Leukemia Inhibitory Factor Requires Concurrent p75^{LNTR} Signaling to Induce Apoptosis of Cultured Sympathetic Neurons

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Apoptosis may result either from positive induction by ligand binding to a plasma membrane receptor or from negative induction attributable to loss of a suppressor signal. For example, apoptosis of developing sympathetic neurons may be induced in culture either by exposure to leukemia inhibitory factor (LIF) or by deprivation of nerve growth factor. This study compared the cell death pathways activated in sympathetic neurons by these two different stimuli. Both types of cell death were developmentally regulated; both were maximal in the immediate postnatal period and disappeared over the next 2 weeks. Both types of cell death were reduced by genetic deletion of Bax or by virally mediated overexpression of Bcl-2. Similarly both were reduced by inhibition of caspase activity or by inhibition of Nedd-2 synthesis with antisense oligonucleotides. Finally, both involved activation of c-Jun N-terminal kinase (JNK) signaling.

Nedd-2 expression by sympathetic neurons declined in parallel with the developmental loss of LIF-mediated cell death, suggesting that downregulation of the caspase during development may underlie the loss of cytokine-mediated apoptosis. Treatment of sympathetic neurons with an antibody that blocks the function of the low-affinity neurotrophin receptor (p75^{LNTR}) prevented LIF-induced cell death. Similarly genetic deletion of p75^{LNTR} prevented apoptosis after LIF treatment. These observations suggest that concurrent p75^{LNTR} signaling is necessary for LIF-induced cell death and that cytokine-mediated cell death and growth factor deprivation appear to activate the same intracellular pathways involving JNK signaling.

Key words: apoptosis; c-JUN N-terminal kinase; gp130; leukemia inhibitory factor; p75; sympathetic neuron

Although neuronal survival in the developing nervous system is regulated by growth factors that inhibit apoptosis, neuron numbers may also be regulated by factors that promote cell death. For example, the cytokine leukemia inhibitory factor (LIF) induces apoptosis of cultured embryonic and neonatal sympathetic neurons in a dose-dependent manner (Nawa et al., 1990; Kessler et al., 1993; Kotzbauer et al., 1994). Similarly there are numerous examples of apoptosis induced by bone morphogenetic proteins both within the nervous system and in other organs (Graham et al., 1996; Furuta et al., 1997; Mabie et al., 1999). Thus apoptosis may result either from positive induction by ligand binding to a plasma membrane receptor or from negative induction attributable to loss of a suppressor signal. The same growth factor may either induce or forestall cell death depending on the cellular context. For example, nerve growth factor (NGF) promotes the survival of sympathetic, sensory, and other neurons via activation of the trkA receptor tyrosine kinase (Bredesen and Rabizadeh, 1997; Dechant and Barde, 1997; see Yoon et al., 1998). However NGF may induce rather than prevent apoptosis in cells that express the low-affinity neurotrophin receptor (p75^{LNTR}) but not the high-affinity (trkA) receptor (Casaccia-Bonnet et al., 1996; Frade et al., 1996). Similarly brain-derived neurotrophic factor (BDNF) induces apoptosis of sympathetic neurons that express p75^{LNTR} but not trkB, the high-affinity neurotrophin receptor that binds BDNF (Bamji et al., 1998). Furthermore, although LIF

induces death of cultured sympathetic neurons, it promotes the survival of a large number of other populations of neurons (Martinou et al., 1992; Thaler et al., 1994; Murphy et al., 1997).

The effects of growth factors on neuronal survival may be restricted to precise developmental periods. For example, developing sympathetic neurons require NGF for survival, but they lose that dependence with time postnatally (Easton et al., 1997). The apoptotic response to LIF is similarly lost with time in culture (Kessler et al., 1993; Kotzbauer et al., 1994). Although there are differences among apoptotic pathways activated by these mechanisms in different cells, there appear to be a number of common features. Activation of cysteine proteases (caspases) is a common feature of many different apoptotic pathways (for review, see Cohen, 1997; Green, 1998; Nunez et al., 1998). Some cysteine proteases are present as zymogens in nonapoptotic cells and are activated by other cysteine proteases, by noncaspase proteases, or by autoproteolysis. The same cell may express more than one caspase, and different caspases may be activated by different proapoptotic events. For example, apoptosis caused by growth factor deprivation of sympathetic neurons is mediated by the cysteine aspartase Nedd-2, whereas apoptosis of the same cells induced by downregulation of superoxide dismutase involves a different protease (Troy et al., 1997). Another point of convergence of different apoptotic pathways involves the Bcl-2 family of proteins (for review, see Kroemer, 1997). Some members of this protein family (e.g., Bcl-2 and Bcl-x_L) tend to suppress apoptosis, whereas others (e.g., Bax, Bad, and Bik) induce apoptosis. Genetic deletion of Bax primarily prevents apoptosis of cultured sympathetic neurons after NGF deprivation (Deckwerth et al., 1998), whereas overexpression of Bcl-2 has similar effects (Garcia et al., 1992). Changes in the expression of Bcl-2 family members may underlie the acquisition or loss of growth factor dependence;

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the postnatal loss of dependence of sympathetic neurons on NGF correlates with downregulation of the expression of Bax (Easton et al., 1997). However the effects of the Bcl-2 family of proteins are also dependent on the nature of the death signal and the cellular context. Thus p75^{LNTN}-mediated death of cultured sensory neurons is actually promoted rather than inhibited by Bcl-2 (Coulson et al., 1999). Although these pathways represent points of convergence of pathways mediating apoptosis evoked by different insults, there are several independent signaling pathways that may initiate apoptosis. For example, activation of JNK is involved in the death of sympathetic neurons or of pheochromocytoma 12 cells after NGF deprivation, p75 activation, or oxidative stress but not after serum deprivation or treatment with cytosine arabinoside (Xia et al., 1995; Aloyz et al., 1998; Eilers et al., 1998; Anderson and Tolkovsky, 1999; Maroney et al., 1999).

In this study we investigated the intracellular mechanisms underlying the acquisition and loss of the proapoptotic and antiapoptotic effects of LIF and NGF on cultured sympathetic neurons. We report that apoptosis because of LIF treatment shares many common features with cell death after NGF deprivation including inhibition either by overexpression of Bcl-2 or by deletion of Bax. Similarly both are primarily prevented by inhibition of caspase activity and, specifically, by decreasing expression of Nedd-2. Furthermore, LIF treatment and NGF withdrawal also both activate JNK. Most notably, p75^{LNTN} is required for LIF-induced apoptosis as well as for cell death after NGF deprivation. These observations suggest that similar mechanisms mediate sympathetic neuron death after LIF treatment and NGF deprivation and that p75^{LNTN} signaling is required for both. Furthermore, we find that downregulation of Nedd-2 expression may underlie, at least in part, the postnatal loss both of NGF dependence and of LIF-mediated apoptosis of sympathetic neurons.

MATERIALS AND METHODS

Cell culture. Sympathetic neurons were cultured from dissociated superior cervical ganglia (SCGs) of postnatal day 1 rats (Sprague Dawley) or mice as described previously (Spiegel et al., 1990). The cells were plated at a density of 2000–3000 cells per well onto collagen-coated 24-well plates in Ham's nutrient mixture F12 (Life Technologies, Gaithersburg, MD) with 10% fetal calf serum (HyClone, Logan, UT), rhNGF (100 ng/ml), penicillin (50 U/ml; Life Technologies), and streptomycin (50 µg/ml; Life Technologies). Cultures were maintained at 37°C in a 95% air/5% CO₂ atmosphere at nearly 100% humidity and were fed three times per week. Ganglion non-neuronal cells were eliminated by treatment on day 1 of culture with cytosine arabinofuranoside (15 µM). The number of phase-positive cells with neuronal morphology was counted after 24 hr for all cultures before LIF treatment or other manipulations. During 10 d in culture, neuronal death in control cultures never exceeded 3–5%. The number of viable cells at the end of all experiments was quantified by trypan blue exclusion. Unless otherwise stated, each experiment contained five samples for each condition, and each experiment was repeated three times. The data are reported as the mean ± SEM. LIF was used at 100 ng/ml unless otherwise stated.

Synthesis of antisense-Nedd and scrambled-Nedd oligonucleotides. Antisense-Nedd oligonucleotides [A-Nedd; sequences described by Troy et al. (1997); bearing an SH group at their 5' end and a NH group at their 3' end] were purchased from Operon (Alameda, CA). The oligonucleotides were coupled to Penetratin 1 (Oncor, Gaithersburg, MD), a peptide that facilitates the movement of oligonucleotides across cell membranes, as described previously (Troy et al., 1997). Briefly the oligonucleotides were resuspended in deionized water, an equimolar ratio of Penetratin 1 was added, and the mixture was incubated at 37°C for 1 hr to allow coupling. The yield of the reaction, estimated by SDS-PAGE followed by Coomassie blue staining, was routinely >50%. A scrambled sequence with the same base composition as the antisense oligonucleotide, defined as S-Nedd, was synthesized and coupled to Penetratin 1 as a control. Cultures were treated with 400 nM Penetratin-coupled antisense oligonucleotides unless otherwise stated.

NGF deprivation. Sympathetic neuronal cultures were deprived of NGF by rinsing once with Ham's nutrient mixture F12 with 10% fetal calf serum, followed by the addition of Ham's mixture containing 1% goat neutralizing anti-mouse NGF antiserum (1:200; courtesy of Eugene Johnson). Control cultures were rinsed with reintroduction of the usual medium without antibody.

Immunohistochemistry for Nedd-2. SCGs from postnatal day 1 (P1), P4, P11, and adult rats were frozen in −25°C isopentane, and 12 µm cryosections were placed onto Superfrost Plus slides (Fisher Scientific, Houston, TX), air-dried for 2 hr, and fixed with cold methanol for 10 min. Primary antibodies were diluted (1:250) in PBS containing 5% heat-inactivated horse serum and were applied at 37°C for 2 hr. Anti-Nedd-2, a polyclonal rabbit antiserum, was a generous gift from Dr. Lloyd Greene. Peroxidase staining was performed using a biotinylated secondary antibody and ABC and VIP substrates (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. For negative controls, secondary antibody was not applied to corresponding sections of P1, P4, and P11 SCGs.

Preparation and titration of adenovirus vectors. Nonreplicative adenovirus deleted in the E1 region, carrying the human wild-type bcl-2 gene under the control of the cytomegalovirus promoter, was generously provided by Dr. Jayanta Roy Chowdhury. The adenovirus carrying the β-galactosidase gene was also provided by Dr. Chowdhury. Recombinant adenovirus was propagated in E1-complementing human embryonic kidney 293 cells. Viral stocks were purified in cesium chloride gradients and titered according to the method of Barr et al. (1995).

Bax knock-out and p75^{LNTN} knock-out mice. Mice heterozygous for the deletion of Bax (Bax +/−) were a generous gift of Dr. S. Korsmeyer. On postnatal day 1, tail DNA was prepared from the offspring of heterozygotes and was screened for both the normal and mutant alleles using a single PCR as described by Deckwerth et al. (1996). Sympathetic neurons were cultured individually from the SCGs of every neonate, and the genotype was determined after the cultures had been established. Breeding pairs of mice homozygous for a null mutation in the p75^{LNTN} gene (Lee et al., 1992) were purchased from The Jackson Laboratory (Bar Harbor, ME). Breeding pairs of control mice with the same background were also purchased from The Jackson Laboratory. Genotypes were confirmed by PCR of tail genomic DNA.

c-Jun N-terminal kinase activity. c-Jun N-terminal kinase (JNK) assays were performed with a glutathione S-transferase (GST)–c-jun (1–79) fusion protein as a substrate after immunoprecipitation of the cell lysates with agarose-conjugated anti-JNK (1:100) antibodies (Yoon et al., 1998). Phosphorylation of GST–c-Jun was evaluated after gel electrophoresis and autoradiography.

Materials. LIF was purchased from Life Technologies. Z-vad-fmk and capthepsin B were obtained from Kamiya Biomedical (Thousand Oaks, CA). rhNGF was generously supplied by Genentech (South San Francisco, CA). The p75^{LNTN} antibody (REX) was generously provided by Dr. Louis Reichardt. The soluble interleukin-6 α receptor was generously provided by Regeneron, and anti-JNK was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

RESULTS

Effects of LIF on cultured neonatal sympathetic neurons

To examine the effects of LIF on sympathetic neuronal survival, cultures from P1 ganglia were treated after 24 hr in culture with 100 ng/ml cytokine or with vehicle (control). Cell numbers were quantified by trypan blue exclusion over the next 48 hr (Fig. 1A). Exposure to LIF led to the death of 60% of the neurons within 2 d of treatment. By comparison, NGF deprivation killed >90% of the neurons. However, if addition of LIF was delayed until day 10 of culture there was no significant reduction in cell number (Fig. 1B), indicating that the proapoptotic effects of the cytokine declined with time in culture. The effects of NGF deprivation also declined with time in culture but to a lesser extent; treatment of P10 neurons with anti-NGF still killed more than one-half of the neurons. However cotreatment with LIF at day 10 prevented the cell death associated with NGF deprivation (Fig. 1B). Thus the proapoptotic effects of LIF converted to antiapoptotic ones with time in culture. The change in neuronal responses to LIF oc-

Figure 1. Effects of LIF on the survival of cultured sympathetic neurons. Sympathetic neurons were cultured from P1 (*A*, *B*) and P12 (*C*) animals at a density of ~2000 neurons per well. *A*, Cultures from P1 rats were treated after 24 hr in culture with 100 ng/ml LIF or vehicle (*Control*). Some cultures were also treated with antiserum to NGF. Cell numbers were quantified 48 hr later by trypan blue exclusion. Note that treatment with LIF led to the death of ~60% of the neurons and that NGF deprivation (anti-NGF) killed >90% of the cells. *B*, Cultures from P1 rats were treated on day 10 of culture with LIF (100 ng/ml) or anti-NGF, and cell numbers were quantified 48 hr later by trypan blue exclusion. Note that after 10 d in culture the neurons did not die in response to LIF but treatment with anti-NGF still killed more than one-half of the cells. However cotreatment with LIF prevented the death associated with NGF deprivation, indicating that LIF now promoted survival. *C*, Cultures from P12 animals were treated with LIF or anti-NGF, and cell numbers were quantified 48 hr later by trypan blue exclusion. Note that exposure to anti-NGF killed >50% of the neurons, whereas LIF treatment promoted survival of cells deprived of NGF. Thus the conversion of sympathetic neuron responses to LIF from proapoptotic ones (*A*) to antiapoptotic ones (*B*) that occurred with time *in vitro* was recapitulated *in vivo*. * $p < 0.05$ compared with control; ** $p < 0.05$ compared with both control and LIF by ANOVA.

curred *in vivo* as well as *in vitro*; LIF exerted only survival effects on ganglion neurons cultured from P12 animals (Fig. 1*C*).

Bcl-2 and Bax in LIF-mediated cell death

Because previous studies demonstrated that LIF-induced neuronal death is apoptotic (Kessler et al., 1993), we questioned whether members of the bcl-2 family might be involved. To address this issue the effects of LIF were examined on neurons lacking Bax. LIF treatment resulted in the death of 74% of sympathetic neurons derived from newborn Bax (+/+) mice on day 3 of cell culture (Fig. 2*A*). By contrast, treatment of neurons from animals deficient in Bax (−/−) resulted in the death of <30% of the cells. The protective effects on survival conferred by the absence of Bax were confirmed by the lack of substantial morphological damage; although Bax (+/+) neurons underwent marked shrinkage, most Bax (−/−) neurons retained viable cell bodies and neurites after LIF exposure. NGF deprivation resulted in the death of <15% of Bax (−/−) neurons after 3 d in culture, whereas virtually all Bax (+/+) neurons died under these conditions. Because deletion of Bax diminished LIF-induced neuronal death, we investigated whether overexpression of Bcl-2

might similarly afford protection against LIF-induced death. Enhancing Bcl-2 levels by gene transfer using an adenoviral vector at a multiplicity of infection (MOI) of 500 virtually prevented neuronal cell death induced by LIF (Fig. 2*B*). By contrast, infection either with an adenovirus expressing the β -galactosidase gene (MOI of 500) or with an adenovirus containing no insert provided no protection, indicating that the infection process by itself did not alter neuronal survival.

Caspases and LIF-mediated cell death

Another point of convergence shared by many apoptotic pathways is the activation of the caspase family. We, therefore, examined the effects of caspase inhibitors on LIF-treated sympathetic neurons (Fig. 3). Treatment with LIF alone significantly reduced neuron numbers by ~80%. Cotreatment with the nonspecific caspase inhibitor z-vad-fmk reduced cell death to only 30% of the neurons (Fig. 3*A*). However concurrent administration of z-vad-fmk with its inhibitor capthepsin B abolished this protective effect. These observations suggest involvement of a caspase in LIF-induced neuronal death. However treatment with y-vad, a more specific inhibitor for caspase 1, was not protective, suggest-

Figure 2. LIF-induced cell death of cultured sympathetic neurons requires Bax. *A*, Sympathetic neurons were cultured from neonatal Bax-deficient ($-/-$) mice and from controls ($+/+$). After 24 hr in culture the cells were treated with LIF or anti-NGF, and cell numbers were determined on day 3 of culture. Note that exposure to LIF resulted in the death of $<30\%$ of the sympathetic neurons derived from newborn Bax-deficient ($-/-$) mice, whereas LIF treatment of neurons from control animals killed 74% of the cells. *Con*, Control. *B*, Sympathetic neurons from P1 animals were infected after 24 hr in culture with a bcl-2-containing adenoviral vector at an MOI of 500 or with a control vector containing the β -galactosidase gene (*lac-z*). Twelve hours later the cells were treated with LIF, and cell numbers were determined 48 hr later. Note that the bcl-2-expressing virus prevented LIF-mediated cell death whereas adenoviral introduction of *lac-z* provided no protection. * $p < 0.05$ compared with control; ** $p < 0.05$ compared with LIF alone by ANOVA.

ing that other members of the caspase family mediate LIF's proapoptotic effects (Fig. 3*B*). Because caspase 2 (Nedd-2) is one of the mediators of sympathetic neuronal death after NGF deprivation (Troy et al., 1997), we sought to determine whether Nedd-2 is necessary for LIF-induced cell death. Pretreatment of sympathetic neurons with A-Nedd for 6 hr provided robust protection against subsequent LIF exposure (Fig. 4). Treatment with LIF alone killed $\sim 80\%$ of the cells, whereas cotreatment with A-Nedd reduced cell death to $<20\%$ of cells. By contrast, treatment with S-Nedd had no effect on survival or death. Because Nedd-2 is required for LIF-induced apoptosis, we questioned whether downregulation of caspase 2 expression during postnatal development might account in part for the loss of the proapoptotic effects of LIF. Nedd-2 expression in the SCG was therefore examined immunohistochemically at different postnatal time points (data not shown). The SCG expressed caspase 2 abundantly on postnatal day 1. However levels of Nedd-2 expression

Figure 3. Inhibition of caspases prevents the LIF-induced death of cultured sympathetic neurons. *A*, P1 sympathetic neurons were treated after 24 hr *in vitro* with LIF, z-vad (Z-VAD; 100 μ M), or capthepsin B (Inh), an inhibitor of z-vad, and cell numbers were determined 48 hr later. Note that cotreatment with z-vad partially prevented the effects of LIF. Concurrent administration of the z-vad inhibitor capthepsin B abolished this protective effect of z-vad. *B*, By contrast, similar experiments using a more specific inhibitor for caspase 1, y-vad (Y-VAD), did not protect neurons from LIF, suggesting the involvement of other caspases. * $p < 0.05$ compared with control; ** $p < 0.05$ compared with LIF alone by ANOVA.

declined substantially by P4, and by P11 and thereafter the caspase was almost undetectable in the ganglia.

LIF requires p75^{LNTR} signaling to induce cell death

Apoptosis of sympathetic neurons is mediated, at least in part, by p75^{LNTR} (Bamji et al., 1998), and it has been suggested that competitive signaling between TrkA and p75^{LNTR} determines cell survival. Because of the striking similarities between LIF-induced apoptosis and neuronal death after NGF deprivation, we questioned whether p75^{LNTR} participates in LIF-induced death. To address this issue, we cultured sympathetic neurons in the presence of LIF and an antibody (REX) that binds to p75^{LNTR} and inhibits NGF binding to the receptor (Weskamp and Reichardt, 1991) (Fig. 5). Treatment with LIF alone resulted in the death of 72% of the neurons. However treatment with both LIF and the antibody killed only 15% of the neurons, suggesting that p75^{LNTR} function is necessary for LIF-induced death. To test this hypothesis further, the effects of LIF were examined on neurons cultured from animals with a null deletion of the p75^{LNTR} gene (Fig. 6). Approximately 73% of neurons expressing p75^{LNTR} ($+/+$) died in the presence of LIF, whereas $<15\%$

Figure 4. LIF-induced cell death is mediated by the caspase Nedd-2. P1 sympathetic neurons were treated after 24 hr *in vitro* with A-Nedd or with S-Nedd for 6 hr. Then the cultures were exposed to LIF, and cell numbers were determined 48 hr later. Note that A-Nedd rescued cells from LIF exposure, whereas S-Nedd provided no protection. * $p < 0.05$ compared with control; ** $p < 0.05$ compared with LIF alone by ANOVA.

Figure 5. p75^{LNTF}-blocking antibody protects against LIF-mediated neuronal death. P1 sympathetic neurons were treated after 24 hr *in vitro* with LIF. Sister cultures were treated with both LIF and a p75^{LNTF}-blocking antibody (p75 Ab; REX; 1:100) or with the antibody alone. Treatment with LIF alone led to the death of 72% of the cells compared with control. However treatment with both LIF and the antibody killed only 15% of the neurons, suggesting that p75^{LNTF} function is necessary for LIF-induced death. *Differs from all other groups at $p < 0.05$ by ANOVA.

of p75^{LNTF}-deficient (–/–) neurons died after treatment with the cytokine.

LIF requires gp130 signaling to induce cell death

The requirement for p75^{LNTF} function for LIF-mediated apoptosis raises the question of whether the effects of the factor are mediated via classical cytokine receptors. To address this issue, we took advantage of the fact that several other cytokines signal via receptors that share the same LIFR and gp130 subunits that constitute the LIF receptor but that also require additional α subunits. The interleukin 6 (IL6) receptor is comprised of two gp130 subunits and an IL6 α subunit (IL6R). Sympathetic neurons do not express significant levels of the IL6 receptor, and treatment of cultured neurons with IL6 does not induce apoptosis (Fig. 7). The IL6R in soluble form can be added to cultured cells that express gp130 to reconstitute functional receptors (Rose-John and Heinrich, 1994). Addition of soluble IL6R alone to

Figure 6. LIF-induced cell death of cultured sympathetic neurons requires p75^{LNTF}. Sympathetic neurons were cultured from neonatal p75^{LNTF}-deficient (–/–) mice and from controls (+/+). After 24 hr in culture the cells were treated with LIF or with vehicle, and cell numbers were determined 48 hr later. Note that exposure to LIF resulted in the death of <25% of the sympathetic neurons derived from newborn p75^{LNTF}-deficient (–/–) mice, whereas LIF treatment of neurons from control animals killed ~73% of the cells. *Differs from all other groups at $p < 0.05$.

Figure 7. Cell death is mediated by classic cytokine receptors. After 24 hr in culture, sympathetic neurons were treated with IL6 (100 ng/ml), soluble IL6 receptor (sIL6R; 200 ng/ml), PI-PLC (1 U/ml), and/or CNTF (100 ng/ml). In PI-PLC-treated cultures, enzyme was added 1 hr before CNTF treatment. Neuron numbers were determined 48 hr later. Note that IL6 induced cell death only in the presence of sIL6R and that PI-PLC pretreatment abolished CNTF-mediated cell death. *These two groups differ from all other groups at $p < 0.025$ by ANOVA.

cultured sympathetic neurons did not alter survival. However, addition of IL6 along with soluble IL6R induced apoptosis, indicating that IL6 only induces apoptosis in the presence of its own receptor. In previous studies we found that ciliary neurotrophic factor (CNTF) exerts the same effects as LIF on sympathetic neuron survival (Kessler et al., 1993) (Fig. 7). The CNTF receptor is comprised of the same gp130 and LIFR subunits as the LIF receptor along with a CNTF α subunit that is anchored by a glycosylphosphatidylinositol linkage. This linkage is sensitive to treatment with phosphatidylinositol-specific phospholipase C (PI-PLC) (Davis et al., 1991; Kessler et al., 1993). Sympathetic neurons were therefore treated with PI-PLC before treatment with CNTF. Treatment with PI-PLC alone did not alter neuronal survival. However pretreatment with the enzyme prevented neuronal death induced by CNTF (Fig. 7) (Kessler et al., 1993). Thus the effects of CNTF on cell death are mediated by a receptor

Figure 8. LIF treatment activates JNK signaling. After 24 hr in culture, sympathetic neurons were treated with LIF or vehicle or deprived of NGF with anti-NGF antibody. At 6 and 12 hr afterward, JNK activity was assayed. Lysates were prepared and subjected to immunoprecipitation/kinase assays using GST-c-jun as a substrate for JNK activity. The experiments were repeated three times with similar results.

sensitive to PI-PLC, suggesting that the classic CNTF receptor is involved.

LIF treatment does not alter trkA expression or signaling but activates the JNK pathway

Because p75^{LNT} activation promotes apoptosis of sympathetic neurons, it was possible that LIF promoted neuronal death by increasing the levels of p75^{LNT}; however Western blot analyses showed no change in the levels of the receptor after LIF treatment (data not shown). Survival of sympathetic neurons depends on trkA signaling with consequent suppression of apoptosis, and inhibition of trkA expression or signaling represented another possible mechanism underlying LIF-induced apoptosis. However LIF treatment did not alter levels of trkA measured by Western blot analysis, and it did not alter trkA phosphorylation (data not shown). Alternatively it was possible that LIF treatment altered activation of mitogen-activated protein kinase (MAPK), but there were no changes in the phosphorylation of MAPK after LIF treatment (data not shown). However activation of JNK, another member of the MAPK family, plays a role both in p75-mediated death of sympathetic neurons and in death after NGF deprivation (Xia et al., 1995; Aloyz et al., 1998). We therefore determined whether LIF treatment of cultured sympathetic neurons leads to JNK activation. JNK activity was not detected in control cultures (Fig. 8) under the assay conditions. However treatment with LIF significantly increased JNK activity at both 6 and 12 hr after LIF exposure (Fig. 8), and JNK activity was similarly increased at these time points after NGF deprivation (Fig. 8).

DISCUSSION

Neuronal survival during development is regulated by target-derived growth factors that act to suppress apoptosis. Increasing evidence, however, suggests that cell number is also regulated by cytokines that activate rather than prevent neuronal death. LIF treatment of cultured sympathetic neurons induces apoptosis even in the presence of NGF (Kessler et al., 1993). Tumor necrosis factor, which kills a number of cell types outside of the nervous system, has been reported to induce cell death of oligodendroglia in culture (Louis et al., 1993). NGF activation of its low-affinity receptor p75^{LNT} also kills cultured oligodendroglia (Casaccia-Bonnet et al., 1996) and may lead to apoptosis of retinal cells (Frade et al., 1996). Similarly BDNF activation of p75^{LNT} induces apoptosis of cultured sympathetic neurons (Bamji et al., 1998). Furthermore, targeted deletion either of the BDNF gene or of the p75^{LNT} increases sympathetic neuron

number (Bamji et al., 1998), indicating that receptor-mediated cell death occurs *in vivo* as well as in culture. Thus, cell survival may be controlled by the combined actions of both proapoptotic and antiapoptotic cytokines.

Although cells undergoing apoptosis share a number of stereotyped morphological features, the intracellular pathways mediating cell death may be quite diverse. For example, Bcl-2 may either promote or inhibit neuronal death depending on the death stimulus and the cellular context. Bcl-2 prevents apoptosis of sympathetic neurons after NGF deprivation (Garcia et al., 1992) and after LIF treatment (Fig. 2B). By contrast, Bcl-2 promotes the p75^{LNT}-mediated death of cultured sensory neurons (Coulson et al., 1999). Furthermore, the same cell may express more than one caspase, and different caspases may be activated by different proapoptotic events. Apoptosis caused by growth factor deprivation of sympathetic neurons is mediated by the cysteine aspartase Nedd-2, whereas apoptosis of the same cells induced by downregulation of superoxide dismutase involves a different protease (Troy et al., 1997). In the present study, we compared some of the intracellular death pathways activated by LIF in sympathetic neurons with those that mediate death after NGF deprivation. The results from this study indicate that these two death signals activate remarkably parallel pathways leading to apoptosis.

Deletion of Bax prevented apoptosis of 85% of the sympathetic neurons in this study and 100% of the neurons in the study by Deckwerth et al. (1996) after NGF deprivation. Furthermore, Bax deletion prevented death of 70% of the sympathetic neurons in this study after LIF treatment (Fig. 24). These findings indicate that Bax is essential to neuronal death activated either by LIF or the absence of NGF. The protection afforded by Bcl-2 overexpression further supports a role for the Bcl-2 family members in cell death induced either by LIF (Fig. 2B) or NGF deprivation (Garcia et al., 1992). Nevertheless 30% of neurons killed by LIF and 15% of neurons deprived of NGF in this study must have died by a Bax-independent pathway. This suggests that each of these insults may activate more than one pathway leading to cell death. There were also striking parallels in caspase activation after LIF treatment and NGF deprivation. The nonspecific caspase inhibitor z-vad was protective against both types of death signal, indicating the participation of these proteases in both processes. More important, downregulation of Nedd 2 (caspase 2) rescued neurons from both LIF-induced apoptosis and NGF deprivation, pointing to a requisite role for Nedd 2 in cytokine-mediated apoptosis as well as death after growth factor deprivation.

Sympathetic neurons lose their dependence on exogenous factors for survival during postnatal development, and this phenomenon is recapitulated in culture. Thus neurons maintained in culture for >10 d begin to develop trophic factor independence. At P1 only ~10% of neurons survive NGF deprivation, whereas ~25% survive this insult at P10 (Fig. 1B). By P20 >90% of cultured neurons survive in the absence of NGF (Easton et al., 1997) (J. A. Kessler, unpublished observations). We found that the apoptotic response to LIF is similarly regulated. Treatment of P1 sympathetic neurons with LIF resulted in death of >80% of the cells. However by day 4 in culture only 40% of the cells died in response to LIF, and by day 10 in culture only 10% died (Fig. 1B). By day 15 in culture LIF did not kill any cells (data not shown). What is the mechanism underlying the increased resistance to LIF treatment and NGF deprivation? Easton et al. (1997) have shown that NGF-independent, sympathetic neurons grown for 23 d *in vitro* do not die without trophic support because

they have a block at the Bax checkpoint. Interruption of the death pathways mediated by Bax could thus account for some of the loss of LIF's proapoptotic effects. However a significant portion of LIF-mediated death was not Bax-dependent (Fig. 2A), indicating that additional mechanisms are involved. Nedd-2 immunoreactivity decreases progressively in SCG neurons from P1 through the adult (data not shown). By P11, expression of Nedd-2 could only be located in a small subpopulation of cells, and in the adult expression of Nedd-2 was almost undetectable in the SCG. Thus the loss of LIF-mediated death is paralleled by a loss of Nedd-2 expression. Because overexpression of Nedd-2 leads to neuronal death even in the absence of a death signal (Kessler, unpublished observations), it could not be determined whether reconstituting Nedd-2 expression would restore LIF-mediated cell death. Easton et al. (1997) restored NGF dependence in mature sympathetic neurons *in vitro* with overexpression of Bax, which suggests that some functional caspase(s) is still present at that age. Nevertheless it seems likely that downregulation during development of multiple components of the cell death pathways including Bax and Nedd-2 underlies the loss of growth factor dependence and cytokine-mediated apoptosis.

Deletion of the gene encoding p75^{LNTF} primarily prevented apoptosis after LIF treatment, implying a requisite role for the receptor in the cell death pathways. Nevertheless several lines of evidence indicate that the proapoptotic effects of LIF were mediated by interactions of the cytokine with its own receptor that includes two subunits, gp130 and LIFR β . Other cytokines that activate gp130 receptor-mediated pathways, such as CNTF, also induce apoptosis of cultured sympathetic neurons (Fig. 7) (Kessler et al., 1993). In the CNTF receptor, the α subunit is anchored by a glycosylphosphatidylinositol linkage that is sensitive to treatment with PI-PLC (Davis et al., 1991). Cleavage of this linkage by treatment of sympathetic neurons with PI-PLC abolished CNTF-mediated apoptosis, indicating that interactions with this receptor mediate the cell death response (Fig. 7) (Kessler et al., 1993). Furthermore, sympathetic neurons do not express detectable levels of the IL6 receptor subunit and do not respond to the factor (Fig. 7). However, addition of soluble IL6 receptor to the cultures along with IL6 induced apoptosis (Fig. 7). Because the IL6 receptor complex includes gp130 but not the LIFR subunit, this indicates that activation of gp130 is involved in the cell death pathway. gp130 is expressed by embryonic as well as neonatal sympathetic ganglia, and its signaling plays a number of roles in the developing ganglia (Wong et al., 1995; Murphy et al., 1997; Geissen et al., 1998). For example, the effects of gp130-mediated signaling on the specification of neurotransmitter phenotype occur *in vivo* as well as in culture (Patterson and Nawa, 1993; Geissen et al., 1998). However multiple cytokines signal via this receptor complex, and effects on cell survival *in vivo* may be dependent on the combined concentrations of all such cytokines. For example, targeted deletion of the gene for either LIF or CNTF leads to only minimal changes in motor neuron survival, whereas knock-out of both genes in the same animal leads to a more extensive phenotype (Sendtner et al., 1996). Therefore delineation of the proapoptotic effects of these cytokines on sympathetic neurons *in vivo* may require more extensive knowledge of all members of the cytokine family that signal via this receptor and of the availability of these factors to sympathetic neurons *in vivo*.

Survival of sympathetic neurons appears to depend on a balance between the antiapoptotic effects of trkA after ligand bind-

ing and the proapoptotic effects of p75^{LNTF} (Bredesen and Rabizadeh, 1997; Dechant and Barde, 1997; Yoon et al., 1998). LIF treatment of sympathetic neurons does not alter levels of either trkA or p75^{LNTF} and does not alter NGF binding (Kessler et al., 1993). Therefore, because all experiments in this study were done in the presence of NGF, there was ligand bound to p75^{LNTF} during LIF-mediated cell death. Treatment with the p75^{LNTF} function-blocking antibody REX (Weskamp and Reichardt, 1991) inhibited LIF-mediated death, implying that NGF binding to the p75^{LNTF} was in fact necessary for the cytokine-mediated apoptosis. However, it is possible that LIF may stimulate the production of another molecule like BDNF, which is known to bind to p75 and cause sympathetic neuronal death (Bamji et al., 1998). Furthermore, deletion of the gene encoding p75^{LNTF} primarily prevented apoptosis after LIF treatment, implying a requisite role for the receptor in the cell death pathways. These observations suggest that p75^{LNTF} signaling is necessary to prime the cells for the proapoptotic effects of LIF. LIF treatment thus can be perceived as a signal that augments the proapoptotic effects of p75^{LNTF}.

Where do LIF signaling and p75^{LNTF} signaling converge to promote apoptosis? Because previous studies have shown that p75^{LNTF}-mediated apoptosis depends on activation of JNK (see Casaccia-Bonnel et al., 1999), the effects of LIF treatment on JNK activation were examined. In fact, LIF treatment activated JNK to an extent similar to that of NGF deprivation (Fig. 8). It is therefore not surprising that the same downstream pathways were activated by LIF and by NGF deprivation and that there was a parallel developmental loss of NGF dependence and the proapoptotic effects of LIF.

There are several reasons why such controls of cell number might be necessary. First, cytokine-mediated cell death might help to achieve a balance among multiple populations of neurons that compete for the same growth factor in a target. For example, the iris is innervated by two NGF-dependent populations of neurons, sympathetic neurons and trigeminal sensory neurons, that compete for NGF in the iris (Kessler et al., 1983). During the developmental period during which LIF or CNTF kills NGF-dependent sympathetic neurons, these cytokines conversely promote survival of NGF-dependent trigeminal sensory neurons (Horton et al., 1998). Thus LIF or CNTF might help to eliminate some sympathetic neurons innervating the iris to achieve an appropriate balance between sympathetic and sensory fibers competing for NGF. Without such additional controls, sympathetic fibers, which compete effectively for NGF in the iris (Kessler et al., 1983), might prevent sensory innervation, or vice versa. Alternatively factors that promote death of some neuronal subpopulations might help to eliminate cells that migrate errantly or that project to incorrect targets. If such aberrant cells encountered NGF, they might persist in the absence of other signals to eliminate them. Although high doses of LIF were used in this study to maximize stimulation of the cell death pathways, much lower doses (~ 1 nM—approximately the same as the concentrations of NGF necessary for optimal survival) are sufficient to promote death of some neurons (Kessler et al., 1993). However some neurons survived even in the presence of the high doses of the cytokine, indicating that there is heterogeneity among sympathetic neurons with respect to responses to LIF. Such heterogeneity could help to explain why some neurons survive and others die in ostensibly similar cellular milieus *in vivo*.

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