Characterization of Apoptosis in Cultured Rat Sympathetic Neurons After Nerve Growth Factor Withdrawal

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Abstract. Sympathetic neurons depend on nerve growth factor (NGF) for their survival both in vivo and in vitro. In culture, the neurons die after NGF withdrawal by an autonomous cell death program but whether these neurons die by apoptosis is under debate. Using vital DNA stains and in situ nick translation, we show here that extensive chromatin condensation and DNA fragmentation occur before plasma membrane breakdown during the death of NGF-deprived rat sympathetic neurons in culture. Furthermore, kinetic analysis of chromatin condensation events within the cell population is consistent with a model which postulates that after NGF deprivation nearly all of the neurons die in this manner. Although the dying neurons display membrane blebbing, cell fragmentation into apoptotic bodies does not occur. Apoptotic events proceed rapidly at around the time neurons become committed to die, regardless of neuronal culture age. However the duration of NGF deprivation required to commit neurons to die, and the rate at which apoptosis occurs, increase with culture age. Thus, within the first week of culture, apoptosis is the predominant form of cell death in sympathetic neurons.

In the developing nervous system, programmed cell death, whereby a cell dies in a self-contained manner without damaging its neighbors, is used for both coordination of neuronal population size and removal of incorrectly connected neurons (for review see Oppenheim, 1991). For some neuronal classes, population size is regulated by the limited availability of trophic factors necessary for cell survival. Such trophic factors have been identified for an increasing number of neuronal populations (Thoenen, 1991; Henderson et al., 1993), and it has been suggested that all cell types may require the continuous presence of survival promoting factors (Raff, 1992). Nerve growth factor (NGF) is the archetypal example of a neuronal trophic factor. During specific critical periods in development, removal of endogenous NGF severely depletes sympathetic and some sensory neuronal populations (Levi-Montalcini and Booker, 1960; Johnson et al., 1980). NGF is synthesized at the target organs of these neurons, in small amounts that are apparently insufficient to promote the survival of all the innervating neurons. In culture, sympathetic neurons from neonatal rats retain this dependence on NGF for survival for up to three weeks (Koike and Tanaka, 1991), providing a model system to study trophic factor dependence and cell death in neurons.

Programmed cell death is an important process in many tissues during both development and maturity (Wyllie et al., 1980). The term "programmed" is used here to signify controlled as opposed to necrotic death, and is not intended to imply genetic pre-determination of death. The most commonly cited form of programmed cell death is apoptosis, which is characterized by DNA fragmentation into oligonucleosomal repeats, chromatin condensation, cytoplasmic shrinkage, and fragmentation of the cell into intact apoptotic bodies (Kerr et al., 1972; Wyllie, 1980). Although the term apoptosis is commonly used synonymously with programmed cell death, multiple forms of programmed cell death have been described (Clarke, 1990; Schwartz et al., 1993). It has also been suggested that the type of programmed cell death in some neurons may be dependent on cell age (Cunningham, 1982). Despite intensive research, the form of programmed cell death which occurs in trophic factor deprived neurons is still under debate.

Established cultures of rat sympathetic neurons are thought to require de novo RNA and protein synthesis in order to die after NGF-withdrawal (Martin et al., 1988). This theory is based on the ability of RNA and protein synthesis inhibitors to prevent neuronal death after trophic factor deprivation, for which alternative explanations have also been proposed (Scott and Davies, 1990). Although a requirement for de novo gene expression is often linked with apoptotic cell death (Bursch et al., 1990), instances of apoptosis insensitive to (e.g., macrophages, Waring et al., 1990), or induced by protein synthesis inhibitors (e.g., HL-60 cells, Martin et al., 1990; primary uterine cells, Gerschenson and Rotello, 1991) have also been demonstrated. Thus prevention of cell death by protein synthesis inhibitors alone cannot be used as a defining characteristic for apoptosis (reviewed by Cohen et al., 1992). As it remains possible that other forms

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of programmed cell death are also sensitive to such inhibitors, their effect on neurons may not indicate a specific mechanism of programmed cell death. In the PC12 cell line, frequently used to study NGF actions, programmed cell death was found to be insensitive to protein synthesis inhibitors (Rukenstein et al., 1991), and was accompanied by extensive DNA fragmentation into the oligonucleosomal repeats typical of apoptosis (Batistou and Greene, 1991). However, the death of PC12 cells differentiated into neurons by pretreatment with NGF, was found to require de novo protein synthesis, and did not appear to involve DNA fragmentation (Mesner et al., 1992). Similarly, in the latter study, the death of established cultures of sympathetic neurons after NGF-deprivation was not found to involve DNA fragmentation. Using a sensitive Southern blotting technique, however, we have previously demonstrated the presence of fragmented DNA, characteristic of apoptosis, in seven-day cultures of rat superior cervical ganglion (SCG)[1] neurons (Edwards et al., 1991). The occurrence of DNA fragmentation in the death of NGF-deprived SCG neurons has also been inferred from the ability of the nuclease inhibitor aurintricarboxylic acid to prevent the death of these neurons (Batistou and Greene, 1991). Thus it is still unclear whether all, or only a small proportion of these neurons die by apoptosis after NGF deprivation. We have therefore investigated the extent of apoptosis in NGF-deprived sympathetic neurons, and the effect of cell age on the death mechanism. Our results demonstrate that dying neurons exhibit chromatin condensation and DNA fragmentation consistent with apoptotic cell death, and that the majority of cells die by apoptosis regardless of cell age.

Materials and Methods

Materials

NGF was purified from adult male mice submaxillary glands (Mobley et al., 1976). Anti-NGF antisera were raised in rabbits, purified by ammonium sulphate precipitation and titrated against the survival promoting effect of the standard NGF in the growth medium (20 ng/ml). Rat serum was obtained from obsolete Wistar breeding stock. Strataclean resin was from Stratagene (La Jolla, CA), Digoxygenin reagents from Boehringer Mannheim (Lewes, UK), DNA Polymerase I from Amersham (UK), and L15 medium and cell culture reagents were supplied by GIBCO BRL (Paisley, Scotland). [^{35}S]methionine or Tran^{35}S-label™ (>1,000 Ci/mmol) was from ICN Radiochemicals (Costa Mesa, CA).

Cell Preparation and Tissue Culture

Sympathetic neurons were isolated from SCG of Wistar rats during the first postnatal day and cultured on a collagen substrate in L15-CO_2 medium containing 3% rat serum with or without 20 ng/ml NGF as described previously (Buckmaster et al., 1991). The neuronal population was enriched by preplating the cells in L15-CO_2 medium containing 5% rat serum on a collagen substrate for 15–30 min. Under these conditions non-neural cells become preferentially attached to the substrate whereas most neurons are retrieved by gentle shaking. For seven-day cultures, non-neuronal cells were also removed by inclusion of 10 μM 5-fluoro-2'deoxyuridine in the culture medium, leaving 5–10% non-neural cells, mainly Schwann cells and fibroblasts. Fluorodeoxyuridine was not present in cultures of newly isolated neurons used to assay DNA fragmentation. For cell counts of newly isolated neurons the culture medium was thickened with 0.6% methyl cellulose stirred into the medium at 4°C overnight, to prevent cells deprived of NGF from floating away from the culture substrate (Hawrot and Patterson, 1979). Neurons were plated under this medium onto the culture substrate using L15 plating medium (Hawrot and Patterson, 1979).

Assay of DNA Fragmentation

Cells from five pups were used for each sample. Cultures to be analyzed were washed once in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) and lysed in TE containing 0.4% Triton X-100 on ice. Lysed cells were collected from the culture plates by scraping, and pooled with free cells collected from the culture medium by centrifugation at 300 *g* for 10 min. Samples were treated with 100 μg/ml RNase for 30 min at 37°C, then with 10 μg/ml Proteinase K at 45°C for 3 h. Samples were treated with Strataclean resin to remove the proteins, and the DNA was recovered by precipitation with sodium acetate and isopropanol followed by an ethanol wash. DNA contained in cell-free culture medium was processed in the same manner. DNA samples were resuspended in TE and 0.1% SDS, and electrophoresed on a 1.5% TBE agarose gel. DNA was visualized by ethidium bromide staining.

Cell Staining Assays

Acridine Orange (AO) was added to the culture medium of unfixed cells at 2 μg/ml, and the cells viewed immediately under blue light (FITC) fluorescence. Alternatively, Hoechst 33342 was added to the culture medium at 2 μg/ml, cells were incubated at room temperature for 5 min and viewed under ultra violet fluorescence. Ethidium bromide was added to the medium to 2 μg/ml. Trypan blue was added to 0.1%, and dye exclusion assessed after a 10-min incubation at 37°C.

In Situ Nick Translation

The method was based on that of Oberhammer et al. (1993). Cells cultured on collagen-coated glass coverslips were fixed in 3% paraformaldehyde for 5 min, washed with PBS and incubated with 0.025% NP-40 in PBS for 20 min at room temperature. After washing in PBS, then TBS pH 7.4, samples were incubated at 37°C for 1 h in 21-μl nick translation buffer (50 mM Tris pH 7.4, 10 mM $MgCl_2$, 50 μg/ml BSA) with 2 μl Digoxygenin-DNA labeling mix, and 2 μl DNA polymerase I (Amersham, 500 U/83 μl). Polymerase activity was stopped with 50 mM Tris, 20 mM EDTA, pH 7.4. Samples were washed extensively with TBS then with digoxygenin kit buffer 1 (100 mM Tris, 150 mM NaCl, pH 7.5). Samples were blocked for 30 min in buffer 1 with 2% sheep serum and 0.3% Triton X-100, incubated with anti-DIG alkaline phosphatase-conjugated Fab fragment at 1:1,000 in buffer 1 containing 1% sheep serum plus 0.3% Triton X-100 for 2 h at room temperature, and washed extensively in buffer 1 plus 0.3% Triton X-100. Color development was carried out in digoxygenin kit buffer 3 (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) in the presence of 2.4 mg/ml levamisole with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate for approximately 5 min and stopped with TE. Controls carried out without either DNA polymerase or anti-DIG antibody were both negative. Samples were counter stained after colour development with Hoechst 33342, and mounted in Gelvatol.

Scanning Electron Microscopy

Cells plated on collagen-coated Thermanox coverslips (Nunc) were cultured in the presence or absence of NGF, then fixed in 2.5% glutaraldehyde in PBS for 1 h. Samples were dehydrated through graded acetones, critical point dried with the Polaron Critical Point Drier Apparatus E3000 using liquid CO_2, and coated in gold using a Polaron Series II Cool Splutter Coater. Samples were viewed with a Jeol scanning electron microscope JSM-T20.

Curve Fitting

Data was fitted to equations derived from a three state model using the Fig-P graph package non-linear curve fitting programme. The model follows the form:

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$

where *A*, *B*, and *C* are "healthy," "apoptotic," and "ghost" profiles, respectively, and A_0 is the total number of cells at time zero. The equations derived from the model are:

1. *Abbreviations used in this paper*: AO, acridine orange; SCG, superior cervical ganglion.

$$A_t = A_0.exp(k_1.t)$$
$$B_t = A_0.[k_1/(k_2-k_1)]\{exp(-k_1.t) - exp(-k_2.t)\}$$
$$C_t = A_0.\{1 - k_2.exp(-k_1.t)/(k_2-k_1) + k_1.exp(-k_2.t)/(k_2-k_1)\}$$

Death Commitment Assays

Newly isolated neurons were cultured immediately into NGF-free medium containing anti-NGF antibodies, then rescued after increasing intervals by addition of NGF (200 ng/ml final concentration) to the medium. Cell survival was measured 72 h after the last rescue time point, either by counting cells or by incorporation of Tran^{35}S-label or [^{35}S]methionine over 1-2 h into TCA precipitable material (Martin et al., 1988). Time zero was defined as the time at which cells were first incubated at 37°C, and percent survival was calculated as a percent of the survival obtained at the earliest rescue point. Deprivation and rescue of 4-d old cultures was carried out by the same method. For these experiments, cultures were simultaneously deprived of NGF at time zero exactly 4 d after plating. NGF rescue studies for 7–8-d-old cultures were carried out as described previously (Edwards et al., 1991): cultures were deprived of NGF at staggered intervals, and allowed to recover for exactly the same time before metabolic labeling. All NGF-deprivations were carried out in the presence of anti-NGF antibodies.

Results

Cell Death in Newly Isolated Neurons

Newly isolated neurons die within 24 h of plating unless provided with NGF (Chun and Patterson, 1977*a*). To investigate whether this death occurs by apoptosis, nuclease activity was assessed by extraction and gel electrophoresis of DNA. Dying neurons were found to contain fragmented DNA in multiples of approximately 185 base pairs, giving rise to the characteristic DNA "ladder" pattern of apoptosis (Fig. 1 *a*) (Wyllie, 1980). DNA ladders were detected in ethidium bromide stained gels by 10 h after plating, and reached a plateau of maximum intensity by 16 h. The high molecular weight band of DNA declined steadily with time, suggesting extensive DNA degradation. The medium in which dying cells were cultured was also found to contain DNA ladders, but no high molecular weight DNA (Fig. 1 *b*). As dying cells which had floated free from the culture substrate were first removed from this medium by centrifugation and pooled with the adherent cell population, it is possible that this extracellular DNA originated from substantial release of DNA or nuclear fragments from dying cells. NGF did not promote the survival of all newly isolated neurons (Chun and Patterson, 1977*b*; Saadat et al., 1989), and low levels of DNA fragmentation were also seen in the presence of NGF, with a similar time course to that found in NGF-deprived cultures.

To establish an assay for apoptosis in individual cells, chromatin condensation, a further hallmark of apoptosis, was assessed in un-fixed cells using the nucleic acid stain acridine orange (AO) (Fig. 2). AO stained neurons cultured with NGF had rounded nuclei containing finely dispersed chromatin with a delicate filamentous appearance, one (or two) nucleoli, and brightly stained cytoplasm, presumably due to the presence of RNA (Fig. 2 *b*). Initially most NGF-deprived neurons also gave this "healthy" staining pattern, but after increasing periods of NGF-deprivation the staining patterns shown in Figs. 2, *d* and *f* appeared. The cells shown in Fig. 2 *d* contain single or multiple bright, round clumps of condensed chromatin and a pale cytoplasm. The chromatin clumps in these "apoptotic" cells appeared to gradually decrease in size, resulting in palely staining "ghost" profiles shown in Fig. 2 *f*. These profiles were the three major AO

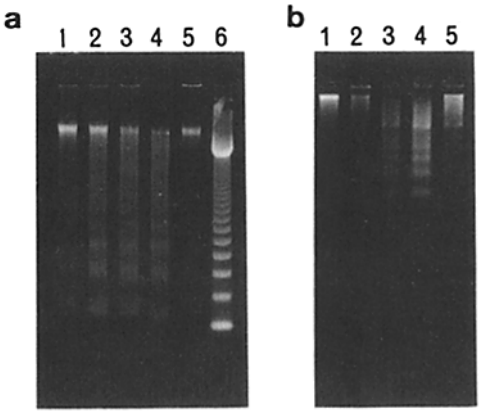

Figure 1. DNA fragmentation in newly isolated neurons. (*a*) Purified neurons from 10 SCG per condition were seeded onto collagen-coated dishes containing growth medium lacking NGF, containing 3% rat serum. Lanes *1–4* show DNA collected from cells at 10, 16, 19, and 24 h, separated on a 1.5% agarose gel. Lane *5* shows DNA from cells maintained for 24 h in the presence of 20 ng/ml NGF. Lane *6* shows a 123-bp DNA ladder. Equivalent cells stained with AO and ethidium bromide showed that at 10 h, 10% of the cells contained fragmented nuclei (2% ethidium bromide positive); at 15 h, 26% had fragmented DNA; at 19 h, 44% had fragmented DNA (9% ethidium bromide positive); and at 24 h, 49% had fragmented DNA (21% ethidium bromide positive) and 20% cells were ghosts. (*b*) Lane *1*, DNA from neurons cultured with NGF for 24 h; Lane *2*, DNA from culture supernatant of cells maintained with NGF. Lanes *3–5*, DNA from culture supernatants of cells seeded in the absence of NGF for 12, 24, and 36 h, respectively.

staining patterns observed in cultures of dying neurons. When compared with phase contrast images, healthy cells corresponded to the phase bright, smooth cells normally seen in cultures containing NGF (Fig. 2 *a*). Apoptotic cells were either phase bright or dark, often containing bright, refractive bodies (Fig. 2 *c*), whereas ghost cells were always phase dark with a rough surface (Fig. 2 *e*). No fragmentation of cells into apoptotic bodies was observed by light microscopy.

NGF-deprived neurons also showed roughening of the plasma membrane or blebbing typical of apoptotic cell death when viewed by scanning electron microscopy. Healthy neurons cultured with NGF were smooth and had neurites (Fig. 3 *a*), whereas NGF-deprived neurons exhibited varying degrees of blebbing (Fig. 3 *b*), or a totally collapsed membrane (Fig. 3 *b*, *arrow*).

To investigate the relationship between chromatin condensation and DNA fragmentation, the relative frequencies of the three AO staining profiles were determined after increasing intervals of NGF-deprivation. All cells with a diffuse nuclear staining were defined as healthy, all cells with visible clumps of condensed chromatin were defined as apoptotic and all cells that had palely stained cell bodies and no discernable nuclear staining were defined as ghosts. Because it was found that dying cells showed an increased tendency to float free from the culture substrate, the viscosity of the medium was increased with methyl cellulose which prevented cells from floating away from the base of the dishes. As shown in Fig. 4 *a*, the proportion of healthy cells declined within hours of the onset of culture. The proportion of apoptotic

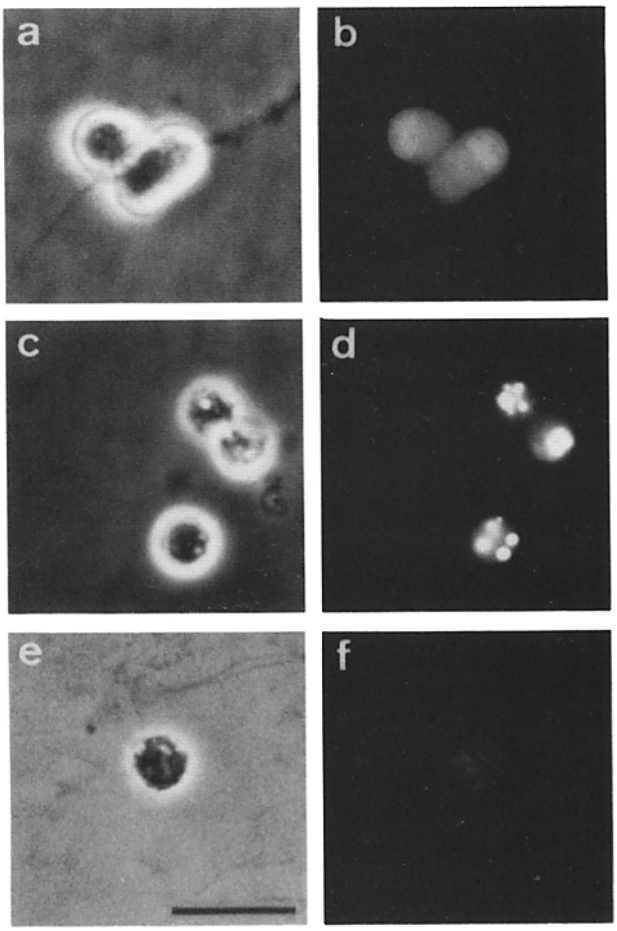

Figure 2. Neurons deprived of NGF can be classified into three distinct phenotypes by AO staining. (*a* and *b*) Healthy phenotype of cells maintained in NGF for 24 h; (*c* and *d*) apoptotic phenotype of cells deprived of NGF for 20 h; (*e* and *f*) ghost phenotype of cells deprived of NGF for 20 h. (*a*, *c*, and *e*) phase contrast micrographs; (*b*, *d*, and *f*) equivalent AO staining patterns. Bar, 25 μm.

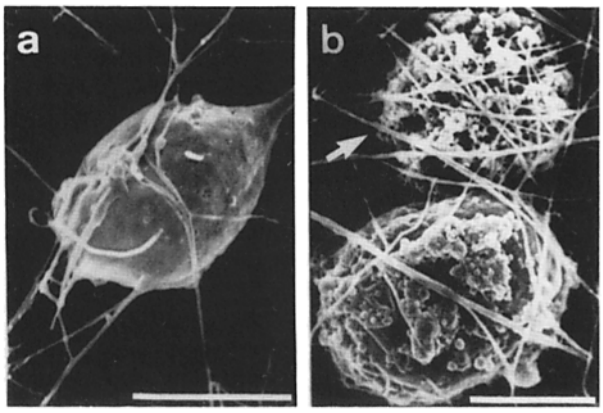

Figure 3. Scanning electron microscopy of neurons maintained in the presence (*a*) or absence of NGF (*b*) for 24 h. In panel *b*, bottom cell shows marked blebbing. Arrow points to a neighboring cell which has lost its plasma membrane. Bar, 5 μm.

cells increased until 20 h of NGF deprivation, and then reached a plateau (Fig. 4 *b*). When compared with the timing of DNA fragmentation it can be seen that the increase in the frequency of apoptotic cells correlates positively with the timing of increased intensity of DNA ladders. Thus cells which give the apoptotic staining profile are thought to be actively undergoing DNA fragmentation. The pattern of chromatin condensation shown in Fig. 2 *d* was highly characteristic of these neurons when deprived of NGF, and was never separable from the phenomenon of DNA laddering.

Because the cells did not enter the apoptotic phase simultaneously, nor remain in this state indefinitely, it could not be directly demonstrated that all the cells died in this manner. Thus to assess whether the data are consistent with all cells passing through an apoptotic phase during cell death, the data were fitted by nonlinear curve fitting to a model which postulates that ghosts are derived from apoptotic cells. This model is characterized by two apparent first order rate constants, k_1 and k_2 which define the transitions from healthy to apoptotic and from apoptotic to ghosts (see Materials and Methods for model and equations). The curves generated by the fit and the 95% confidence intervals are shown in Fig. 4. The values of the apparent rate constant k_1 derived independently from the healthy, apoptotic and ghost sets of data were very similar (0.066 ± 0.004, 0.054 ± 0.007 and 0.059 ± 0.09 h^{-1}, respectively, SE, $n = 14$) as were the independently derived values of k_2 (0.034 ± 0.005 and 0.035 ± 0.041 h^{-1} from apoptotic and ghost data, respectively). Using this model, the best fit, giving an extrapolated value of 99 ± 3.8% for the total number of cells at time zero (A_0 in the equation) was obtained when the fit was begun at 5 h, suggesting a small lag phase between before the beginning of the visible transition to the apoptotic state. From the values of k_1 and k_2, the half life of the healthy and apoptotic states was calculated as 11.6 and 19.8 h. The data are consistent with the majority of healthy cells passing through an apoptotic phase, before becoming ghosts. Alternative models postulating the transition of cells from the healthy to ghost state independently of the apoptotic state gave significantly poorer fit (using an F test for goodness of fit). However based on these models, the possibility that 10% or less of the cells change directly from healthy to ghost profiles could not be excluded.

During the late stages of death, dying neurons were observed to lose plasma membrane integrity, measured using trypan blue or ethidium bromide exclusion. As rat serum was recently reported to contain DNase I capable of producing DNA ladders (Peitsch et al., 1993), the possibility that DNA fragmentation and chromatin condensation were a result of exogenous nuclease activity was investigated. In cultures containing methyl cellulose to prevent cell loss into the medium, trypan blue exclusion assays showed that loss of plasma membrane integrity occurred well after the onset of chromatin condensation (Fig. 4 *d*), with a $t_{1/2}$ of ~24 h. This observation was confirmed on a cell by cell basis by co-staining unfixed cells with AO and ethidium bromide, which will not permeate intact membranes. Nuclei of permeant cells were stained red by ethidium bromide, displacing the green AO staining. In many apoptotic cells the condensed chromatin remained green after co-staining (~70% at 16 h), even in some cells which were close to becoming ghosts. Thus in most cells plasma membrane disruption was pre-

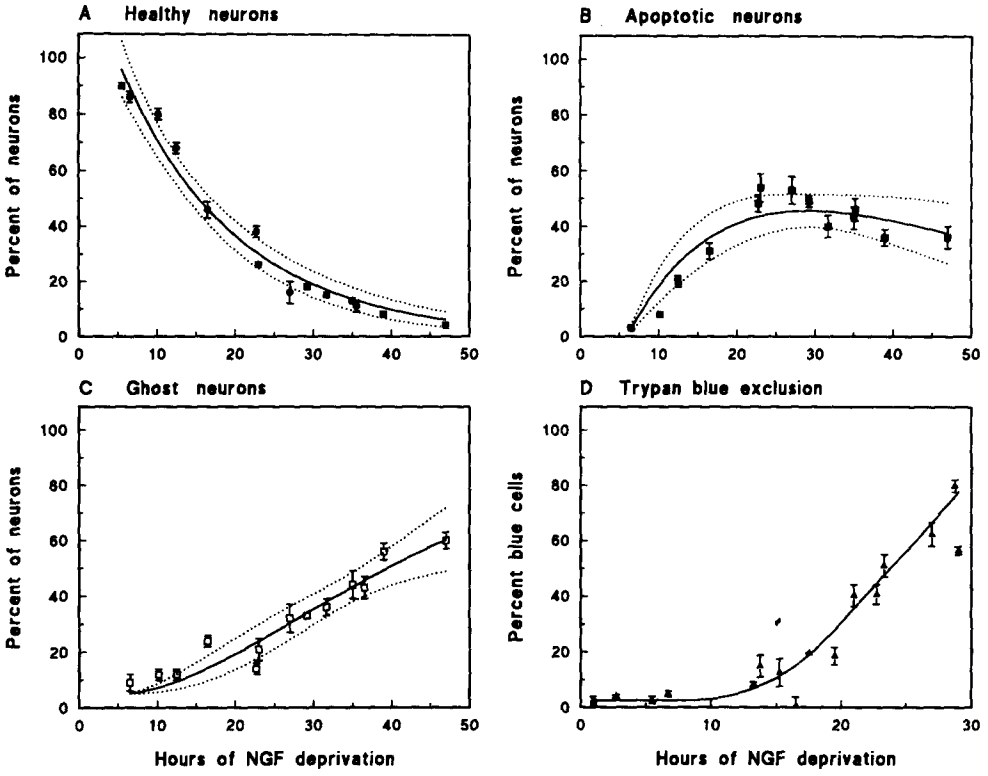

Figure 4. The majority of the newly isolated neurons die by apoptosis after NGF withdrawal. Neurons were deprived of NGF and at the times indicated, stained with AO and assigned to three groups using the morphological criteria shown in Fig. 2. (*A*) Healthy; (*B*) apoptotic; (*C*) ghosts. The data are derived from four independent experiments in which 3–4 replicate wells were scored, counting at least 100 cells per well (the average ± SD for each determination is shown by the error bars). The solid lines were derived from non-linear curve fitting of the data (using the average values) to three equations that predict the proportion of cells in each group according to a model that assumes the following transitions: healthy → apoptotic → ghosts, and the dotted lines show the calculated 95% confidence intervals. The fit was started from 5 h, and the equations are given in the Materials and Methods section. The parameters derived (± SE) are: (*A*) A_o = 99 ± 3.8%, k_1 = 0.066 ± 0.004 h^{-1}; (*B*) k_1 = 0.054 ± 0.007 h^{-1}, k_2 = 0.034 ± 0.005 h^{-1}; (*C*) k_1 = 0.059 ± 0.09 h^{-1}, k_2 = 0.035 ± 0.041 h^{-1}. (*D*) At the times indicated, cells were incubated in the presence of 0.03% trypan blue for 10 min at 37°C, and the percent of cells not able to exclude the dye are shown. (The line is drawn by eye).

ceded by chromatin condensation and (presumably) DNA fragmentation, which is not therefore due to influx of exogenous nucleases. Furthermore, the presence of rat serum in the culture medium was not required for DNA fragmentation in NGF-deprived neurons (Fig. 5 *a*). To determine if the rat serum in our culture media could induce DNA fragmentation in the cells, newly isolated neurons were permeabilized with 0.05% NP-40. Neither chromatin condensation, nor DNA ladders were observed (Fig. 5 *b*). A deliberate addition of 1 μg/ml DNase I, however, caused complete digestion of the nuclear DNA.

As in the case of older cultures (Martin et al., 1988; Edwards et al., 1991), the death and DNA fragmentation of newly isolated neurons deprived of NGF (Fig. 5 *c*) were prevented by the protein synthesis inhibitor cycloheximide. Cycloheximide, however, was unable to promote long term survival in the absence of NGF, and chronic exposure for more than 24 h was toxic to newly isolated cells.

Cell Death in Established Cultures

Although we had previously detected DNA fragmentation in neurons cultured with NGF for several days before NGF-deprivation (Edwards et al., 1991), the inability of other investigators to find evidence of DNA fragmentation in these cells suggested that only a small proportion of these neurons die by apoptosis. To investigate this on a cell by cell basis, chromatin condensation was examined using the DNA stain Hoechst 33342 in neurons cultured with NGF for 7–8 d be-

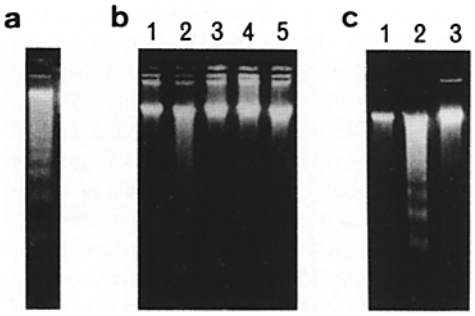

Figure 5. DNA fragmentation is not due to a DNase in the rat serum, and can be prevented by cycloheximide. (*a*) Purified, newly isolated neurons were plated in the absence of both NGF and rat serum and DNA was harvested after 20 h. (*b*) Purified, newly isolated neurons were plated in the presence (lane *1*) or absence of NGF (lane *2*) in the presence of 3% rat serum and DNA was collected after 15 h. In lanes *3–5*, cells were plated in the presence of 3% rat serum and NGF, and permeabilized with 0.05% NP-40, which caused the dissolution of the plasma membrane and left cells permeable to ethidium bromide. The nuclei were incubated in the presence of 3% rat serum and NGF under growth conditions for 12 (lane *3*), 15 (lane *4*), and 24 h (lane *5*). (*c*) Purified neurons were plated in the presence (lane *1*) or absence (lane *2*) of NGF, or in the absence of NGF and the presence of 2 μg/ml cycloheximide (lane *3*). DNA was harvested after 20 h.

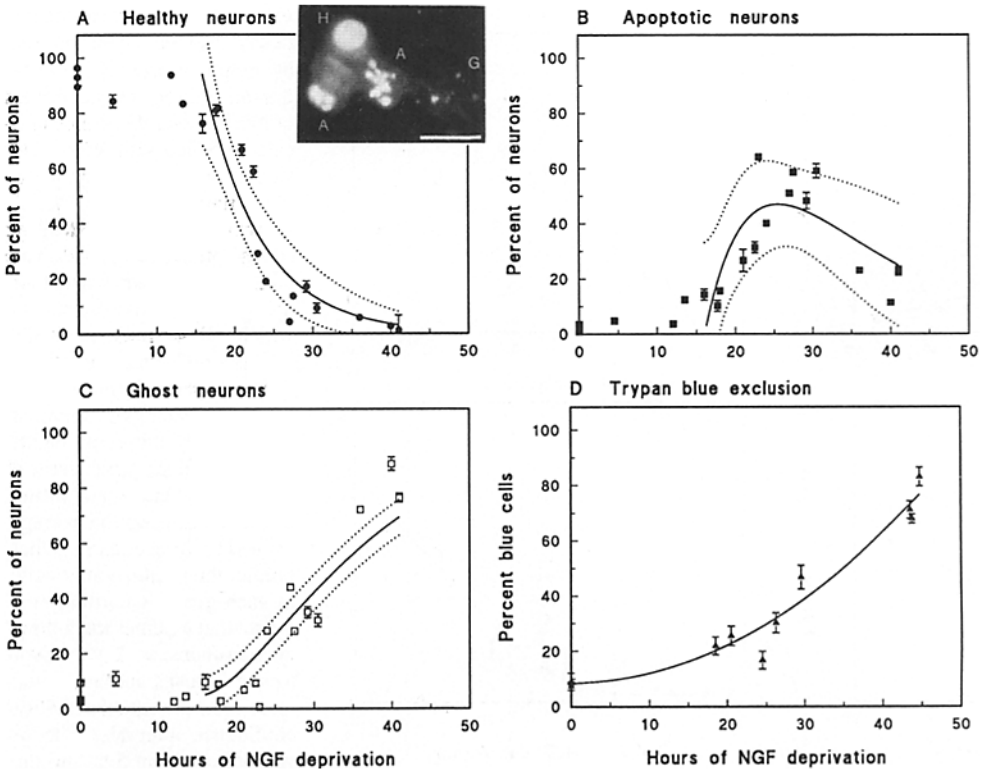

Figure 6. The majority of the 7–8-d cultured neurons die by apoptosis after NGF withdrawal. Neurons were deprived of NGF rapidly using ant-NGF antibody and at the times indicated, stained with Hoechst 33342 and assigned to three groups using the morphological criteria established in Fig. 2. The inset shows one healthy (*H*) cell, two apoptotic (*A*) cells and a ghost (*G*). The data are derived from four independent experiments (2 with 4 replicate wells, 2 with single wells), counting 100–300 cells per well (the average ± SD for each determination is shown by the error bars). The solid lines were derived from the non-linear curve fitting of the data (using the average values) to the three equations given in the Materials and Methods section (see legend to Fig. 4) and the dotted lines show the calculated 95% confidence intervals. The fit was started from 15 h. The parameters derived (± SE) are: (*A*) A_o = 107 ± 12%, k_1 = 0.134 ± 0.02 h^{-1}; (*B*) k_1 = 0.136 ± 0.05 h^{-1}, k_2 = 0.077 ± 0.03 h^{-1}; (*C*) k_1 = 0.13 ± 0.0 h^{-1}, k_2 = 0.06 ± 0.0 h^{-1}. (*D*) At the times indicated, cells were incubated in the presence of 0.03% trypan blue, and the percent of cells not able to exclude the dye are shown. (The line is drawn by eye).

fore NGF-deprivation. AO is unsuitable for the analysis of chromatin condensation in older cells as intense cytoplasmic staining masks the nucleus. Using the Hoechst dye three staining patterns, identical to those seen in newly isolated neurons, were observed (Fig. 6, *inset*). Few dying cells detached from the culture substrate presumably because of neurite attachment, thus the relative frequencies of the three profiles were determined after increasing intervals of NGF-deprivation in the absence of methyl cellulose (Fig. 6, *A-C*). Neurons cultured for 7–8 d died more slowly than newly isolated neurons. Approximately 15 h of NGF deprivation were required before any significant reduction in the proportion of healthy cells occurred, after which rapid loss of these cells was observed (Fig. 6 *A*). The frequency of apoptotic cells peaked at ~25 h after NGF-withdrawal (Fig. 6 *B*) after which it declined markedly, concomitantly with a rapid appearance of the ghost phenotype. From these observations, the older cells appeared to both enter and pass through the apoptotic state more rapidly than newly isolated cells and this was reflected in the greater apparent k_1 and k_2 values which were obtained when the data were fitted to the same model used for the newly isolated neurons. For the older cells, the estimated start point of the model had to be shifted to 15–16 h (giving an A_0 value of 107 ± 12%). The scatter of the data between experiments, especially notable in the apoptotic profile, was considerable. Nevertheless, the values of k_1 (0.134 ± 0.02, 0.136 ± 0.05, and 0.13 ± 0.05 h^{-1}, ± SE, n = 15) and k_2 (0.077 ± 0.03 and 0.06 ± 0.05 h^{-1}, ± SE, n = 15) derived from the fit to the data sets representing each cell state were closely matched, indicating a good fit to the model and suggesting that the majority of 7–8-d neurons pass through an apoptotic phase. The calculated $t_{1/2}$ for apoptotic cells was 11.5 ± 3.8 h. Again, alternative models postulating independent transitions from healthy to ghost states gave significantly poorer fit (F test for goodness of fit) but the possibility that 10% or less of the cells change directly from healthy to ghost profiles could not be eliminated.

Ethidium bromide and Hoechst 33342 co-staining of unfixed cells again showed that chromatin condensation preceded loss of membrane integrity in the majority of cells. Trypan blue exclusion also showed loss of membrane permeability occurring after chromatin condensation (Fig. 6 *D*), although it appears to occur more rapidly after condensation than in newly isolated neurons.

DNA extracted from NGF-deprived 7–8-d-old neurons or the medium in which these cells were cultured also produced DNA ladders in ethidium bromide stained gels (results not shown). These ladders showed variable reproducibility and many samples contained smeared DNA, even in healthy cultures. To confirm that DNA fragmentation does occur in the neurons undergoing chromatin condensation, we used an in situ nick translation assay to detect DNA breakage (Oberhammer et al., 1993) (Fig. 7). After NGF-deprivation, an increasing proportion of neurons stained positive for DNA breaks (Fig. 7 *b*). Co-staining with Hoechst 33342 showed that nearly all cells positive for DNA breaks also contained condensed chromatin (Fig. 7 *a*). Not all apoptotic cells showed positive staining, however the proportion of positive cells was greater than the proportion of ethidium bromide permeable cells in parallel studies, confirming the intracellu-

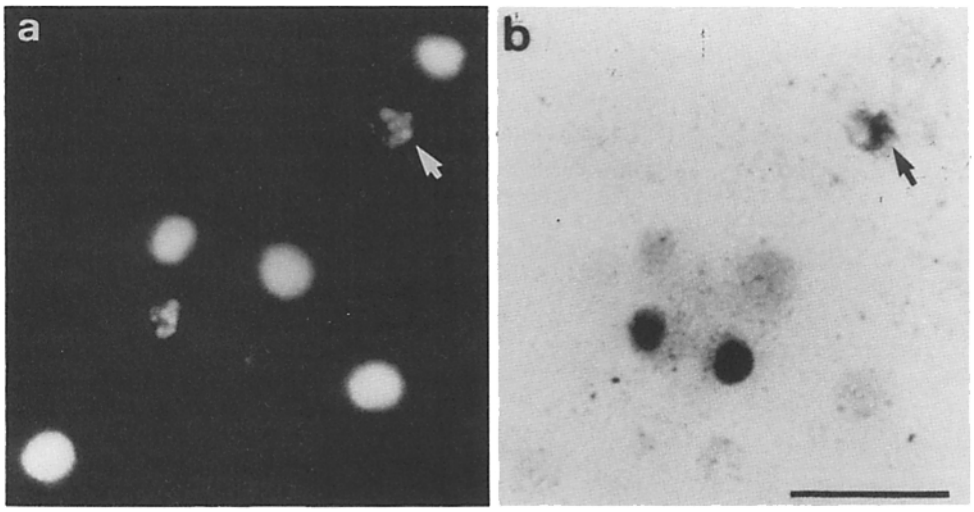

Figure 7. 7–8-d cultured neurons fragment their DNA during apoptosis. Neurons were fixed, permeabilized and incubated with DNA polymerase I in the presence of digoxygenin-labeled UTP for an in situ nick translation assay as described in the Materials and Methods section. (*a*) Neurons stained with Hoechst 33342 and viewed under UV light. Three cells show clear nuclear condensation with the typical apoptotic profile. (*b*) The same neurons are shown to be labeled after in situ nick translation. Note the exclusion of staining (*arrow*) from the center of the DNA "blobs." Bar, 50 μm.

lar origin of nuclease activity. Therefore nuclease activity is a feature of death in these 7–8-d neurons as well as in newly isolated neurons.

Commitment to Cell Death

As shown in Figs. 4 and 6, there is a greater delay between the time of NGF withdrawal and the onset of chromatin condensation in 7–8-d cultures compared to newly isolated neurons. To investigate whether the difference in this delay was due to slower activity of the death program preceding visible detection of cell death, or to an increase in the duration of NGF-deprivation required to induce death in the older cultures, the time at which neurons became committed to die was compared for both ages of culture. Neuronal commitment to cell death was determined by re-adding NGF to rescue neurons after increasing intervals of prior NGF-deprivation (Martin et al., 1988). Fig. 8 shows that for both culture ages, a phase of rapid commitment to cell death was preceded by a lag phase during which few cells became committed to die. This lag phase was prolonged by ~10 h for 7–8-d cultures as compared to newly isolated neurons. Thus there is an increase in the duration of NGF-deprivation required to induce the step which commits older neurons to die.

To determine whether this difference in the duration of NGF deprivation required to commit newly isolated and 7–8-d cultures to die was a sudden or gradual switch, death commitment was examined in 4-d-old cultures (Fig. 8 *A*). The rescue curve for 4-d neurons can be seen to be intermediate between that for 0 and 7–8-day old cultures. The 50% death commitment point is estimated to be 12, 20, and 26 h for 0, 4, and 7–8 day cultures, respectively. Thus under constant culture conditions the duration of NGF deprivation required to commit neurons to die increases gradually with increasing culture age. To examine whether this response to NGF-deprivation could also be modulated by culture conditions other than cell age, rat serum, which is not required for neuronal survival in the presence of NGF, was removed from cultures at the same time as NGF. It was found that co-deprivation followed by co-rescue with both NGF and rat serum reduced the 50% death commitment point from 26 to ~17 h (Fig. 8 *B*). Thus unidentified factors present in rat serum are also able to modulate the neurons' response to NGF-deprivation.

To examine how well the timing of death commitment correlates with onset of chromatin condensation, the rescue data (Fig. 8 *A*) re-plotted with the data showing the decline in healthy cells (Figs. 4 *A* and 6 *A*) are shown in Fig. 9, *A* and *B*. For 7–8-d cultures, there was a strong correlation between the fraction of neurons rescued with NGF and the remaining fraction of healthy cells throughout the entire time course of NGF deprivation (Fig. 9 *B*). For newly isolated neurons, death commitment was again initially correlated with loss of healthy cells, however, from 15 h of NGF deprivation onwards this correlation deteriorated. Commitment to cell death then appeared to occur more rapidly than loss of healthy cells, such that by 24 h ~20% of neurons still scored as healthy but no neurons were rescued by NGF. Thus, chromatin condensation in sympathetic neurons appears to proceed rapidly after death commitment although the match for newly isolated neurons is not complete.

Discussion

NGF-deprived Sympathetic Neurons Die by Apoptosis

From the data presented here, it is clear that NGF-deprived neurons undergo the processes of DNA fragmentation, chromatin condensation and membrane blebbing typically seen during apoptosis. DNA fragmentation occurs simultaneously in a sufficiently large population of cells, such that it can be detected using ethidium bromide staining of the DNA in agarose gels. The neurons were not observed to complete the apoptotic programme seen in some other cells by fragmenting into apoptotic bodies, but instead undergo plasma membrane breakdown. (The death of some newly isolated neurons in the presence of NGF may be due to loss of responsiveness to NGF due to damage incurred during cell preparation).

In newly isolated neurons deprived of NGF, DNA ladders were easily detected, using ~10^5 cells. Precise DNA ladders from 7–8-d cultures were more difficult to obtain. This may reflect the greater speed at which older cultures appear

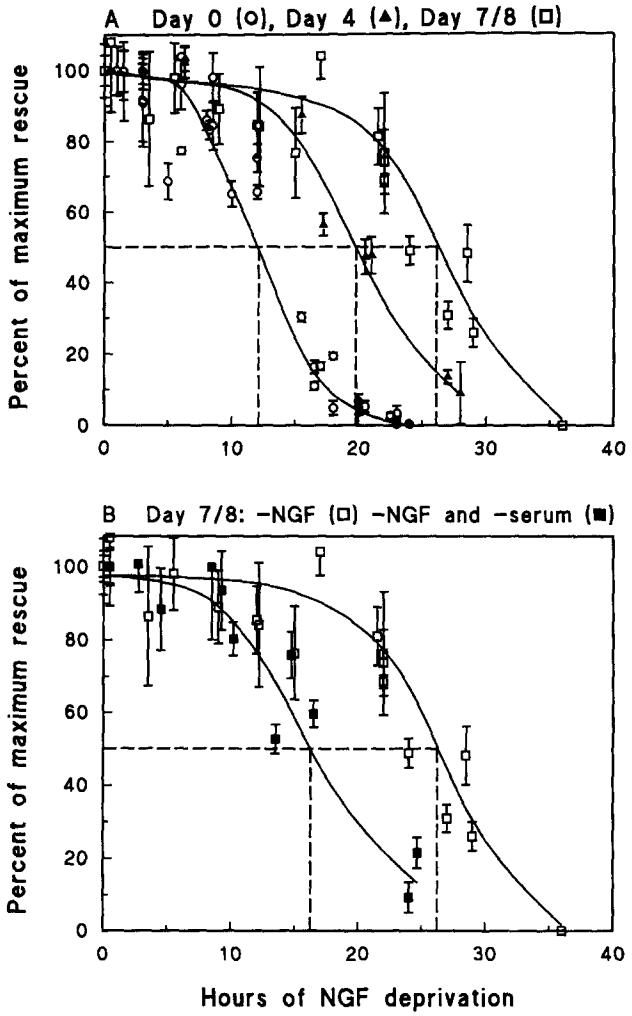

Figure 8. The timing of death commitment onset increases with time in culture. (*A*) Neurons were either plated in medium containing 3% rat serum in the absence of NGF (with anti-NGF antibody added) immediately after their isolation (o), or were cultured in the presence of 3% rat serum and 20 ng/ml NGF for 4 d (▲) or for 7–8 d (□) before deprivation of NGF at time zero by one wash in NGF-free medium followed by the addition of anti-NGF antibody. At the times shown by the points, NGF was added to 200 ng/ml and the proportion of neurons surviving 72 h later was calculated as described in the Materials and Methods section. Each point shows the mean ± SD of triplicate wells: (o—o), five experiments, two with both cell counting and [^{35}S]methionine labeling; (▲—▲), two experiments using [^{35}S]methionine labeling; (□—□), five experiments, one with both cell counting and [^{35}S]methionine labeling. The curves are drawn by eye and the estimated 50% death commitment points are shown as dashed lines. (*B*) 7–8-d cultures were deprived of NGF (□) or deprived of NGF and serum (■) and rescued with NGF or with NGF and serum, respectively, at the times after deprivation indicated (□—□ is the same data shown in *A*. ■—■, three experiments, mean ± SD of triplicate wells). The curves are drawn by eye and the estimated 50% death commitment point is shown as dashed lines.

to flux through the apoptotic state, or the more rapid deterioration of the plasma membrane relative to chromatin condensation. Nevertheless, DNA ladders were still detected using ethidium bromide staining and it is therefore not clear why Mesner et al. (1992) were unable to find any DNA ladders in sympathetic neurons, especially as we have detected DNA breaks using in situ nick translation in NGF-deprived neurons cultured under their conditions (results not shown). In our study, death of 7–8-d cultures by apoptosis was further confirmed by use of Hoechst 33342 staining, to show that cells condense their chromatin, and by demonstrating DNA fragmentation in situ using a nick translation assay.

The highly characteristic pattern of chromatin condensation revealed by AO or Hoechst 33342 staining provides a simple assay for apoptosis which can be applied to low cell numbers. By assigning cells to three states, defined phenotypically as healthy, apoptotic, and ghosts (Fig. 2), it was possible to follow the progress of apoptosis in the cell population. This data showed that the fraction of cells displaying chromatin condensation was never >50%. To assess whether the data were consistent with each cell passing through an apoptotic state, the data were fitted to a model which postulates that cells pass through three states by two apparent first order, sequential steps (Figs. 4 and 6). Alternative models which postulate that a significant fraction of ghost cells are derived directly from the healthy cells in one or two steps, rather than from apoptotic cells, were also tested (giving models with two or three apparent rate constants). These models were rejected either because of significantly poorer fits, or because they failed to fit the data. However, the possibility that 10% of the neurons pass directly into the ghost state could not be excluded. Thus the data is consistent with the majority of neurons at both culture ages dying by apoptosis. It should be noted, however, that this model does not provide a complete description of the death commitment time course, whose kinetics are much more complex (Tolkovsky, A. M., manuscript in preparation).

In a previous electron microscopical study on 1-wk cultures of sympathetic neurons dying after NGF deprivation, very few neurons were found which appeared to be dying by apoptosis (Martin et al., 1988). During the later stages of cell death (30–48 h), a minority of neurons showed substantial cell shrinkage and fragmentation considered by the authors to be apoptotic, however the majority of cells had undergone rupture of their plasma membranes. This is consistent with our findings that loss of cell integrity occurs rapidly in the older cells, and with the virtual absence of fragmenting cells. Although the earliest changes apparent in the cell soma (18–24 h) were nuclear shrinkage and irregularity, and a diffuse increase in chromatin density, cells with severe heterochromatin clumping were not seen. Whether the extent of nuclear changes recorded in this electron microscopical study is sufficient to account for our observations using the Hoechst dye is not yet known. Interestingly, Wright et al. (1983) show electron micrographs of chromatin clumping and nucleolar dispersion which may indicate apoptosis in the early stages of neuron degeneration in the intact rat SCG. It remains to be demonstrated, however, whether DNA fragmentation is involved in the natural death of these neurons in vivo.

Death Commitment Is Correlated with Chromatin Condensation

Chromatin condensation during apoptosis has been suggested to be a direct result of nuclease activity in the dying cell (Arends et al., 1991). However, chromatin condensation has also been observed in dying cells in the absence of inter-

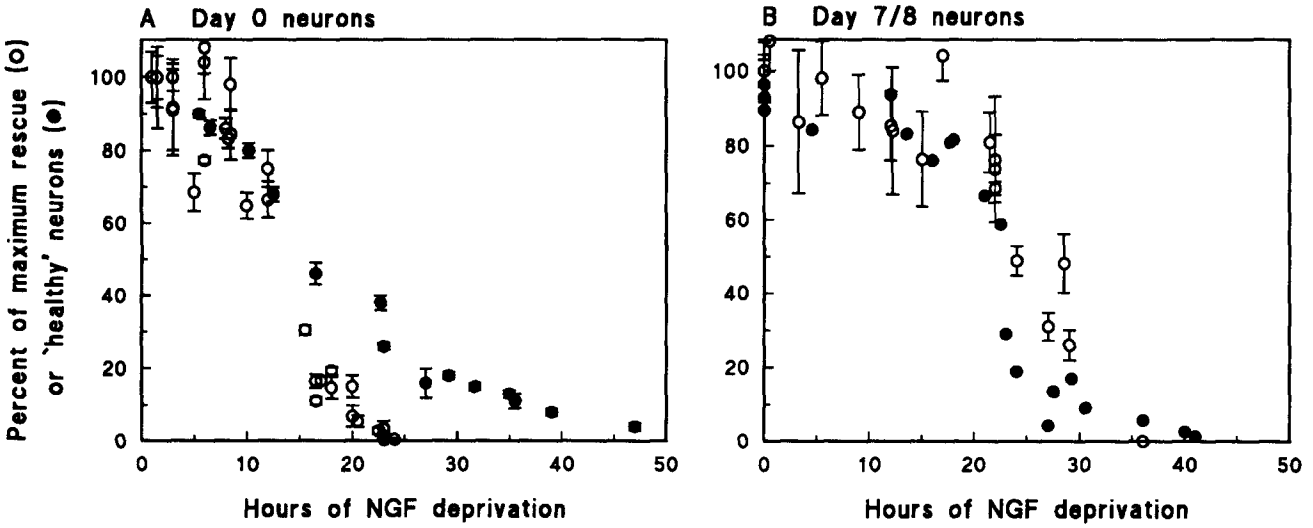

Figure 9. Comparison of timing of death commitment and decline of the healthy phenotype. In *A* the data shown in Fig. 8 *A* for newly isolated cells (○) is plotted together with the data from Fig. 4 *A* and in *B* the data shown in Fig. 8 *A* for 7–8-d cells (□) is plotted together with the data from Fig. 6 *A*.

nucleosomal DNA fragmentation (Oberhammer et al., 1993; Cohen et al., 1992). In 7–8-d cultures, some cells with condensed chromatin were seen in which no labeling with DNA polymerase was detected. This may be due to chromatin condensation preceding DNA fragmentation as suggested by Oberhammer et al. (1993), or instead to limited access for DNA polymerase I in highly condensed areas of chromatin. In support of the latter, cells positive for DNA breaks often showed staining around the edges of the chromatin clumps (Fig. 7, *arrow*). Alternatively, this may also reflect preferential sites of nuclease activity in areas of less condensed chromatin. Although the nature of the relationship between chromatin condensation and DNA fragmentation in sympathetic neurons is not clear, because of the strong correlation seen, chromatin condensation may be used to assess the timing of DNA fragmentation where the latter is known to occur.

Comparison of the time at which SCG neurons became committed to die (defined by the time that NGF can no longer rescue the cells) and the onset of chromatin condensation suggests that changes in DNA occur rapidly around the time of death commitment. In 7–8-d cultures, the time courses describing the loss of rescue by NGF and the loss of healthy cells (which matches the time of condensation of DNA) appeared to coincide quite closely ($t_{1/2}$ 23–25 h). In newly isolated neurons the ability to rescue the neurons with NGF coincides with the rate of decline in healthy cells up to 15 h, when nearly 60% of the newly isolated neurons were committed to die. However, this correlation appeared to break down later yielding fewer rescued cells compared with the apparent proportion of healthy cells, a difference of ~20% at 20 h. The possibility that this discrepancy was due to undercounting of AO stained ghost cells, leading to increasing overestimation of the healthy population, was eliminated by viewing cells under both AO and phase contrast. Thus the reason for this discrepancy is unknown. It may be that death commitment occurs prior to chromatin condensation and DNA fragmentation, the gap between these events increasing in the latter part of the time course. Alternatively this discrepancy could be due to NGF deprivation gradually reducing the efficiency of NGF signal transduction, such that it takes longer for NGF addition to promote a survival response. Neurons cultured with NGF for 7–8 d might have built up their NGF signal transduction machinery, for example, by increasing the number of *trk* receptors per cell (we find *trk* receptors on the cell bodies and over the entire neurite surface), by upregulation of *trk* expression, as shown for PC12 cells (Holtzman et al., 1992), or by increasing intracellular calcium as discussed below, such that its efficiency is not reduced by NGF-deprivation.

Whether nuclease activation is the step which commits sympathetic neurons to die, or is merely involved in removing the dead cell thus remains unclear. Our results demonstrate that chromatin condensation and presumably DNA fragmentation generally occur rapidly around the time of death commitment, but cannot prove that nuclease activity is, or is not, the critical step. Although purported nuclease inhibitors such as aurintricarboxylic acid can prevent the death of these neurons (Batistou et al., 1991; Edwards, S. N., unpublished observations), it cannot be assumed that this effect is solely due to nuclease inhibition. Furthermore, instances where nuclease activity was shown to be unnecessary for cell death have been described for both apoptosis (Cohen et al., 1992) and CTL-mediated programmed cell death where DNA-fragmentation normally occurs (Ucker et al., 1992). The de novo expression of death genes is unlikely to be the death commitment step, as NGF has been shown to rescue neurons after the putative onset of death protein synthesis (Edwards et al., 1991). Thus which event or combination of events commit the neurons to die remains to be determined. The timing of death commitment and chromatin condensation are closely matched such that even if chromatin condensation merely represents an early event in the execution program, the events that enable it to occur will be worth exploring further.

A Pre-commitment Phase Varies with Age of Culture

It is already known that newly isolated neurons die more rap-

idly after NGF deprivation than established cultures (Chun and Patterson, 1977*a*; Martin et al., 1988). Our results show that there is a lag period before apoptosis takes place which increases with time in culture and the rescue experiments suggest that this is due to an increase in the duration of NGF deprivation required to commit neurons to die. It is still not clear whether this is a function of absolute cell age, or is a result of culturing these cells in vitro. The presence of serum during NGF deprivation was also found to modulate the duration of this lag period. Whether the effects of serum and culture age are related remains to be investigated, however the finding that the response of neurons to NGF deprivation can be modulated in this manner is itself of interest.

One possible explanation for the effect of culture age is that endocytosis of the NGF-receptor complex provides a residual store of NGF signal, the extent of which is determined by neurite length (Claude et al., 1982). Other survival factors produced during the week in culture may also help delay death commitment after NGF withdrawal. Alternatively some intracellular factor which modulates responsiveness to NGF may change. For example, the basal intracellular calcium concentration has been shown to increase gradually as cultures age, and this has been suggested to result in NGF independence (Koike and Tanaka, 1991). The basal intracellular free calcium level of these neurons is also increased by acute exposure to rat serum (Tolkovsky et al., 1990). It will be interesting to examine whether the gradual increase in the duration of NGF deprivation required to trigger cell death in older cells is related to the mechanism by which these neurons lose NGF dependence.

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