Cytosine arabinoside rapidly activates Bax-dependent apoptosis and a delayed Bax-independent death pathway in sympathetic neurons

CG Besirli[1], TL Deckwerth[2], RJ Crowder[3], RS Freeman[4] and EM Johnson Jr*[,1]

[1] Departments of Neurology and Molecular Biology & Pharmacology, Washington University School of Medicine, Saint Louis, MO 63110, USA
[2] Idun Pharmaceuticals, Inc., San Diego, CA 92121, USA
[3] Department of Pathology, Washington University School of Medicine, Saint Louis, MO 63110, USA
[4] Department of Pharmacology and Physiology, University of Rochester School of Medicine and Dentistry, Rochester, NY 14642, USA
* Corresponding author: EM Johnson Jr., 660 South Euclid, Campus Box 8103, St Louis, MO 63110, USA. Tel.: 314 362 3926; fax: 314 747 1772; E-mail: ejohnson@pcg.wustl.edu

Abstract

Cytosine arabinoside (ara-C) is a nucleoside analog used in the treatment of hematologic malignancies. One of the major side effects of ara-C chemotherapy is neurotoxicity. In this study, we have further characterized the cell death induced by ara-C in sympathetic neurons. Similar to neurons undergoing trophic factor deprivation-induced apoptosis, ara-C-exposed neurons became hypometabolic before death and upregulated c-_myb_, c-_fos_, and Bim. Bax deletion delayed, but did not prevent, ara-C toxicity. Neurons died by apoptosis, indicated by the release of mitochondrial cytochrome-_c_ and caspase-3 activation. p53-deficient neurons demonstrated decreased sensitivity to ara-C, but neither p53 nor multiple p53-regulated genes were induced. Mature neurons showed increased ara-C resistance. These results demonstrate that molecular mechanisms underlying ara-C-induced death are similar to those responsible for trophic factor deprivation-induced apoptosis. However, substantial differences in neuronal death after these two distinct stress stimuli exist since ara-C toxicity, unlike the developmental death, can proceed in the absence of Bax.
Cell Death and Differentiation (2003) **10**, 1045–1058. doi:10.1038/sj.cdd.4401259

Introduction

The nucleoside analog cytosine arabinoside, (1-β-D-arabino-furanosylcytosine, ara-C), is one of the most effective chemotherapeutic agents in the treatment of acute myelogenous leukemia and a variety of other hematologic malignancies. The exact mechanism of ara-C toxicity to proliferating cells still remains to be elucidated. Ara-C can inhibit DNA synthesis in proliferating cells by incorporating into elongating DNA strands and causing retardation of DNA elongation as well as chain termination. Moreover, ara-C inhibits several enzymes involved in DNA synthesis and repair. In addition to its ability to interfere with DNA metabolism, ara-C also increases the generation of reactive oxygen species, alters the synthesis of membrane lipids and glycoproteins, and affects the intracellular levels of lipid second messengers (see review in Grant[1]).

Besides killing mitotic cells, ara-C is also toxic to postmitotic cells such as neurons.[2–7] Neurotoxicity is a major dose-limiting factor in high-dose ara-C treatment for refractory leukemias. Neuronal death occurs in several regions of the central nervous system, including the cerebellum, brainstem, medulla, and spinal cord, and includes motor neurons and Purkinje cells.[8–12] *In vitro*, ara-C is toxic to postmitotic sympathetic, parasympathetic and sensory neurons of the peripheral nervous system as well as cerebellar and cortical neurons of the central nervous system.[2–4,7,13] This neurotoxicity is dose-dependent and can be completely prevented by deoxycytidine (dC), which is the preferred substrate for most metabolic processes that are affected by ara-C.[2]

In sympathetic neurons of the superior cervical ganglion (SCG), cell death after ara-C exposure shows some similarities to the programmed cell death of these neurons after the withdrawal of their trophic factor, nerve growth factor (NGF).[4] As in NGF deprivation, potassium depolarization and elevation of cyclic AMP prevent or retard ara-C-induced sympathetic neuronal death. Moreover, cell death in neurons after ara-C exposure is an active process that requires the synthesis of new proteins and can be prevented by the inhibitors of RNA and protein syntheses. Additional findings, including fragmentation of neuronal genomic DNA and retardation of death by caspase inhibitors, strongly suggest that ara-C kills sympathetic neurons by apoptosis.[6,14]

Inhibition of ara-C neurotoxicity by the protein synthesis inhibitor, cycloheximide, suggests that synthesis of new protein(s) is required for the execution of neuronal death. One strong candidate is p53, an important mediator of DNA-damage-induced apoptosis after exposure to ionizing irradiation or other DNA-damaging agents.[15–18] p53 protein has been reported to accumulate in ara-C-treated sympathetic neurons.[5] Ara-C is also toxic to cerebellar granule neurons *in vitro*.[19] This cell death appears to be apoptotic and has been

reported to depend on the presence of a functional *p53* allele.[20,21] These findings suggest that ara-C may kill postmitotic neurons by a DNA-damage-activated, p53-dependent pathway.

Enhanced sensitivity of neuronal cells to DNA damage is demonstrated by genetic studies in mice. Mutant mice lacking several proteins involved in DNA repair show severe and selective loss of postmitotic neurons.[22–25] This may be caused by incomplete DNA repair pathways in neurons or increased dependence on functional DNA repair as a result of higher basal DNA damage. Therefore, postmitotic neurons may have low tolerance to additional DNA damage and secondary DNA damage may be an important factor in neuronal cell death seen in several neurodegenerative disorders.

In this study, we have addressed several issues related to the mechanism(s) of the neuronal cell death induced by ara-C in sympathetic neurons *in vitro*. After ara-C treatment, sympathetic neurons underwent metabolic changes that are very similar to those observed after NGF deprivation, including a decrease in protein synthesis as well as total protein levels. Moreover, ara-C exposure caused the induction of genes known to be important in neuronal cell death caused by NGF deprivation. Mitochondrial cytochrome-*c* release and caspase activation demonstrated that sympathetic neurons die by apoptosis after ara-C exposure. This relatively rapid process depended on the presence of at least one functional allele of *bax* or *p53*. However, neither Bax nor p53 was induced in neurons after ara-C exposure. Our results demonstrate that ara-C kills sympathetic neurons by inducing an apoptotic pathway that has similarities to the programmed cell death of sympathetic neurons after trophic factor deprivation. However, when this apoptotic pathway is blocked, ara-C can activate a Bax- and caspase-independent death pathway that kills sympathetic neurons more slowly.

Results

Toxicity of ara-C in sympathetic neurons is dose dependent

As we and others have reported previously, ara-C is toxic to sympathetic neurons *in vitro*.[2–4] Rat sympathetic neurons exposed to 100 μM of ara-C for 24 h did not show any morphological changes (Figure 1a). By 48 h, however, ara-C toxicity was easily visible such that neurons showed morphological signs of cell death including neurite disintegration and soma degeneration. These changes are very similar to those observed in sympathetic neurons after trophic factor deprivation.[6] Most neurons appeared dead after 72 h of ara-C treatment.

The dose-response and time course of ara-C toxicity had been measured previously in sympathetic neuronal cultures by using the adenylate kinase assay as qualitative measure of neuronal viability.[4] To obtain quantitative viability data and to determine the optimal ara-C dose for cell death under the current culture conditions, we exposed neurons to various doses of ara-C for 84 h and viability was measured after crystal violet staining (Figure 1b). Ara-C-induced death was apparent at 10 μM, at which concentration only half of the neurons survived. A 100 μM of ara-C showed the highest
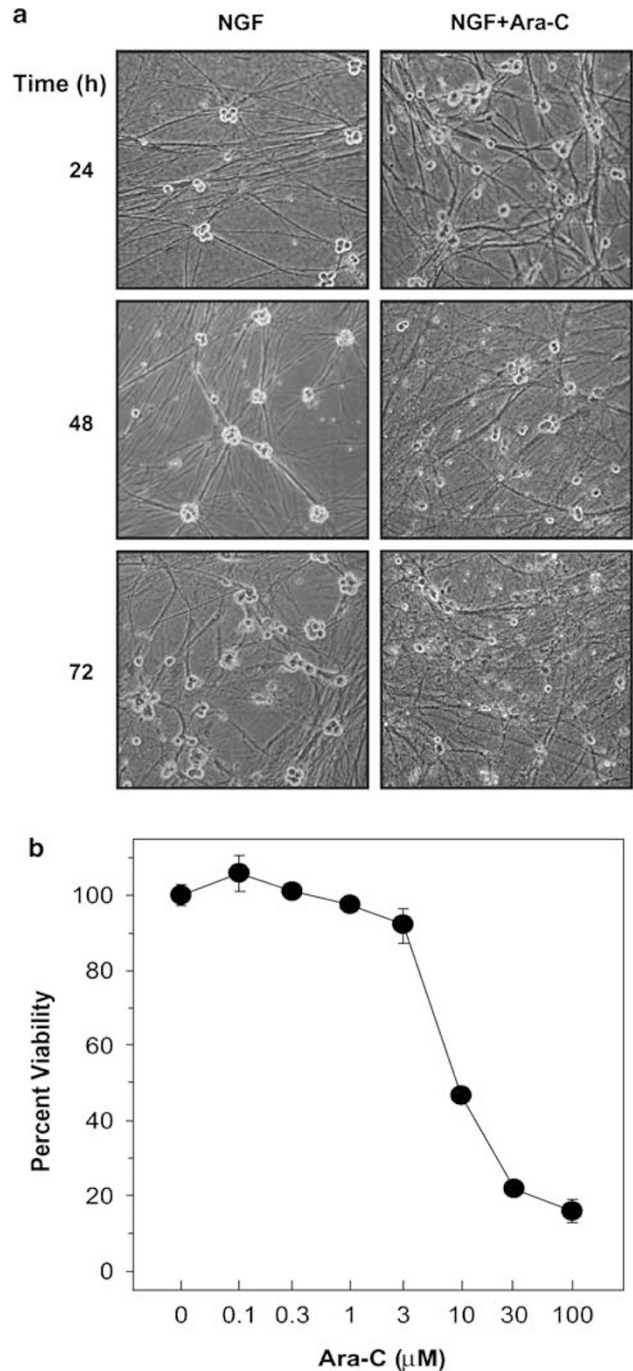
Figure 1 Dose-dependent toxicity of ara-C in sympathetic neurons. (**a**) Phase-contrast images were taken of NGF-maintained, untreated rat sympathetic neurons (NGF) or neurons from sister cultures exposed to 100 μM ara-C (NGF+Ara-C) at 5 DIV for the indicated times. Untreated neurons have round and phase-bright cell bodies and intact neurites. However, ara-C-treated neurons show neurite disintegration and soma degeneration by 48 h. By 72 h, most ara-C-exposed neurons are dead and have lost their phase-bright appearance. (**b**) Sympathetic neurons maintained in NGF for 7 DIV were exposed to different concentrations of ara-C for 84 h and the fraction of surviving neurons was determined. Half the neurons died at a concentration of 10 μM ara-C. Mean $\pm$ S.E.M. from two independent experiments

toxicity and killed over 80% of the neurons by 84 h. Therefore, we selected 100 μM of ara-C concentration for subsequent experiments.

Commitment-to-die after ara-C exposure is significantly earlier than loss of viability in sympathetic neurons

We next determined a detailed time course of neuronal death after ara-C exposure. Rat sympathetic neurons kept in culture for 5 days were treated with 100 μM of ara-C and the number of viable neurons was determined every 24 h (Figure 2a). All neurons exposed to ara-C were viable by 24 h. At 48 h, however, about half of the neurons lost their viability and most neurons were dead by 96 h. These results are consistent with the time course of visible degeneration of neurons as shown in Figure 1a. This time course of neuronal death is delayed by about 24 h compared to the time course of neuronal degeneration after NGF deprivation,[6] suggesting that a significant window occurs during which neurons have to be exposed to ara-C before they sustain enough damage necessary to trigger cell death. Moreover, once cell death starts, it appears to proceed with somewhat slower kinetics compared to NGF deprivation since ara-C-treated neurons require 48 h for complete degeneration, whereas the majority of NGF-deprived neurons die within 30 h.[6]

As ara-C-induced death had a delayed time course compared with programmed cell death of sympathetic neurons after NGF deprivation, we examined whether neurons also committed to die more slowly in the presence of ara-C. As we have shown previously, ara-C-induced death can be completely prevented by deoxycytidine (dC) in sympathetic neurons.[2] At each rescue time point, the population of ara-C-exposed neurons that has sustained enough irreversible damage will commit to die. Therefore, the commitment assay identifies the number of neurons that have already started the death process, some of which may still appear viable. Sympathetic neurons were exposed to 100 μM of ara-C for various times, at the end of which they were rescued by replacing the medium with fresh AM50 containing 100 μM dC. Surprisingly, sympathetic neurons treated with ara-C committed to die with an accelerated time course compared to the viability time course (Figure 2a). Although 100% of neurons were still viable at 24 h after ara-C exposure, about half of the neurons had already committed to die at this time (Figure 2a). Moreover, sympathetic neurons had all committed to die at 36 h, at which time the majority of neurons still appeared to be viable. This time course indicates that ara-C rapidly caused irreversible damage to neurons, but the execution of cell death was slower and required another 24–36 h to complete. The commitment time course of ara-C-exposed sympathetic neurons was more similar to the commitment of neurons after NGF deprivation, lagging only by 6–12 h.[6]

Sympathetic neurons become hypometabolic after ara-C exposure

Programmed cell death in sympathetic neurons after trophic factor deprivation is preceded by a decrease in metabolic events such as protein and RNA synthesis.[6] We investigated whether ara-C exposure also led to hypometabolism by measuring the rate of protein synthesis. Upon exposure of sympathetic neurons to 100 μM of ara-C, the rate of protein synthesis did not change during the first 12 h, decreased
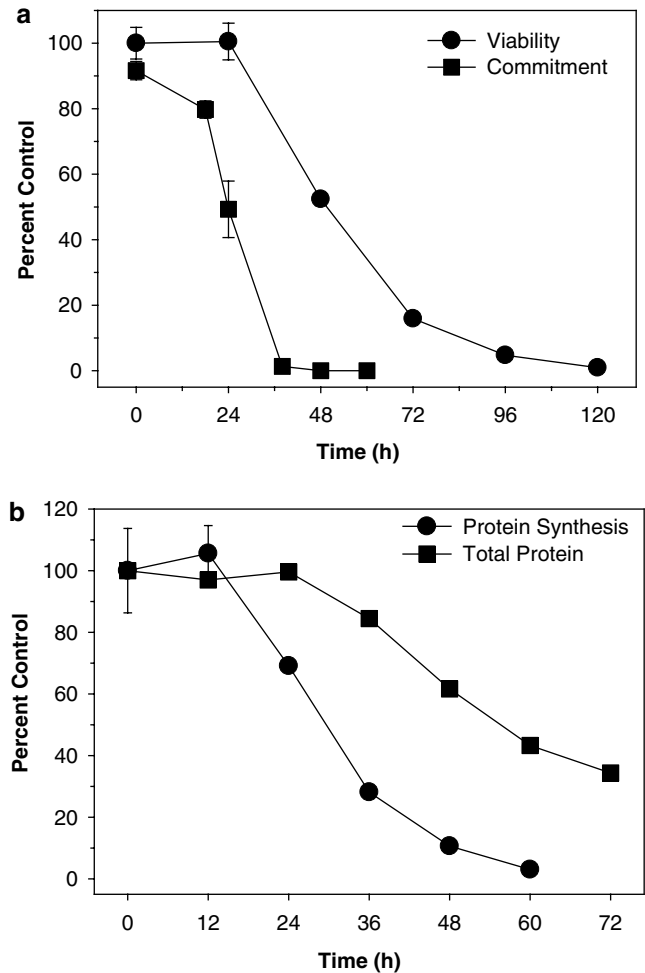
Figure 2 Time course of ara-C-induced neuronal death and decrease in protein synthesis. (**a**) Sympathetic neuronal cultures maintained in the presence of NGF for 5 DIV were exposed to 100 μM ara-C for different times, and the fraction of surviving neurons was determined by measuring viability or commitment as described. Half the neurons lost their viability at 48 h and 100% of neurons were dead by 120 h. Sympathetic neurons committed to die 24–48 h before losing viability. The mean $\pm$ S.D. from a representative experiment of three independent trials with 3–4 wells per condition is shown. (**b**) Time course of the change in the rate of neuronal protein synthesis and total neuronal protein after ara-C exposure. NGF-maintained neuronal cultures were exposed to 100 μM ara-C for different times prior to determination of protein synthesis as measured by the incorporation of L-[4,5-^{3}H]leucine into protein on 9 DIV or measuring total neuronal protein by the bicinchoninic acid method. After a lag time of 12 h, neuronal protein synthesis declined with only 30% remaining after 36 h of ara-C exposure. Total neuronal protein declined after a lag of 24 h, concurrent with the loss of viability. The data represent the mean $\pm$ S.E.M. from 2 to 3 independent experiments

during the next 24 h by 30%, and dropped to 10% after 48 h (Figure 2b). The time course of the decline in protein synthesis rates was somewhat delayed compared to that of commitment to die after ara-C exposure (Figure 2a). This delay may suggest that once a neuron is committed to die, metabolic processes needed to keep a cell functioning normally were no longer sustained. However, the time course of protein synthesis reflects the changes in a population of sympathetic neurons, so one cannot determine whether decreased protein synthesis at any time point is because of every neuron

generating less protein or a complete stop of protein synthesis in a subpopulation of neurons that have committed to die. A combination of these two potential scenarios likely contributes to the decreased protein synthesis seen after ara-C exposure. Association of ara-C-induced death with neuronal hypometabolism was very similar to that after NGF deprivation. However, compared to the time course of NGF deprivation,[6] the decrease in the rate of protein synthesis showed a delay in onset of 12–18 h after exposure to ara-C. This delay probably indicated a significant difference in the activation phase preceding the onset of the decrease in protein synthesis after NGF deprivation and ara-C exposure. In addition, the decline in the rate of protein synthesis after ara-C exposure occurred more slowly than after NGF withdrawal (Figure 2b and [6]). This difference most likely arose from the variability of ara-C-induced damage in individual neurons, making the cell death and the onset of the decline in metabolic events more desynchronized in the neuronal population compared to NGF deprivation.

Decline in protein synthesis rate was also demonstrated by the decreased amount of total protein in sympathetic neurons after ara-C treatment. No reduction in the level of neuronal protein occurred during the first 24 h after ara-C treatment (Figure 2b). Concurrent with the onset of loss of viability at 36 h, neuronal protein content decreased. At later times, the decrease in neuronal protein lagged behind the decrease in loss of viability, similar to that after removal of NGF.[6] The lack of a decrease in neuronal protein prior to loss of viability was consistent with the less-impressive neuronal atrophy seen after addition of ara-C compared to the atrophy seen upon removal of NGF (Figure 1a and [6]). The lack of proportionality between the time courses of viability and the loss of total protein after 48 h of ara-C treatment was because of the accumulation of protein in neuronal debris, which cannot be separated from the protein associated with living neurons by the washing step.

Genes induced after NGF deprivation are also upregulated upon ara-C exposure in sympathetic neurons

Sympathetic neurons show increased levels of certain gene products at both mRNA and protein level before the execution of NGF-deprivation-induced apoptosis. We have analyzed whether similar changes in gene expression occurred during ara-C-induced neuronal death. One of the earliest genes that is upregulated in trophic factor deprivation in sympathetic neurons is c-myb.[26] Similarly, ara-C treatment led to a robust increase in c-myb mRNA level in neurons, which peaked at 49 h (Figure 3a). Recent evidence suggests that this increase may be a result of derepression of genes regulated by the E2F transcription factor family.[27] NGF deprivation also leads to an increased level of c-fos mRNA in neurons.[26,28] c-fos was also induced in sympathetic neurons after ara-C exposure as measured by reverse transcription-polymerase chain reaction (RT-PCR) of c-fos mRNA (Figure 3a).

The Bcl-2 family of pro- and antiapoptotic proteins is critical for the regulation of apoptosis in many cell types.[29,30] One of the best examples of the role of Bcl-2 family in apoptosis is observed in sympathetic neurons. Bim, a BH-3-only member
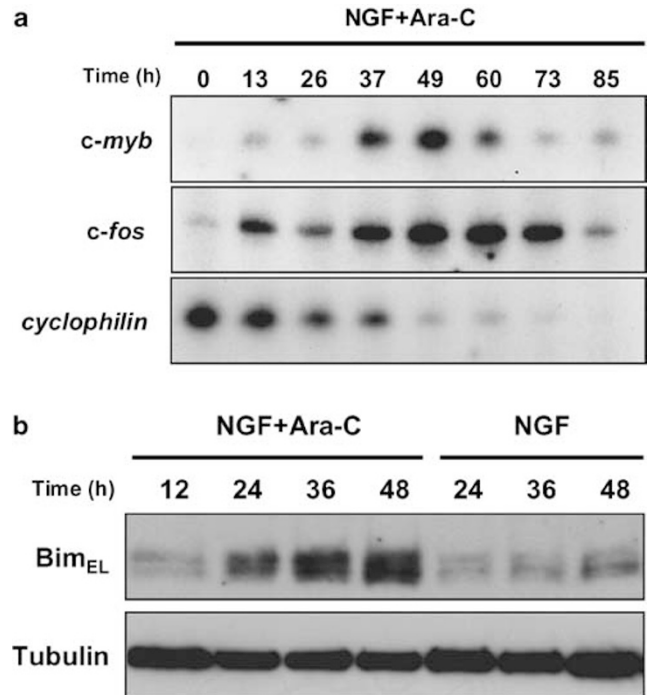
Figure 3 Ara-C-induced upregulation of genes associated with neuronal apoptosis. (**a**) c-myb and c-fos mRNAs are induced in sympathetic neurons after ara-C exposure. The 5 DIV sympathetic neurons were treated with 100 μM ara-C and neuronal mRNA was isolated at various times. RT-PCR was performed with primers specific for c-myb and c-fos. Similar results were obtained in an independent time course. RT-PCR result for the ubiquitous gene cyclophilin is shown for control. (**b**) Bim$_{EL}$ is upregulated after ara-C treatment. Sympathetic neuronal cultures were treated with 100 μM ara-C (NGF+Ara-C) for the indicated times and Bim levels were determined by immunoblotting. Bim$_{EL}$ levels increase in ara-C-exposed neurons by 24 h and are stable overtime. Blots was stripped and reprobed with Tubulin antibody for loading control. Identical results were obtained in at least three independent trials

of the Bcl-2 family, is critical for neuronal cell death after trophic factor deprivation.[31,32] Both Bim mRNA and protein levels increase in neurons before the release of cytochrome-c from the mitochondria and the activation of caspases.[31–33] Similarly, ara-C-induced death of sympathetic neurons was associated with increased levels of Bim protein, in particular Bim$_{EL}$, the extra-long isoform of Bim (Figure 3b). The increase of Bim$_{EL}$ was apparent by 24 h and preceded the loss of cell survival, but not commitment (Figure 2a). Bim$_{EL}$ protein increased with greater time of ara-C exposure, peaking at 48 h. Lysates obtained from cultures containing only non-neuronal cells showed no change in Bim$_{EL}$ levels after DNA damage, thus confirming that Bim$_{EL}$ induction was neuron-specific (data not shown). Induction of Bim correlated with ara-C-induced neuronal death, thus suggesting that similar to NGF deprivation, Bim may also be an important regulator of neuronal death after ara-C exposure,

Bid, another BH-3-only Bcl-2 family member, is a proapoptotic protein that is involved in extrinsic cell death pathway.[34,35] Cleavage of Bid by caspase 8 generates the active form, tBid, which then localizes to the mitochondria and induces cytochrome-c release. Ara-C-treated sympathetic neurons, however, did not show any induction or Bid cleavage

by Western analysis (data not shown). This is consistent with the recent findings showing that in Bid-deficient mice, telencephalic neural precursors and neurons die normally after ara-C exposure,[36] and the Bid-deficient sympathetic neurons die normally and do not demonstrate Bid cleavage after NGF deprivation.[37] We also analyzed any potential changes in the levels of antiapoptotic Bcl-2 family members. Antiapoptotic Bcl-2 or Bcl-X_L showed a decline in protein levels after ara-C exposure (data not shown), similar to that seen after NGF deprivation.

Bax is important in ara-C-induced neuronal death

The proapoptotic Bcl-2 family member, Bax, has been shown to be required for neuronal apoptosis after trophic factor deprivation. Neurons with no functional *bax* allele do not die after NGF deprivation *in vitro* and, moreover, *bax*-null mice have increased numbers of sympathetic neurons in the SCG.[38] Bax deletion in sympathetic neurons arrests the cell death pathway at the mitochondrial checkpoint, preventing the release of cytochrome-*c* into the cytosol and subsequent activation of caspases.[39] To determine whether Bax is also required for neuronal death induced by ara-C, we analyzed the survival of *bax*-deficient sympathetic neurons after ara-C treatment. Wild-type and *bax* heterozygous sympathetic neurons died with a time course similar to that of rat neurons after ara-C exposure (Figure 4). However, *bax*-null neurons showed no evidence of cell death up to 48 h. Furthermore, whereas ara-C killed most of the wild-type and heterozygous neurons by 72 h, 90% of homozygous *bax*-deficient neurons were still viable at this time point. Thus, Bax had an important function in ara-C-induced death of sympathetic neurons. However, when *bax*-null neurons were exposed to ara-C for longer times, they eventually lost their viability with 60% of neurons dying by 120 h and no neurons surviving after 168 h. Therefore, although Bax was required for the normal ara-C-induced death, continued exposure to ara-C induced neuronal death via a Bax-independent pathway.

Antiapoptotic protein, Bcl-2, protects neurons from NGF-deprivation-induced programmed cell death by acting against the proapoptotic members of the Bcl-2 family, such as Bax in neurons.[39–41] Since Bax was important in ara-C-induced neuronal death, we examined the role of Bcl-2 using *bcl-2* knockout mice. Deletion of *bcl-2* accelerated ara-C-induced cell death in sympathetic neurons. While 33 ± 5 and $41 \pm 2\%$ of wild-type and *bcl-2*-heterozygous neurons, respectively, survived after 48 h of ara-C treatment, only $14 \pm 3\%$ of *bcl-2*-null neurons were still viable at the same time point ($P < 0.001$, one-way ANOVA). The increased sensitivity of *bcl-2*-deficient sympathetic neurons to ara-C treatment is similar to that seen after NGF deprivation.[41] Therefore, like Bax, Bcl-2 is also important in ara-C-induced neuronal death.

Ara-C treatment causes the release of cytochrome-*c* into the cytosol

In NGF-deprivation-induced apoptosis of sympathetic neurons, Bax translocates from the cytosol to mitochondria, followed by the release of cytochrome-*c*.[39] Cytochrome-*c*
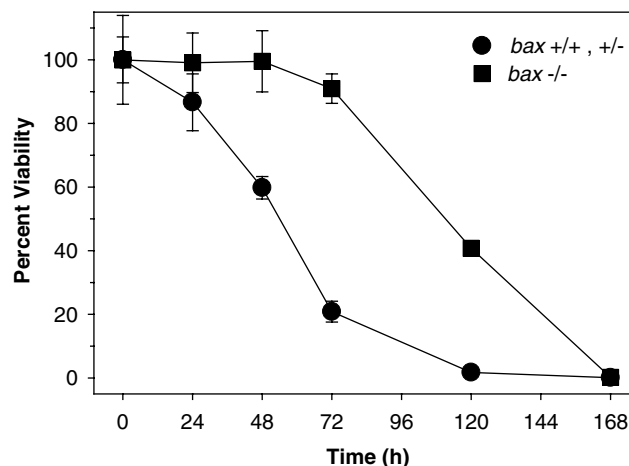
Figure 4 Bax deficiency delays ara-C-induced neuronal death. Sympathetic neurons were isolated from the SCGs of *bax*^{+/+}, *bax*^{+/−}, or *bax*^{−/−} P0 littermate mice. The 5 DIV neurons were exposed to 100 μM of ara-C and number of surviving neurons was determined at the indicated times by measuring viability. Percentage of viability was calculated by dividing the number of viable neurons at each time point by the total number of neurons in NGF-maintained, untreated sister cultures. The data for *bax*^{+/+} and *bax*^{+/−} neurons were combined since no significant difference occurred between these two groups (data not shown). *bax* deficiency prevented the majority of ara-C-induced death in the first 72 h, after which *bax*-null neurons started to degenerate and were dead by 168 h. Mean ± S.D. from 3 to 4 cultures for each genotype; data shown are representative of two independent experiments

release is necessary for the formation of the apoptosome and activation of caspases, the final step of apoptosis.[42] Since Bax deficiency caused a significant delay in ara-C-induced death of sympathetic neurons, we examined whether ara-C causes the release of mitochondrial cytochrome-*c* into the cytosol. The five days *in vitro* (DIV) sympathetic neurons were treated with ara-C for 48 h and cytochrome-*c* was localized in neurons by using immunocytochemistry. As seen in Figure 5, NGF-maintained neurons showed bright cytochrome-*c* staining confined to the cytosol; this staining was punctate in appearance because of mitochondrial localization of cytochrome-*c*. In contrast, neurons exposed to ara-C demonstrated an altered cytochrome-*c* staining. Whereas some neurons still showed cytochrome-*c* distribution similar to that of untreated cells, others had completely lost their staining or displayed only a very faint signal. In neurons, cytochrome-*c* released into the cytosol is unstable and quickly degraded.[43] The same event occurred in ara-C-exposed neurons, demonstrating that ara-C treatment led to the release of cytochrome-*c*. Cytochrome-*c* release was most likely because of Bax translocation to the mitochondria, as *bax*-null neurons failed to release cytochrome-c after ara-C exposure (data not shown).
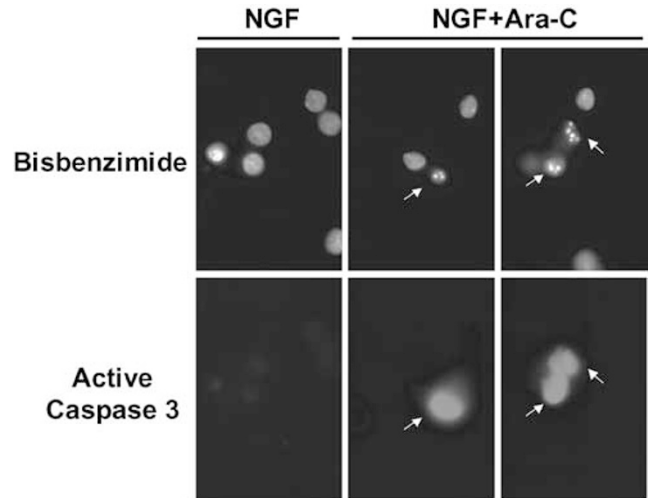
Caspase-3 is activated in sympathetic neurons after ara-C exposure

Caspases are cysteine aspartate proteases that are activated in apoptosis and execute the orderly death of cells after various apoptotic stress stimuli. Programmed cell death of NGF-deprived neurons is accompanied by caspase activation and can be attenuated by caspase inhibitors.[44,45] The
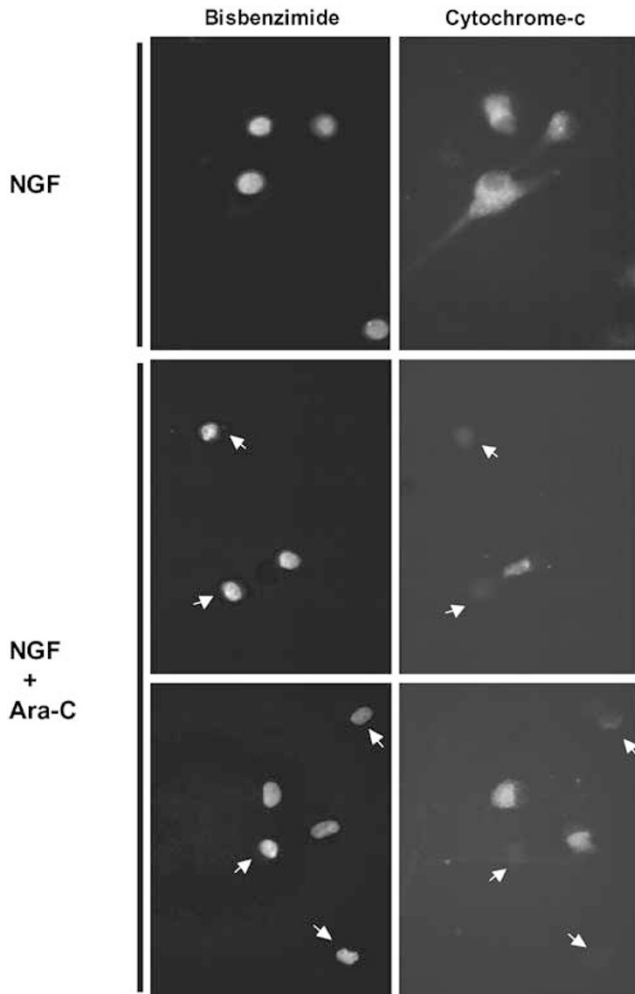
Figure 5 Cytochrome-c is released into the cytosol of sympathetic neurons upon ara-C exposure. The 5 DIV rat sympathetic neurons were treated with 100 μM of ara-C for 48 h (NGF+Ara-C) or left untreated (NGF), and cytochrome-*c* localization was determined by immunocytochemistry. A measure of 50 μM of broad-spectrum caspase inhibitor BAF was included in all treatment conditions to inhibit neuronal death. Concurrent staining with bisbenzimide was performed to detect the nuclei of neurons. NGF-maintained, untreated neurons show bright cytochrome-c staining confined to the cytosol. Mitochondrial localization of cytochrome-*c* is indicated by the punctate appearance of this staining. In some sympathetic neurons (arrows), ara-C-exposure for 48 h induces the release of cytochrome-*c*, which is indicated by the loss of cytochrome-*c* signal caused by the rapid degradation of cytochrome-*c* in the cytosol after its release. Diffuse staining for cytochrome-*c* is also detected in ara-C-exposed sympathetic neurons, albeit in a very small population (data not shown). Identical results were obtained in at least three independent trials

dependence of ara-C-induced death on the presence of Bax and the release of cytochrome-*c* after ara-C exposure suggest that sympathetic neurons may die by apoptosis after ara-C treatment. To determine whether ara-C-induced damage kills neurons by apoptosis, we checked for the possible activation of caspases by immunocytochemistry using the CM1 antibody, which detects activated caspase-3 and does not crossreact with the uncleaved zymogen, procaspase-3.[46] Although CM1 antibody has been reported to show weak crossreactivity to caspase-7, caspase-3-deficient apoptotic neurons did not show any CM1 staining, indicating that caspase-3 activation is required for cellular CM1 reactivity.[46]


Figure 6 Ara-C causes caspase-3 activation in sympathetic neurons. Rat sympathetic neurons were treated with 100 μM of ara-C for 48 h (NGF+Ara-C) on 5 DIV or left untreated (NGF). Activation of caspase-3 was determined by immunocytochemistry with the CM1 antibody that specifically detects the active form of this protease. Identical results were obtained with Promega active caspase-3 antibody. Neuronal cultures were costained with bisbenzimide to detect the nuclei. Untreated neurons have no detectable staining for activated caspase-3. In contrast, ara-C-treated sister cultures contain neurons that show robust staining for activated caspase-3 (arrows). Nuclei of these neurons are pyknotic, indicating genomic DNA condensation. Identical results were obtained in at least three independent trials

Untreated sympathetic neurons maintained in NGF showed no staining with the active caspase-3 antibody (Figure 6). On the other hand, neurons that were exposed to ara-C for 48 h displayed robust activation of caspase-3, demonstrating that caspase activation accompanied ara-C-induced death in neurons. Cells that had activated caspase-3 also showed pyknotic nuclei indicating the condensation of cellular DNA, another hallmark of apoptosis. Although these results clearly demonstrate that ara-C killed sympathetic neurons by apoptosis, the possibility that some neurons may have died through a nonapoptotic pathway is not excluded.

Commitment-to-die in ara-C-exposed sympathetic neurons is independent of the mitochondrial cytochrome-*c* release or caspase activation

During NGF deprivation-induced sympathetic neuronal apoptosis, the time course of cytochrome-*c* release from the mitochondria immediately precedes that of commitment-to-die.[47] Cytochrome-*c*-mediated caspase activation appears to be the molecular basis for the commitment event since blocking caspase activity by pan-caspase inhibitor boc-aspartyl oMe)-fluoromethylketone (BAF) significantly delays the commitment time point after NGF deprivation.[47] Therefore, we determined whether commitment-to-die in ara-C-treated sympathetic neurons is also associated with cyto-chrome-c-mediated caspase activation. In contrast to trophic factor deprivation, the time course of cytochrome-*c* release after ara-C exposure was significantly delayed compared to that of commitment (Figure 7a). In all, 51±9% of rat
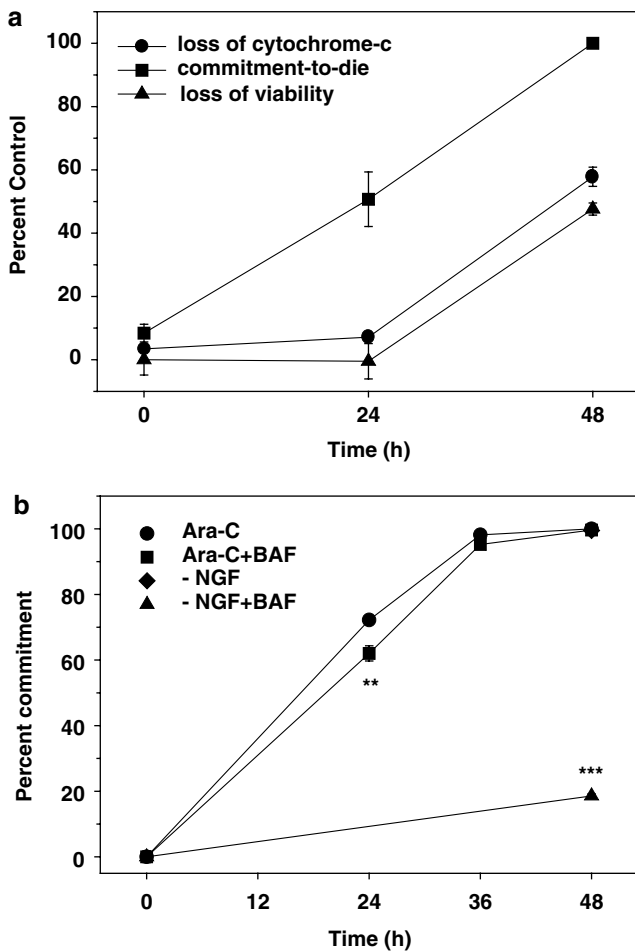
Figure 7 Ara-C-treated sympathetic neurons commit to die before mitochondrial cytochrome-*c* release or caspase activation. (**a**) Sympathetic neurons were treated with 100 μM of ara-C or left untreated in the presence of 50 μM BAF. The number of cytochrome-*c* positive neurons was determined by a blinded observer. Concurrent staining with bisbenzimide was performed to detect the nuclei of neurons. Neurons showing only bright and punctate cytochrome-*c* staining confined to the cytosol were counted as positive (see Figure 5). Data represent mean $\pm$ S.E.M. from two independent trials with 4–6 cultures for each condition. Commitment and viability data from Figure 2 are included for comparison. (**b**) Sympathetic neurons were exposed to 100 μM ara-C (Ara-C) or deprived of NGF (−NGF) for different times with or without 50 μM BAF and the fraction of surviving neurons was determined by measuring commitment as described (**: $P < 0.01$; ***: $P < 0.001$, Student's *t*-test). Data represent mean $\pm$ S.D. of three cultures for each treatment condition. Similar results were obtained in an independent experiment

sympathetic neurons had already committed to die after 24 h of ara-C treatment (Figure 2), while only 7 $\pm$ 1% of the neurons released their cytochrome-*c* at the same time point. Similarly, in 42 $\pm$ 3% of sympathetic neurons, cytochrome-*c* staining was still exclusively mitochondrial after 48 h of ara-C exposure at which time point all neurons had already committed to die. Unlike commitment, the time course of neuronal death immediately followed that of cytochrome-*c* release (Figure 7a). These results suggest that although cytochrome-*c* release appeared to be critical for the execution of cell death, it was not important for commitment to death after ara-C exposure. Similarly, blocking caspase activity in sympathetic neurons by BAF did not significantly affect the time course of

commitment-to-die after ara-C exposure (Figure 7b). This was, again, in contrast to commitment-to-die after NGF deprivation, which was significantly prevented by caspase inhibition (Figure 7b). These results demonstrated that ara-C-exposed neurons committed to die before cytochrome-*c* release and caspase activation.

Loss of Bax function delays the time course of commitment after ara-C treatment

Since both cytochrome-*c* release and caspase activation occurred after the commitment of neurons, we tested the hypothesis that deletion of *bax* in neurons should have no affect on the commitment time course after ara-C treatment. Sympathetic neurons isolated from *bax*$^{+/+}$, *bax*$^{+/-}$, and *bax*$^{-/-}$ littermate mice were treated with 100 μM ara-C for various times, followed by a rescue period with 100 μM dC for 5–7 days. Surprisingly, Bax-null neurons showed a significant delay in commitment-to-die after ara-C exposure (Figure 8). Fewer *bax*$^{-/-}$ neurons committed to die after 36 and 48 h of ara-C exposure compared to *bax*$^{+/+}$ and *bax*$^{+/-}$ neurons. However, similar to the delayed death of *bax*$^{-/-}$ neurons (Figure 4), neuronal commitment was only temporarily prevented after loss of Bax function and majority of the *bax*$^{-/-}$ sympathetic neurons eventually committed to die after 72 h of ara-C treatment (Figure 8).

Absence of p53 attenuates ara-C-induced neuronal cell death

p53 is a tumor suppressor that has been implicated in DNA-damage-induced cell-cycle arrest and apoptosis.[15–18] Since
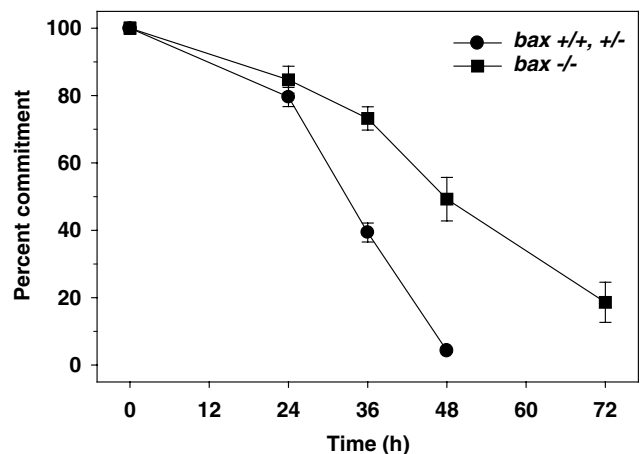

Figure 8 Bax deficiency delays commitment-to-die in ara-C-exposed sympathetic neurons. Sympathetic neurons were isolated from the SCGs of *bax*$^{+/+}$, *bax*$^{+/-}$, or *bax*$^{-/-}$ P0 littermate mice. The 5 DIV neurons were exposed to 100 μM of ara-C, and number of surviving neurons was determined at the indicated times by measuring commitment as described. Percentage of commitment was calculated by dividing the number of viable neurons at each time point by the total number neurons in NGF-maintained, untreated sister cultures. The data for *bax*$^{+/+}$ and *bax*$^{+/-}$ neurons were combined since no significant difference occurred between these two groups (data not shown). Data represent mean $\pm$ S.E.M. from two independent experiments with 5–12 wells per condition

ara-C might have been killing sympathetic neurons by causing DNA damage,[7] we analyzed the role of p53 in sympathetic neurons after ara-C exposure. Survival of neurons with two, one, or no functional copies of *p53* was determined after 72 h of ara-C treatment (Figure 9a). While the majority of neurons were dead by 72 h of ara-C exposure in both wild-type and *p53* heterozygotes, neurons isolated from *p53*-null animals showed about 80% survival at this time. Therefore, *p53* deficiency significantly retards the death of neurons after ara-C exposure. In addition, both copies of *p53* had to be deleted to acquire resistance to ara-C, since neurons with one copy of *p53* are as sensitive to ara-C as the neurons with two *p53* copies.

In neuronal cells, the role of p53 in apoptosis induced by trophic factor withdrawal has been controversial, with some studies indicating p53 involvement while others showing no significant effect of p53 within the same neuronal death paradigms.[48–52] When we analyzed *p53*-deficient neurons, a small, but significant, protection after NGF deprivation occurred. Over 90% of sympathetic neurons committed to die after 35–39 h of NGF deprivation (Figure 9b). This number was decreased slightly to about 80% in *p53*-null neurons.

Since p53 deficiency provided neurons with significant resistance to ara-C treatment, we examined whether p53 levels were increased in ara-C-exposed sympathetic neurons. Accumulation of p53 in cells may be accomplished by increased gene transcription and/or increased protein stability. RT-PCR analysis of p53 mRNA in neurons treated with ara-C showed no significant increase in the amount of p53 transcript (data not shown). In contrast, p53 mRNA levels declined with time, similar to what is observed in neurons after NGF deprivation (data not shown).[28] Analysis of p53-protein levels in neurons by immunoblotting showed that p53 was readily detectable in sympathetic neurons maintained in NGF (Figure 10a). In contrast to a previous report,[5] we found that the amount of p53 protein in neurons declined with increased ara-C exposure (Figure 10a), presumably as a result of decreased protein synthesis as well as loss of viability (Figure 2b). We also examined any potential phosphorylation of neuronal p53 after ara-C exposure and were unable to detect any Ser[15] phosphorylation, a residue shown to be phosphorylated after DNA damage and critical for p53 stabilization (data not shown). Although p53-protein levels in NGF-maintained and NGF-deprived neurons seemed to increase at 24 h compared to 12 h, this finding was not reproducible. Similar to ara-C treatment, NGF deprivation led to decreased levels of p53 protein (Figure 10a, data not shown).

As no detectable increase in p53 levels was seen, we investigated whether the expression of p53-regulated genes changed in sympathetic neurons after ara-C treatment. First, we analyzed the pro-apoptotic protein Bax, which is important for ara-C-induced neuronal death (Figure 4). p53 has been shown to be a direct transcriptional regulator of Bax mRNA.[53–55] The amount of Bax in sympathetic neurons after ara-C exposure or NGF deprivation decreased with time (Figure 10b), demonstrating that p53 did not activate *bax* transcription in neuronal apoptosis. Another transcriptional target of p53 is the CDK inhibitor p21.[56,57] p21 induction has previously been reported in sympathetic neurons after NGF deprivation.[48] Immunoblot analysis of protein lysates obtained from ara-C-
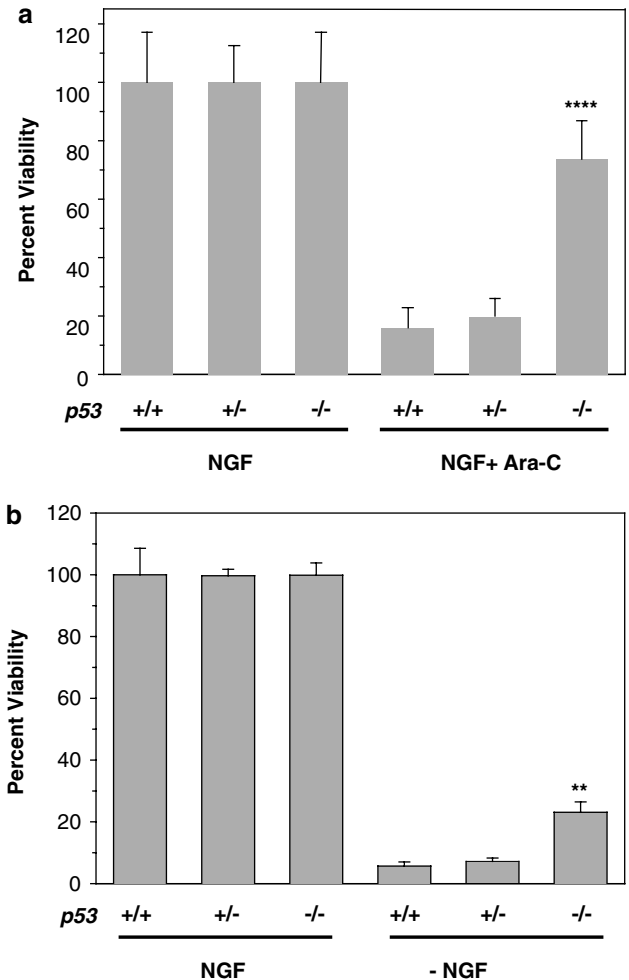
Figure 9 p53 deletion attenuates ara-C-induced neuronal death. Sympathetic neurons were isolated from the SCGs of *p53*$^{+/+}$, *p53*$^{+/-}$, or *p53*$^{-/-}$ P0 mice that were littermates. (**a**) Neurons were exposed to 100 μM of ara-C (NGF+Ara-C) on 5 DIV or left untreated (NGF), and number of surviving neurons was determined at 72 h by measuring viability. The difference between cultures of *p53*$^{-/-}$ neurons and either *p53*$^{+/+}$ or *p53*$^{+/-}$ neurons is statistically significant ($P < 0.0001$, two-sided Mann–Whitney test). Mean $\pm$ S.D. from 3 to 14 sister cultures per each genotype. Data shown are representative of five independent experiments. (**b**) Effect of *p53*-gene dose on NGF-deprivation-induced death. Murine sympathetic neurons from littermates with two, one, or no *p53* allele were deprived of NGF for 35–39 h, after which they were rescued by readdition of NGF for 2–3 days. All three NGF rescue experiments taken together demonstrate a significant ($P < 0.01$, Student's *t*-test), but subtle retardation of neuronal death in neurons lacking *p53*. The data are graphed as the mean $\pm$ S.E.M. from three independent experiments

treated sympathetic neurons for p21 expression indicated that p21 was not expressed at detectable levels in neurons before or after ara-C treatment (Figure 10c). The entire p21 signal came from the non-neuronal cells that are present in these cultures at very low numbers ($< 5\%$, Figure 10c). These results demonstrate that p21 was not induced in sympathetic neurons after ara-C exposure. Therefore, neither an increase in p53 levels nor any positive change in two p53-regulated genes was observed in ara-C-induced apoptosis of sympathetic neurons. Although there may be other genes that could potentially be regulated by p53 after ara-C exposure, we examined the ones that have been suggested to be
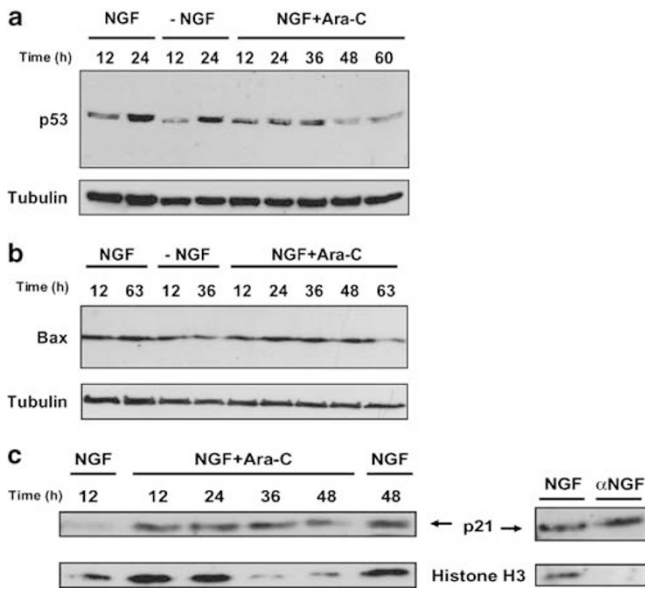
Figure 10 Neither p53 nor p53-regulated genes are induced in sympathetic neurons after ara-C treatment. 5 DIV rat sympathetic neurons were treated with 100 μM of ara-C (NGF+Ara-C), deprived of NGF (−NGF), or left untreated (NGF), and the neuronal protein was isolated at the indicated times. Levels of p53, Bax, and p21 were determined by immunoblotting. (**a**) NGF-maintained, untreated neurons show high basal levels of p53, which decrease with time in both NGF-deprived and ara-C-treated neurons. (**b**) Similar to p53, Bax levels also decline in ara-C-exposed or NGF-deprived neurons. (**c**) p21 is not expressed in NGF-maintained neurons and expression is not detected after ara-C exposure. The right panel shows that p21 signal in neuronal lysates comes from the small number of non-neuronal cells. αNGF represents protein extracts isolated from cultures established in the absence of NGF. These cultures contain only non-neuronal cells since sympathetic neurons do not survive without NGF. Blots were stripped and reprobed with Tubulin antibody in A and B, and Histone H3 antibody in C for loading control. Identical results were obtained in at least two independent trials

transcriptional targets of p53 in sympathetic neurons, and contrary to previous reports, we found no changes in these genes.[48]

Maturation increases resistance to ara-C death in sympathetic neurons

Mature sympathetic neurons do not depend acutely on trophic support for survival.[58,59] After NGF deprivation, mature neurons undergo many metabolic and molecular changes observed in immature sympathetic neurons, but the apoptotic pathway is arrested at the Bax checkpoint since cytochrome-*c* is not released into the cytosol and caspases are not activated.[43,60] Since Bax is also important in ara-C-induced death, we investigated whether maturation had any effect on ara-C sensitivity of sympathetic neurons. Similar to Bax-deficient neurons, mature sympathetic neurons showed a delay in cell death after ara-C exposure (Figure 11). At 48 h of ara-C exposure, half of the neurons in immature cells were killed (Figure 2a), while all mature neurons were still alive (Figure 11). Neuronal death was apparent in mature neurons by 72 h (50% survival), but at any given time, mature neurons had increased survival compared with immature neurons.

Ara-C killed all mature neurons by 168 h, demonstrating a 24–48 h delay compared to immature neurons.

Discussion

In this study, we have examined the molecular mechanism(s) underlying ara-C-induced death of sympathetic neurons. Our results demonstrate that ara-C induced an apoptotic pathway in sympathetic neurons that strongly resembles NGF-deprivation-induced programmed cell death. However, when this apoptotic pathway was blocked by *bax* deletion, ara-C was capable of activating a novel, Bax-independent death pathway, which killed sympathetic neurons more slowly and was independent of caspase activity. We found that genes known to be associated with apoptosis, that is, transcription factors c-*fos* and c-*myb* and proapoptotic BH3-only Bcl-2 family member *bim* were induced in sympathetic neurons during ara-C-induced apoptosis. Induction of these genes was followed by Bax-dependent cytochrome-*c* release, leading to caspase activation and execution of neuronal death. Although Bax function was important for neuronal commitment to death after ara-C-exposure, time course analysis (Figure 7) showed that this function was independent of the Bax-mediated cytochrome-*c* release and caspase activation. Our data confirm the role of p53 in neuronal apoptosis after DNA damage. However, unlike most other DNA-damaged cells, neurons did not upregulate p53 or certain classical p53 target genes after ara-C treatment, suggesting either a transcription-independent role of p53 during neuronal death or induction of atypical p53 target genes that remain to be identified. In addition, we confirm that p53 is not important in neuronal apoptosis after trophic factor deprivation. Finally, this study demonstrates that maturation increases resistance to DNA damage induced by ara-C in sympathetic neurons.

Sympathetic neuronal death induced by ara-C had many similarities to that after NGF deprivation. Neurons treated with ara-C showed a decline in the rate of protein synthesis, indicating that they became hypometabolic before cell death, like NGF-deprived neurons (Figure 2b). Similar to NGF-deprived neurons, ara-C-exposed sympathetic neurons increased the expression of genes associated with apoptosis, despite a global decrease in protein and mRNA synthesis (Figure 3). Both c-*fos* and c-*myb*, two transcription factors that are induced after NGF deprivation,[26] were induced in neurons by ara-C. A recent study by Liu and Greene[27] suggests that induction of c-*myb* is caused by the derepression of genes regulated by the E2F transcription factor family. In addition, another member of the *myb* family, b-*myb*, is induced in neurons after DNA damage and trophic factor deprivation.[27] These results suggest that derepression of E2F-regulated genes is a consequence of ara-C exposure in sympathetic neurons and may be crucial for neuronal death induced by ara-C.

Analysis of the Bcl-2 family proteins identified two members as potential mediators of ara-C toxicity in sympathetic neurons. First, as in NGF deprivation, we observed an induction of Bim, a proapoptotic BH-3-only member of the Bcl-2 family (Figure 3b). This result demonstrates Bim induction in neurons by a stress stimulus other than trophic
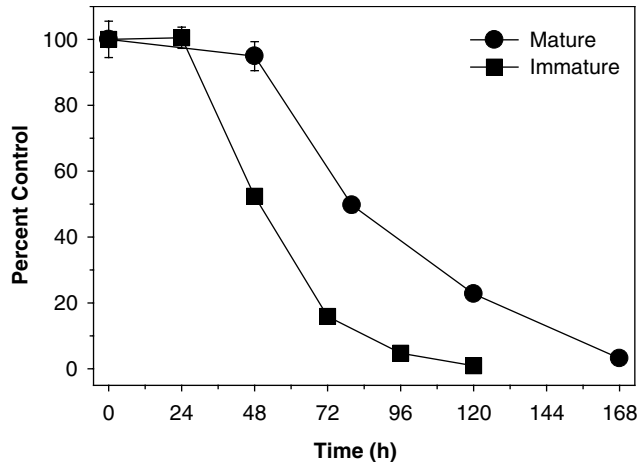
Figure 11 Mature sympathetic neurons show increased resistance to ara-C-induced death. Sympathetic neurons isolated from P0 rat SCGs were maintained in NGF for 3 weeks before being exposed to 100 μM of ara-C. Viability data for ara-C-exposed immature neurons from Figure 2a are shown for comparison. Ara-C-induced death is delayed 24–48 h in mature sympathetic neurons compared to the immature neurons. Mean $\pm$ SEM, $n=3$–4. Similar results were obtained in two other experiments

factor withdrawal. The importance of Bim for neuronal apoptosis has recently been shown,[31,32] but whether Bim is also required for ara-C-induced neuronal death remains to be determined with further studies. Second, we identified Bax as an essential regulator of neuronal apoptosis after ara-C exposure. Although Bax expression was unaltered in neurons after ara-C treatment (Figure 10b), Bax deficiency prevented death in the first 72 h of ara-C treatment, which killed the vast majority of wild-type neurons (Figure 4). Bax activates apoptosis by mediating the release of mitochondrial cytochrome-*c*, which is necessary for formation of the cytosolic apoptosome and activation of the initiator caspase-9. Accordingly, we observed Bax-dependent cytochrome-*c* release into the cytosol in ara-C-exposed sympathetic neurons (Figure 5 and data not shown). Moreover, we detected caspase-3 activation in dying sympathetic neurons upon ara-C treatment (Figure 6). These findings indicate that the cell death pathway activated after ara-C treatment in sympathetic neurons was very similar to the apoptotic pathway initiated after NGF deprivation and involved Bax-dependent cytochrome-*c* release and caspase activation.

Despite these similarities in the molecular events underlying both ara-C-exposure and NGF-deprivation-induced death of sympathetic neurons, these cell death pathways show substantial differences. Time courses of sympathetic neuronal death and the associated metabolic and molecular events were delayed and protracted after ara-C treatment compared to NGF deprivation. This difference is presumably because of variability of ara-C-induced damage in individual neurons, making most events less synchronous in the whole population. In striking contrast to NGF-deprived sympathetic neurons, ara-C-exposed neurons committed to die before cytochrome-*c* release and caspase activation (Figure 7). Unlike the complete protection of *bax*-deficient neurons after NGF deprivation, loss of Bax function was only protective in the first 3 days of ara-C exposure; sympathetic neurons

eventually degenerated after prolonged ara-C treatment (Figure 4). Similarly, ara-C-induced death was delayed in mature neurons, which had complete resistance to NGF deprivation (Figure 11). Furthermore, like Bax deficiency, a broad-spectrum caspase inhibitor, BAF, could only delay ara-C-induced death in sympathetic neurons (unpublished observations).[14] Therefore, Bax-dependent apoptosis was the 'default' mechanism of neuronal degeneration after ara-C treatment, but ara-C also caused neuronal death through an alternative pathway after *bax* deletion. This Bax-independent cell death was caspase independent since we did not observe any caspase-3 activation in Bax-deficient neurons after ara-C exposure. After 96 h of ara-C treatment, which kills about 40% of $bax^{-/-}$ neurons (Figure 4), there was no detectable active caspase-3 staining in dying sympathetic neurons (data not shown). In contrast, many $bax^{+/+}$ and $bax^{+/-}$ neurons had robust active caspase-3 staining after 46 h of ara-C exposure ($\sim$40% death, Figure 4), which corresponds to the 96 h time point of ara-C-treated $bax^{-/-}$ neurons (data not shown). Moreover, caspase inhibitor BAF could not protect the majority of $bax^{-/-}$ neurons from ara-C-induced death (data not shown). These results are consistent with the previously published data reporting that similar to *bax* deletion, BAF could delay ara-C-induced death of rat sympathetic neurons only up to 3 days, indicating that the delayed cell death seen after day 3 is caspase-independent.[14] Retardation of ara-C-induced death after *bax* deletion suggests that a more intense and prolonged DNA damage is required to trigger the Bax-independent death pathway. Under normal conditions, neurons can presumably sense a much lower amount of DNA damage and activate a rapid, Bax-dependent apoptotic death when this damage exceeds a threshold level. Caspase-independent cell death seen in ara-C-treated Bax-null sympathetic neurons is similar to the caspase-independent death of embryonic cortical neurons evoked by topoisomerase-I inhibitor camptothecin.[61,62] Therefore, activation of caspase-independent death pathways in neurons may be a common consequence of DNA damage induced by multiple agents.

Another noticeable difference between ara-C and NGF-deprivation induced apoptosis of sympathetic neurons was the potential role of p53. p53 has been suggested to have a role in sympathetic neuronal apoptosis after ara-C exposure as well as NGF deprivation.[5,48] Analysis of sympathetic neurons isolated from p53-mutant mice demonstrated that p53 deficiency conferred significant resistance to ara-C-induced death (Figure 9a). This finding is consistent with similar results obtained in cerebellar granule cells after ara-C exposure and in sympathetic neurons after campthothecin-induced DNA damage.[20,21,61] In contrast, NGF-deprivation-induced neuronal death was only slightly affected by p53 deficiency (Figure 9b). Deletion of *p53* in mice has been reported to reduce the basal Bax expression as detected by immunohistochemistry in many neuronal populations including sympathetic neurons.[54] Therefore, we favor the idea that the slight delay of apoptosis in $p53^{-/-}$ neurons after NGF deprivation was most likely because of reduced Bax levels since Bax-heterozygous neurons also die more slowly in response to NGF deprivation than cells from wild-type littermates.[38] Although inconsistent with the report of Aloyz

et al.,[48] the subtle effect of p53 deficiency on NGF-deprivation-induced death we observe is consistent with the previous studies that could not identify a significant effect of p53 on trophic factor deprivation-induced death.[50–52]

Although p53 deletion delayed ara-C-induced death significantly, neither an increase in p53 levels nor any positive change in two p53-regulated genes was observed in ara-C-exposed sympathetic neurons. It has been suggested that transcriptional activity of p53 can be dissociated from its apoptotic activity;[63] this suggestion is supported by the results reported here. Furthermore, p53 has been reported to translocate to mitochondria and activate apoptosis independent of its transcriptional activity after DNA damage or hypoxia.[64,65] Our results regarding the role of p53 in ara-C-induced neuronal death could be explained by a similar mechanism, in which p53 may be acting at the mitochondria, and its transcriptional activity may be dispensable for neuronal death. Since neurons already contain high basal levels of p53 (Figure 10a), p53 induction may not be necessary for mitochondrial translocation. Both Bax and p53 deletion delay ara-C death to a similar extent, possibly suggesting that one may be compensating for the loss of other. In view of this possibility, bax and p53 double-knockout neurons may demonstrate significantly more resistance to ara-C compared to single knockouts.

Susceptibility of neurons to ara-C-induced damage and subsequent death may be a consequence of the higher dependence of neurons on DNA repair. Neurons may have elevated basal DNA damage as a result of free radicals produced by higher oxygen metabolism.[66] Increased DNA damage would lead to higher ara-C incorporation into genomic DNA during DNA repair. Alternatively, neurons may have less than optimal capacity to repair DNA damage compared to other cells. Postmitotic neurons are especially vulnerable to the lack of proteins that are part of the DNA repair machinery, including nonhomologous DNA-end-joining pathway, homologous recombination, and base-excision repair. In mutant mice that have impaired DNA repair, cell death is increased significantly in neurons soon after they become postmitotic.[24,66,67] A combination of both increased ara-C incorporation and decreased tolerance to DNA damage may make neurons sensitive to ara-C. Reduced tolerance of neurons to DNA damage may have an important physiological significance since secondary DNA damage and subsequent neuronal degeneration may potentially be the final common pathway in many neurological disorders. Therefore, understanding the molecular mechanisms underlying neuronal cell death induced by DNA damage may suggest new therapeutic targets for the treatment of neurodegeneration.

Materials and Methods

Animals and materials

All reagents were purchased from Sigma (St Louis, MO, USA) unless otherwise stated. Timed-pregnant Sprague Dawley rats were obtained from Harlan (Indianapolis, IN, USA). Mutant male and female bcl-2,[68] bax,[69] or p53 (TSG-p53, Genpharm Intl., Mountain View, CA, USA) mice were bred locally. Collagenase and trypsin were purchased from Worthington Biochemical Corporation (Freehold, NJ, USA). Mouse 2.5S NGF was from Harlan Bioproducts (Indianapolis, IN, USA). The goat anti-mouse 2.5S NGF neutralizing antiserum has been characterized previously.[70]

Genotyping of mutant mice

Genotypes of bcl-2 and bax mutant mice were determined as described previously.[38,41] To determine the genotype of p53 mutant mice, the tail DNA was isolated and subjected to PCR with gene-specific primers. Cycling parameters were 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C for 35 cycles, followed by a 10-min extension at 72°C. The p53 forward primer (1 μM, 5′-TCGGATCCGGACAGCCAAGTCTGTTATGT-3′) and the reverse primer (1 μM, 5′-TCGAATTCTTATTGAGGGGAGGAGAGTA-3′) led to an 840-bp fragment while the same p53 forward primer with the murine RNA polymerase 2 promoter reverse primer (0.2 μM, 5′-TGGAATTC-TAGTAGCTTTACGGAGCCCT-3′) generated a 520-bp fragment.

Neuronal culture

Primary sympathetic neuronal cultures were prepared from P0–P1 rat or mouse superior cervical ganglion (SCG) by using previously described methods.[71–73] Briefly, SCGs were dissected from newborn animals and incubated for 30 min each with 1 mg/ml collagenase and 2.5 mg/ml trypsin at 37°C. The ganglia were dissociated by triturating through a 200-μl micropipet tip; cells were plated on collagen-coated plastic tissue culture plates at the appropriate densities. Cultures were maintained in AM50 medium (90% minimum essential medium (Invitrogen, Carlsbad, CA, USA), 2 mM glutamine, 10% fetal bovine serum (Hyclone, Logan, UT, USA), 50 ng/ml 2.5S NGF, 20 μM fluorodeoxyuridine, 20 μM uridine, 100 U/ml penicillin, and 100 U/ml streptomycin), supplemented with 3.3 μg/ml aphidicolin (AG Scientific, San Diego, CA, USA) for the first 5 DIV to reduce the number of non-neuronal cells. Culture medium was replaced every 3–4 days. Cultures devoid of neurons but containing equivalent amounts of non-neuronal cells were established by plating cells in a medium lacking NGF (AM0) supplemented with 0.01% anti-NGF antiserum.

To deprive neuronal cultures of NGF, 5 DIV immature or 3-week-old mature neuron cultures were washed three times with AM0 and fed with fresh AM0 containing 0.01% anti-NGF antiserum. For ara-C treatment, the culture medium was replaced with fresh AM50 containing ara-C. In neuronal cultures used to harvest protein, 25–50 μM of broad-spectrum caspase inhibitor, boc-aspartyl(OMe)-fluoromethylketone (BAF, Enzyme Systems, Livermore, CA, USA), was included in all treatment conditions to inhibit neuronal death.

Neuronal survival

The number of viable cells was assessed after fixing the cultures with 4% paraformaldehyde (Fischer Scientific, Pittsburgh, PA, USA) in PBS and staining with crystal violet. Neurons were scored as viable if the crystal violet-positive cells had large, well-defined cellular outlines. Dead neurons and debris stain faintly or show no staining with crystal violet. Percent viability was calculated by dividing the number of crystal violet–positive neurons at each time point by the total number of neurons in NGF-maintained, untreated sister cultures. In most of the experiments with bcl-2 and p53 mutant neurons, viability was measured by determining the fraction of surviving neurons from serial phase-contrast images taken before onset of NGF deprivation and at the end of the experimental period.[74] A neuronal soma was counted as dead when it had lost the bright refractile ring. In one p53 experiment, the fraction of surviving neurons was determined by comparing the phase-contrast image of a field of neurons

taken prior to onset of NGF deprivation to the epifluorescence image taken at the end of the experimental period after staining the living cultures with 5 μM of the vital dye calcein AM (Molecular Probes Inc., Eugene, OR, USA). All three techniques to measure viability yielded comparable results.

Rate of protein synthesis

This procedure has been described previously.[6] L-[4,5-³H]leucine (Amersham Corp., Arlington Heights, IL, USA) at 10 μCi/ml was used in the presence of 10 μM unlabeled L-leucine instead of L-[³⁵S]methionine. The change of the label did not affect the time course of the rate of protein synthesis after NGF deprivation.[6]

Neuronal protein

Neuronal protein was measured by the bicinchoninic acid method (Pierce, Rockford, IL, USA) as described previously.[6]

RT-PCR analysis

RT-PCR analysis of SCG neuronal cultures has been previously described.[26,28] In summary, half of the mRNA isolated at specified times after ara-C treatment was converted into cDNA by RT with random hexamers (10 μM) as primers. For PCR analysis, 1% of the cDNA was used in a 50 μl PCR reaction including [α-³²P]dCTP, half of the PCR reaction was separated on an 8% polyacrylamide gel; the PCR product was visualized with autoradiography. No PCR product was amplified when purified mRNA was used in the PCR reaction. Each gene was tested twice in at least two independent time courses. The sequences of the PCR products were confirmed by DNA sequencing. The primer sequences were described previously.[28,75]

Immunocytochemistry

Cultures were fixed with fresh 4% paraformaldehyde in PBS, washed with Tris-buffered saline (TBS: 0.1 M Tris-HCl, pH 7.6, 0.9% NaCl), and incubated in blocking solution (5% normal goat serum (NGS) in TBS, containing 0.3% Triton X-100) for 1 h at room temperature. The cultures were then incubated with primary antibodies in antibody solution (1% NGS in TBS, containing 0.3% Triton X-100) overnight at 4°C. The following primary antibodies were used: cytochrome-c (0.5 μg/ml, BD Pharmingen, San Diego, CA, USA), CM1 (1 : 5000, IDUN, San Diego, CA, USA), active caspase-3 (1:250, Promega, Madison, WI, USA). The cultures were next washed three times with TBS and incubated in antibody solution containing Cy-3-labeled secondary antibodies (1 : 400, Jackson Immu-noResearch, West Grove, PA, USA) for 2 h at 4°C and counterstained with 1 μg/ml bisbenzimide (Hoechst 33258, Molecular Probes, Eugene, OR, USA). After four washes with TBS, the cultures were mounted for fluorescence microscopy.

Western analysis

Neuronal cultures were rinsed twice with cold PBS, lysed in reducing sample buffer (125 mM Tris-HCl, pH 6.8, 10% 2-mercaptoethanol, 4% SDS, 0.1% bromophenol blue, and 20% glycerol), boiled for 5 min, and stored at −20°C until use. Proteins were separated with SDS-PAGE on Tris-glycine mini-gels (Invitrogen) and transferred to Immobilon-P PVDF membrane (Millipore, Bedford, MA, USA). Blots were blocked for 1 h at room temperature with TBST (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.1% Tween 20) containing 5% nonfat dry milk and incubated overnight at 4°C with primary antibody diluted in blocking solution recommended by the manufacturer. The following primary antibodies were used: Bim (1 μg/ml, Stressgen, Victoria, BC, Canada), p53 (1 μg/ml, Stressgen), p21 (C-19, 1 : 500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Bax (1 μg/ml, Upstate Biotechnology, Lake Placid, NY, USA), Tubulin (Clone DM 1A, 1 : 50,000, Sigma). After washing, blots were incubated for 1 h at room temperature with HRP-linked secondary antibodies (Cell Signaling) diluted 1 : 2,500–1 : 10,000 in blocking solution. The blots were finally washed 3 times with TBST and developed with a chemiluminescent substrate (Supersignal, Pierce). To strip and reprobe blots, the membranes were incubated in 100 mM glycine, pH 2.5, twice for 25 min and then washed with TBST. The Western analysis was then repeated.

References

1. Grant S (1998) Ara-C: cellular and molecular pharmacology. Adv. Cancer Res. 72: 197–233
2. Wallace TL and Johnson Jr. EM (1989) Cytosine arabinoside kills postmitotic neurons: evidence that deoxycytidine may have a role in neuronal survival that is independent of DNA synthesis. J. Neurosci. 9: 115–124
3. Tomkins CE, Edwards SN and Tolkovsky AM (1994) Apoptosis is induced in post-mitotic rat sympathetic neurons by arabinosides and topoisomerase II inhibitors in the presence of NGF. J. Cell Sci. 107 (Part 6): 1499–1507
4. Martin DP, Wallace TL and Johnson Jr. EM (1990) Cytosine arabinoside kills postmitotic neurons in a fashion resembling trophic factor deprivation: evidence that a deoxycytidine-dependent process may be required for nerve growth factor signal transduction. J. Neurosci. 10: 184–193
5. Anderson CN and Tolkovsky AM (1999) A role for MAPK/ERK in sympathetic neuron survival: protection against a p53-dependent, JNK-independent induction of apoptosis by cytosine arabinoside. J. Neurosci. 19: 664–673
6. Deckwerth TL and Johnson Jr. EM (1993) Temporal analysis of events associated with programmed cell death (apoptosis) of sympathetic neurons deprived of nerve growth factor. J. Cell Biol. 123: 1207–1222
7. Geller HM, Cheng KY, Goldsmith NK, Romero AA, Zhang AL, Morris EJ and Grandison L (2001) Oxidative stress mediates neuronal DNA damage and apoptosis in response to cytosine arabinoside. J. Neurochem. 78: 265–275
8. Winkelman MD and Hines JD (1983) Cerebellar degeneration caused by high-dose cytosine arabinoside: a clinicopathological study. Ann. Neurol. 14: 520–527
9. Sylvester RK, Fisher AJ and Lobell M (1987) Cytarabine-induced cerebellar syndrome: case report and literature review. Drug Intell. Clin. Pharm. 21: 177–180
10. Resar LM, Phillips PC, Kastan MB, Leventhal BG, Bowman PW and Civin CI (1993) Acute neurotoxicity after intrathecal cytosine arabinoside in two adolescents with acute lymphoblastic leukemia of B-cell type. Cancer 71: 117–123
11. Vogel H and Horoupian DS (1993) Filamentous degeneration of neurons. A possible feature of cytosine arabinoside neurotoxicity. Cancer 71: 1303–1308
12. Lazarus HM, Herzig RH, Herzig GP, Phillips GL, Roessmann U and Fishman DJ (1981) Central nervous system toxicity of high-dose systemic cytosine arabinoside. Cancer 48: 2577–2582

13. Dessi F, Pollard H, Moreau J, Ben-Ari Y and Charriaut-Marlangue C (1995) Cytosine arabinoside induces apoptosis in cerebellar neurons in culture. J. Neurochem. 64: 1980–1987

14. Park DS, Morris EJ, Stefanis L, Troy CM, Shelanski ML, Geller HM and Greene LA (1998) Multiple pathways of neuronal death induced by DNA-damaging agents, NGF deprivation, and oxidative stress. J. Neurosci. 18: 830–840

15. Clarke AR, Purdie CA, Harrison DJ, Morris RG, Bird CC, Hooper ML and Wyllie AH (1993) Thymocyte apoptosis induced by p53-dependent and independent pathways. Nature 362: 849–852

16. Lotem J and Sachs L (1993) Hematopoietic cells from mice deficient in wild-type p53 are more resistant to induction of apoptosis by some agents. Blood 82: 1092–1096

17. Lowe SW, Ruley HE, Jacks T and Housman DE (1993) p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell 74: 957–967

18. Lowe SW, Schmitt EM, Smith SW, Osborne BA and Jacks T (1993) p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature 362: 847–849

19. Courtney MJ and Coffey ET (1999) The mechanism of Ara-C-induced apoptosis of differentiating cerebellar granule neurons. Eur. J. Neurosci. 11: 1073–1084

20. Chen RW, Saunders PA, Wei H, Li Z, Seth P and Chuang DM (1999) Involvement of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and p53 in neuronal apoptosis: evidence that GAPDH is upregulated by p53. J. Neurosci. 19: 9654–9662

21. Enokido Y, Araki T, Aizawa S and Hatanaka H (1996) p53 involves cytosine arabinoside-induced apoptosis in cultured cerebellar granule neurons. Neurosci. Lett. 203: 1–4

22. Deans B, Griffin CS, Maconochie M and Thacker J (2000) Xrcc2 is required for genetic stability, embryonic neurogenesis and viability in mice. EMBO J. 19: 6675–6685

23. Gilmore EC, Nowakowski RS, Caviness Jr. VS and Herrup K (2000) Cell birth, cell death, cell diversity and DNA breaks: how do they all fit together? Trends Neurosci. 23: 100–105

24. Chun J and Schatz DG (1999) Rearranging views on neurogenesis: neuronal death in the absence of DNA end-joining proteins. Neuron 22: 7–10

25. Gao Y, Sun Y, Frank KM, Dikkes P, Fujiwara Y, Seidl KJ, Sekiguchi JM, Rathbun GA, Swat W, Wang J, Bronson RT, Malynn BA, Bryans M, Zhu C, Chaudhuri J, Davidson L, Ferrini R, Stamato T, Orkin SH, Greenberg ME and Alt FW (1998) A critical role for DNA end-joining proteins in both lymphogenesis and neurogenesis. Cell 95: 891–902

26. Estus S, Zaks WJ, Freeman RS, Gruda M, Bravo R and Johnson Jr. EM (1994) Altered gene expression in neurons during programmed cell death: identification of c-jun as necessary for neuronal apoptosis. J. Cell Biol. 127: 1717–1727

27. Liu DX and Greene LA (2001) Regulation of neuronal survival and death by E2F-dependent gene repression and derepression. Neuron 32: 425–438

28. Freeman RS, Estus S and Johnson Jr. EM (1994) Analysis of cell cycle-related gene expression in postmitotic neurons: selective induction of Cyclin D1 during programmed cell death. Neuron 12: 343–355

29. Gross A, McDonnell JM and Korsmeyer SJ (1999) BCL-2 family members and the mitochondria in apoptosis. Genes Dev. 13: 1899–1911

30. Korsmeyer SJ (1999) BCL-2 gene family and the regulation of programmed cell death. Cancer Res. 59: 1693s–1700s

31. Whitfield J, Neame SJ, Paquet L, Bernard O and Ham J (2001) Dominant-negative c-Jun promotes neuronal survival by reducing BIM expression and inhibiting mitochondrial cytochrome c release. Neuron 29: 629–643

32. Putcha GV, Moulder KL, Golden JP, Bouillet P, Adams JA, Strasser A and Johnson EM (2001) Induction of BIM, a proapoptotic BH3-only BCL-2 family member, is critical for neuronal apoptosis. Neuron 29: 615–628

33. Harris CA and Johnson Jr. EM (2001) BH3-only Bcl-2 family members are coordinately regulated by the JNK pathway and require Bax to induce apoptosis in neurons. J. Biol. Chem. 276: 37754–37760

34. Gross A, Yin XM, Wang K, Wei MC, Jockel J, Milliman C, Erdjument-Bromage H, Tempst P and Korsmeyer SJ (1999) Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. J. Biol. Chem. 274: 1156–1163

35. Wang K, Yin XM, Chao DT, Milliman CL and Korsmeyer SJ (1996) BID: a novel BH3 domain-only death agonist. Genes Dev. 10: 2859–2869

36. Leonard JR, D'Sa C, Cahn BR, Korsmeyer SJ and Roth KA (2001) Bid regulation of neuronal apoptosis. Brain Res. Dev. Brain Res. 128: 187–190

37. Putcha GV, Harris CA, Moulder KL, Easton RM, Thompson CB and Johnson Jr. EM (2002) Intrinsic and extrinsic pathway signaling during neuronal apoptosis: lessons from the analysis of mutant mice. J. Cell Biol. 157: 441–453

38. Deckwerth TL, Elliott JL, Knudson CM, Johnson Jr. EM, Snider WD and Korsmeyer SJ (1996) BAX is required for neuronal death after trophic factor deprivation and during development. Neuron 17: 401–411

39. Putcha GV, Deshmukh M and Johnson Jr. EM (1999) BAX translocation is a critical event in neuronal apoptosis: regulation by neuroprotectants, BCL-2, and caspases. J. Neurosci. 19: 7476–7485

40. Garcia I, Martinou I, Tsujimoto Y and Martinou JC (1992) Prevention of programmed cell death of sympathetic neurons by the bcl-2 proto-oncogene. Science 258: 302–304

41. Greenlund LJ, Korsmeyer SJ and Johnson Jr. EM (1995) Role of BCL-2 in the survival and function of developing and mature sympathetic neurons. Neuron 15: 649–661

42. Liu X, Kim CN, Yang J, Jemmerson R and Wang X (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell 86: 147–157

43. Putcha GV, Deshmukh M and Johnson Jr. EM (2000) Inhibition of apoptotic signaling cascades causes loss of trophic factor dependence during neuronal maturation. J. Cell Biol. 149: 1011–1018

44. Deshmukh M, Vasilakos J, Deckwerth TL, Lampe PA, Shivers BD and Johnson Jr. EM (1996) Genetic and metabolic status of NGF-deprived sympathetic neurons saved by an inhibitor of ICE family proteases. J. Cell Biol. 135: 1341–1354

45. Deshmukh M and Johnson Jr. EM (1997) Programmed cell death in neurons: focus on the pathway of nerve growth factor deprivation-induced death of sympathetic neurons. Mol. Pharmacol. 51: 897–906

46. Srinivasan A, Roth KA, Sayers RO, Shindler KS, Wong AM, Fritz LC and Tomaselli KJ (1998) In situ immunodetection of activated caspase-3 in apoptotic neurons in the developing nervous system. Cell Death Differ. 5: 1004–1016

47. Deshmukh M, Kuida K and Johnson Jr. EM (2000) Caspase inhibition extends the commitment to neuronal death beyond cytochrome c release to the point of mitochondrial depolarization. J. Cell Biol. 150: 131–143

48. Aloyz RS, Bamji SX, Pozniak CD, Toma JG, Atwal J, Kaplan DR and Miller FD (1998) p53 is essential for developmental neuron death as regulated by the TrkA and p75 neurotrophin receptors. J. Cell Biol. 143: 1691–1703

49. Slack RS, Belliveau DJ, Rosenberg M, Atwal J, Lochmuller H, Aloyz R, Haghighi A, Lach B, Seth P, Cooper E and Miller FD (1996) Adenovirus-mediated gene transfer of the tumor suppressor, p53, induces apoptosis in postmitotic neurons. J. Cell Biol. 135: 1085–1096

50. Sadoul R, Quiquerez AL, Martinou I, Fernandez PA and Martinou JC (1996) p53 protein in sympathetic neurons: cytoplasmic localization and no apparent function in apoptosis. J. Neurosci. Res. 43: 594–601

51. Martinou I, Fernandez PA, Missotten M, White E, Allet B, Sadoul R and Martinou JC (1995) Viral proteins E1B19 K and p35 protect sympathetic neurons from cell death induced by NGF deprivation. J. Cell Biol. 128: 201–208

52. Davies AM and Rosenthal A (1994) Neurons from mouse embryos with a null mutation in the tumour suppressor gene p53 undergo normal cell death in the absence of neurotrophins. Neurosci. Lett. 182: 112–114

53. Selvakumaran M, Lin HK, Miyashita T, Wang HG, Krajewski S, Reed JC, Hoffman B and Liebermann D (1994) Immediate early up-regulation of bax expression by p53 but not TGF beta 1: a paradigm for distinct apoptotic pathways. Oncogene 9: 1791–1798

54. Miyashita T, Krajewski S, Krajewska M, Wang HG, Lin HK, Liebermann DA, Hoffman B and Reed JC (1994) Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. Oncogene 9: 1799–1805

55. Miyashita T and Reed JC (1995) Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 80: 293–299

56. el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW and Vogelstein B (1993) WAF1, a potential mediator of p53 tumor suppression. Cell 75: 817–825

57. el-Deiry WS, Harper JW, O'Connor PM, Velculescu VE, Canman CE, Jackman J, Pietenpol JA, Burrell M, Hill DE, Wang Y (1994) WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. Cancer Res. 54: 1169–1174

58. Goedert M, Otten U and Thoenen H (1978) Biochemical effects of antibodies against nerve growth factor on developing and differentiated sympathetic ganglia. Brain Res. 148: 264–268

59. Angeletti PU, Levi-Montalcini R and Caramia F (1971) Analysis of the effects of the antiserum to the nerve growth factor in adult mice. Brain Res. 27: 343–355

60. Easton RM, Deckwerth TL, Parsadanian AS and Johnson Jr. EM (1997) Analysis of the mechanism of loss of trophic factor dependence associated with neuronal maturation: a phenotype indistinguishable from Bax deletion. J. Neurosci. 17: 9656–9666

61. Morris EJ, Keramaris E, Rideout HJ, Slack RS, Dyson NJ, Stefanis L and Park DS (2001) Cyclin-dependent kinases and P53 pathways are activated independently and mediate Bax activation in neurons after DNA damage. J. Neurosci. 21: 5017–5026

62. Stefanis L, Park DS, Friedman WJ and Greene LA (1999) Caspase-dependent and independent death of camptothecin-treated embryonic cortical neurons. J. Neurosci. 19: 6235–6247

63. Bissonnette N, Wasylyk B and Hunting DJ (1997) The apoptotic and transcriptional transactivation activities of p53 can be dissociated. Biochem. Cell. Biol. 75: 351–358

64. Marchenko ND, Zaika A and Moll UM (2000) Death signal-induced localization of p53 protein to mitochondria. A potential role in apoptotic signaling. J. Biol. Chem. 275: 16202–16212

65. Sansome C, Zaika A, Marchenko ND and Moll UM (2001) Hypoxia death stimulus induces translocation of p53 protein to mitochondria. Detection by immunofluorescence on whole cells. FEBS Lett. 488: 110–115

66. Karanjawala ZE, Murphy N, Hinton DR, Hsieh CL and Lieber MR (2002) Oxygen metabolism causes chromosome breaks and is associated with the neuronal apoptosis observed in DNA double-strand break repair mutants. Curr. Biol. 12: 397–402

67. Sugo N, Aratani Y, Nagashima Y, Kubota Y and Koyama H (2000) Neonatal lethality with abnormal neurogenesis in mice deficient in DNA polymerase beta. EMBO J. 19: 1397–1404

68. Veis DJ, Sorenson CM, Shutter JR and Korsmeyer SJ (1993) Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. Cell 75: 229–240

69. Knudson CM, Tung KS, Tourtellotte WG, Brown GA and Korsmeyer SJ (1995) Bax-deficient mice with lymphoid hyperplasia and male germ cell death. Science 270: 96–99

70. Ruit KG, Elliott JL, Osborne PA, Yan Q and Snider WD (1992) Selective dependence of mammalian dorsal root ganglion neurons on nerve growth factor during embryonic development. Neuron 8: 573–587

71. Johnson MI and Argiro V (1983) Techniques in the tissue culture of rat sympathetic neurons. Methods Enzymol. 103: 334–347

72. Deshmukh M and Johnson Jr. EM (1998) Evidence of a novel event during neuronal death: development of competence-to-die in response to cytoplasmic cytochrome c. Neuron 21: 695–705

73. Moulder KL, Narita M, Chang LK, Bu G and Johnson Jr. EM (1999) Analysis of a novel mechanism of neuronal toxicity produced by an apolipoprotein E-derived peptide. J. Neurochem. 72: 1069–1080

74. Deckwerth TL and Johnson Jr. EM (1994) Neurites can remain viable after destruction of the neuronal soma by programmed cell death (apoptosis). Dev. Biol. 165: 63–72

75. Miller TM, Moulder KL, Knudson CM, Creedon DJ, Deshmukh M, Korsmeyer SJ and Johnson Jr. EM (1997) Bax deletion further orders the cell death pathway in cerebellar granule cells and suggests a caspase-independent pathway to cell death. J. Cell Biol. 139: 205–217