Nerve Growth Factor Withdrawal-induced Cell Death in Neuronal PC12 Cells Resembles That in Sympathetic Neurons

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Abstract. Previous studies have shown that in neuronal cells the developmental phenomenon of programmed cell death is an active process, requiring synthesis of both RNA and protein. This presumably reflects a requirement for novel gene products to effect cell death. It is shown here that the death of nerve growth factor–deprived neuronal PC12 cells occurs at the same rate as that of rat sympathetic neurons and, like rat sympathetic neurons, involves new transcription and translation. In nerve growth factor–deprived neuronal PC12 cells, a decline in metabolic activity, assessed by uptake of [^{3}H]2-deoxyglucose, precedes the decline in cell number, assessed by counts of trypan blue–excluding cells. Both declines are prevented by actinomycin D and anisomycin. In contrast, the death of nonneuronal (chromaffin-like) PC12 cells is not inhibited by transcription or translation inhibitors and thus does not require new protein synthesis. DNA fragmentation by internucleosomal cleavage does not appear to be a consistent or significant aspect of cell death in sympathetic neurons, neuronal PC12 cells, or nonneuronal PC12 cells, notwithstanding that the putative nuclease inhibitor aurintricarboxylic acid protects sympathetic neurons, as well as neuronal and nonneuronal PC12 cells, from death induced by trophic factor removal. Both phenotypic classes of PC12 cells respond to aurintricarboxylic acid with similar dose–response characteristics. Our results indicate that programmed cell death in neuronal PC12 cells, but not in nonneuronal PC12 cells, resembles programmed cell death in sympathetic neurons in significant mechanistic aspects: time course, role of new protein synthesis, and lack of a significant degree of DNA fragmentation.

ATTAINMENT of proper cell number in each region of the organism is an essential aspect of development and morphogenesis. To accomplish this, not only is the rate of cell proliferation tightly regulated, but there is also a program of cell death which results in the elimination of specific subsets of cells (28, 43, 46). Programmed cell death (PCD)[1] provides a rapid and efficient method for achieving proper tissue mass and cell number as well as for eliminating supernumerary or dysfunctional cells. During neuronal development, PCD operates to ensure precise quantitative matching of presynaptic and postsynaptic pools of cells (28). Determination of neuronal survival appears to occur via an intercellular competition for a limited supply of target-derived neurotrophic support (28). Thus, an augmented supply of nerve growth factor (NGF) reduces in vivo death of sympathetic and sensory neurons while NGF deprivation, in vivo or in vitro, causes them to die (21). PC12 cells, grown in serum-containing medium, do not require NGF for their survival although treatment with NGF induces neuronal differentiation (11). In serum-free medium, NGF alone is sufficient for PC12 cell survival (10). This allows the neurotrophic effect of NGF to be revealed as subsequent removal of the NGF from the serum-free medium causes the PC12 cells to die.

PCD in a variety of systems appears to involve a requirement for new gene expression, evidenced by the ability of transcriptional or translational inhibitors to slow or block PCD (17, 22, 31, 34, 39, 41). Presumably, specific proteins, the function of which is to effect cell death, are induced under conditions which necessitate PCD. In particular, the death of sympathetic neurons that ensues from NGF withdrawal is dependent on protein synthesis: addition of translational or transcriptional inhibitors upon withdrawal of NGF significantly reduces the extent of cell death (23). Similarly, cell death in trophic factor–deprived motor neurons (29), parasympathetic neurons (36), and sensory neurons (36) also requires protein synthesis. Neurotoxin-induced neuronal death can also require protein synthesis (19).

Although cell death programs often share a requirement for activation of gene expression, there is evidence to support the conclusion that there is no universal mechanism for PCD. Rather, diverse mechanisms appear to account for cell death in different cell types. Even in a particular cell type, PCD may occur by different mechanisms when induced by

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1. *Abbreviations used in this paper*: 2DG, 2-deoxyglucose; AT, aurintricarboxylic acid; NGF, nerve growth factor; PCD, programmed cell death; SCG, superior cervical ganglion.

different means. For instance, ectopic expression of the PCD-inhibiting oncogene *bcl*-2 in immature thymocytes protects the cells from lymphotoxins but not from PCD-mediated elimination of autoreactive lymphocytes (38, 40).

Another illustration of the disparity among cell death mechanisms is the appearance of DNA fragmentation in most, but not all, examples of cell death. Wyllie (45) showed that the death of thymocytes due to glucocorticoid exposure is accompanied by fragmentation of cellular DNA into ~180-bp fragments and integer multiples thereof. Such cleavage, which presumably occurs in regions of "linker" DNA between nucleosomes, produces a characteristic "ladder" pattern of fragments on electrophoretic gels. This phenomenon has since been observed in a number of instances of PCD (20, 27, 32). Since inhibitors of RNA or protein synthesis also inhibit apoptotic DNA fragmentation (7, 19, 25, 47), an endonuclease may be among the novel gene products induced during apoptosis. PCD can require DNA fragmentation in some cases; this is further supported by the finding that glucocorticoid-induced thymocyte PCD is prevented by the putative nuclease inhibitor aurintricarboxylic acid (AT) (25). Schwartz (34) has indicated that nucleolytic degradation does not always occur but rather that PCD can occur by distinct nucleolytic (apoptotic) or proteolytic (autophagic) mechanisms. Masters et al. (24) have shown that glucocorticoid-induced cell death of cultured rat hippocampal cells does not involve DNA fragmentation, unlike glucocorticoid-induced PCD in thymocytes. On the other hand, nucleolytic cell death, inhibited by AT, has been observed during glutamate-induced PCD in both cultured rat cortical neurons and hippocampal cells in vivo (19) and in trophic factor–deprived PC12 cells (2).

The use of PC12 cells to investigate neuronal PCD holds much promise for future studies as with these cells it is possible to obtain large homogenous cell populations to facilitate molecular and biochemical analysis. In the present study we use the PC12 cell line and primary cultures of newborn rat superior cervical ganglion (SCG) neurons to further investigate the mechanisms of neuronal PCD. Our results support a conclusion that at least two different mechanisms can mediate cell death in trophic factor–deprived PC12 cells; the mechanism chosen depends on the differentiated state of the cells. Nonneuronal PC12 cells, which possess a chromaffin-like but transformed phenotype, die when deprived of trophic factor and do so by a mechanism that is independent of transcription and translation. In contrast, neuronal PC12 cells resemble sympathetic neurons in that PCD occurring upon withdrawal of NGF is inhibited by transcriptional or translational inhibitors. Although AT does inhibit PCD in sympathetic neurons, neuronal PC12 cells, and nonneuronal PC12 cells, DNA fragmentation is not detectable during PCD in any of these cells. With regard to PC12 cells, this finding is in contrast to the results referenced above (2). The present results reveal similarities in PCD between neuronal PC12 cells and sympathetic neurons establishing neuronal PC12 cells as a suitable model system for biochemical and molecular studies of neuronal PCD.

Materials and Methods

Materials

NGF was prepared from male mouse submaxillary glands essentially as described by Mobley et al. (26). Actinomycin, anisomycin, and AT were purchased from Sigma Chem. Co. (St. Louis, MO). Donor horse serum was purchased from JRH Biosciences (Lenexa, KS); all other media and reagents were from Gibco Laboratories (Grand Island, NY).

Cell Culture

PC12 cells (passages 29–38) were maintained as described in Greene et al. (13) in RPMI 1640 medium containing 5% FBS and 10% heat-inactivated donor horse serum + 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were cultured on plastic culture dishes coated with rat tail collagen prepared as described previously (13). For serum deprivation, cells were washed by 5× repeated centrifugation in serum-free RPMI 1640. Neuronal PC12 cell cultures were prepared by treatment with NGF at a concentration of 50 ng/ml for 14 d in RPMI 1640 medium containing 1% heat-inactivated donor horse serum. Cells from which NGF was withdrawn to initiate cell death were washed as above but, after the washes, rabbit polyclonal antimouse NGF (Sigma Chem. Co.) was added at a dilution of 1:1,000.

Cultures of neurons from newborn rat (Wistar-Kyoto) SCG were prepared essentially as described by Johnson and Argiro (15). Before their use in an experiment, the SCG cells were maintained for 7–10 d in RPMI 1640 medium containing 10% FBS, 5% heat-inactivated donor horse serum, and 50 ng/ml NGF. 5-Fluorodeoxyuridine (10 μM) was added to reduce the number of nonneuronal cells. Visual inspection of the cultures by phase-contrast microscopy revealed that by the time the cells were used for an experiment >95% of the cells were neurons.

Micrographs were taken with a Diaphot inverted microscope equipped with a 20× phase-contrast objective (Nikon Inc., Melville, NY). A Nikon model F 35-mm camera was fitted to the microscope and exposures were made using a green interference filter and a T-Max 100 film (Eastman Kodak Co., Rochester, NY).

Thymocyte cultures were prepared from neonatal rats and cultured as described in McConkey et al. (25).

Assay of [^{3}H]2-Deoxyglucose Uptake

PC12 cells were plated in equal numbers in 6-well plates in control or in experimental conditions for 6–72 h. The culture medium was removed and the cells were washed with PBS. The cells were then incubated for 2 h at 37° in 1 ml of PBS containing 1 μCi of [^{3}H]2-deoxyglucose (2DG, purchased from Amersham Corp., Arlington Heights, IL). After washing with PBS, the cells were scraped up in 0.5 ml PBS containing 1% Triton X-100 and 0.1% SDS. Scintillation fluid (Budget-Solve) was added and the lysate was assayed for tritium in a scintillation counter. The significance of differences in [^{3}H]2DG uptake among different cell populations was determined statistically using Student's *t* test.

Cell Counts

PC12 cells were plated in equal numbers in 6-well plates in control or in experimental conditions for 6–72 h. The culture medium was removed and reserved. The cells were removed from culture dishes by treatment with 0.1% trypsin in Ca^{+2}/Mg^{+2}-free PBS. After trypsinization, cells were gently triturated to separate clumps and the original culture medium was added back. After brief centrifugation (1,000 *g* for 5 min) to concentrate the cells, the supernatant fluid was removed and an equal volume of trypan blue solution (0.4% trypan blue, 0.81% NaCl, and 0.06% K_2HPO_4) was added. Triplicate samples were counted using a hemacytometer. The significance of differences in cell number among different cell populations was determined statistically using ANOVA or Student's *t* test.

DNA Preparation and Analysis

Cells were harvested by scraping and trituration in their original culture medium (to prevent loss of detached cells), and were collected by centrifugation (1,000 *g* for 5 min). Typically, SCG from 12 rat pups were used for a single determination of sympathetic neuronal DNA fragmentation (~600,000 neurons). For nondenaturing electrophoresis, total genomic DNA (5 μg/lane), prepared as described by Trauth et al. (42), was separated for 1.5 h at 100 V in 1% agarose gels containing 40 mM Tris-acetate, and 1 mM EDTA, pH 8.0 as running buffer. A high concentration of agarose was used to maximize resolution of potential low molecular weight fragments. DNA was depurinated, denatured, and neutralized by transferring for 3 min in each of the following solutions: 0.2 N HCl, 0.5 M NaOH/1.5 M NaCl, and 0.5 M Tris-Cl/3 M NaCl (pH 5.0). The DNA was transferred to ZetaProbe GT nylon (Bio-Rad Laboratories, Richmond, CA) and

cross-linked to the filters by baking at 80°C for 1 h. Filters were prehybridized overnight at 42°C in 25 mM potassium phosphate buffer (pH 7.4), 5× SSC, 5× Denhardt's, 50 mg/ml salmon sperm DNA, and 50% formamide. EcoRI- or HindIII-digested rat genomic DNA was used to make a [^{32}P]-dCTP-labeled probe using random hexamer primers (Bethesda Research Laboratories, Gaithersburg, MD) according to the manufacturer's protocol. The probe was added to the prehybridization buffer and allowed to hybridize overnight at 42°C. The filters were washed for 15 min in 2× SSC/0.1% SDS, 15 min in 0.5× SSC/0.1% SDS, 15 min in 0.1× SSC/0.1% SDS, and 30 min at 42°C in 0.1× SSC/1.0% SDS. Autoradiograms were made by overnight exposure to preflashed X-OMAT AR film (Eastman Kodak Co.) with dual Lightning Plus intensifying screens (Dupont Co., Wilmington, DE) at −86°C.

Denaturing ("alkaline") electrophoresis was performed essentially as described in Sambrook et al. (33). Alkaline gels (also 1% agarose) were run for 4–5 h at 40–50 V. Preparation of the DNA and its transfer to nylon filters was as described above except that 0.4 N NaOH was the sole transfer solution. Cross-linking, prehybridization/hybridization, washes, and autoradiography were all performed as described above.

Results

Nonneuronal PC12 Cells Die by Mechanisms That Are Transcription and Translation Independent

Cell Morphology. Serum deprivation has previously been shown to result in the rapid (24–48 h) death of nonneuronal PC12 cells (11). To assess the involvement of transcription and translation in this process, PC12 cell cultures were deprived of serum in the presence of the transcriptional inhibitor actinomycin D or the translational inhibitor anisomycin and cultured for various times up to 72 h. Assays of [^{35}S]methionine incorporation into TCA-precipitable material (data not shown) showed that actinomycin D at 3 μg/ml reduced PC12 cell protein synthesis to 2% of control levels and, at 1 μg/ml, to 6% of control levels. Anisomycin at 3 μg/ml reduced PC12 cell protein synthesis to 2% of control levels and, at 1 μg/ml, to 15% of control levels.

After 48 h, cultures containing serum (Fig. 1 *A*) exhibited attached cells, normal in appearance, with phase-bright margins and little debris. Within 12 h of serum deprivation, cells began to lose their phase-bright appearance and debris began to appear in the dish. After 48 h serum-derived cultures (Fig. 1 *B*) consisted entirely of floating debris with few surviving cells. Visual inspection of serum-free cultures treated with either anisomycin or actinomycin (Fig. 1, *C* and *D*), both at 3 μg/ml, revealed that the inhibition of translation or transcription (respectively) provided no protective effect. Treatment with 3 μg/ml anisomycin or 3 μg/ml actinomycin for up to 12 h before serum deprivation also did not delay or inhibit loss of viability (data not shown).

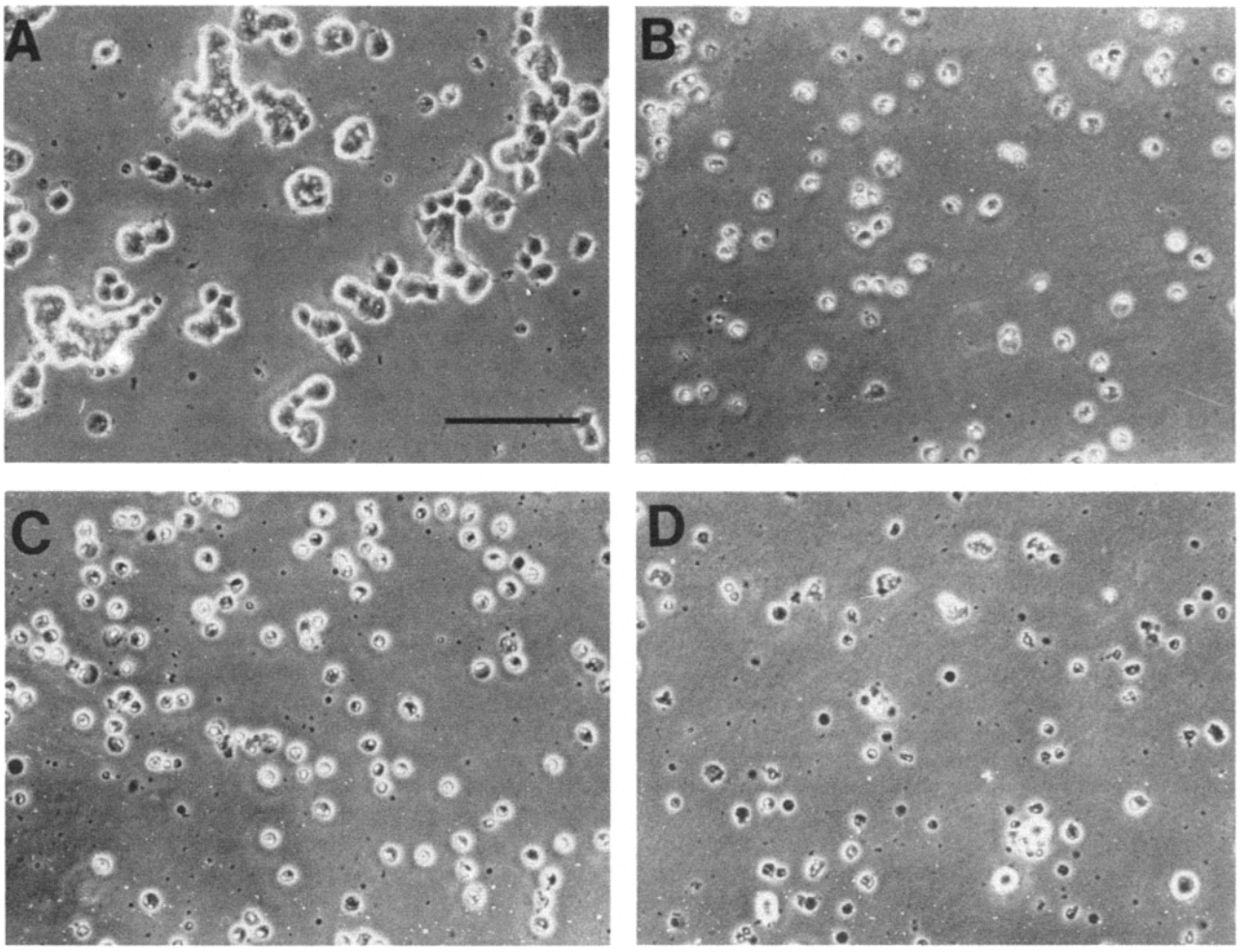

Figure 1. Morphological assay of nonneuronal PC12 cell survival. Nonneuronal PC12 cells were washed by centrifugation in RPMI 1640 medium and then maintained for 48 h in RPMI 1640 containing (*A*) 5% FBS and 10% donor horse serum, (*B*) no additives, (*C*) anisomycin (3 μg/ml), or (*D*) actinomycin (3 μg/ml). Phase-contrast optics. Bar, 100 μm.

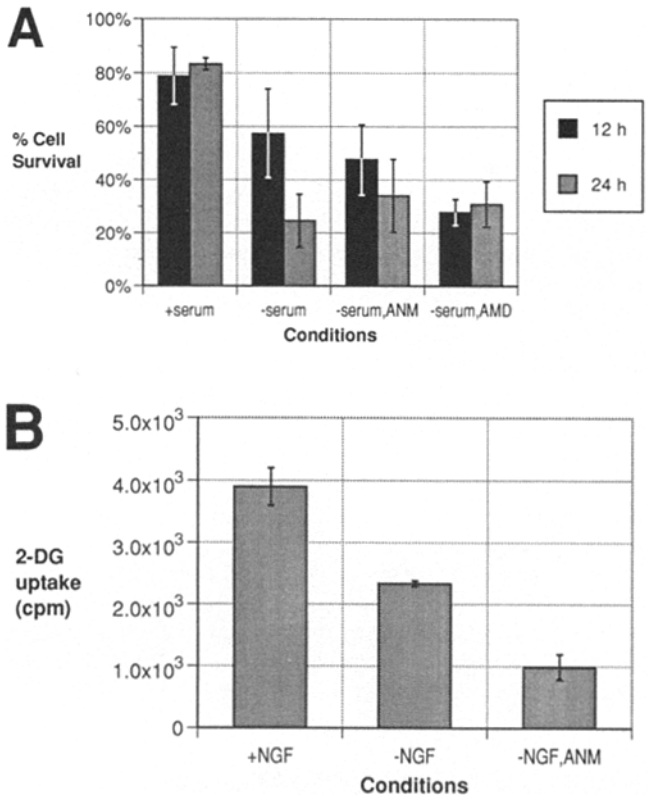

Figure 2. Quantitative assays of nonneuronal PC12 cell survival. (*A*) Effects of anisomycin (*ANM*) or actinomycin D (*AMD*) (both at 3 μg/ml) on the viability of cultures deprived of serum, determined by trypan blue dye exclusion assays. Cells were washed in serum-free RPMI 1640 and then plated in RPMI 1640 containing 5% FBS + 10% donor horse serum (*+serum*) or were plated in serum-free RPMI 1640. Cell counts were performed at 12 and 24 h after transfer to serum-free medium, by which time loss of viability was clearly evident. (*B*) Effects of anisomycin (3 μg/ml) on the viability of trophic factor–deprived cells, measured as uptake of [³H]2DG. Cells were washed and then transferred to serum-free medium (*−NGF*) or to NGF-supplemented serum-free medium (*+NGF*). Viability was assayed 24 h after transfer to experimental conditions.

Cell Number. The effects of actinomycin D and anisomycin on nonneuronal PC12 cell death were quantified by counting viable and necrotic cells using trypan blue dye exclusion as a marker for live cells. Relative to serum-containing controls, cultures deprived of serum exhibited a rapid loss of cells that was largely unaltered by treatment with inhibitors (Fig. 2 *A*). Within 12 h of transfer to serum-free medium, cell number was reduced to ~60% of cultures replated in serum-containing medium. Cell number in cultures plated for 12 h in serum-free medium containing actinomycin D or anisomycin declined, respectively, to ~30 or 50% of cultures replated in serum-containing medium, showing no protective effect of the inhibitors. After 24 h in serum-free medium, cell number declined to ~30% of that in cultures replated with serum, regardless of treatment with protein synthesis inhibitors. We conclude that cell death in serum-deprived nonneuronal PC12 cells does not depend upon transcription and translation.

[³H]2DG Uptake. Cell viability was also assessed by measuring uptake of [³H]2DG, a metabolic marker (Fig. 2 *B*). Uptake of 2DG was reduced by >40% within 24 h of transfer to serum-free medium. The magnitude of 2DG uptake is a function of both cell number and cellular metabolic activity. In this case, 2DG uptake declined in parallel with the decline in cell number assessed by cell counts (Fig. 2 *A*) indicating that 2DG uptake is strictly proportional to cell number. Thus, no significant reduction in cellular metabolic activity is apparent before cell death.

Inclusion of anisomycin in the medium did not reduce the decline in 2DG uptake that occurred after withdrawal of serum (Fig. 2 *B*). To the contrary, viability of anisomycin-treated cells was reduced relative to the controls, presumably because of toxicity of the drug (see below).

Neuronal PC12 Cells Die by Mechanisms That Require RNA and Protein Synthesis

Cell Morphology. Neuronally differentiated PC12 cells were generated by treatment with NGF for 2 wk (11). Such treatment gave rise to cultures containing flattened, attached, phase-bright cells with an extensive network of robust neurites (Fig. 3 *A*). Subsequent withdrawal of NGF, and culture in serum-free medium supplemented with anti-NGF antibody, led to cell death within 48–72 h. This was detectable as dramatic neuritic shrinkage and degeneration, and the appearance of phase-dark cells and abundant floating debris (Fig. 3 *B*). This time course of cell degeneration contrasted sharply with that observed in nonneuronal PC12 cells which exhibited marked degeneration in just 24 h. Neuronal PC12 cells cultured for 48 h in serum-free/NGF-free medium containing actinomycin were phase-bright and otherwise healthy in appearance, although some neuritic deterioration was evident (Fig. 3 *D*). Control cultures treated with actinomycin + NGF (Fig. 3 *C*) were indistinguishable from NGF-treated controls (Fig. 3 *A*). These results indicate that actinomycin prevents the PCD that typically follows withdrawal of the neurotrophic factor but not the deterioration of the neurites. Thus, programmed cell death requires RNA synthesis but neurite maintenance by NGF requires at least one RNA synthesis–independent component.

Translation is likewise required for PCD induced by NGF withdrawal but is not sufficient for maintenance of neurites by NGF. Cultures treated for 48 h with anisomycin in the absence of NGF and serum (Fig. 3 *F*) were protected from PCD for at least 72 h, although some neuritic degeneration was observed in these cultures as compared with cultures treated with anisomycin in the presence of NGF (Fig. 3 *E*).

Cell Number. The morphological data showing that inhibitors of RNA and protein synthesis protect neuronal PC12 cells from PCD is further supported by quantitative assays of cell survival (Fig. 4). Cell viability, as measured by direct counting of viable and necrotic cells using trypan blue dye exclusion to distinguish between them, was not significantly reduced relative to control cells maintained in NGF in the first 24 h after replating in NGF-free/serum-free RPMI 1640 (Fig. 4 *A*). By 48 h after NGF/serum withdrawal, viability had declined to 65% of control cells and this difference was highly significant ($p = 0.009$). Viability continued to decline with time in NGF-free/serum-free medium: declining, in 72 h, to 36% of the viability of cells maintained in NGF.

Actinomycin D and anisomycin were somewhat toxic to PC12 cells. This could be revealed by a 36% loss of viability

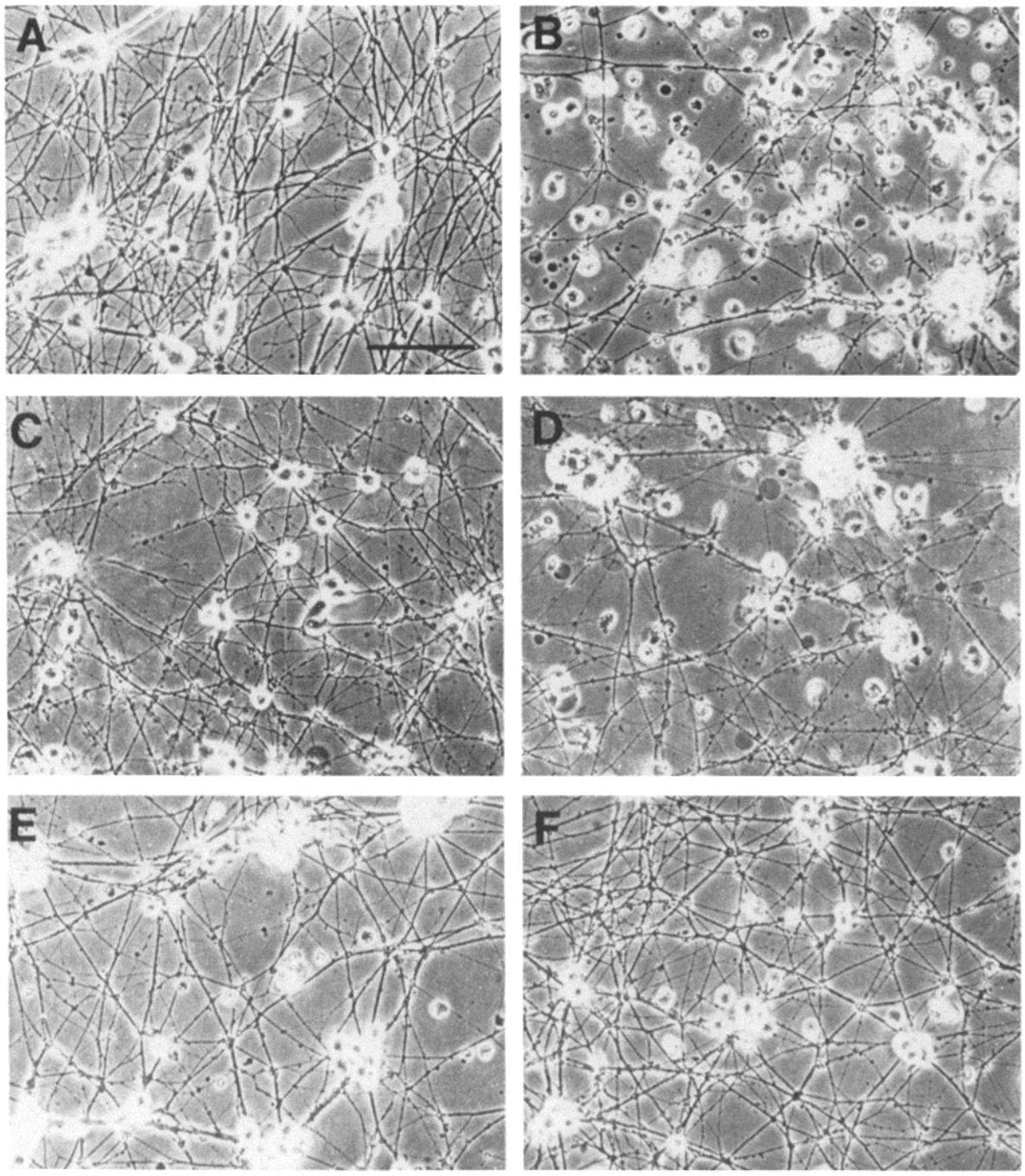

Figure 3. Morphological assay of neuronal PC12 cell survival. Neuronal PC12 cells (pretreated for 2 wk with 50 ng/ml NGF) were washed in RPMI 1640 medium and then maintained for 48 h in serum-free RPMI 1640 medium containing (*A*) NGF (50 ng/ml), (*B*) no additives, (*C*) NGF and actinomycin (3 μg/ml), (*D*) actinomycin only, (*E*) NGF and anisomycin (3 μg/ml), or (*E*) anisomycin only. NGF-free cultures included anti-mouse NGF (1:1,000). Phase-contrast optics. Bar, 100 μm.

in NGF-treated PC12 cells maintained for 72 h in 3 μg/ml actinomycin D and a 50% loss of viability in NGF-treated PC12 cells maintained for 72 h in 3 μg/ml anisomycin (Fig. 4 *A*). The loss of viability at 72 h of inhibitor treatment was large and significant ($p = 0.015$ for anisomycin, $p = 0.006$ for actinomycin) but there was no significant loss of viability due to treatment with actinomycin or anisomycin before 72 h (Fig. 4 *A*).

In spite of the toxicity of actinomycin and anisomycin, they promoted survival of neuronal PC12 cells deprived of NGF, as evidenced by increased viability of inhibitor-treated cells relative to untreated cells in serum-free/NGF-free medium (Fig. 4 *A*). The difference in cell viability between actinomycin-treated and actinomycin-untreated NGF-deprived cells—1.4× at 48 h and 1.7× at 72 h after NGF withdrawal—is significant ($p < 0.02$) by 48 h after NGF withdrawal which is also when loss of cells due to NGF withdrawal is first significant. No significant difference in cell viability was observed between actinomycin-treated cultures maintained in NGF and those deprived of NGF, implying that loss of cells

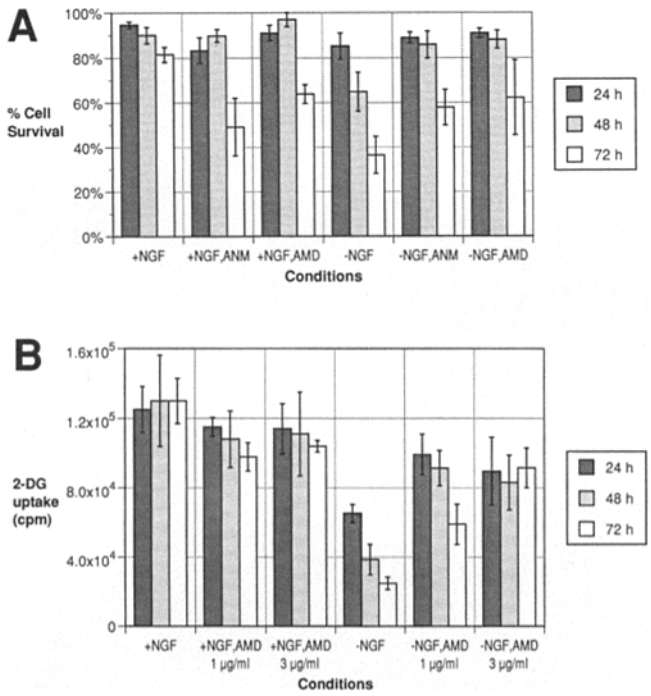

Figure 4. Quantitative assays of neuronal PC12 cell survival. (*A*) Effects of anisomycin (*ANM*) or actinomycin-D (*AMD*) (both at 3 μg/ml) on the viability of NGF-deprived cells, determined by trypan blue dye exclusion assays. Cells were washed and then plated in serum-free RPMI 1640 with NGF (*+NGF*) or without NGF (*−NGF*). NGF-free cultures contained anti-mouse NGF (1:1,000). Cell counts were performed at 24, 48, or 72 h after transfer to experimental conditions. (*B*) Effects of 1 μg/ml and 3 μg/ml actinomycin D on the viability of NGF-deprived cells, measured as uptake of [^{3}H]2DG. Cells were washed and then transferred to serum-free medium or to NGF-supplemented serum-free medium. Viability was assayed 24, 48, or 72 h after transfer to experimental conditions.

in NGF-deprived/actinomycin D–treated cultures is a result of drug toxicity.

These data additionally show that anisomycin, a translation inhibitor, maintains survival of neuronal PC12 cells in serum-free/NGF-free medium (Fig. 4 *A*). Cell viability is 1.6× greater at 72 h after NGF withdrawal and 1.3× greater at 48 h in anisomycin-treated cells, relative to anisomycin-untreated cells. As with actinomycin D, this difference is significant ($p < 0.04$) at both of these times. Furthermore, there is no significant difference between anisomycin-treated cultures maintained in NGF and those deprived of NGF, implying that loss of cells in NGF-deprived/anisomycin-treated cultures is a result of drug toxicity.

[^{3}H]2DG Uptake. Uptake of [^{3}H]2DG was assessed in neuronal PC12 cell cultures maintained in, or deprived of, NGF. Uptake of 2DG can be influenced by both cell number and cell metabolic rate. In the case of NGF/serum-deprived neuronal PC12 cells, 2DG uptake declined much more rapidly than did cell number. Within 24 h of replating in NGF-free/serum-free RPMI 1640, 2DG uptake was significantly ($p < 0.005$) reduced to 53% of the uptake of control cells maintained in NGF (Fig. 4 *B*). This contrasts with the insignificant decline in cell number after 24 h in NGF-free/serum-free medium and indicates that the decline in 2DG uptake in the first 24 h is due to a decline in cellular metabolic rate. Uptake of 2DG continued to decline with time in NGF-free/serum-free medium: at 48 h it was 30% and at 72 h it was 19% of the viability of cells maintained in NGF (Fig. 4 *B*). Comparison with the cell counts described above and depicted in Fig. 4 *A* reveals that the NGF withdrawal–induced decline in 2DG uptake is due not only to a decline in cell number but also to a decline in metabolic rate in cells that are still alive.

Actinomycin D toxicity could be revealed by a 20–25% reduction of 2DG uptake in NGF-treated PC12 cells maintained for 72 h in 1 μg/ml or 3 μg/ml actinomycin D (Fig. 4 *B*). As with decline in cell number, decline in 2DG uptake due to actinomycin treatment was judged to be statistically significant at 72 h ($p < 0.03$) but was not significant before 72 h.

In spite of the toxicity of actinomycin, it prevented the decline in cell viability and the decline in metabolic rate exhibited by PC12 cells deprived of NGF. Cells replated in serum-free/NGF-free medium in the presence of 3 μg/ml actinomycin showed no significant decline in 2DG uptake relative to cultures similarly treated with actinomycin but maintained in NGF (Fig. 4 *B*). This was in marked contrast to the fate of neuronal PC12 cells deprived of NGF in the absence of actinomycin. These cells manifested a highly significant ($p < 0.003$) decline in 2DG uptake relative to cells maintained in NGF or to cells maintained in 3 μg/ml actinomycin D. For cells deprived of NGF for 48 h, 2DG uptake in cells treated with 3 μg/ml actinomycin D was 2.2× that of cells plated without actinomycin D. For cells deprived of NGF for 72 h, 2DG uptake in cells treated with 3 μg/ml actinomycin D was 3.7× that of cells plated without actinomycin D. All of the difference in viability between NGF-treated cells and NGF-deprived/actinomycin-treated cells appears to be due to toxicity of the drug: no significant difference in viability could be detected between NGF-treated cells and NGF-deprived cells if both were treated with 3 μg/ml actinomycin (Fig. 4 *B*).

Comparison of the effects of 1 μg/ml actinomycin D with those of 3 μg/ml actinomycin D (Fig. 4 *B*) revealed that maintenance of cell viability by actinomycin D was dose dependent, with the higher dose of actinomycin D more effective in preventing a decline in 2DG uptake after NGF withdrawal. For neuronal PC12 cells deprived of NGF for 72 h, 2DG uptake in cells treated with 3 μg/ml actinomycin D was 1.6× that of cells treated with 1 μg/ml actinomycin D (although no significant difference in 2DG uptake was detected between cells in 1 μg/ml actinomycin D and those in 3 μg/ml actinomycin D after 48 h of NGF deprivation). The difference in 2DG uptake between NGF-deprived cells treated with 3 μg/ml actinomycin D and those treated with 1 μg/ml actinomycin D was significant ($p = 0.026$). Nevertheless, the maintenance of 2DG uptake due to 1 μg/ml actinomycin was still highly significant ($p = 0.009$) when comparing actinomycin-treated to actinomycin-untreated NGF-deprived cells. The dependence of the effects of actinomycin D on its dosage implies that maintenance of NGF-deprived neuronal PC12 cells by actinomycin D is directly attributable to addition of the drug.

Maintenance of neuronal PC12 cells by actinomycin D becomes more evident with increasing time after NGF withdrawal as 2DG uptake declines. However, the difference in

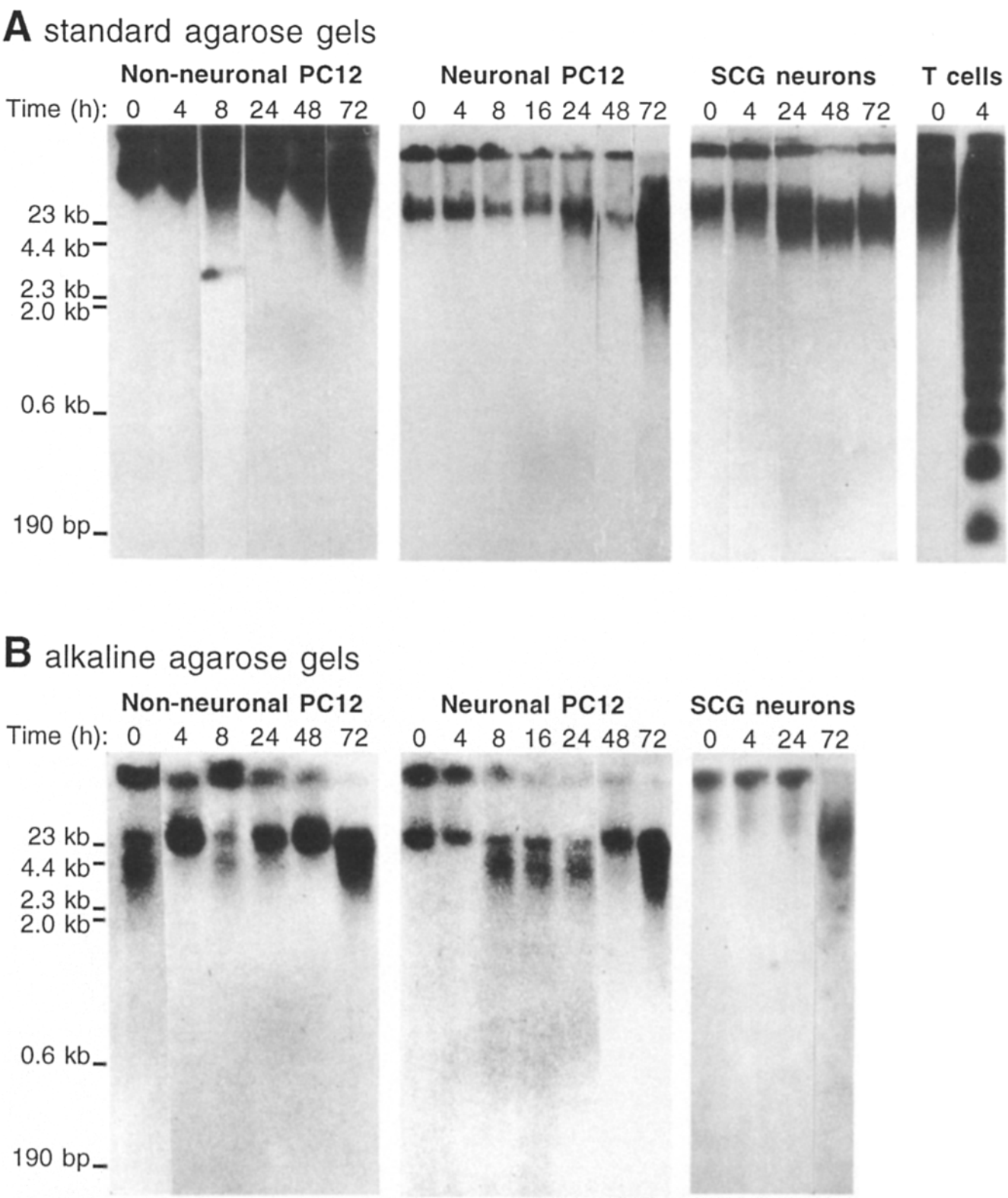

Figure 5. Genomic Southern blots. Nonneuronal PC12 cells, neuronal PC12 cells, or SCG sympathetic neurons were deprived of trophic factor for various times as indicated. Neonatal rat thymocytes (*T cells*) were treated with glucocorticoids for 4 h to induce apoptosis or were untreated (0). (*A*) Samples of whole genomic DNA (5 μg/lane) were prepared by ethanol precipitation and size separated on 1% agarose gels in TAE buffer. After vacuum transfer to nylon filters, the DNA was hybridized with a ^{32}P-labeled probe consisting of HindIII-digested rat genomic DNA. The filter was exposed to x-ray film at −86°C to obtain the autoradiogram shown. (*B*) Total genomic DNA was processed as in *A*, except that the DNA was run on 1% alkaline agarose gels for 4 h at 50 V to permit strand separation. Experiments with different cell types were performed at different times, with probes of different specific activities, and were exposed for different times. Therefore, the densities of the lanes are not comparable between sets. Overexposing autoradiograms of PC12 and neuronal DNA did not reveal any DNA fragmentation.

2DG uptake between actinomycin-treated (1 μg/ml or 3 μg/ml) and actinomycin-untreated NGF-deprived cells is highly significant ($p < 0.003$) at 48 h after NGF withdrawal and is even significant ($p < 0.05$) 24 h after NGF withdrawal (Fig. 4 *B*).

PC12 Cell and Sympathetic Neuronal PCD Is Not Associated with Significant DNA Fragmentation

Cultures of nonneuronal or neuronal PC12 cells or cultures of SCG neurons were deprived of trophic support for various times. Samples of DNA were analyzed by gel electrophoresis and visualized, in initial experiments, with ethidium bromide. DNA fragmentation was not detected in these experiments even though similar methods have been used previously to detect DNA fragmentation in these and other cells (2, 7, 20, 25, 27, 45).

To exclude the possibility that significant amounts of DNA fragmentation could be overlooked, we turned to a method for visualizing DNA more sensitive than using ethidium bromide. Samples of DNA were analyzed by gel electrophoresis followed by transfer to nylon membranes and Southern-type hybridization with ^{32}P-labeled rat genomic DNA for subsequent autoradiography. Presumably, repetitive elements in the genome drive the hybridization. To determine the sensitivity of the assay system, we first analyzed dexamethasone-treated thymocyte DNA which, as previously reported, is extensively fragmented after brief exposure to glucocorticoid hormones (45). After 4 h of treatment with 1 μM dexamethasone, analysis of 5 μg samples of thymocyte genomic DNA clearly revealed the characteristic ladder of DNA fragments commonly associated with T lymphocyte PCD (Fig. 5 *A*). This result indicates that detection of DNA fragmentation is within the sensitivity range of our assay system. We densitometrically quantified both the extent of thymic DNA degradation and the lower limit of our ability to detect fragments (data not shown). Approximately 30% of the DNA in apoptotic thymocytes existed as fragments of 2 kbp or less. Titration of our assay system with decreasing quantities of genomic DNA from apoptotic thymocytes established the lower limit of fragment detection at ~125 ng of thymocyte DNA per lane. Finally, to eliminate the possibility that fragmentation was occurring in the form of single-stranded nicks, samples were analyzed by a combination of denaturing (alkaline) gel electrophoresis and autoradiography.

Genomic DNA isolated from PC12 cells was analyzed on gels using sample sizes of 5 μg. This mass is 40× greater than the amount that we had established as minimal for detection of DNA fragmentation in apoptotic thymocytes. Of 24 independent experiments, each using DNA from control nonneuronal PC12 cells and nonneuronal PC12 cells deprived of serum for various durations, only one case showed a pattern of nucleolytic degradation consistent with possible internucleosomal cleavage. Fig. 5 *A* shows a typical example of these experiments. DNA from nonneuronal PC12 cells deprived of serum for 4, 8, 24, 48, and 72 h did not exhibit fragmentation consistent with internucleosomal cleavage, nor was any increase in low molecular weight detected in these samples relative to control cells maintained with serum. Quantitation by densitometry showed that <1% of the radioactivity in samples from dying cells was in the region of the autoradiogram corresponding to sizes of 2 kbp or less. This is comparable to the amount of radioactivity in the <2 kbp region of the autoradiogram in the lane containing DNA from control cells maintained in serum.

Fig. 5 *A* shows a small amount of degradation apparent in DNA from cells 72 h after removal of trophic factor. This is unlikely to be causal to cell death as it appeared only after most of the cells had already died and it was unaffected by the putative nuclease inhibitor AT which inhibits cell death (see below). Also, it appeared to be a generalized degradation not involving internucleosomal cleavage – no characteristic "190-bp ladder" was observed – nor did it lead to the production of low molecular weight (<2 kbp) fragments. This apparent DNA degradation is therefore likely to be the result of deterioration of the debris accumulating in the dishes as the cells die.

Similar results were obtained from an analysis of neuronal PC12 cells. In 14 independent experiments, each using DNA from control neuronal PC12 cells and neuronal PC12 cells deprived of NGF for various durations, no instances of possible internucleosomal cleavage were detected. Fig. 5 *A* shows a typical example of these experiments. DNA from neuronal PC12 cell cultures deprived of serum and NGF for 4, 8, 16, 24, 48, and 72 h did not exhibit fragmentation consistent with internucleosomal cleavage, nor was any increase in low molecular weight DNA detected in these samples relative to control cells maintained with NGF. The same was true of cultured rat SCG sympathetic neurons which were similarly deprived of NGF for 4, 24, 48, and 72 h (Fig. 5 *A*). In eight independent experiments, no significant DNA fragmentation was detected in SCG neurons.

As shown above (Fig. 2), the viability of nonneuronal PC12 cells was dramatically reduced after 12–24 h of culture in serum-free medium, while neuronal PC12 cell viability in serum and NGF-free medium was greatly reduced after 48–72 h (see Fig. 4 *A*). Thus, if DNA fragmentation was involved to a significant degree in the death of the cells in the present study, it should have been detected within the time covered by our observations. Our failure to detect fragmentation in PC12 cells or in sympathetic neurons therefore suggests that it is not an essential component of neuronal PCD, although it may occur in many instances.

It is possible that a nuclease activity is activated during PCD in trophic factor–deprived neurons and PC12 cells although a 190-bp ladder like that occurring in glucocorticoid-treated thymocytes does not occur because cleavage of the DNA is random and not restricted. The data in Fig. 5 *A* indicates that there is no significant increase in the occurrence of double-stranded breaks in the DNA of NGF-deprived cells, regardless of whether the breaks are internucleosomal or randomly distributed. Randomly distributed breaks in the DNA would have given rise to a smear of increased density in the autoradiogram in the lower molecular weight region and such a smear was not detected. However, putative increased nuclease activity might have caused only randomly distributed single-strand breaks which might not have caused a detectable change in DNA motility on nondenaturing gels such as the ones used in Fig. 5 *A*. We therefore ran samples of DNA from control and from NGF-deprived cells on denaturing, alkaline (strand separating) gels to determine whether there was an increase in single-strand breaks. Again, no increased density in the autoradiogram in the lower molecular weight region was observed (Fig. 5 *B*) supporting the conclusion that there was no significant increase in nuclease activity in NGF-deprived cells.

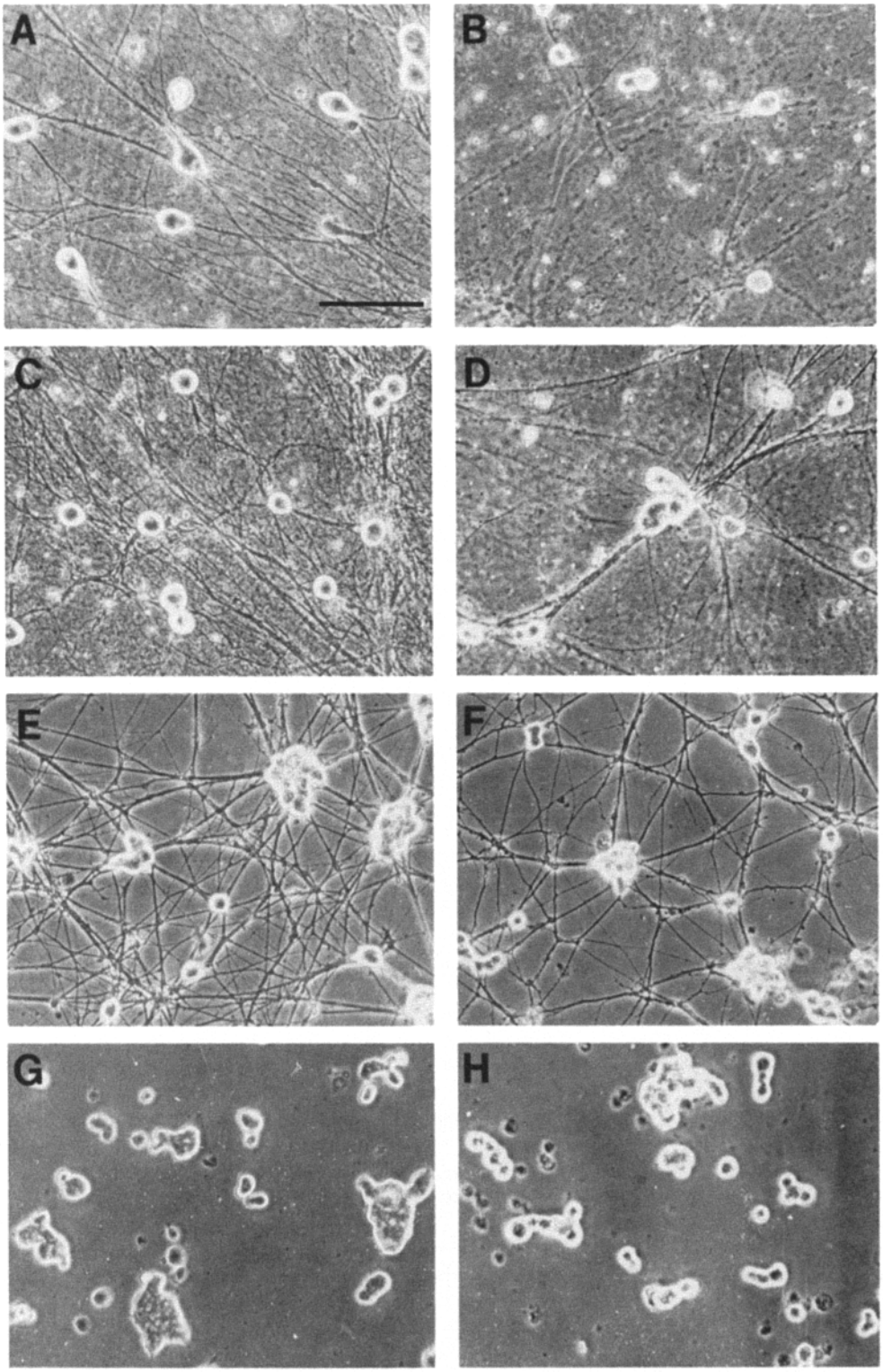

Figure 6. Effects of AT on neuronal and on PC12 cell survival. The effects of AT on the viability of SCG neurons (*A–D*), neuronal PC12 cells (*E* and *F*), or nonneuronal PC12 cells (*G* and *H*) were determined by visual inspection of the cells: SCG neurons cultured for 7–10 d were washed extensively with RPMI 1640 medium and then maintained for 72 h in the same medium containing (*A*) NGF (50 ng/ml), (*B*) no additives, (*C*) NGF and 100 μM AT, or (*D*) 100 μM AT only. Neuronal PC12 cells washed as previously described were maintained for 72 h in RPMI 1640 medium containing (*E*) NGF + 100 μM AT or (*H*) 100 μM AT only. Nonneuronal PC12 cells washed as described previously were maintained for 72 h in RPMI 1640 medium containing (*G*) serum + 100 μM AT or (*H*) 100 μM AT only. NGF-free cultures contained anti-mouse NGF (1:1,000). Phase-contrast optics. Bar, 100 μm.

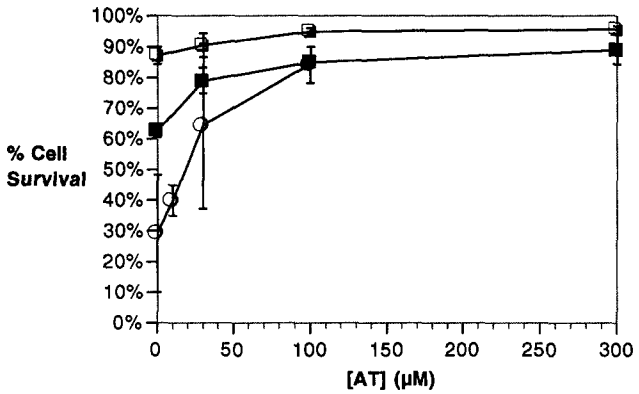

Figure 7. Quantitative assay of the effects of AT on PC12 cell survival. Neuronal or nonneuronal PC12 cells were deprived of trophic support, respectively, NGF or serum. Neuronal cells were washed and then cultured in serum-free RPMI 1640 for 24 (□) or 72 h (■), and nonneuronal cells for 24 h (○). The viability of the cells was then determined by counting trypan blue dye-excluding and -nonexcluding cells.

Aurintricarboxylic Acid Prevents the Programmed Death of Cells That do Not Experience DNA Fragmentation

Previous studies have shown that the putative endonuclease inhibitor aurintricarboxylic acid (AT) prevents the death of cells that die apoptotically with accompanying DNA fragmentation (19, 25). In the present study we show that AT also strongly inhibits the death of cells in which DNA fragmentation is not a consistent or significant feature of cell death. Cultures of SCG neurons treated with 100 μM AT in the absence of serum and NGF for 72 h (Fig. 6 *D*) appeared similar to control cultures maintained in trophic factor with 100 μM AT (Fig. 6 *C*) or without AT (Fig. 6 *A*). Such cultures contained phase-bright cells, in contrast to serum and NGF-deprived cultures which contained degenerating cells with fragmented neurites and abundant debris (Fig. 6 *B*). Likewise, both neuronal and nonneuronal PC12 cells cultured for 3 d in serum-free/NGF-free medium containing 100 mM AT (Figs. 6, *F* and *H*, respectively) were identical to control cultures not deprived of trophic factors (see Figs. 3 *A* and 1 *A*), or to control cultures treated with AT (Fig. 6, *E* and *G*). These data suggest that AT strongly inhibits the death of SCG neurons, neuronal PC12 cells, and nonneuronal PC12 cells by a mechanism that is independent of its ability to block nucleolytic DNA fragmentation.

Suppression of PC12 cell death by AT was quantified by directly counting viable and necrotic cells using trypan blue dye exclusion as a marker for live cells (Fig. 7). For nonneuronal PC12 cells, cell number in serum-deprived cultures declined by 71% relative to control cultures containing serum. Cell number declined by only 16% if AT, at concentrations ≥100 μM, was added to the serum-free medium, a decline 23% as great. Half-maximal suppression of nonneuronal PC12 cell death was observed at ~30 μM AT. In cultures of NGF-deprived neuronal PC12 cells, cell number declined by 13% relative to NGF-treated controls at 24 h after replating. While this represented only a small loss of viability, suppression of cell death by AT was nonetheless observed. At 72 h after replating in NGF-free/serum-free medium, a larger decline in cell number was observed but in cultures also treated with ≥100 μM AT, the decline was 39% as great: cell number declined by 15% as opposed to 38%. As with nonneuronal cells, maximal suppression of cell death was observed at AT concentrations ≥100 μM and half-maximal suppression at ~30 μM AT. These data suggest that AT suppresses cell death in trophic factor–deprived neuronal and nonneuronal PC12 cells by a similar mechanism, in spite of the differences between these cell types regarding the role of transcription in their death.

Discussion

The present study describes mechanistic aspects of the cell death that ensues upon withdrawal of trophic factors from sympathetic neurons or PC12 cells. These aspects include the time course of cell death, the requirement for protein synthesis for cell death, the involvement of DNA fragmentation, and the effects of AT. We find that cell death induced in neuronal PC12 cells by NGF withdrawal resembles previously described (23) cell death in NGF-deprived sympathetic neurons with respect to time course and dependence on protein synthesis. In addition, we show that, while AT suppresses cell death in PC12 cells and sympathetic neurons as Batistatou and Greene (2) have observed, nevertheless, DNA fragmentation does not accompany cell death in the PC12 cells or in sympathetic neurons. These observations are particularly relevant to the question of the appropriateness of the use of PC12 cells as a model for neuronal cell death. Insofar as we find that cell death in NGF-deprived neuronal PC12 cells resembles that of NGF-deprived sympathetic neurons in several critical aspects, these results support the use of neuronally differentiated PC12 cells as a model for the study of cell death in neurons.

While cell death in neuronal PC12 cells is suppressed by actinomycin D and anisomycin—inhibitors, respectively, of transcription and translation—a striking observation is that this is not the case for nonneuronal PC12 cells. This implies that at least two different mechanisms can account for cell death in trophic factor–deprived PC12 cells with the mechanism used dependent upon the differentiated state of the cells. Apparently, among the many phenotypic changes accompanying neuronal differentiation of PC12 cells, there appears to be a shift in the mechanism by which the cells die upon withdrawal of trophic factor. Nonneuronal cells die rapidly upon withdrawal of serum from the medium, with the majority of the cells dead within 24 h. The time course of death of these cells is unaffected by transcription and translation inhibitors and is thus unlikely to involve protein synthesis. The mechanism that accounts for cell death of serum-deprived nonneuronal PC12 cells is not evident from these studies but it is distinctly different from that operating in the death of NGF-deprived neuronal PC12 cells.

PC12 cells are neuronally differentiated after 2 wk or more of treatment with NGF (11, 12). Such neuronal PC12 cells die in serum-free medium after removal of NGF and the rate of their cell death is slow relative to that of nonneuronal PC12 cells: at least 72 h is required for death of a majority of the cells. Moreover, the death of NGF-deprived neuronal PC12 cells is significantly, if not completely, suppressed by the addition of translational or transcriptional inhibitors, in-

dicating that the process of cell death requires protein synthesis.

Fig. 4 shows that inhibition of neuronal PC12 cell death by actinomycin or anisomycin may not be complete. Even in the presence of these drugs at concentrations that almost entirely eliminate protein synthesis, trophic factor–deprived cells have a lower viability than cells maintained in serum or NGF (although their viability is significantly greater than that of trophic factor–deprived cells maintained without these inhibitors). In contrast, Martin et al. (23) reported that viability of NGF-deprived sympathetic neurons maintained in transcription or translation inhibitors was the same as control neurons maintained with NGF. While we can't completely rule out the possibility that there exists a transcription- and translation-independent mechanism for cell death in neuronal PC12 cells in addition to the mechanism dependent on transcription and translation, our data support an alternative explanation. The decline in cell number in actinomycin- or anisomycin-treated cultures was about the same whether NGF was present or not. Therefore, the decline in cell number is likely to be due to the toxicity of the inhibitors, and the neuronal PC12 cells differ from sympathetic neurons mainly in their greater sensitivity to this toxicity.

Like survival, neurite outgrowth in sympathetic neurons is dependent on the continued presence of NGF (6). These actions of NGF on neurites appear to involve a protein synthesis–independent step. While cell death caused by NGF deprivation is inhibited by actinomycin and anisomycin, neurite degeneration is apparently not prevented. This is consistent with previous reports (6, 37) showing local effects of NGF on the growth cone independent of its effects at the soma.

The characteristics of cell death in NGF-deprived neuronal PC12 cells that are described here are comparable to those reported by Martin et al. (23) in their study of PCD in sympathetic neurons: with regard both to time course and dependence on protein synthesis, the death of NGF-deprived neuronal PC12 cells resembles that of sympathetic neurons. These results suggest that the mechanism mediating PCD in trophic factor–deprived neurons involves the specific induction of cellular proteins, the function of which is to carry out the cell death program. This mechanism appears to operate in neuronal PC12 cells as it does in sympathetic neurons. Such a suggestion is consistent with the results of several other recent studies (19, 29, 36) and has been recently reviewed (28, 34). Identifying these proteins and their precise functions is necessary for understanding the mechanism of PCD. While no such proteins have been identified in neurons, expression of nucleases (7), polyubiquitin (35), hsp70, c-*fos*, c-*myc* (4), and TRPM-2 (5) have been associated with cell death in various nonneuronal instances of PCD. Mutations affecting PCD have been described in the nematode (9), allowing a molecular genetic approach to the identification of proteins required for cell death. Furthermore, Owens et al. (30) have used differential screening of cDNA libraries to identify novel mRNAs specifically induced during PCD in thymocytes—although the absolute requirement for these mRNAs for PCD has not yet been demonstrated. The observation that NGF-deprived neuronal PC12 cells require protein synthesis to undergo cell death allows the use of this versatile cell line for a similar molecular approach to the identification of the cellular proteins that effect neuronal PCD.

In addition to dependence on transcription, an additional common feature of PC12 cell and sympathetic neuronal PCD is revealed by these studies: in both cases the cells appear to die without significant DNA fragmentation. Since the observations of Wyllie (45) of DNA fragmentation in glucocorticoid-treated thymocytes, DNA fragmentation has been found to accompany cell death in a variety of cell types (1, 7, 8, 16, 20, 27, 32, 42, 44, 48) including neuronal cells (2, 19). In contrast, we do not detect DNA fragmentation during PCD in sympathetic neurons and PC12 cells. These results indicate that DNA fragmentation is not an essential component of cell death in trophic factor–deprived PC12 cells or sympathetic neurons. Similarly, DNA fragmentation does not occur during the death of hippocampal neurons induced to die by exposure to glucocorticoids (24). We conclude from this that cell death can involve at least two different mechanisms, one of which involves DNA fragmentation and one which does not. Occurrence of DNA fragmentation may be dependent on the particular neuronal type undergoing cell death and on the particular signal that is inducing cell death. This conclusion is consistent with similar conclusions regarding PCD in neuronal and nonneuronal cells (34), and a demonstration of *bcl*-2–inhibitable (apoptotic) and *bcl*-2–noninhibitable PCD in thymocytes (38).

It is surprising in this regard that Batistatou and Greene (2) do observe DNA fragmentation in PC12 cells induced to die by trophic factor withdrawal in essentially the same way as we have done in the present study. That study did not use sympathetic neurons as we have done here but did arrive at different conclusions with regard to PC12 cells. An inability to detect DNA fragmentation because of insensitivity of our detection method is ruled out by our use of radiolabeled probes instead of ethidium bromide to detect DNA fragments and our quantitative comparison of genomic DNA from PC12 cells and thymocytes undergoing cell death. In the latter case, authentic internucleosomal cleavage of DNA can be detected by our methods with >30% of the total genomic DNA appearing in a clearly defined "ladder" of fragments >2 kbp in size with sizes that are multiples of ~190 bp. Such fragments are detected in thymocyte DNA after loading only 125 ng of genomic DNA. No such fragments could be detected in PC12 cells or sympathetic neuronal DNA after loading 5 μg of DNA, a 40× greater mass. We conclude that if internucleosomal cleavage is occurring in these cells then it can account for, at most, 0.75% of the total DNA content of the cells. This is in marked contrast to typical apoptosis, such as that occurring in thymocytes, where most of the DNA is present in the 190-bp repeat "ladder" and >30% of the DNA is found in fragments <2 kbp in size. The differences between the two sets of results with regard to the occurrence of DNA fragmentation may derive from divergence between different PC12 cell populations, but the present results demonstrate that, at least in the PC12 cells and primary cultures of rat sympathetic neurons used here, cell death need not involve fragmentation of a significant fraction of genomic DNA.

Lack of significant DNA fragmentation during PCD implies that suppression of cell death by AT need not be a result of inhibition of nuclease activity as has been previously suggested (2, 25). Pleiotrophic actions of AT have been reported elsewhere (14, 18), including a generalized ability to block protein–nucleic acid interactions (3). Consideration of these findings in light of data on the importance of transcription

in neuronal PCD suggests that the crucial event affected by AT may be one involving gene expression, although AT is apparently not a general inhibitor of transcription (2).

In conclusion, PCD in neuronal PC12 cells and sympathetic neurons are alike in several critical respects. In both cell types, PCD is a relatively slow process that does not involve DNA fragmentation but does require protein synthesis. Our results strongly support the use of neuronal PC12 cells as a model system for the further investigation of the biochemical and molecular aspects of neuronal PCD.

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