Inhibitors of Cyclin-dependent Kinases Promote Survival of Post-mitotic Neuronally Differentiated PC12 Cells and Sympathetic Neurons*

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Previous studies have demonstrated that multiple agents that promote survival of PC12 cells and sympathetic neurons deprived of trophic support also block cell cycle progression. Presently, we address whether inhibition of cell cycle-related cyclin-dependent kinases (CDKs) prevents neuronal cell death. We show that two distinct CDK inhibitors, flavopiridol and olomoucine, suppress the death of neuronal PC12 cells and sympathetic neurons. In addition, we demonstrate that inhibitor concentrations required to promote survival correlate with their ability to inhibit proliferation. Promotion of survival, however, does not correlate with inhibition of extracellular signal-regulated kinase or c-Jun kinase activities or with interference with the activation of c-Jun kinase that accompanies serum/nerve growth factor deprivation. In contrast to their actions on nerve growth factor-differentiated PC12 cells, the CDK inhibitors do not prevent the death of proliferation-competent PC12 cells and, in fact, promote their cell death. These findings support the hypothesis that post-mitotic neuronal cells die after removal of trophic support due to an attempt to re-enter the cell cycle in an uncoordinated and inappropriate manner. We speculate that cycling PC12 cells are not saved by these agents due to a signaling conflict between an inherent oncogenic signal and the inhibition of CDK activity.

Neuronal apoptosis is an important aspect of nervous system development and a component of neuronal injury and disease. The most generally accepted model of the developmental regulation of neuronal death states that limiting quantities of target-derived neurotrophic support control the optimum number of neuron-target interactions (1). Neurotrophins also play a role in ameliorating the effects of oxidative stress and many forms of neuronal injury (2, 3).

In an effort to define the mechanisms of neurotrophin action in neuronal survival, two model systems, the PC12 pheochromocytoma cell line and cultured primary sympathetic neurons, have been exploited. The PC12 cell line was initially derived from a rat adrenal medullary pheochromocytoma (4). When grown in serum-containing medium, PC12 cells divide and resemble precursors of adrenal chromaffin cells and sympathetic neurons. Upon addition of NGF, these "naive" cells gradually attain the phenotypic properties of sympathetic neurons. Both naive and neuronally differentiated PC12 cells undergo apoptosis upon removal of trophic support (i.e. serum or serum/NGF) (5, 6). The response of PC12 cells to withdrawal of trophic support is quite analogous to that of sympathetic neurons. In vivo (7–9) and in vitro (10, 11) evidence demonstrates that sympathetic neurons require NGF for survival. Studies in this laboratory (6, 12) and by others (13, 14) have shown that both PC12 cells and sympathetic neurons undergo apoptotic death upon NGF deprivation.

Although the mechanisms by which neurotrophins suppress apoptosis are not fully understood, it has been hypothesized that neurotrophins prevent apoptotic death by acting to coordinate cell cycle progression and/or prevent inappropriate cell cycle re-entry (12, 15–17). Accordingly, this hypothesis predicts that cells that attempt to enter or traverse the cell cycle without a set of proper mitogenic signals will undergo apoptosis. In support of this model, numerous observations of apoptosis in the presence of conflicting cell cycle signals have been reported in non-neuronal systems (18–21). We have applied the cell cycle/apoptosis hypothesis to interpret the characteristics of apoptotic death in PC12 cells, sympathetic neurons, and other cells of neuronal origin (15, 22). In this view, withdrawal of serum from naive proliferating PC12 cells leads to an uncoordinated and disastrous attempt to continue to cycle, whereas in post-mitotic differentiated PC12 cells and sympathetic neurons, withdrawal of NGF results in an inappropriate attempt to re-enter the cell cycle and consequent death.

Findings from this and other laboratories have provided some evidence for this interpretation and for the cell cycle/apoptosis model in neuronal cells. While apoptotic death of sympathetic neurons and post-mitotic PC12 cells is delayed by protein synthesis inhibitors (11, 14, 23), such inhibitors do not block cell death of naive PC12 cells (24). One interpretation of this discrepancy is that the proteins needed for apoptosis are regulators of the general cell cycle mechanism. Since naive PC12 cells continually synthesize cell cycle proteins, they may utilize a pre-existing pool of cell cycle regulators to enter the cell cycle even in the absence of new protein synthesis. Without appropriate coordinating mitogenic signals such as provided by growth factors, apoptosis would result. In contrast, post-mitotic cells would require de novo synthesis of cell cycle proteins prior to inappropriate cell cycle re-entry. In accordance with this

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1 The abbreviations used are: NGF, nerve growth factor; JNK, c-Jun kinase; GST, glutathione S-transferase; ERK, extracellular signal-regulated kinase.
view, Freeman et al. (17) showed that NGF removal from sympathetic neurons results in an induction of the cell cycle regulatory protein cyclin D1 along with transcription factors c-Fos and c-Jun (25), typically induced prior to cell division. Furthermore, the activation of another cell cycle protein, Cdc2, has been reported in differentiated PC12 cells as a consequence of NGF withdrawal (26). It has also been reported that expression of SV40 T antigen in Purkinje cells results in apoptotic death (27) concurrent with DNA synthesis.\(^2\)

Initial attempts to test the cell cycle/apoptosis model by blocking cell cycle progression have produced additional support for this hypothesis. Induction of dominant-negative Ras expression in both naïve and post-mitotic PC12 cells inhibits cell cycle progression and death induced by withdrawal of trophic support (15). A similar correlation between survival and blockade of the cell cycle has been shown in PC12 cells and sympathetic neurons treated with N-acetylcysteine (28). In addition, we have recently reported that the G1/S blocker’s mimosine, ciclopirox, and deferoxamine are effective in preventing cell death of both post-mitotic and dividing PC12 cells as well as of sympathetic neurons (29). In these cases, the mechanisms by which the cell cycle is inhibited are unknown.

The cyclin-dependent kinase (CDK) family, which among others includes Cdk2-4/6 and Cdc2 (Cdk1), is an important group of cell cycle regulatory molecules whose inhibition represents a more defined means to block cell cycle progression or re-entry. Cdc2 is a well characterized M phase regulator and may also serve to mediate progression through the S phase (30). Cdk2 and Cdk3 activities are required for progression through the G1/S phases of the cycle (30, 31). In the present studies, we investigated whether inhibitors of the CDK family of kinases would prevent apoptotic death induced by trophic factor withdrawal from PC12 cells and sympathetic neurons. We report that the CDK inhibitors flavopiridol and olomoucine are effective in blocking the death of trophic factor-deprived, post-mitotic PC12 cells and sympathetic neurons, but not of dividing PC12 cells. These observations thus support and further refine the cell cycle/apoptosis model.

**EXPERIMENTAL PROCEDURES**

Materials—Human recombinant NGF was kindly provided by Genentech. Flavopiridol (L86-8275, (−)-4,5,7-trihydroxy-2-(2-chlorophenyl)-8-[4-(3-hydroxy-1-methyl)piperidinyl]-4H-benzopyran-4-one) and PD98059 were generous gifts from Dr. Peter Worland (National Institutes of Health) and Parke-Davis, respectively. Olomoucine (2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine) and isoolomoucine was purchased from LC Laboratories. Flavopiridol and olomoucine/isoolomoucine were dissolved in dimethyl sulfoxide as stock solutions at 50 and 200 mM, respectively. Mouse NGF and anti-mouse NGF anti-serum were obtained from Sigma.

PC12 Cell Culture—Naïve PC12 cells were cultured and passaged as described previously (4) in collagen-coated dishes with RPMI 1640 medium supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum. Neuronally differentiated PC12 cells were obtained by washing PC12 cells and replating them onto collagen-coated dishes at ~1 × 10^5 cells/100-mm dish in the presence of 100 ng/ml NGF for a period of 8–9 days in serum-free RPMI 1640 medium.

PC12 Cell Survival Assay—For survival experiments, naïve or neuronally differentiated PC12 cells were washed extensively in serum- and NGF-free medium as described previously (6, 24) and replated onto collagen-coated 24-well tissue culture dishes at a density of ~2 × 10^4 cells/well. The final volume of medium in each well, including all drugs, was 1 ml. To allow time for blockage of DNA synthesis, naive PC12 cells were, in some cases, pretreated with drug for 16 h prior to serum withdrawal. NGF-treated neuronally differentiated cells were not pre-treated with drug prior to serum deprivation. At appropriate times of culture under the desired conditions, cells were lysed, and the numbers of viable cells were determined as described previously (24). The number of intact nuclei was determined by counting in a hemocytometer. All counts were performed in triplicate, and survival data are expressed as a percentage of cells plated on day 0 ± S.E.

**FIG. 1.** Flavopiridol inhibits [3H]thymidine incorporation by dividing PC12 cells and promotes survival of neuronally differentiated PC12 cells in serum-free medium following withdrawal of NGF. The neuronal PC12 cell phenotype was obtained by treatment with NGF in serum-free medium for 8 days. A, relationship between the drug dose required for promotion of day 2 survival of NGF-deprived neuronally differentiated PC12 cells and inhibition of thymidine incorporation by dividing NGF-untreated (naive) PC12 cells. Naive PC12 cells were pretreated with the indicated concentrations of flavopiridol for 16 h in serum- or insulin-containing RPMI 1640 medium prior to measurement of thymidine incorporation (determined as described under “Experimental Procedures”). Cell survival data are normalized so that survival without flavopiridol (51%) is defined as zero and 100% survival is defined as the number of cells initially present. B, effect of flavopiridol (3 μM) on the time course of survival of neuronally differentiated PC12 cells following withdrawal of NGF. Each data point is the mean ± S.E. of three samples.

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\(^2\) Orr, H. T., Clark, B., and Fedderson, R. M., Society for Neuroscience Short Course "Mitosis to Apoptosis," Miami Beach, FL, Nov. 13, 1994 (abstr.).
described previously (32). The cells were plated in 0.5 ml of medium/well in collagen-coated 24-well dishes at a density of 0.5 ganglia/well. The growth medium was RPMI 1640 medium supplemented with 10% heat-inactivated horse serum and 60 ng/ml mouse NGF. To eliminate non-neuronal cells, a mixture of uridine and 5-fluorodeoxyuridine (10 μM each) was added to the cultures on the following day. On the third day after plating, the neurons were deprived of NGF by washing the cultures three times with RPMI 1640 medium containing 10% heat-inactivated horse serum. The appropriate drug and/or NGF or anti-mouse NGF antibody (1:200 dilution) was added in a final volume of 0.5 ml of the same medium described above. At appropriate times, the numbers of viable phase-bright neurons were determined by strip counting as described previously (33). All survival data are expressed as relative to the original number of cells counted for each well. All experimental points were performed in triplicate and are reported as means ± S.E.

FIG. 2. Olomoucine inhibits [3H]thymidine incorporation by dividing PC12 cells and promotes survival of neuronally differentiated PC12 cells in serum-free medium following withdrawal of NGF. A, relationship between the drug dose required for promotion of day 2 survival of NGF-deprived neuronally differentiated PC12 cells and inhibition of thymidine incorporation by naive PC12 cells. Naive PC12 cells were pretreated with the indicated concentrations of olomoucine for 16 h in serum- or insulin-containing medium prior to measurement of thymidine incorporation. Cell survival data are normalized so that survival without olomoucine (43%) is defined as zero and 100% survival is defined as the number of cells initially present. B, effect of olomoucine (200 μM) on the time course of survival of neuronally differentiated PC12 cells following withdrawal of NGF. Each data point is the mean ± S.E. of three samples.

FIG. 3. Isoolomoucine is less effective than olomoucine in inhibiting [3H]thymidine incorporation and maintaining survival of neuronally differentiated PC12 cells following withdrawal of NGF. A, comparison of inhibition of thymidine incorporation by olomoucine and isoolomoucine. Naive PC12 cells were pretreated with the indicated concentrations of olomoucine/isoolomoucine for 16 h in serum- or insulin-containing medium prior to measurement of thymidine incorporation. B, time course of survival of neuronally differentiated PC12 cells following withdrawal of NGF. Each data point is the mean ± S.E. of three samples.

CDK Inhibitors Promote Survival of Neuronal Cells

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pretreated with the appropriate drug for a period of 16 h prior to the addition of 1 μCi/well [3H]thymidine (DuPont NEN). The cells were exposed to thymidine for 1–2 h and then washed three times with cold phosphate-buffered saline. Each well was extracted with 1 ml of 10% trichloroacetic acid at 4°C for 1 h, and the insoluble material was solubilized overnight at room temperature with 0.3 ml of 1N NaOH. After neutralization with an equal volume of 1N HCl, the solution was quantified by scintillation counting. The level of background counts was determined by treating cells with 10 μM aphidicolin. All experimental points were performed in triplicate. All data are presented relative to untreated control samples as means ± S.E.

Determination of Rate of Protein Synthesis—Neuronally differentiated PC12 cells and sympathetic neurons were plated on 24-well dishes as described above. After the cultures were pretreated with the appropriate drug for 1 h, 5μCi of [3H]leucine was added per well, and the cells were allowed to incorporate the isotope for 10 h. The cultures were washed and prepared for scintillation counting as described above for thymidine incorporation. Background levels of radioactivity was determined by treating wells with 5 μg/ml cycloheximide. All experimental points were performed in triplicate, and the data are presented relative to untreated control samples as means ± S.E.

Determination of c-Jun Kinase Activity—c-Jun kinase (JNK) was affinity-purified from PC12 cell extract with GST-c-Jun (a gift from Dr. Michael Karin, University of California, San Diego, CA) bound to GSH-agarose beads (Sigma) by methods previously described (34). c-Jun kinase activity was determined by an in vitro solid-phase kinase assay as described previously (34). Activated c-Jun kinase was isolated from PC12 cells treated with 0.5 mM sodium arsenite for 30 min. To determine the potential inhibitory effects of flavopiridol or olomoucine on c-Jun kinase activity in vitro, the indicated concentrations of inhibitor were added to constant amounts of activated c-Jun kinase during the solid-phase kinase assay. In vivo effects of the CDK inhibitors on c-Jun kinase activation following NGF withdrawal from neuronally differentiated PC12 cells were determined by treatment of NGF-deprived cell cultures with flavopiridol or olomoucine for various times, affinity purification of c-Jun kinase activity from the cell extract (normalized for protein content as quantified by the Bio-Rad protein assay), and determination of c-Jun kinase activity as described above. GST-c-Jun was resolved on an 8.5% SDS gel, and incorporation of 32PPO4 was quantified by autoradiography and densitometry.

RESULTS

CDK Inhibitors Flavopiridol and Olomoucine Inhibit PC12 Cell Cycle Progression and Death of Post-mitotic Neuronally Differentiated PC12 Cells—Two CDK inhibitors were utilized to study the effects of cell cycle blockade on survival of PC12 cells and sympathetic neurons. Flavopiridol was first reported to inhibit immunoprecipitated Cdk1 activity from breast carcinoma cells with an IC50 of 0.4 mM (assayed at an ATP concentration of 375 μM) (35). Subsequently, this flavonoid derivative was demonstrated to inhibit the G1/S phase-related Cdk2/4 enzymes with similar potency (36). In addition, Kaur et al. (36) reported that flavopiridol blocks progression from both G1 to S and G2 to M in several breast carcinoma cell lines. Flavopiridol has been shown to be a relatively poor inhibitor of all other protein kinases examined, including cAMP-dependent protein kinase, epidermal growth factor receptor kinase, and protein kinase C (35). Olomoucine, a purine derivative, has been demonstrated to be a relatively specific inhibitor for Cdk1/2/4 enzymes with similar potency (37). In addition, Kaur et al. (36) reported that flavopiridol blocks progression from both G1 to S and G2 to M in several breast carcinoma cell lines. Flavopiridol has been shown to be a relatively poor inhibitor of all other protein kinases examined, including cAMP-dependent protein kinase, epidermal growth factor receptor kinase, and protein kinase C (35). Olomoucine, a purine derivative, has been demonstrated to be a relatively specific inhibitor for Cdk1/2/4 enzymes with similar potency (37). Examination of approximately 30 other protein kinases revealed that olomoucine had poor inhibitory actions on these enzymes (37). Tests of the cell cycle inhibitory actions of this

3 P. Worland, submitted for publication.
agent on 60 tumor cell lines demonstrated that olomoucine arrests cells at both the G1 to S and G2 to M borders (37).

We first examined whether these inhibitors inhibit DNA synthesis by naive PC12 cells. As shown in Figs. 1A and 2A, flavopiridol and olomoucine inhibited [3H]thymidine incorporation with IC₅₀ values of ~0.3 and ~100 μM, respectively.

We next determined the ability of flavopiridol and olomoucine to block death induced by NGF withdrawal from PC12 cells that had been neurally differentiated by pre-exposure to NGF in serum-free medium. Maximal protection was observed at a concentration of 1 μM for flavopiridol and 200 μM for olomoucine. Flavopiridol was more effective in long-term protection of NGF-differentiated PC12 cells than olomoucine, with good maintenance of survival even 5 days after NGF withdrawal (Figs. 1B and 2B). Both drugs showed progressive toxicity even in the presence of NGF, which appears to limit their long-term efficacy. As shown in Figs. 1 and 2, both drugs significantly delayed death, and this correlated well with inhibition of thymidine incorporation.

As a control for nonspecific effects of olomoucine, the analog isoolomoucine was also tested for its ability to inhibit cell cycle progression and neuronal death. This derivative is identical to olomoucine with the exception of the location of a substituent methyl group on the imidazole ring of the purine backbone. As reported by the manufacturer (51), this change severely reduces inhibition of Cdk1 activity (IC₅₀ > 500 μM isoolomoucine versus IC₅₀ = 7 μM olomoucine). As shown in Fig. 3, isoolomoucine was far less effective than olomoucine in inhibiting both thymidine incorporation and cell death.

Fig. 4 shows the morphology of neurally differentiated PC12 cells treated with the CDK inhibitors in serum-free medium with and without NGF. The cells rescued by the drugs showed the typical phase-bright morphology of living cells, but did not regenerate neurites. In the presence of NGF, the drugs appeared to partially suppress neurite regeneration. Potential reasons for this effect on regeneration include inhibition of Cdk5, a kinase linked to neurite formation (38, 39), and/or of ERK1 kinase, activation of which also appears to be required for neurite outgrowth (40). Olomoucine is reported to inhibit GST-ERK1 activity in vitro (IC₅₀ = 30 μM).

To test whether ERK inhibition by olomoucine might contribute to its actions on survival (by either promoting or blocking death), we treated naive and neurally differentiated PC12 cells with PD98059 (20–100 μM), an inhibitor of the mitogen-activated protein kinase/ERK kinase that mediates NGF-promoted activation of ERKs (41). We then examined the behavior of the cells when passaged into serum-free medium with or without NGF. Although the drug effectively suppressed NGF-stimulated neurite regeneration (as anticipated from its inhibition of ERK activation), it neither blocked nor mimicked the capacity of NGF to promote survival of naive or neurally differentiated PC12 cells (data not shown). These findings suggest that inhibition of ERK activity does not account either for the survival-promoting actions of olomoucine in the absence of NGF or for its toxicity in the presence of NGF. They also indicate that ERK activation is not required for NGF-promoted survival.

Flavopiridol and Olomoucine Do Not Prevent Activation of c-Jun Kinase Activity in NGF-deprived Neuronally Differentiated PC12 Cells—Recent findings suggest that activation or activity of JNK family members may be involved in apoptosis of NGF-deprived sympathetic neurons and PC12 cells (42). In accordance with these findings, we find that c-Jun un kinase activ-

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4 Virdee, K., and Tolkovsky, A. M., Cold Spring Harbor Programmed Cell Death Meeting, (Cold Spring Harbor, NY, Sept. 20–24, 1995, p. 177 (abstr.).
moucine inhibited GST-c-j un phosphorylation with an IC$_{50}$ of $-100$ $\mu$m. In contrast, a $12$ $\mu$m concentration of flavopiridol (12-fold higher than that required for survival of neuronal cultures) had no effect on the in vitro phosphorylation of GST-c-j un, while $50$ $\mu$m flavopiridol significantly inhibited phosphorylation (Fig. 5C). These observations indicate that it is unlikely that the CDK inhibitors used here promote survival by preventing activation of JNK and that at least flavopiridol does not work by inhibiting JNK activity.

Flavopiridol and Olomoucine Prevent Death of NGF-deprived Sympathetic Neurons—To extend our observations with post-mitotic PC12 cells, we evaluated the efficacy of flavopiridol and olomoucine on sympathetic neurons deprived of NGF. Sympathetic neurons were obtained from 1-day-old rats and cultured in the presence of NGF for 3 days prior to NGF withdrawal. Typically after NGF deprivation, $>80\%$ of the neurons die within 48 h. Consistent with the observations with post-mitotic PC12 cells, both agents effectively inhibited death of the NGF-deprived neurons (Fig. 6). Moreover, in accordance with the proposed involvement of inappropriate cell cycle entry in neuronal death, the dose-response relationship for both compounds paralleled the ability of flavopiridol and olomoucine to inhibit thymidine incorporation in cycling PC12 cells (compare Figs. 1A and 2A) with Fig. 6 (B and D). Fig. 6 also shows that both drugs promoted good long-term survival of NGF-deprived neurons. At a concentration of $1$ $\mu$m, flavopiridol promoted full survival for at least 4 days post-NGF depletion (Fig. 6A). Quantification at time periods longer than this was not possible since the neurons (which still appeared to be viable) tended to lift from the tissue culture dish in sheets. However, we observed that those neurons that remained in contact with the dish after this period maintained a viable appearance (Fig. 7). Olomoucine (200 $\mu$m) also promoted long-term survival so that $\sim 75\%$ of the neurons were still viable by 7 days post-NGF depletion. Again, quantitation of surviving neurons after this period was compromised by their losing contact with the tissue culture dish. In the cases of long-term cultures maintained with either flavopiridol or olomoucine, sheets of neuritic processes and healthy-appearing cell bodies were clearly visible floating in the medium. In contrast to olomoucine, isoolomoucine was ineffective in promoting survival of NGF-deprived sympathetic neurons (Fig. 6C).

Fig. 7 illustrates the morphology of NGF-deprived neurons cultured with flavopiridol and olomoucine. We have previously noted that a number of agents that support the survival of such neurons do not maintain neurites and that these degenerate within several days (6, 28). However, as shown in Fig. 7, healthy processes are clearly visible in the drug-treated cultures 6 days after NGF depletion. In contrast, as with most other agents that rescue sympathetic neurons after NGF withdrawal, somatic hypertrophy was not maintained by the drugs (Fig. 7).

Because inhibition of protein synthesis promotes survival of neurally differentiated PC12 cells and sympathetic neurons (11, 14, 23), we examined the effect of flavopiridol (Fig. 8A) and olomoucine (Fig. 8B) on leucine incorporation. As shown in Fig. 8A, $1$ $\mu$m flavopiridol inhibited leucine incorporation by $\sim 25\%$ in cultures of either neurally differentiated PC12 cells or sympathetic neurons. Olomoucine (200 $\mu$m) inhibited leucine incorporation in sympathetic neuron cultures by $\sim 30\%$, but had no effect on protein synthesis in PC12 cell cultures (Fig. 8B). Martin et al. (44) reported that at least $80\%$ inhibition of protein synthesis is required to protect sympathetic neurons from NGF withdrawal. Accordingly, it is therefore unlikely that
the mechanism by which flavopiridol and olomoucine rescue post-mitotic neurons and PC12 cells is by inhibition of protein synthesis.

Flavopiridol and Olomoucine Do Not Promote Survival of Serum-deprived Naive PC12 Cells—In contrast to their survival-promoting effects on neuronally differentiated PC12 cells and sympathetic neurons, flavopiridol and olomoucine failed to promote survival of naive PC12 cells deprived of trophic support by serum withdrawal. To ensure that the naive PC12 cells were cell cycle-arrested, naive PC12 cell cultures were pretreated with the CDK inhibitors for 16 h. Under these conditions, serum-deprived naive PC12 cells exposed to flavopiridol died somewhat more quickly than control cells without flavopiridol, whereas PC12 cells treated with olomoucine died at about the same rate as control cells (Fig. 9A). Naive cell cultures exposed to inhibitor only immediately following serum deprivation (that is, without pretreatment) also showed no increased survival over control cells (data not shown). Interestingly, flavopiridol and olomoucine proved to cause death of naive PC12 cells cultured in the presence of serum (75% death by day 3; see Fig. 9B). This level of death was considerably greater than that induced by drugs in cultures of post-mitotic neuronally differentiated PC12 cells in the presence of NGF (−20% death at day 3; see Figs. 1B and 2B).

**DISCUSSION**

We have hypothesized that trophic factors such as NGF prevent the death of proliferating neuroblasts by guiding them through the cell cycle, as is the case with naive PC12 cells, and inhibit the death of post-mitotic neurons by suppressing their inappropriate re-entry into the cell cycle. Testing this model has involved examining whether agents that are known to prevent cell cycle progression promote neuronal survival. In previous studies, we showed that multiple agents that induce cell cycle arrest, including G1 blockers (29), N-acetylcysteine (28), and chlorphenylthio-cAMP (33), as well as induction of dominant-negative Ras (15) all suppress the death of neuronal cells caused by withdrawal of trophic support. Although these findings support the cell cycle/apoptosis model, the mechanisms by which such agents block the cell cycle remain largely unclear, and in each case, there was the possibility that alternative actions might be responsible for preventing death. To further and more directly evaluate the effects of cell cycle inhibition on death of neurons, we employed two known inhibitors of the cell cycle-related CDK family of kinases.

As predicted from the cell cycle/apoptosis model and consistent with previous findings with agents that induce cell cycle arrest, both flavopiridol and olomoucine suppressed the death of post-mitotic PC12 cells and sympathetic neurons caused by NGF deprivation. Significantly, the dose relationship for inhibition of thymidine incorporation by flavopiridol and olomoucine correlates very closely with their abilities to block cell death. In accordance with these observations, several lines of evidence support the potential role of cyclins and CDKs in the process of neuronal death. Brooks et al. (26) reported that elevated Cdc2 activity is observed concurrent with NGF deprivation and death of neuronally differentiated PC12 cells. In addition, NGF treatment of PC12 cells leads to a reduction of both Cdk2 and Cdc2 activities (45) and increases the levels of
p21, a G1 phase CDK inhibitor (46). Outside the nervous system, Cdc2 activity has been shown to be required for lymphocyte granule protease-mediated cell death (21). Furthermore, cyclin A induction and an increase in cyclin A/Cdc2 activity occur in association with apoptosis induced pharmacologically or by Myc overexpression (47, 48).

Surprisingly, the CDK inhibitors were not effective in preventing the death of proliferation-competent naive PC12 cells after withdrawal of trophic support. This is in sharp contrast to our earlier observations with other cell cycle-blocking agents. The G1/S blockers mimosine, deferoxamine, and ciclopirox as well as N-acetylcysteine are equally effective in protecting naive PC12 cells, post-mitotic neuronally differentiated PC12 cells, and sympathetic neurons from loss of trophic support (28, 29). This indicates that the manner in which the cell cycle is blocked may be critical in determining whether an agent promotes neuronal survival. For instance, we showed that G1 blockers, and not S, G2, or M phase blockers, promote survival of neuronal cells (29). This, however, does not imply that cells die in a cell cycle stage-specific manner, as Lindenboim et al. (49) have shown that apoptosis of PC12 cells occurs at each phase of the cell cycle.

How might flavopiridol and olomoucine act to save postmitotic cells and not proliferation-competent cells? It is possible that nonspecific toxicity may negate any potential attenuation of cell death in naive PC12 cultures. The increased degree of CDK inhibitor-induced toxicity when compared with neuronal PC12 cells, however, suggests additional causes for this difference. One attractive explanation is that inhibition of CDKs in cycling PC12 cells forces a conflict between this aspect of cell cycle arrest and one or more endogenous proliferative signals. Naive PC12 cells, by origin, are transformed and therefore must possess oncogenic signals that have yet to be defined. The cell cycle/apoptosis hypothesis would predict that the resulting conflict would lead to apoptotic death. In support of this view, flavopiridol promotes the death not only of naive PC12 cells, but also of other transformed cell lines even in the presence of trophic support. After long-term exposure to NGF, PC12 cells attain a nondividing phenotype lacking the oncogenic cue present in proliferating cultures and thereby eliminate the signaling conflict present in naive cultures.

Although the presently demonstrated effects of flavopiridol and olomoucine on survival are consistent with their ability to

5 D. Park, unpublished observations.
prevent cell cycle progression and are therefore consistent with the cell cycle/apoptosis theory, alternative actions could account for these observations. The most likely of these is the inhibition of kinases other than CDKs. For instance, as discussed above, olomoucine inhibits ERKs only somewhat less potently than CDKs. However, our data presented here, as well as those of Virdee and Tolkovsky (50), indicate that ERKs are as those of Virdee and Tolkovsky (50), indicate that ERKs are not required for evocation or prevention of neuronal cell death. Another possibility is that these agents inhibit either the activation or activity of JNK family members. This possibility, however, does not appear to account for the differences we observed here between post-mitotic and naive PC12 cells. In addition, we show that flavopiridol and olomoucine do not prevent the intracellular activation of JNK. While olomoucine does suppress JNK activity in vitro at concentrations similar to those at which it blocks death, flavopiridol proved to be a relatively poor inhibitor of JNK (IC$_{50}$ > 12 μM). Thus, it seems unlikely that inhibition of either JNK activation or activity accounts for the protective effects of flavopiridol or olomoucine.

In summary, we have found that two distinct CDK inhibitors prevent the death of post-mitotic neuronal cells. Proliferating PC12 cells, however, were not saved by these agents. These results are consistent with the model that conflicts between concurrent proliferative and nonproliferative signals may be one important factor in apoptosis due to trophic withdrawal. These findings may have important implications for developing therapeutic strategies for the treatment of neuronal injury and degeneration.

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