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Gangliosides Rescue Neuronal Cells from Death after Trophic Factor Deprivation

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Serum-free cultures of PC12 cells have been used as a model system for studying neuronal death occurring after neurotrophic factor deprivation. In this system, NGF rescues cells from death and prevents apoptotic DNA fragmentation. We report here that GM1 also promotes long-term survival of naive and NGF-pretreated PC12 cells in serum-free medium and prevents internucleosomal cleavage of PC12 cell DNA. In contrast to NGF, GM1 does not promote neurite outgrowth or somatic hypertrophy. The survival effects of GM1 are concentration dependent, with maximal activity at 30–50 μM. Optimal promotion of survival is obtained with multiple additions of GM1. Asialo-GM1 and sialic acid do not mimic these actions, indicating a requirement for the intact GM1 molecule. Prevention of serum-free PC12 cell death is also obtained with di-, tri-, and tetrasialogangliosides. The ganglioside effects on survival and DNA fragmentation appear to be independent of macromolecular synthesis. GM1 is also effective under conditions in which cellular protein kinase C activity is downregulated by preexposure to high concentration of 12-*O*-tetradecanoylphorbol-13-acetate. Furthermore, GM1 promotes long-term survival of cultured rat sympathetic neurons after withdrawal of NGF. These findings complement prior observations that gangliosides protect cerebellar granule neurons from neurotoxicity caused by exposure to excitatory amino acids and extend the actions of gangliosides to rescue of neuronal cells deprived of neurotrophic factor support.

[Key words: PC12 cells, sympathetic neurons, cell death, GM1, DNA fragmentation, apoptosis]

During development of the nervous system, neurons undergo a period of "naturally occurring cell death" in which about 20–80% die (Hamburger and Oppenheim, 1982; Purves and Lichtman, 1985; Oppenheim, 1991). This appears, at least in part, to result from the failure of certain neurons to acquire an adequate supply of neurotrophic factors from their targets (Oppenheim, 1985; Barde, 1989). The requirement for trophic support has also been shown in the adult. For example, disruption of connections with targets results in neuronal degeneration and this process can be reversed by application of purified trophic factors (Hendry and Campbell, 1976; Rich et al., 1987). The presently best characterized neurotrophic factor is NGF (for review, see Levi-Montalcini, 1987; Thoenen et al., 1987). Both during development and in the adult, loss of sympathetic neurons occurs when endogenous NGF is removed by exposure to NGF antibodies (Levi-Montalcini and Booker, 1960; Gorin and Johnson, 1979, 1980). The survival-promoting actions of NGF can also be observed *in vitro* (Levi-Montalcini and Angeletti, 1963; Martin et al., 1988). For instance, sympathetic neurons can be maintained in culture if NGF is present but die after NGF withdrawal. The mechanisms by which NGF and other trophic factors promote survival are not yet known, nor is it clear by which mechanisms neurons die.

Recently, it has been reported that the PC12 line of rat pheochromocytoma cells can be used as a model system to study the mechanism by which neurotrophic factors prevent neuronal cell death (Rukenstein et al., 1991). PC12 cells respond to NGF by undergoing morphological and biochemical differentiation similar to that of sympathetic neurons (Greene and Tischler, 1976, 1982). Unlike sympathetic neurons, PC12 cells do not require NGF for survival in serum-containing medium. However, when serum is removed from the culture medium, PC12 cells die (Greene, 1978) and exhibit the characteristic pattern of DNA fragmentation associated with a type of cell death designated apoptosis (Batistatou and Greene, 1991). In this system, NGF rescues PC12 cells from death (Greene, 1978) and prevents DNA fragmentation (Batistatou and Greene, 1991). Because PC12 cells possess properties associated both with neuroblasts and mature neurons, serum-free cultures of these cells have also proved useful to screen various agents for their capacities to promote neuronal survival (Rukenstein et al., 1991).

There is growing evidence that substances in addition to macromolecular growth factors can affect neuronal survival (Martin et al., 1988; Rydel and Greene, 1988; Koike et al., 1989; Wallace and Johnson, 1989; Chang et al., 1990; Batistatou and Greene, 1991). One intensively investigated group of such agents is the gangliosides, a class of naturally occurring sialoglycosphingolipids (Ledeen, 1985; Varon et al., 1986; Cuello et al., 1989; Skaper et al., 1989a). In this regard, evidence is now accumulating that certain pathological forms of neuronal death (i.e., the death of cells deprived of oxygen or exposed to high levels of specific neurotoxic agents) can be reduced by pretreatment with gangliosides (Favaron et al., 1988; Skaper et al., 1989b; Facci et al., 1990; Karpiak et al., 1990; Manev et al., 1990; Seren et al., 1990; Nakamura et al., 1992). In the present study we employed PC12 cells in serum-free medium and cultured sympa-

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thetic neurons deprived of NGF to test whether the actions of gangliosides also extend to rescue of neuronal cells deprived of trophic factor support.

Materials and Methods

Materials. Mouse NGF was prepared from adult male submaxillary glands as previously described (Mobley et al., 1976) and was used at a concentration of 100 ng/ml. Gangliosides GM1, GD1b, GT1b, asialo-GM1 and neuraminic acid were purified according to methods described by Tettamanti et al. (1973) and were provided by Fidia Research Laboratories (Abano Terme, Italy). The purity of the compounds was >99% as determined by high-performance thin-layer chromatography. Gangliosides were dissolved in PBS at a concentration of 10^{-2} M and diluted to the desired concentration directly in the culture medium. All other reagents were purchased from Sigma.

Cell culture. PC12 cells were cultured as previously described on collagen-coated dishes in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum (Greene and Tischler, 1976). For the experiments in serum-free medium, cells were extensively washed in serum-free RPMI 1640 as previously described (Batistatou and Greene, 1991; Rukenstein et al., 1991).

For cell survival experiments, washed cells were resuspended in RPMI 1640 medium and plated in 0.5 ml at a density of 8–10 × 10^4 per well in 24-well plastic culture dishes previously coated with rat tail collagen. To feed, but to avoid loss of floating cells, fresh medium (0.2 ml) was added to the cultures on days 1, 5, and 10. For experiments involving "primed" PC12 cells, cultures were pretreated for 1–2 weeks with NGF in RPMI 1640 medium supplemented with 1% heat-inactivated horse serum. The cells were then washed and passaged into serum-free RPMI 1640 medium as described above.

Primary cultures of dissociated sympathetic neurons were prepared from the superior cervical ganglia of postnatal day 2 or 3 rats (Lee et al., 1980). Cells were plated at a density of approximately 0.4 ganglion per well in 24-well plastic culture dishes precoated with collagen. Cultures were grown in RPMI 1640 culture medium supplemented with 10% heat-inactivated horse serum and 100 ng/ml NGF. Fluorodeoxyuridine (10 μM) and uridine (10 μM) were added on days 1–3 and 5–7 to eliminate non-neuronal cells. To effect rapid and total NGF deprivation, the cultures were washed three times with RPMI 1640 medium containing 10% horse serum and then maintained in the same medium (0.5 ml/well) in the presence of affinity-purified anti-NGF polyclonal rabbit antibodies (provided by Fidia Research Laboratories) at a dilution of 1:250 with or without different concentrations of GM1. At this dilution, the anti-NGF antibodies completely antagonize the effect of NGF (20 ng/ml) when tested on neurite regeneration of NGF-primed PC12 cells. One day later, an aliquot of concentrated GM1 was added to the medium to bring about a doubling of the original GM1 concentration. Thereafter, cultures were fed every 2 d.

Cell counts. To avoid loss of floating cells, the culture medium was removed, centrifuged (500 × *g*, 5 min), and replaced with 0.2 ml of a solution that lyses the cell membrane but leaves the nuclei intact (Soto and Sonnenschein, 1985). The nuclei were counted in a hemacytometer. Counts were performed on triplicate wells and are presented as means ± SEM. The results are presented relative to the cell number initially plated per well (designated as 100). For sympathetic neurons, neuronal cell number was determined by strip counts (Greene et al., 1990). The neuronal cell bodies, as seen by phase-contrast microscopy, are round and phase bright and thus are easily distinguishable in the cultures. Cells in triplicate wells were scored and the counts are presented as means ± SEM. The results are presented relative to the cell number in NGF-treated cultures (designated as 100).

DNA fragmentation. Experiments were performed by minor modification of the techniques described by Edwards et al. (1991) and Batistatou and Greene (1991; unpublished observation). In brief, PC12 cells were washed and plated (4–8 × 10^6 per 100 mm dish) in RPMI 1640 medium with or without additives. After incubation for 3 hr at 37°C, the cells were triturated off the substrate and centrifuged at 800 × *g* for 5 min, and the pellet washed twice with ice-cold PBS (Ca^{2+}/Mg^{2+}-free). Soluble DNA was extracted and resuspended in TE buffer (10 mM Tris, pH 8; 1 mM EDTA). Subsequently the samples were incubated with 50 mg/ml DNase-free RNase (Boehringer-Mannheim) at 37°C for 30 min. DNA samples were subjected to electrophoresis on a 1.2% agarose gel, blotted onto Gene Screen Plus Membrane (Dupont/New England Nuclear) and analyzed with radioactive probes from digested total PC12 cell genomic DNA. Probe labeling was performed according to Sambrook et al. (1989) and hybridization according to the manufacturer's protocol (Dupont/New England Nuclear).

Results

GM1 promotes long-term survival of serum-deprived PC12 cells

To test the ability of GM1 to prevent PC12 cell death after growth factor deprivation, cultures were extensively washed and plated in serum-free RPMI 1640 medium with no additives, NGF, or ganglioside GM1. As shown in Figure 1*A*, about half of the untreated cells died in RPMI 1640 medium within one d and most were dead by five d. In contrast, GM1, like NGF, promoted the long-term (2 weeks) survival of PC12 cells in serum-free medium. GM1 did not, however, cause a small increase in cell number as is sometimes observed with NGF (Greene, 1978; Rukenstein et al., 1991). GM1 was added 1 hr after plating and optimal survival was obtained by refeeding on days 1, 5, and 10. Although quantification was not systematically carried out, good survival of the GM1-treated cells was observed up to three weeks in serum-free medium. The GM1 effect is concentration dependent (Fig. 1*B*), with maximal activity at 30–50 μM and an ED_{50} of approximately 10 μM. Similar effects were also obtained with 50 μM GD1b, GT1b, and GQ1b (data not shown), which differ from one another in number of neuraminic acid residues (two, three, and four, respectively). In contrast, asialo-GM1 and neuraminic acid (Fig. 1*C*) were inactive, indicating a requirement for the intact ganglioside molecule. The survival-promoting effects of GM1 persisted three d after the cultures were washed and switched to RPMI 1640 medium alone. However, under such conditions the cells died within seven d.

As shown in Figure 2, the GM1-treated cells, although smaller than those treated with NGF, were phase bright, and in contrast to those with no additives, the cultures did not contain debris. GM1, unlike NGF, did not promote neurite outgrowth or increase in cell size. At GM1 concentrations above 30 μM, the cells attached more loosely to the collagen substratum than in the presence of NGF or serum. When serum and NGF were readded to 7 d GM1-treated cultures, the cells increased their size, grew neurites, and attached more firmly to the substratum.

As shown in Figure 3, the survival-promoting action of GM1 was not limited to "naive" PC12 cells, but extended to "NGF-primed" cells pretreated with NGF in serum-containing medium and then subcultured without NGF in serum-free medium. As with naive cells, GM1 affected survival at concentrations between 10 and 50 μM; however, unlike NGF, it did not promote neurite regeneration.

GM1 prevents PC12 cell DNA fragmentation

Previous studies have shown that PC12 cells cultured in serum-free RPMI 1640 medium for 3 hr or more exhibit a pattern of internucleosomal DNA fragmentation characteristic of apoptotic cell death (Batistatou and Greene, 1991). Moreover, this fragmentation is prevented by NGF. To determine whether GM1, in addition to promoting cell survival, inhibits the fragmentation of DNA, PC12 cells were deprived of serum and incubated for 3 hr in the presence of either GM1, NGF, asialo-GM1, neuraminic acid, or no additives. Figure 4 shows that GM1, like NGF, prevented the internucleosomal cleavage of PC12 cell DNA. In contrast, the "ladder-like" pattern of DNA fragmen-

tation was not prevented when the cultures were incubated with asialo-GM1, neuraminic acid, or no additives.

The effect of PKC downregulation on the ability of GM1 to promote survival and prevent DNA fragmentation

A correlation has been described in cerebellar granule neurons between GM1-induced inhibition of protein kinase C (PKC) translocation and prevention of glutamate neurotoxicity (Favaron et al., 1988). We therefore tested whether PKC plays a role in the effect of GM1 in our system. PC12 cultures were pretreated with 1 μM 12-*O*-tetradecanoylphorbol-13-acetate (TPA) for 3 d in serum-containing medium, and then exposed to either GM1 or no additives in serum-free medium containing TPA (1 μM) and tested for cell survival and appearance of DNA fragmentation. Treatment of PC12 cells with this concentration of TPA has been reported to induce a significant downregulation of PKC levels (Matthies et al., 1987). As shown in Figure 5, the extents of cell death (1 week) and DNA fragmentation (3 hr) were unaffected by TPA pretreatment. Moreover, despite the TPA pretreatment and PKC downregulation, GM1 retained the ability to prevent both cell death and DNA fragmentation.

Role of macromolecular synthesis

The ability of GM1 to prevent DNA fragmentation was also tested in the presence of inhibitors of protein and RNA synthesis. For this purpose, we used cyclohexamide at a concentration known to inhibit protein synthesis by approximately 90% (Greenberg et al., 1986) and camptothecin at a concentration that inhibits PC12 cell RNA synthesis by 85% (Burstein and Greene, 1978). PC12 cells were preincubated with the inhibitors for 1 hr in serum-containing medium, and then deprived of serum by washing and incubated for 3 hr in RPMI 1640 with the inhibitors in the presence or absence of GM1. As shown in Figure 6, the inhibitors did not substantially prevent GM1 from inhibiting internucleosomal DNA cleavage. Thus, the ganglioside effect on DNA fragmentation appears to be independent of macromolecular synthesis.

GM1 promotes long-term survival of sympathetic neurons after NGF deprivation

Sympathetic neurons established *in vitro* in the presence of NGF die after NGF withdrawal (Levi-Montalcini and Angeletti, 1963; Martin et al., 1988). Since sympathetic neurons share many properties with PC12 cells, we tested whether GM1 is able to rescue them from cell death after NGF deprivation. Neonatal rat superior cervical ganglionic neurons were cultured for seven d with NGF in serum-containing medium and then deprived of the factor by treatment with anti-NGF antibodies (anti-NGF) in the presence or absence of GM1. Before deprivation and during the initial phase of anti-NGF treatment, neurons maintained phase-bright, round perikarya and a dense neurite network (Fig. 7*A*). After four d of treatment with anti-NGF alone, both the neuronal cell bodies and neurite network underwent marked degeneration (Fig. 7*B*); by nine d only neuritic debris was present (Fig. 7*E*). In contrast, GM1 rescued a substantial portion of the neurons from NGF deprivation. Although smaller

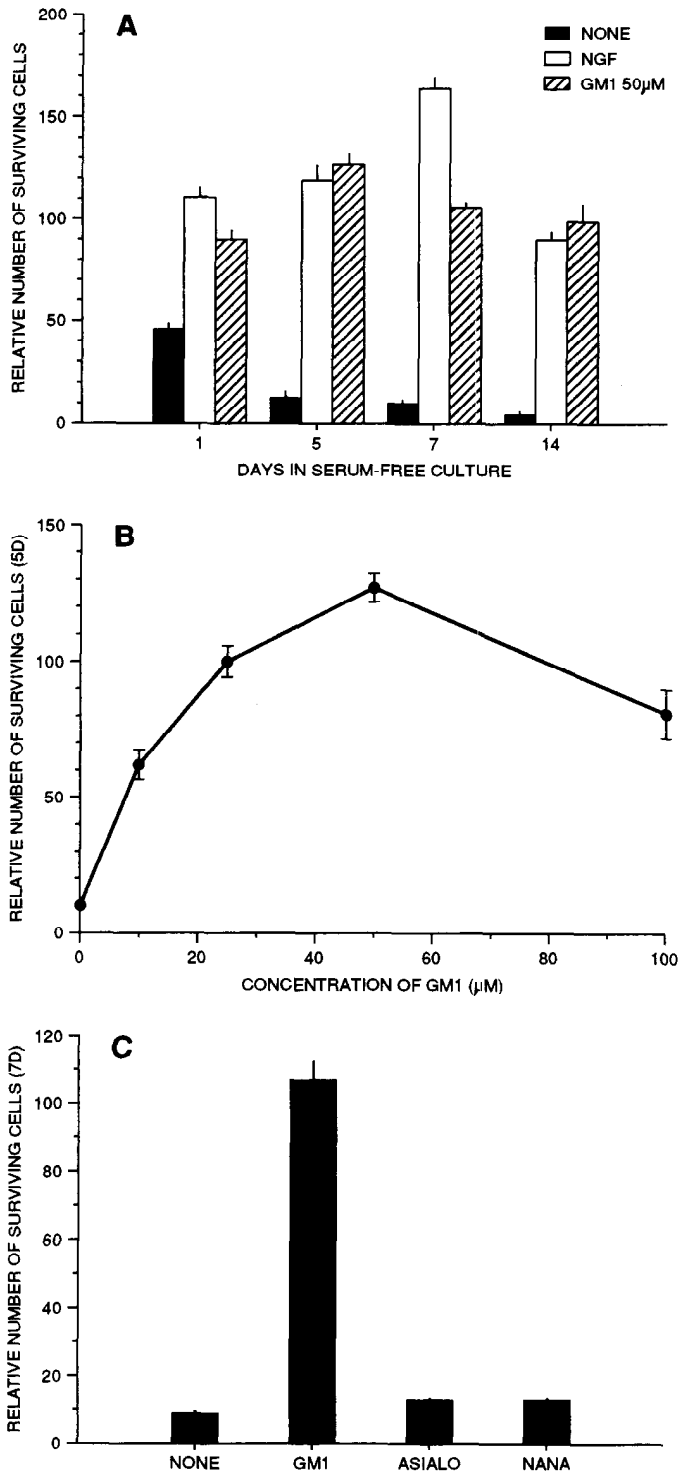

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Figure 1. Effect of GM1 on long-term survival of PC12 cells in serum-free medium. Cells were washed and plated in 0.5 ml of RPMI 1640 medium containing either no additive or the indicated agents. On days 1, 5, and 10 (if applicable) the cultures received 0.2 ml of the same medium. Viable cells were counted on the indicated days as described in Materials and Methods. The number of surviving cells is presented relative to the number initially plated (8–10 × 10^4; designated 100). Error bars represent SEM (n = 6). *A*, Time course of GM1 and NGF effects. *Solid bars* represent control cultures in the absence of additive (*NONE*); *open bars* represent cultures maintained with NGF (100 ng/ml); *hatched bars* represent cultures treated with GM1 (50 μM). *B*, Dose–response curve for GM1 (5 d in serum-free medium). *C*, Molecular specificity of the GM1 effect (7 d in serum-free medium). Concentrations were 50μM for *GM1*, asialo-GM1 (*ASIALO*), and neuraminic acid (*NANA*). Comparable results were achieved in three independent experiments.

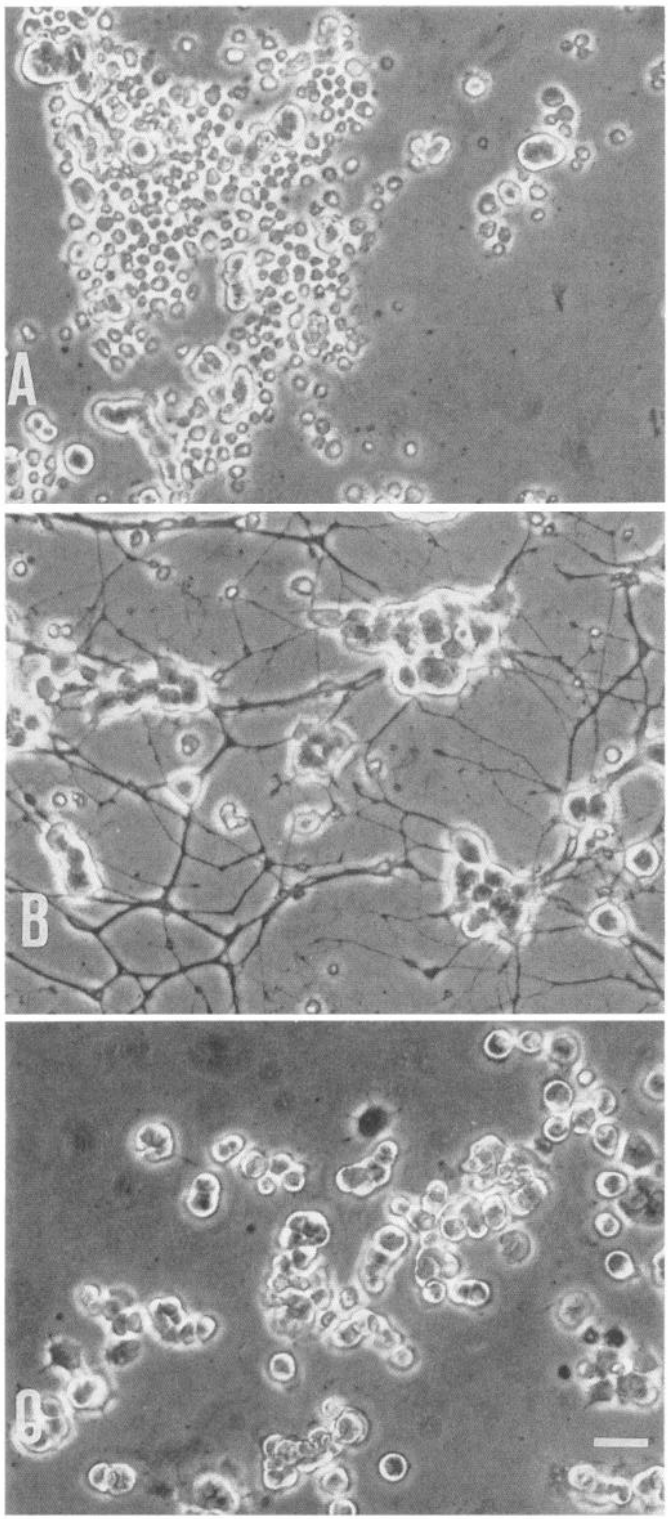


Figure 2. Morphology of PC12 cells maintained in serum-free medium with or without NGF or GM1. PC12 cells were maintained for seven days in serum-free RPMI 1640 medium supplemented with either no additive (*A*), 100 ng/ml NGF (*B*), or 50 μM GM1 (*C*). Cells were washed and plated in RPMI 1640 medium as described in Materials and Methods. Phase-contrast optics. Scale bar, 13.5 μm.

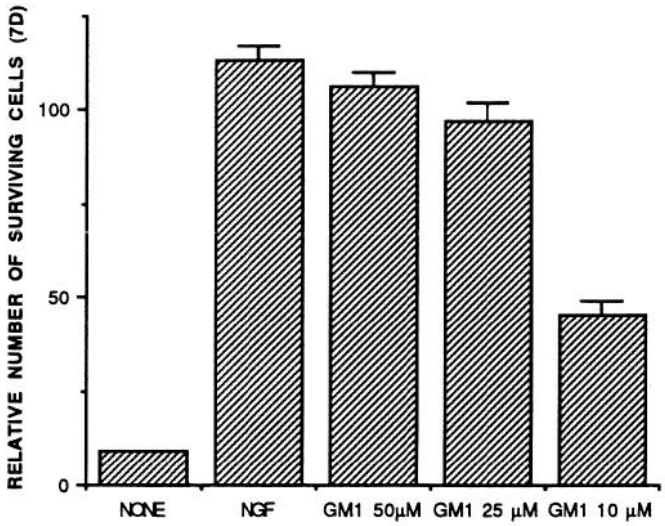


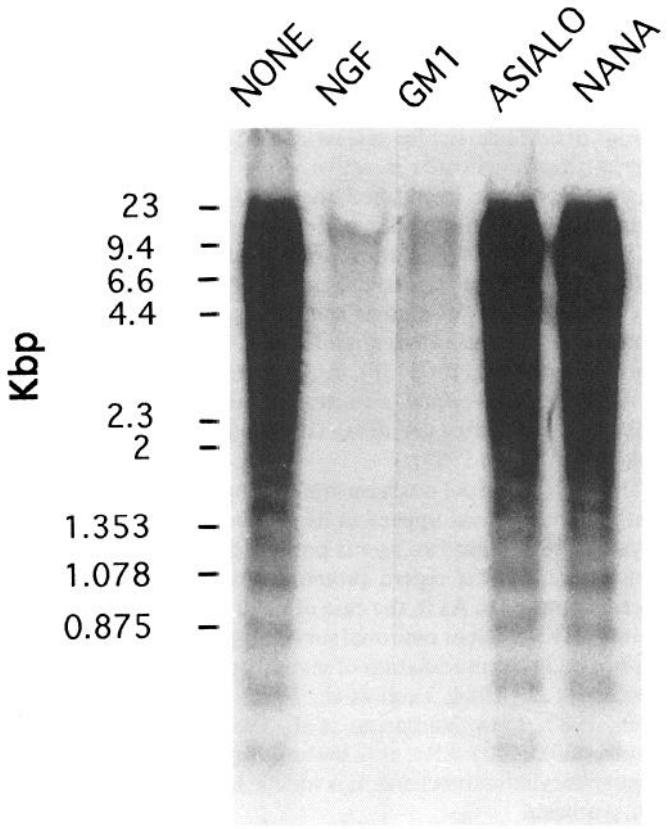
Figure 3. Effect of GM1 on long-term survival of NGF-primed PC12 cells in serum-free medium. Cells were pretreated with NGF in RPMI 1640 medium containing 1% heat-inactivated horse serum for 10 d, and then washed and passaged into serum-free RPMI 1640 medium and maintained for seven d with the indicated additives (*NONE*, no additive). The cultures were fed on days 1 and 5 by adding 0.2 ml of the same medium. Cell numbers are expressed relative to those initially plated (8–10 $\times$ 10^4). Concentration for NGF was 100 ng/ml. Error bars represent SEM (n = 3). Comparable results were achieved in three independent experiments.

than NGF-treated controls, the GM1-treated neuronal cell bodies remained phase bright and appeared to be healthy (Fig. 7*C,F*). Neurite degeneration, although present, occurred more slowly than in the absence of GM1. Readdition of NGF to cultures maintained with GM1 for nine d resulted in the reappearance of healthy neurites and an increase of cell size (data not shown). A quantitative evaluation of the number of surviving neurons in the variously treated cultures is given in Figure 8. At four and 11 d, the numbers of surviving neurons in cultures treated with 120 μM GM1 and anti-NGF were about 75% and 50%, respectively, of those maintained with NGF and not exposed to anti-NGF. At four d of NGF deprivation, similar results were obtained using GM1 at 200 μM, while half-maximal effects on survival were observed at approximately 60 μM. In some experiments, addition of GM1 was delayed for 24 hr after addition of anti-NGF. Even in this case, there was a substantial rescue from cell death (data not shown).

In contrast to its effects on established cultures, GM1 did not support survival of rat sympathetic neurons when applied directly after dissociation and in the absence of NGF. These findings suggest that the maturity or the developmental stage of the neurons might be critical factors for their responsiveness to GM1.

Discussion

Neuronal cell death occurs not only during development but also as a consequence of insult or degenerative disorders (Appel, 1981; Ellis et al., 1991; Oppenheim, 1991). Glutamate-induced toxicity has been implicated to play a critical role in the neuronal loss associated with acute insults such as hypoglycemia and hypoxia (Olney et al., 1989; Choi and Rothman, 1990). Accumulating evidence indicates that neuronal death occurring as a consequence of protracted abusive activation of excitatory amino acid receptors can be reduced by treatment with gangliosides



(Favaron et al., 1988; Skaper et al., 1989b; Facci et al., 1990; Karpiak et al., 1990; Seren et al., 1990). In this context, gangliosides have been termed "receptor abuse–dependent antagonists" because their positive effects on glutamate neurotoxicity occur without inhibition of transmitter function (Manev et al., 1990), but reside in an antagonism of the consequences (i.e., protracted elevation of free cytosolic Ca^{2+} and sustained activation of PKC) of abusive glutamate receptor stimulation (Favaron et al., 1988; De Erausquin et al., 1990; Milani et al., 1991). With regard to neurodegenerative disorders, a recent report indicates that GM1 can protect neurons of the substantia nigra from death caused by exposure to the specific neurotoxic agent 7-methyl-4-phenyl-1,2,3,6-tetrahydropyzidine (MPTP) (Schneider et al., 1992).

The present study extends the actions of gangliosides to rescue of neuronal cells deprived of neurotrophic factor support. Using serum-free cultures of PC12 cells as a model system, we showed that GM1, like NGF, promotes long-term survival and prevents

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Figure 4. Prevention of internucleosomal cleavage of DNA by GM1. PC12 cells were washed free of serum and cultured in the presence of either no additive (*NONE*), 100 ng/ml NGF (*NGF*), 50 μM GM1 (*GM1*), 50 μM asialo-GM1 (*ASIALO*), or 50 μM neuraminic acid (*NANA*) for 3 hr. Soluble DNA was extracted from the cells, separated on a 1.2% agarose gel, blotted onto Gene Screen Plus membrane, and analyzed as described in Materials and Methods. Comparable results were achieved in two independent experiments. Molecular size markers at *left* are in kilobase pairs (*Kbp*).

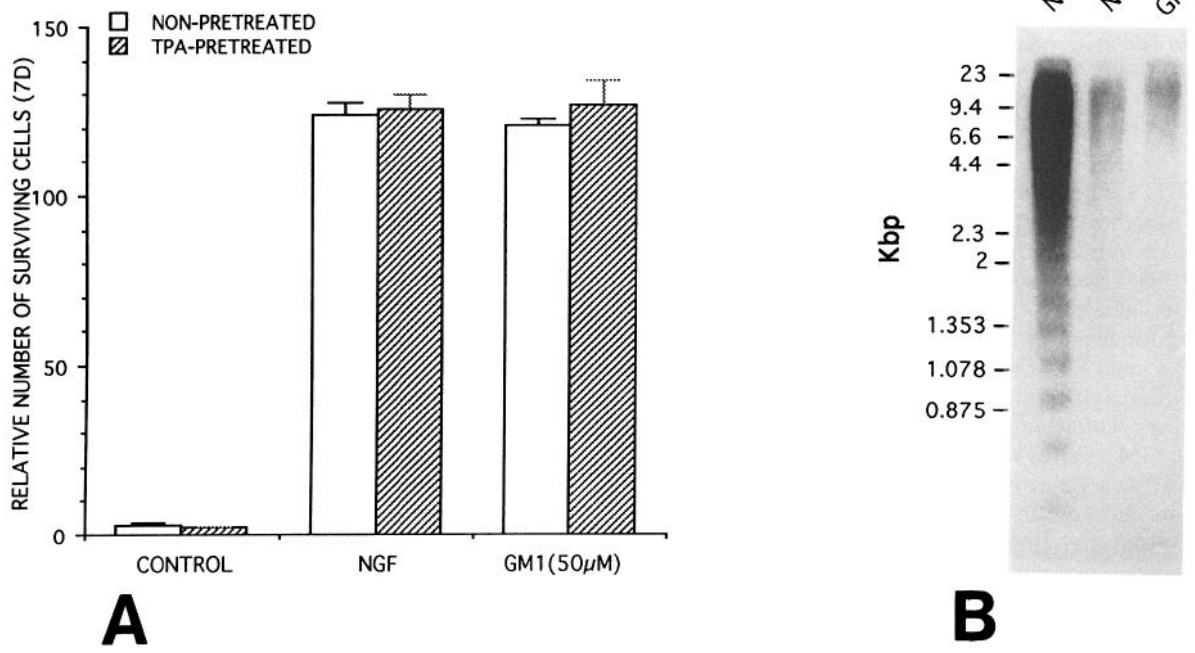

Figure 5. GM1 prevents both cell death (*A*) and DNA fragmentation (*B*) in PC12 cells pretreated with phorbol ester. PC12 cells were pretreated for three d with or without 1 μM TPA in serum-containing RPMI 1640 medium, and then washed and passaged into serum-free RPMI 1640 medium in the presence of 1 μM TPA with or without 50 μM GM1 or NGF (100 ng/ml). *A,* On days 1 and 5 the cultures received 0.2 ml of the same medium. Cell numbers were determined after 7 d and are expressed relative to those initially plated (8×10^4). Error bars represent SEM ($n = 6$). Comparable results were achieved in two independent experiments. *B,* TPA-pretreated cells, after washing, were treated with 1 μM TPA (*NONE*), 1 μM TPA + 100 ng/ml NGF (*NGF*), or 1 μM TPA + 50 μM GM1 (*Gm1*) for 3 hr. Soluble DNA was extracted from the cells, separated on a 1.2% agarose gel, blotted onto Gene Screen Plus membrane, and analyzed as described in Material and Methods.

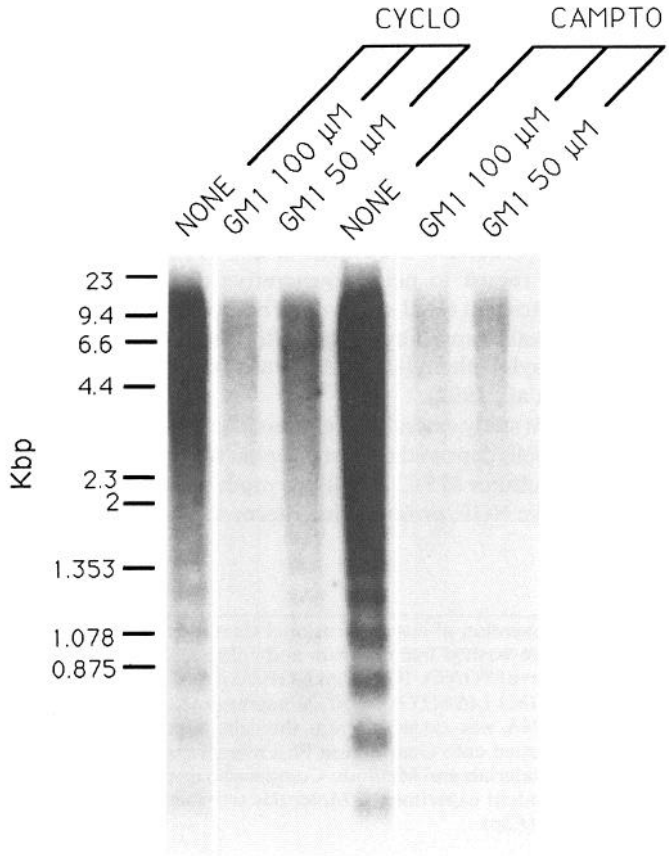

Figure 6. Role of inhibitors of protein and RNA synthesis in GM1-induced prevention of DNA fragmentation. PC12 cells were pretreated where indicated for 1 hr in serum-containing medium with 10 μg/ml cyclohexamide (*CYCLO*) or 20 μM camptothecin (*CAMPTO*). Cells were then washed and plated in RPMI 1640 serum-free medium with the indicated inhibitors with or without 100 or 50 μM GM1 for 3 hr. DNA fragmentation was analyzed as described in Materials and Methods.

apoptotic DNA fragmentation. These effects of GM1 are concentration dependent, are mimicked by different ganglioside species, and require the intact ganglioside molecule. We also found that GM1 promotes long-term survival of sympathetic neurons after NGF deprivation. In this system, the concentration needed for maximal effect was higher than that required for PC12 cultures. This might be related to the presence in the neuronal cultures of serum, which is known to affect the free ganglioside concentration (Tomasi et al., 1980).

Although GM1 shares with NGF the ability to prevent cell death, the two were not equivalent in their actions. Unlike NGF, GM1 did not promote PC12 cell neurite generation or regeneration and although it slowed degeneration of neurites in NGF-deprived neuronal cultures, it did not prevent this process. These findings confirm previous observations that separate mechanistic pathways are responsible for maintenance of survival and for promotion of neuronal differentiation (Greene, 1978; Greene et al., 1990; Rukenstein et al., 1991). Another major contrast between NGF and GM1 was that the ganglioside did not induce somatic hypertrophy of PC12 cells or sympathetic neurons. The difference in effects of NGF and GM1 also rules out the possibility that the actions of the ganglioside were due to potentiation of subthreshold levels of NGF produced or remaining within the cultures.

Although the mechanisms by which GM1 prevents neuronal cell death are unknown, our findings raise several aspects relevant to this issue. The observations that GM1 is more effective when applied in multiple doses, and that the survival-promoting actions of GM1 persist for at least several days after its washout, suggest that ganglioside is active when incorporated into the plasma membrane. In addition, the use of appropriate inhibitors revealed that the mechanism underlying prevention by GM1 of PC12 cell DNA fragmentation, and therefore probably of PC12 cell death, does not require ongoing RNA or protein synthesis. These results support studies showing that inhibition of macromolecular synthesis does not interfere with the ability of NGF, fibroblast growth factor (FGF), insulin-like growth factors (IGFs), a cAMP derivative, and a nuclease inhibitor to rescue PC12 cells from serum-free cell death (Batistatou and Greene, 1991; Rukenstein et al., 1991).

Although the initial mechanisms employed by NGF and GM1 to maintain survival appear to be distinct, the signaling pathways by which these two agents prevent death may converge at some point. In this regard protein phosphorylation is an attractive candidate. As in the case of most of the substances that maintain PC12 cell or neuronal survival, gangliosides have been implicated in the modulation of several protein kinase activities (Bremer et al., 1984; Tsuji et al., 1985; Kreutter et al., 1986; Chan, 1987, 1988; Vaccarino et al., 1987; Yates et al., 1989; Ferrari et al., 1992). Also, as in the case of promotion of survival, a phosphorylation mechanism is independent of RNA and protein synthesis.

One protein kinase of relevance to ganglioside actions is PKC. This kinase is reported to be a relevant target in cerebellar granule neurons (Vaccarino et al., 1987; Favaron et al., 1988). However, we found that, in PC12 cells, GM1 is effective under conditions in which a large proportion of cellular PKC activity is downregulated. This result argues against a major role for PKC in the survival mechanism of GM1 in PC12 cells. However, we cannot rule out a requirement for the activity that remains after pre-exposure to high levels of phorbol ester.

A second potential candidate for the mechanism by which gangliosides prevent neuronal cell death is modulation of an endonuclease activity. Our experiments with GM1 are consonant with previous findings that NGF and serum inhibit in PC12 cell cultures the characteristic pattern of internucleosomal DNA fragmentation that is the biochemical hallmark of apoptosis (Wyllie et al., 1980; Batistatou and Greene, 1991). Both temporal considerations and experiments with the nuclease inhibitor aurintricarboxylic acid have suggested that DNA fragmentation may be a cause for neuronal cell death (Batistatou and Greene, 1991). In addition to trophic factor–deprived PC12 cells, such fragmentation has been found to occur in sympathetic neurons in which cell death is caused by NGF deprivation (Edwards et al., 1991), and in rat cortical neurons *in vitro* and in dorsal hippocampus *in vivo* after exposure to excess glutamate (Kure et al., 1991). These findings raise the possibility that DNA fragmentation might be the common denominator in neuronal death due to various causes such as neurotrophic factor deprivation and exposure to excess excitatory neurotransmitter. A corollary of this is that agents that lead to prevention of DNA fragmentation might exhibit a general ability to rescue neurons from death triggered by different causes. The present study suggests that gangliosides may belong to the above-mentioned category. In this regard, it is of interest that neurotrophic factors such as NGF, basic FGF, and IGFs, which are known to promote neuronal survival, are also able to protect neurons from

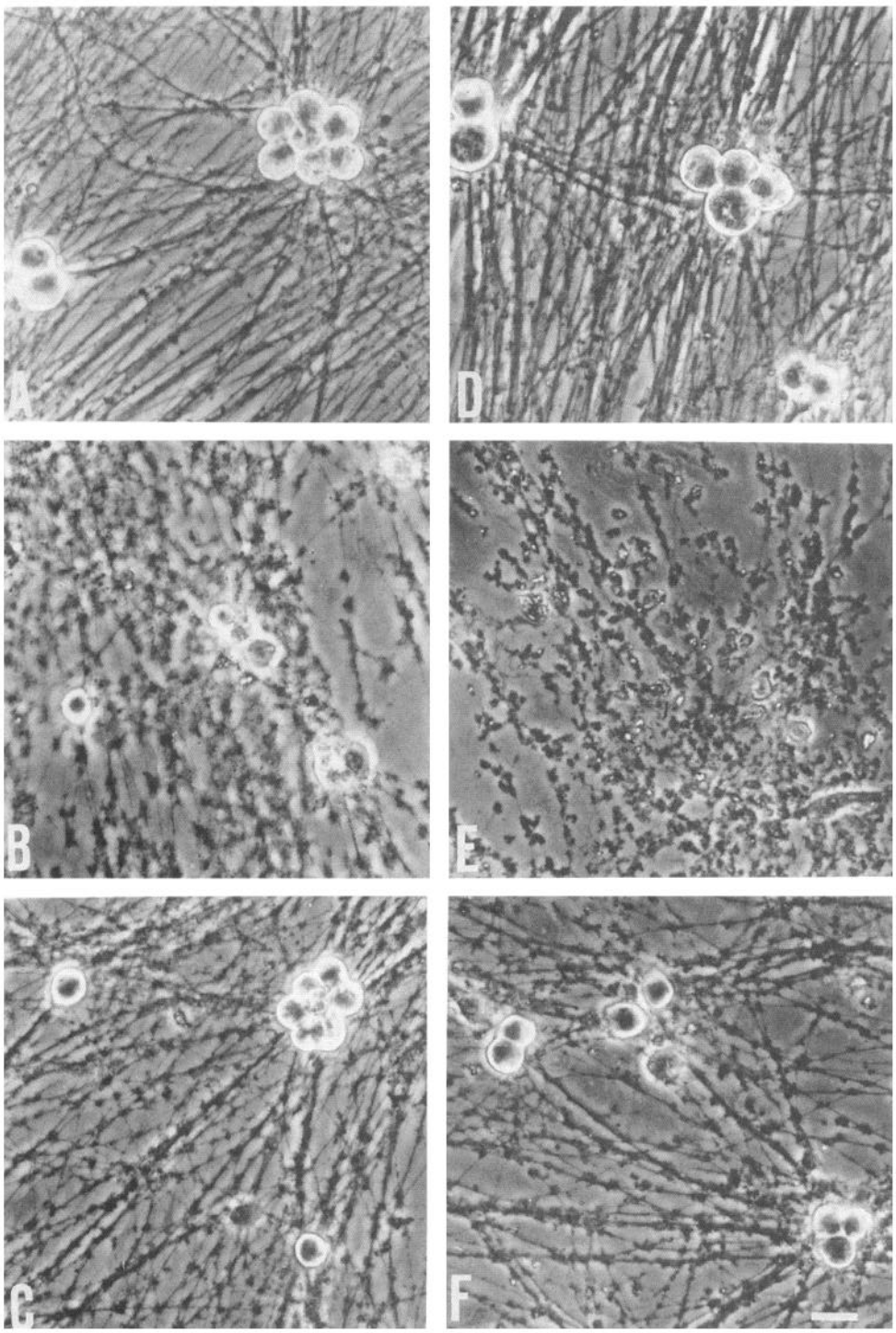

Figure 7. Effect of GM1 on cultured rat sympathetic neurons deprived of NGF. Newborn sympathetic neurons were cultured for seven d with NGF and then for 4 d (*A–C*) or 9 d (*D–F*) as follows: *A* and *D*, with NGF present (100 ng/ml); *B* and *E*, without NGF and with anti-NGF; *C* and *F*, without NGF, with anti-NGF and GM1 (120 μM). Scale bar, 13.5 μm.

hypoglycemic/excitotoxic insult (Cheng and Mattson, 1991, 1992). It is thus possible that growth factors and gangliosides prevent neuronal death under a variety of circumstances by the same mechanism, that is, by leading to inhibition of endonuclease activity.

An additional possible convergent regulator of neuronal survival or cell death is calcium. In this regard, the toxic effects of excitatory neurotransmitters appear to be mediated via elevation of intracellular calcium levels (Choi, 1988). Pretreatment of cells with GM1 has been reported to reduce this late increase of $[Ca]_i$ as well as the resulting neurotoxic effects (De Erausquin et al., 1990; Milani et al., 1991). Similar findings have been reported with trophic factors (Cheng and Mattson, 1991, 1992). Moreover, it has been proposed that survival of sympathetic neurons requires an optimal range of intracellular free calcium (Koike et al., 1989; Koike and Tanaka, 1991). The above results support the notion that regulation of intracellular Ca^{2+} levels may play a common role in the mechanisms by which ganglioside and trophic factors promote neuronal survival and/or prevent neuronal cell death.

In summary, we have shown that GM1 promotes long-term survival and prevents DNA fragmentation in serum-deprived PC12 cell cultures. In addition, GM1 rescues sympathetic neurons from death after NGF deprivation. These findings extend

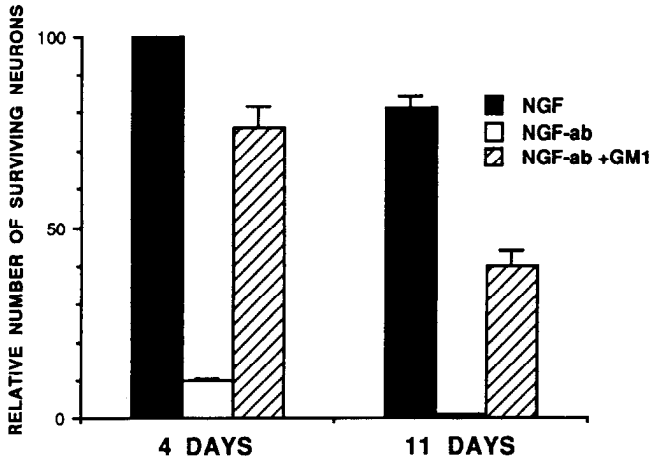

Figure 8. Quantitation of the effect of GM1 on survival of cultured rat sympathetic neurons deprived of NGF. Neurons were maintained in the presence of NGF for 1 week, deprived of the factor, and cultured for the indicated times in the presence of anti-NGF with or without 120 μM GM1. Living, phase-bright neurons were counted and the values are presented relative to the number present in NGF-treated cultures on day 4 of treatment (100 = 143 ± 10 neurons per strip count). Error bars represent SEM (n = 3 independent cultures). Comparable results were obtained in three independent experiments.

the actions of gangliosides to rescue of neuronal cells deprived of trophic support. They therefore suggest that gangliosides may have a pharmacologic potential in pathologies related to trophic factor restriction. Moreover, these studies provide an additional system in which to uncover the mechanism of ganglioside actions.

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