Chem.* 255:10481–10492.