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DNA damage-induced neural precursor cell apoptosis requires p53 and caspase 9 but neither Bax nor caspase 3

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SUMMARY

Programmed cell death (apoptosis) is critical for normal brain morphogenesis and may be triggered by neurotrophic factor deprivation or irreparable DNA damage. Members of the Bcl2 and caspase families regulate neuronal responsiveness to trophic factor withdrawal; however, their involvement in DNA damage-induced neuronal apoptosis is less clear. To define the molecular pathway regulating DNA damage-induced neural precursor cell apoptosis, we have examined the effects of drug and γ-irradiation-induced DNA damage on telencephalic neural precursor cells derived from wild-type embryos and mice with targeted disruptions of apoptosis-associated genes. We found that DNA damage-induced neural precursor cell apoptosis, both in vitro and in vivo, was critically dependent on p53 and caspase 9, but neither Bax nor caspase 3 expression. Neural precursor cell apoptosis was also unaffected by targeted disruptions of *Bclx* and *Bcl2*, and unlike neurotrophic factor-deprivation-induced neuronal apoptosis, was not associated with a detectable loss of cytochrome c from mitochondria. The apoptotic pathway regulating DNA damage-induced neural precursor cell death is different from that required for normal brain morphogenesis, which involves both caspase 9 and caspase 3 but not p53, indicating that additional apoptotic stimuli regulate neural precursor cell numbers during telencephalic development.

Key words: Apoptosis, Neurodevelopment, Caspase, Bcl2, p53, Mouse

INTRODUCTION

Apoptotic death critically regulates normal nervous system development (Oppenheim, 1991). Recently, the developmental significance of neural precursor cell (NPC) apoptosis has been recognized. NPCs reside within the proliferative ventricular zone and consist of self-renewing pluripotent stem cells and lineage-restricted progenitor cells (Svendsen et al., 1999). They persist into postnatal life and may give rise to both neurons and glial cells in the adult brain (McKay, 1997; Johansson et al., 1999; Doetsch et al., 1999). Defining the molecular pathways that regulate NPC death is imperative for understanding normal brain development and a variety of neuropathological conditions.

Genetic and biochemical studies have identified Bcl-X_L (Bcl2l – Mouse Genome Informatics), Bax, Apaf1, caspase 9 (Casp9 – Mouse Genome Informatics) and caspase 3 (Casp3 – Mouse Genome Informatics) as important regulators of neuronal programmed cell death (Kuan et al., 2000). Interactions between Bcl-X_L and Bax regulate cytochrome c release from mitochondria and establish baseline sensitivity to apoptotic stimuli (Finucane et al., 1999). Cytosolic cytochrome c propagates the apoptotic signal by binding to Apaf1, in the presence of ATP or dATP, and converting caspase 9 zymogen into an active caspase (Zou et al., 1999). Caspase 9 activates caspase 3, which then enzymatically cleaves a variety of intracellular targets giving rise to the biochemical and cytological changes recognized as apoptosis (Woo et al., 1998). Mice lacking Bcl-X_L exhibit massive death of immature neurons in the embryonic brain (Motoyama et al., 1995) whereas Bax-deficient mice show decreased neuronal programmed cell death (Deckwerth et al., 1996; Shindler et al., 1997). We have previously shown that Bax deficiency, as well as caspase 9 or caspase 3 deficiency, eliminates the increased neuronal apoptosis caused by Bclx deficiency indicating that Bax, Bcl-X_L, caspase 9 and caspase 3 function in a linear pathway to regulate apoptosis of immature neurons (Shindler et al., 1997; Roth et al., 2000; Zaidi et al., 2001). However, unlike Bax-deficient embryos, mice lacking the pro-apoptotic molecules Apaf1, caspase 9 or caspase 3, exhibit an expanded

ventricular zone and gross brain malformations (Kuida et al., 1996; Cecconi et al., 1998; Kuida et al., 1998; Hakem et al., 1998; Yoshida et al., 1998). These observations suggest that still to be defined signals in the embryonic ventricular zone activate Apaf1-, caspase 9- and caspase 3-dependent, Bax- and Bcl-X_L-independent NPC apoptosis and that failure to engage this pathway results in NPC expansion and neurodevelopmental pathology.

Targeted disruptions of the genes for Xrcc4 and DNA ligase IV have identified irreparable DNA damage as an important trigger of neuronal programmed cell death (Gao et al., 1998; Frank et al., 1998). These mutant mice, like Bcl-X_L-deficient embryos, exhibit markedly increased apoptosis of immature neurons in the developing nervous system. However, it is unclear if irreparable DNA damage plays a role in limiting NPC numbers, similar to the pro-apoptotic molecules Apaf1, caspase 9 and caspase 3. To define the molecular pathway of DNA damage-induced NPC apoptosis and its possible relationship to normal brain development, we have used a combination of gene-disrupted mice and in vivo and in vitro models of NPC apoptosis. Our results demonstrate that DNA damage triggers a p53- (Trp53 – Mouse Genome Informatics)- and caspase 9-dependent, Bax- and caspase 3-independent NPC death pathway that is distinct from that regulating nervous system morphogenesis.

MATERIALS AND METHODS

Mice

Timed-pregnant Swiss Webster mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN). Generation of *Bcl2*$^{-/-}$ (Nakayama et al., 1994), *Bclx*$^{-/-}$ (Motoyama et al., 1995), *Bax*$^{-/-}$ (Shindler et al., 1997), *Casp3*$^{-/-}$ (Kuida et al., 1996) and *Casp9*$^{-/-}$ (Kuida et al., 1998) mice has been described previously. *p53*$^{+/-}$ and *p53*$^{-/-}$ mice were purchased from Taconic, Germantown, NY. Heterozygous mice were crossed to generate wild-type, heterozygous and homozygous gene-disrupted mice. Endogenous and disrupted genes were detected by PCR analysis of tail DNA extracts as previously described (Timme and Thompson, 1994; Kuida et al., 1996; Shindler et al., 1997; Kuida et al., 1998). The morning on which a vaginal plug was seen was designated as embryonic day 0.5 (E0.5).

Primary telencephalic cultures

Primary cell cultures were prepared as described previously (Flaris et al., 1995; Shindler and Roth, 1996a). Briefly, embryos were removed between gestational days 12 and 13. Telencephalic vesicles were isolated and cells were dissociated for 15 minutes at 37°C in HBSS (Gibco, Grand Island, NY) containing 0.01% trypsin with 0.004% EDTA, 0.02 mg/ml DNase I and 0.1% BSA (all purchased from Sigma, MO). Trypsinization was stopped by adding an equal volume of HBSS containing 10% fetal calf serum (FCS). Cells were further dissociated by three rounds of trituration with a fire-polished Pasteur pipette and washed once with HBSS. A small sample was stained with Trypan Blue and counted. Approximately two million viable cells per embryo were collected. For induction of apoptosis, freshly dissociated cells at a concentration of 1.0×10^6 cells/ml were treated with 100 μM cytosine arabinoside (AraC; Sigma) or γ-irradiated (10 Gy) and incubated at 37°C in humidified 5% CO_2/95% air atmosphere. Six hours later, cells were consecutively labeled with 75 nM MitoTracker Red CMXRos (Molecular Probes, Eugene, OR) and 2.5 nM SYTOX Green (Molecular Probes) or with MitoTracker Red and 625 ng/ml fluorescein-conjugated cholera toxin B subunit (CTB-FITC, LIST Biological Laboratories, Campbell, CA) and analyzed by flow cytometry as described previously (D'Sa-Eipper and Roth, 2000). In some experiments, the ability of the broad-spectrum caspase inhibitor, Boc-Asp (OMe)-Fluoro-methyl-Ketone (BAF, Enzyme Systems Products, Livermore, CA) to inhibit DNA damage-induced death was also tested.

In vivo AraC/γ-irradiation treatment

Pregnant mice between gestational day 12 and 13 were intraperitoneally injected with AraC at a dose of 25 mg/kg body weight or γ-irradiated (1 Gy). Embryos were isolated 6 hours later and placed in cold Bouin's fixative overnight, followed by paraffin embedding. Telencephalic cells were also isolated from embryos 6 hours after AraC injection and apoptosis assessed by flow cytometry following labeling with MitoTracker Red and SYTOX Green.

Immunocytochemistry

Bouin's fixed embryos were washed several times in 70% ethanol, embedded in paraffin and cut into 4 μm sagittal sections. Sections from several levels were Hematoxylin and Eosin (H and E) stained and viewed by light microscopy. Alternatively for semi-thin sections, 2% (vol/vol) glutaraldehyde-fixed embryos were embedded in plastic and 1 μm sections were cut and stained with 1% Toluidine Blue. Immunostaining of sections or primary cell cultures with antibodies to activated caspase 3, cytochrome c, proliferating cell nuclear antigen (PCNA) and microtubule-associated protein 2 (MAP2) were done using previously described methods (Motoyama et al., 1995; Shindler et al., 1997; Srinivasan et al., 1998; Yin et al., 1999). Briefly, sections were deparaffinized and endogenous peroxidase activity blocked with 3% hydrogen peroxide in PBS (10 mM phosphate buffered saline, pH 7.2). This was followed by incubation in PBS-blocking buffer (PBS with 1% BSA, 0.2% powdered milk and 0.3% Triton X-100) for 30 minutes at room temperature and primary antibodies (diluted in PBS-blocking buffer) overnight at 4°C. Sections were then washed with PBS and incubated with horseradish peroxidase-conjugated secondary antibodies (diluted in PBS-blocking buffer) for 1 hour at room temperature. Antigen-antibody complexes were subsequently detected by direct tyramide signal amplification (TSA, NEN Life Science Products, Boston, MA) using either fluorescein- or cyanine 3-conjugated tyramide according to the manufacturer's instructions. Activated caspase 3 was detected with an affinity purified rabbit polyclonal antiserum, CM1, which recognizes the p18 subunit of cleaved caspase 3 (Srinivasan et al., 1998) and was used at a dilution of 1:40,000. Mouse monoclonal antibodies against PCNA (CALBIOCHEM, La Jolla, CA), MAP2 (Sigma), nestin (Rat-401, Developmental Studies Hybridoma Bank, Iowa City, IA) and cytochrome c (Pharmingen, San Diego, CA) were used at dilutions of 1:1000, 1:500,000, 1:10 and 1:1000 respectively. Dual CM1 and cytochrome c immunostaining was performed as previously described (Yin et al., 1999). Cell nuclei were stained by incubating sections for 10 minutes in a 0.2 μg/ml solution of bisbenzimide (Hoechst 33258; Sigma) and visualized on a Zeiss-Axioskop microscope equipped with epifluorescence.

TUNEL staining

Tissue sections were deparaffinized and permeabilized with 0.5% Triton X-100 in PBS for 10 minutes at room temperature. TUNEL reactions were performed with slight modifications of a method described previously (Shindler et al., 1997). Briefly, sections were incubated with terminal deoxynucleotidyl transferase (TDT; 3.125 U/100 μl buffer; Roche Molecular Biochemicals, Indianapolis, IN) and digoxigenin-conjugated deoxyuridine triphosphate (0.125 nmol/100 μl buffer; Roche Molecular Biochemicals) for 60 minutes at 37°C in TDT buffer (30 mM Tris-base pH 7.2, 140 mM sodium cacodylate and 1 mM cobalt chloride). Reactions were stopped by incubating tissues for 15 minutes in a solution of 300 mM sodium chloride and 30 mM sodium citrate followed by an overnight incubation at 4°C with horseradish peroxidase-conjugated sheep anti-

digoxigenin antiserum (Roche Molecular Biochemicals) diluted 1:1000 in PBS-blocking buffer. Following washes with PBS, labeled cells were visualized by tyramide signal amplification with cyanine 3 tyramide (NEN Life Science Products). Tissue was counterstained with bisbenzimide and visualized on a Zeiss-Axioskop microscope equipped with epifluorescence.

Preparation of subcellular fractions and assay for cytochrome c

Pregnant mice were intraperitoneally injected with 25 mg/kg AraC at gestational day E12. Telencephalic cells were isolated from embryos 6 hours after injection and washed twice with PBS. Cells were homogenized in isotonic fractionation buffer (250 mM sucrose, 0.5 mM EDTA, 20 mM HEPES pH 7.4, 500 μM Na_3VO_4) supplemented with the protease inhibitor cocktail Complete (Roche Molecular Biochemicals), using a ballbearing homogenizer and centrifuged at 900 ***g*** for 5 minutes to remove nuclei and intact cells. The post-nuclear supernatant was transferred to a microfuge tube and centrifuged at 25,000 ***g*** for 10 minutes to collect the heavy membrane (HM) fraction, followed by centrifugation of the post-HM supernatant at 100,000 ***g*** for 10-20 minutes to obtain the microsomal and cytosolic fractions. All pellets were resuspended in a volume of fractionation buffer equivalent to the cytosolic volume. All fractions were then resuspended to equivalent volumes with 2× SDS buffer. Samples were separated by SDS-PAGE, transferred to PVDF membranes and probed with anti-cytochrome c (1:1000, Pharmingen), anti-cytochrome oxidase subunit IV (1:1000, Molecular Probes) and anti-lactate dehydrogenase (1:1000, Rockland Immunochemicals, Gilbertsville, PA). The blots were developed using the SuperSignal chemiluminescent system (Pierce, Rockford, IL) according to the manufacturer's protocol.

RESULTS

DNA damage induces caspase 3 activation and NPC apoptosis in vivo and in vitro

Our previous studies demonstrated that freshly isolated E12-E13 telencephalic cells consist of approx. 70% nestin immunoreactive NPCs and 25% MAP2 immunoreactive neurons, the majority of which have yet to extend neurites (D'Sa-Eipper and Roth, 2000). Treatment of E12-E13 telencephalic cells with the nucleoside analog, AraC, or the protein kinase inhibitor, staurosporine, for 6 hours induced caspase 3 activation and apoptotic death that were completely blocked by the broad-spectrum caspase inhibitor, BAF (D'Sa-Eipper and Roth, 2000). To verify that the apoptosis inducing effect of AraC occurred predominantly in the NPC subpopulation of E12-E13 telencephalic cells, we performed dual CTB labeling, a property of postmitotic neurons (Flaris et al., 1995; Shindler and Roth, 1996b), and MitoTracker Red staining in control and AraC-treated telencephalic cells. MitoTracker Red detects functionally active mitochondria, its labeling is decreased in apoptotic cells and virtually lost in necrotic cells. Flow cytometric quantitation revealed that AraC caused a dramatic increase in the low MitoTracker Red-labeled (apoptotic) population in the CTB low (NPC) subpopulation of E12-E13 telencephalic cells (net increase in apoptotic cells, mean±s.e.m. 45±1%; n=4). In contrast, the CTB high subpopulation (neurons) was minimally affected by AraC (net increase in apoptotic cells 5±6%; n=4).

AraC is thought to induce cell death secondary to DNA damage and disrupted DNA replication (Grant, 1998); studies with the DNA damaging agents etoposide (1 μM) and camptothecin (10 μM) also showed BAF-inhibitable NPC apoptosis (data not shown). Similarly, 6 hours after γ-irradiation of freshly isolated E12-E13 telencephalic cells, there was approximately a 50% decrease in the percentage of viable cells identified by high MitoTracker Red and low SYTOX Green labeling (Figs 2, 5). SYTOX Green is a nucleic acid stain that easily penetrates cells with compromised plasma membranes but not cells with intact membranes (D'Sa-Eipper and Roth, 2000). Viable cells and cells in the early stages of apoptotic death are only weakly labeled with SYTOX Green; in contrast, necrotic cells show intense SYTOX Green labeling. The death promoting effect of γ-irradiation was completely blocked by concomitant treatment with 300 μM BAF (data not shown). In total, these studies indicate that DNA damage, whether caused by chemical or physical insult, produces caspase-dependent NPC death.

To determine if DNA damage causes NPC death in vivo, pregnant mice were intraperitoneally injected with AraC (25 mg/kg body weight) on gestational day 12 and embryos were harvested 6 hours later. Apoptosis was assessed with TUNEL, bisbenzimide (Hoechst 33258), and H and E staining of paraffin-embedded sections, and with Toluidine Blue staining of one micron thick plastic sections. Caspase 3 activation was detected in situ using an antiserum (CM1) that specifically recognizes 'activated' caspase 3 and not caspase 3 zymogen (Srinivasan et al., 1998). Untreated embryos showed only rare apoptotic (Fig. 1A) and/or CM1 immunoreactive NPCs in the ventricular zone (data not shown). H and E stained sections of AraC-treated embryos showed numerous cells in the ventricular zone with apoptotic features (Fig. 1B). AraC-induced apoptosis was accompanied by intense immunoreactivity for activated caspase 3 (Fig. 1C). Abnormal bisbenzimide stained nuclei and TUNEL-positive cells (Fig. 1D) were observed in the ventricular zone which was defined by the presence of PCNA and nestin immunoreactivity and the paucity of MAP2 immunoreactive cells (data not shown).

To quantitate the extent of in vivo apoptosis induced by AraC, the percentage of E12-E13 telencephalic cells with apoptotic nuclear features was determined in bisbenzimide stained sections of control and AraC-exposed mice. Six hours post-AraC injection, 23% of telencephalic nuclei appeared apoptotic (22.9±2.3%, n=5) compared with less than 1% in control sections (0.5±0.1%, n=5). Objective measurements of in vivo AraC-induced death were also made using flow cytometric detection of MitoTracker Red and SYTOX Green labeling in single cell suspensions as previously described (D'Sa-Eipper and Roth, 2000). AraC exposure caused an approximate 40% decrease in the viable telencephalic cell subpopulation (% viable: 59±2; n=10) and a corresponding increase in the apoptotic cell population, defined by low MitoTracker Red and low SYTOX Green fluorescence intensity (data not shown). Similar results were obtained when a second indicator of mitochondrial potential, $DiOC_6(3)$, was used instead of MitoTracker Red (data not shown). In total, these results indicate that DNA damage induces caspase 3 activation and NPC apoptosis both in vitro and in vivo.

p53 or caspase 9 deficiency protects against AraC-induced NPC apoptosis

DNA damage in a variety of cell types leads to p53-dependent

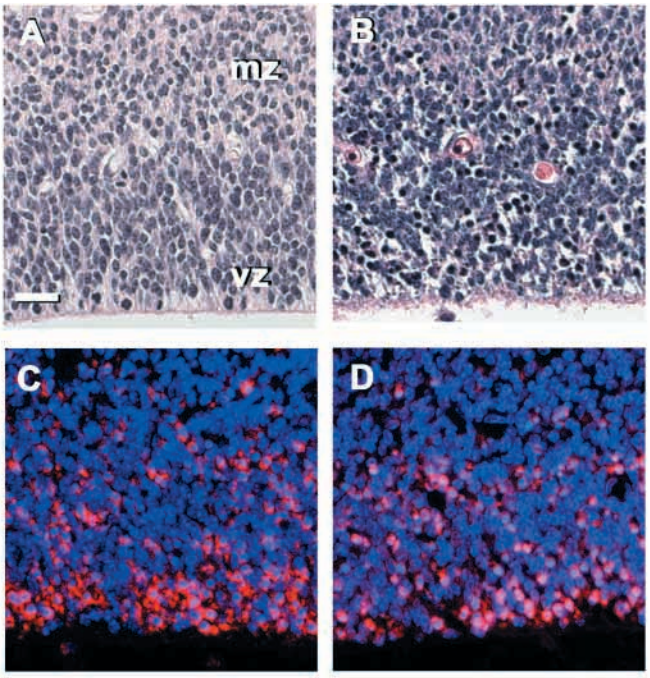

Fig. 1. Transplacental AraC exposure induces NPC apoptosis and Caspase-3 activation. Six hours post transplacental AraC exposure, embryos were fixed in Bouin's solution and sections processed for histological and immunohistochemical evaluation. Compared with an untreated embryo (A), a Hematoxylin and Eosin stained section of telencephalon from an AraC-treated embryo (B) showed numerous pyknotic nuclei and karyorrhectic debris. (C) Extensive caspase 3 activation, as indicated by CM1 immunoreactivity (red), was observed in the telencephalon and numerous TUNEL-positive cells (red; D) were detected in the ventricular zone 6 hours after AraC exposure. Sections in C and D were counterstained with bisbenzimide (blue). All sections were obtained from E13 embryos. mz, marginal zone; vz, ventricular zone. Scale bar: 25 μm.

apoptosis that may also require *Casp9* expression (Evan and Littlewood, 1998; Soengas et al., 1999). We therefore examined whether DNA damage-induced NPC apoptosis was critically dependent on p53 and caspase 9. E12-E13 telencephalic cells isolated from p53-and caspase 9-deficient mice were either untreated or treated with AraC or γ-irradiation in vitro, and apoptosis was assessed 6 hours later by flow cytometry using MitoTracker Red and SYTOX Green labeling.

Treatment of wild-type telencephalic cells with AraC or γ-irradiation decreased the percentage of viable cells by approximately 40%. Both $p53^{-/-}$ (Fig. 2A) and $Casp9^{-/-}$ (Fig. 2B) cells were resistant to DNA damage-induced apoptosis. When one copy of the *p53* gene was present, an intermediate level of apoptosis was found (Fig. 2A). A gene dosage effect was not seen in $Casp9^{+/-}$ embryos (Fig. 2B). The requirement for p53 and caspase 9 in DNA damage-induced NPC apoptosis in vivo was examined in AraC or γ-irradiation treated embryos. In contrast to the massive apoptosis observed within 6 hours of AraC exposure in wild-type embryos (Fig. 1B), neither p53- nor caspase 9-deficient embryos showed an increase in TUNEL reactivity (data not shown), activated caspase 3 immunoreactivity (Fig. 3A), or cytologically apoptotic cells at 6 (Fig. 4A,B) or 24 hours (data not shown) after transplacental AraC exposure. Similarly, p53- and caspase 9-deficient embryos showed no increased telencephalic cell death 6 hours after γ-irradiation (data not shown). Ex vivo flow cytometric quantitation of telencephalic cell viability six hours post in vivo AraC exposure revealed increased apoptosis in wild-type preparations but not in p53- or caspase 9-deficient preparations (data not shown). These results demonstrate that p53 and caspase 9 are critically required for both γ-irradiation and AraC-induced apoptosis of NPCs both in vivo and in vitro.

We have previously shown that targeted disruptions of the pro-apoptotic genes *Casp9*, *Casp3* and *Bax* cause a marked reduction in the number of activated caspase 3-immunoreactive and TUNEL-positive cells in the developing nervous system (Shindler et al., 1997; Srinivasan et al., 1998; Kuida et al., 1998). To determine if p53 is also involved in the apoptotic pathway(s) regulating naturally occurring neuronal cell death, we quantitated the number of activated caspase 3-immunoreactive and TUNEL-positive cells in wild-type and

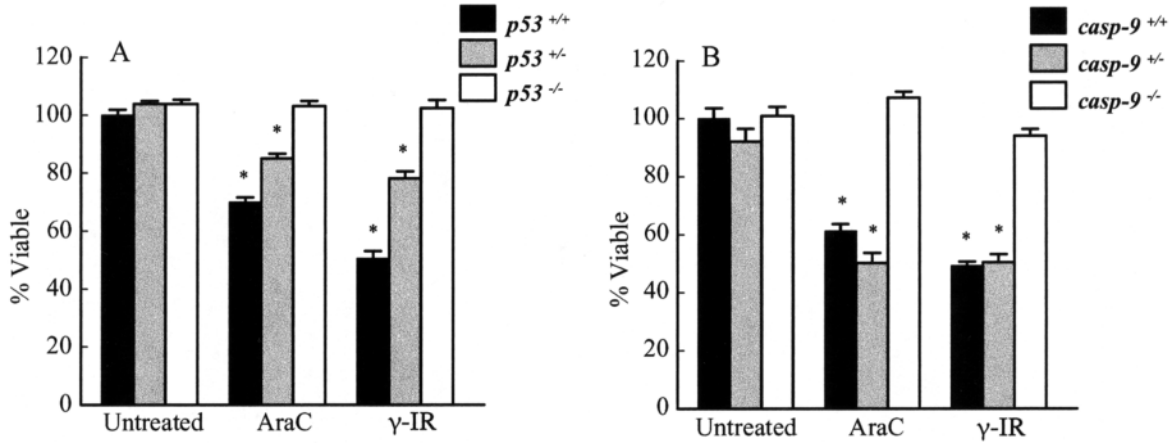

Fig. 2. Requirement for p53 and caspase 9 in DNA damage-induced NPC apoptosis. E12-E13 telencephalic cells were isolated from wild-type, heterozygous or homozygous mutant *p53* (A) or *Casp9* (B) embryos, treated with 100 μM AraC or 10 Gy γ-irradiation, and viability assessed 6 hours later using MitoTracker Red and SYTOX Green labeling. Each data point represents mean±s.e.m. (n=4-10 for the different genotypes). Data are expressed as a percentage of untreated wild-type cells *P<0.05 versus untreated of like genotype (Mann-Whitney Rank Sum Test).

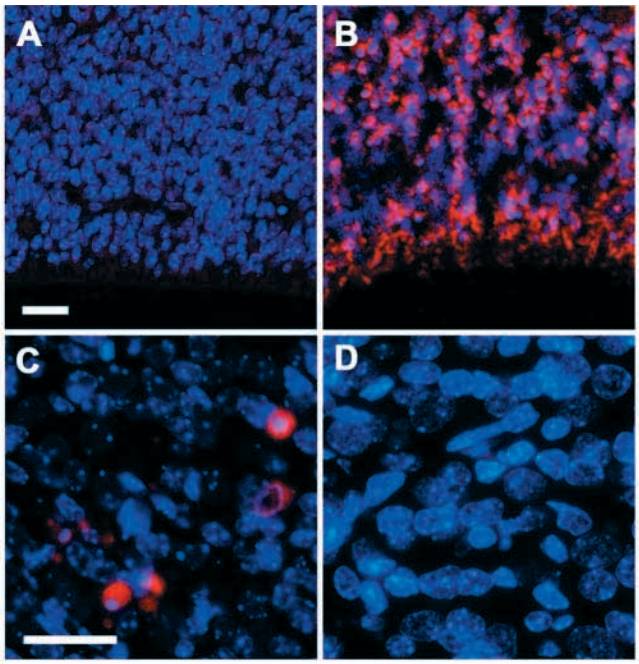

Fig. 3. p53 and Bax regulate stimulus-specific apoptotic pathways. (A) Six hours post transplacental AraC exposure, p53-deficient E13 embryos show no evidence of activated caspase 3 immunoreactivity (red) in the telencephalic ventricular zone. (B) In contrast, Bax-deficient E12 embryos exhibit numerous activated caspase 3-immunoreactive cells. (C,D) Naturally occurring neuronal cell death and caspase 3 activation in the E13 DRG was unaffected in p53-deficient mice (CM1 immunoreactive cells, red; C) but virtually eliminated in Bax-deficient mice (D). All sections were counterstained with bisbenzimide (blue). Scale bars: 25 μm.

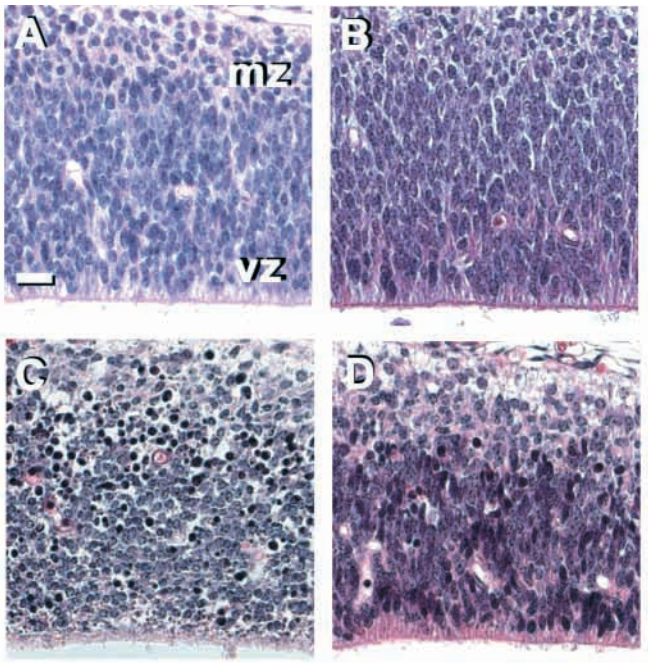

Fig. 4. p53 and caspase 9-deficient embryos, but not Bax or caspase 3-deficient embryos, are resistant to AraC-induced NPC apoptosis in vivo. p53-deficient (A) or caspase 9-deficient (B) E12-E13 telencephalon 6 hours after AraC exposure, showed no histological evidence of apoptosis in the ventricular zone (vz). In contrast, Bax-deficient E12 embryos exhibited extensive cell death in the ventricular zone following AraC treatment (C) and caspase 3-deficient E13 embryos showed numerous telencephalic cells with abnormal nuclear features (D). Scale bar: 25 μm. mz, marginal zone.

p53-deficient E12-E13 dorsal root ganglia. Activated caspase 3-immunoreactive cells were readily detected in p53-deficient DRG (Fig. 3C) and there was no reduction in their number, or that of TUNEL-positive cells, in p53-deficient embryos, compared with $p53^{+/-}$ and $p53^{+/+}$ mice (data not shown). For comparison, Bax-deficient embryos exhibited a nearly complete abolition of activated caspase 3 immunoreactive (Fig. 3D) and TUNEL positive cells in the E12-E13 DRG (Shindler et al., 1997).

Neither Bax nor caspase 3 is required for AraC-induced NPC apoptosis

p53 is a direct transcriptional activator of *Bax* (Miyashita and Reed, 1995) and this action may mediate p53-dependent apoptosis in some cell types (McCurrach et al., 1997; Johnson et al., 1998). Since $p53^{-/-}$ NPCs were resistant to AraC- and γ-irradiation-induced apoptosis, we reasoned that if *Bax* expression was upregulated by p53 and was important for DNA damage-induced NPC apoptosis, then Bax-deficient NPCs should also be resistant to apoptosis. In vitro exposure of Bax-deficient NPCs to AraC or γ-irradiation resulted in a significant loss of cell viability (Fig. 5A). Similarly, 6 hours after transplacental exposure of Bax-deficient embryos to AraC or γ-irradiation, there was massive caspase 3 activation (Fig. 3B) and apoptosis within the telencephalic ventricular zone (Fig. 4C). These results indicate that Bax is not critical for p53-dependent DNA damage-induced NPC apoptosis.

The significance of caspase 3 activation to AraC- and γ-irradiation-induced NPC death was assessed in caspase 3-deficient cells both in vitro and in vivo. Caspase 3 deficiency produces a variably severe neurodevelopmental phenotype that can be grossly appreciated by E12 (Kuida et al., 1996; Zheng et al., 1999). To determine if differential responsiveness to DNA damage underlies the variable penetrance of the neurodevelopmental phenotype, we compared the effects of AraC on telencephalic cells isolated from abnormal (n=6) and normal appearing (n=5) $Casp3^{-/-}$ embryos and their +/– and +/+ littermates. AraC incubation produced a significant decrease in the viable cell population across all *Casp3* genotypes (Fig. 5B) and was completely unaffected by the severity of the neurodevelopmental phenotype (% viable in abnormal $Casp3^{-/-}$ was 68±2 versus 65±1 in normal $Casp3^{-/-}$). γ-irradiation also caused a loss of NPC viability in caspase 3-deficient embryos (Fig. 5B) and DNA damage-induced apoptosis in both Bax- and caspase 3-deficient cells was completely blocked by co-incubation with 300 μM BAF (% viable cells (100 μM AraC + 300 μM BAF); $Bax^{-/-}$, 107±3, n=9; $Casp3^{-/-}$, 107±3, n=11). Caspase 3-deficient embryos exposed to AraC or γ-irradiation in utero showed no caspase 3 activity (as expected from an effective gene disruption) and few cells with apoptotic nuclear features (Fig. 4D). However, occasional TUNEL-positive cells were present (data not shown) and light microscopic examination of one micron thick Toluidine Blue-stained sections of AraC exposed wild-type and caspase 3-deficient ventricular zone showed typical apoptotic

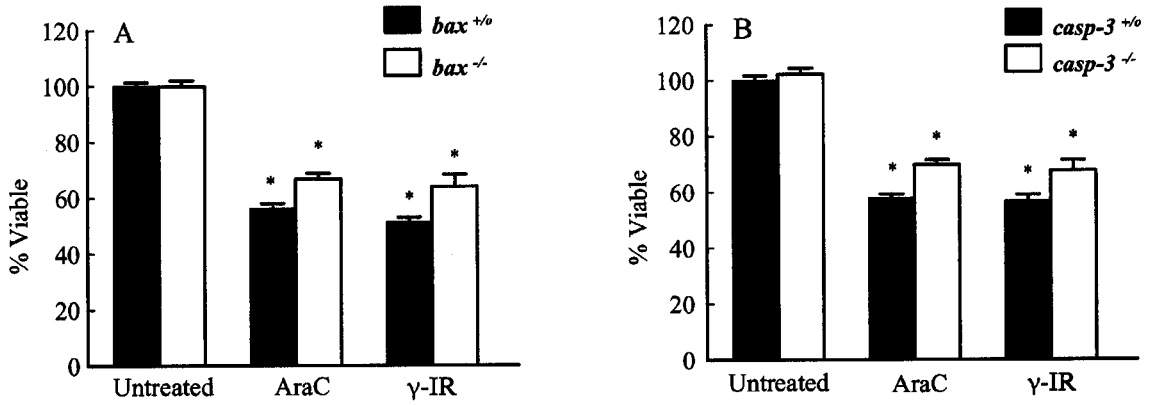

Fig. 5. DNA damage-induced NPC apoptosis does not require Bax or caspase 3 expression. E12-E13 telencephalic cells were isolated from control (+/o includes +/+ and +/–) and homozygous mutant *Bax* (A) or *Casp3* (B) embryos and treated with 100 μM AraC or 10 Gy γ-irradiation. Viability was assessed by MitoTracker Red and SYTOX Green labeling. Each data point represents mean±s.e.m. (n=3-15 for the different genotypes). Data are expressed as a percentage of untreated control cells. *P<0.05 versus untreated of like genotype (Mann-Whitney Rank Sum Test).

nuclei in wild-type (Fig. 6A), but not caspase 3-deficient embryos (Fig. 6B). In these latter embryos, the telencephalic ventricular zone contained large numbers of cells with hyperchromatic abnormally shaped NPC nuclei and cytoplasmic vacuolization (Fig. 6B). Ex vivo flow cytometric analysis of caspase 3-deficient telencephalic cells 6 hours post-transplacental AraC exposure showed decreased MitoTracker Red staining comparable with that observed in AraC-exposed wild-type cells (data not shown).

In contrast to caspase 9 deficiency, caspase 3 deficiency did not prevent DNA damage-induced loss of NPC viability as assessed by MitoTracker Red labeling. Caspase 3 deficiency did however alter the phenotypic appearance of presumably dying cells. To verify that caspase 3-deficient cells exposed to DNA damage had indeed lost their viability and that caspase 9 deficiency protected NPCs from DNA damage-induced death, telencephalic cells from *Casp3*$^{-/-}$, *Casp9*$^{-/-}$, and control (*Casp3*$^{+/+\text{ or }-}$/*Casp9*$^{+/+\text{ or }-}$) embryos were treated with AraC for 6 hours in vitro, washed and plated in chemically defined enriched media for 24 hours. Both control and *Casp3*$^{-/-}$ cells showed a marked decrease in viability over the ensuing 24 hours (Fig. 7). In contrast, AraC-treated *Casp9*$^{-/-}$ cultures were markedly resistant to AraC-induced death (Fig. 7) indicating that the neuroprotective effect of caspase 9 deficiency extended for at least 24 hours. These results indicate that caspase 3 activation is essential for the cytological features of NPC apoptosis but not for DNA damage-induced loss of mitochondrial potential or NPC death per se; a conclusion that is consistent with our previous observations on caspase 3-deficient hepatocytes and thymocytes (Zheng et al., 1998). Furthermore, there is no obvious relationship between neurodevelopmental pathology and the response of NPCs to DNA damage in *Casp3*$^{-/-}$ embryos.

Endogenous Bcl-X_L and Bcl2 do not protect against AraC-induced NPC apoptosis

To determine the possible roles of endogenous Bcl-X_L and Bcl2 in NPC apoptosis, we first examined the baseline level of cell viability in freshly isolated E12 telencephalic cell preparations from wild-type, *Bclx*$^{-/-}$ and *Bcl2*$^{-/-}$ mice. We have previously shown that these preparations contain approximately 20-25% post-mitotic neurons and that Bcl-X_L does not appear to affect NPC programmed cell death in vivo (Roth et al., 2000; D'Sa-Eipper and Roth, 2000). Consistent with these observations, untreated *Bclx*$^{-/-}$ telencephalic cell preparations contained significantly fewer viable cells (%viable; control: 100±2, n=11; *Bclx*$^{-/-}$: 79±4%, n=4) reflecting increased apoptosis in the Bcl-X_L-deficient immature neuron population. However, the net decrease in viable cells on treatment with 100 μM AraC for 6 hours in vitro was not significantly different between control and *Bclx*$^{-/-}$ cells (control: 29±2, n=11; *Bclx*$^{-/-}$: 30±4, n=4). In addition, no effect of Bcl2 deficiency was detected at baseline or with AraC exposure (data not shown).

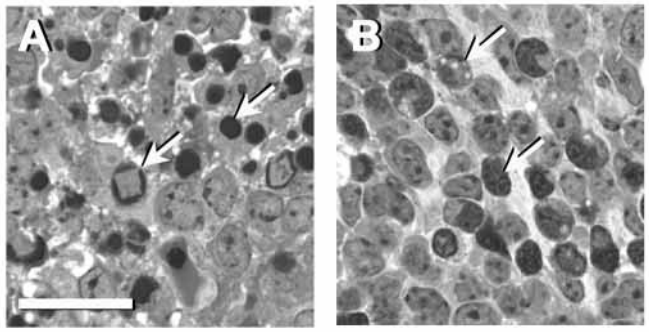

Fig. 6. AraC-treated wild-type, but not caspase 3-deficient, E13 embryos exhibit apoptotic nuclear features in the telencephalic ventricular zone. Light microscopic examination of a 1 μm plastic section from an AraC-treated E13 embryo shows large numbers of shrunken, marginated, hyperchromatic and/or fragmented nuclei in the wild-type telencephalic ventricular zone (A, examples indicated by arrows). In contrast, the AraC-treated *Casp3*$^{-/-}$ telencephalon shows abnormal hyperchromatic nuclei in the ventricular zone (B, examples indicated by arrows) but fragmented or shrunken nuclei are rare. Scale bar: 25 μm.

AraC-induced NPC apoptosis occurs without detectable loss of mitochondrial cytochrome c

In many, but not all, apoptotic cells, loss of mitochondrial potential is preceded by cytochrome c release from mitochondria

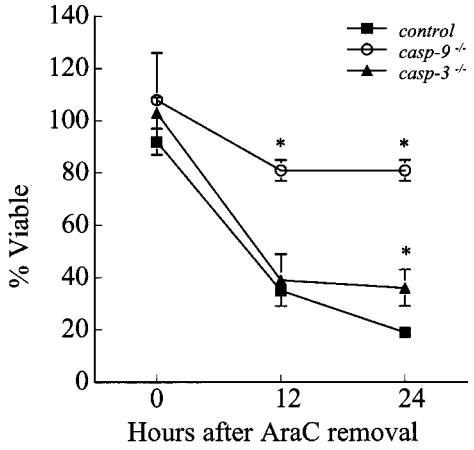

Fig. 7. Caspase 9 deficiency, but not caspase 3 deficiency, promotes telencephalic cell survival post-AraC exposure. E12-E13 telencephalic cells from control, *Casp3*$^{-/-}$ and *Casp9*$^{-/-}$ embryos were treated with 100 μM AraC for 6 hours. Cells were then washed and placed in a chemically defined serum-free medium consisting of DMEM/F12 supplemented with 5 mg/ml insulin, 100 mg/ml transferrin, 100 μM putrescine, 30 nM selenium and 20 mM progesterone. Viability was determined by SYTOX Green (500 nM) exclusion and all data are presented as a percentage of untreated cells of like genotype at the identical time point. Each data point represents mean±s.e.m. (n=11 for control (*Casp3*$^{+/+ \text{ or } -}$ and *Casp9*$^{+/+ \text{ or } -}$), 4 for *Casp3*$^{-/-}$ and 7 for *Casp9*$^{-/-}$). The asterisks represent a significant increase (P< 0.05) in cell viability versus AraC-treated control cells at the same time point (Wilcoxon Signed Rank Test).

(Kluck et al., 1997; Yang et al., 1997). To determine if cytochrome c is released from the mitochondria during AraC-induced NPC apoptosis, the subcellular localization of cytochrome c was assessed by both cell fractionation studies of telencephalic cells isolated from control and AraC-exposed E12.5 embryos and by dual immunocytochemical detection of cytochrome c and activated caspase 3 immunoreactivity. Western blot analysis revealed that cytochrome c immunoreactivity was present in the mitochondria-enriched heavy membrane fraction of control preparations and there was no detectable decrease in the level of cytochrome c protein in this fraction after treatment with AraC (Fig. 8A). This was in contrast to a clear loss of mitochondrial cytochrome c in sympathetic neurons deprived of NGF (data not shown). Immunocytochemical studies of control and AraC-treated telencephalic cells also failed to demonstrate a loss of cytochrome c immunoreactivity 6 hours following AraC exposure despite the presence of intense activated caspase 3 immunoreactivity (Fig. 8B,C) and apoptotic nuclear features (Fig. 8D,E). These results suggest that cytochrome c release may not be required for caspase 9 activation during AraC-induced apoptosis of NPCs.

DISCUSSION

Many studies of programmed cell death have focused on neurons and their response to limited supplies of target-derived trophic factor(s). This process has been well documented and may serve to ensure appropriate matching of neuron number to target size (Oppenheim, 1991). In contrast, estimates of the extent of NPC programmed cell death vary widely (Thomaidou et al., 1997; Blaschke et al., 1998) and only recently, has the critical role of NPC death in development been recognized. Based on the results of targeted gene disruptions, we have proposed that naturally occurring cell death of NPCs is regulated by a Bax-, Bcl-X_L-independent, Apaf1-, caspase 9- and caspase 3-dependent death pathway and is critical for normal brain morphogenesis (Kuan et al., 2000). The developmental stimuli that trigger NPC apoptosis are largely unknown but may involve limited supplies of locally expressed growth factors, such as fibroblast growth factor 2, activation of cell-surface death receptors, or irreparable DNA damage.

DNA damage-induced NPC apoptosis

To test the hypothesis that irreparable DNA damage is a major stimulus regulating naturally occurring NPC death, we compared the molecular pathway of genotoxic NPC apoptosis with that defined above for normal nervous system development. We found that DNA damage-induced NPC apoptosis was critically dependent on p53 and caspase 9, but not caspase 3, Bax or Bcl-X_L. This apoptotic pathway is different from that controlling nervous system morphogenesis. p53 and caspase 9 are expressed at high levels in the ventricular zone and p53 is known to play an important role in the cellular response to DNA damage (Schmid et al., 1991; Komarova et al., 1997). Our results with drug-induced DNA damage, and the observation that γ-irradiation of embryonic mice activates a p53-dependent apoptotic pathway in the developing brain in vivo (Frenkel et al., 1999), demonstrates the general significance of p53 in the NPC apoptotic response to DNA damage. We have also identified a critical role for caspase 9 in this response and shown that caspase 3 and Bax are not necessary for DNA damage-induced NPC death. These studies also indicate that irreparable DNA damage to NPCs is not the major apoptotic stimulus controlling normal brain development. This conclusion is supported by the observation that mice deficient in Xrcc4 and DNA ligase IV do not exhibit increased NPC apoptosis; rather, they show a relatively selective increase in immature neuron death (Gao et al., 1998; Frank et al., 1998).

In addition to regulating NPC apoptosis in response to DNA damage, p53 may be involved in selective aspects of nervous system development since a subset of p53-deficient embryos show neural tube defects and hindbrain exencephaly (Armstrong et al., 1995; Sah et al., 1995). However, it is unlikely that p53 is a critical component of the developmentally triggered NPC programmed cell death pathway involving Apaf1, caspase 9 and caspase 3. The severe p53-deficient neurodevelopmental phenotype is observed in only a fraction of *p53*$^{-/-}$ mice, approximately 15%, and involves exclusively female embryos. This phenotype is distinct from that observed in Apaf1-, caspase 9- or caspase 3-deficient embryos and has not been associated with an expanded ventricular zone. Finally, TUNEL-positive and activated caspase 3-immunoreactive cells are seen in the p53-deficient ventricular zone and developing dorsal root ganglia at comparable levels with those detected in wild-type embryos which contrasts with a reduction in both these parameters in Apaf1-, caspase 9- and caspase 3-deficient mice.

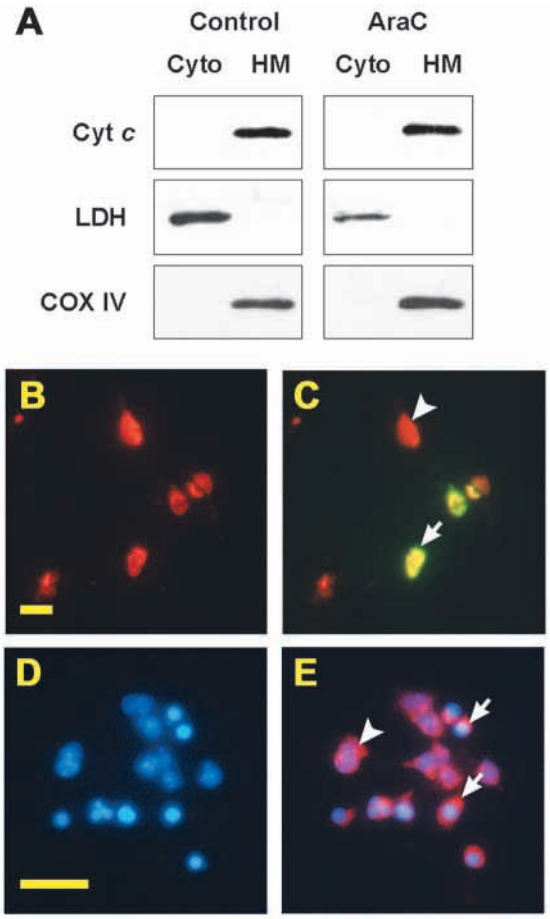

Fig. 8. Mitochondrial cytochrome c redistribution is not observed in AraC-treated NPCs. (A) E12-E13 telencephalic cells were isolated from untreated and transplacentally AraC-exposed embryos. Subcellular fractions containing either cytosol or the mitochondria-enriched heavy membrane (HM) fraction were prepared and cytochrome c, lactate dehydrogenase (LDH) and cytochrome oxidase (COX) reactivity was determined by immunoblotting. LDH was specifically found in the cytosolic fraction whereas COX was detected only in the mitochondrial fraction. The results shown are representative of three independent experiments. (B-E) Immunocytochemical detection of cytochrome c reactivity in freshly isolated E12-E13 telencephalic cells 6-8 hours after AraC exposure showed intense cytochrome c reactivity (B; red) both in cells without (arrowheads) and with (arrows) activated caspase 3 immunoreactivity (C, dual labeled cells appear yellow). Similarly, both cells with (arrows) and without (arrowheads) apoptotic nuclear features (D; bisbenzimide stained) exhibited cytochrome c immunoreactivity (E; red). Scale bars: 25 μm.

Is NPC apoptosis independent of Bcl2 family members?

The systematic evaluation of mice with targeted gene disruptions has defined two distinct apoptotic pathways in NPCs and yet a third in immature neurons (Table 1). Interestingly, a role for Bcl2 family regulation of either NPC death pathway has yet to be demonstrated. Neither ventricular zone expansion nor neurodevelopmental phenotypes resembling Apaf1-, caspase 9- or caspase 3-deficient embryos have been described in mice with targeted gene disruptions of pro-apoptotic Bcl2 family members or in Bcl2 overexpressing transgenic mice (Martinou et al., 1994). Similarly, targeted disruptions of anti-apoptotic Bcl2 family members have not resulted in increased ventricular zone death (Antonsson and Martinou, 2000). Since targeted gene disruptions of all Bcl2 family members have not been reported, and owing to the fact that Bcl2 family members may have redundant functions requiring multiple simultaneous gene disruptions, it is virtually impossible to exclude the Bcl2 family in its entirety from a role in NPC apoptosis. However, several lines of evidence suggest that Bcl2 family-independent apoptosis is possible. Both naturally occurring and DNA damage-induced NPC apoptosis require caspase 9 activation. Bcl2 family-independent, caspase-dependent apoptotic death has been demonstrated in several apoptotic paradigms so there is not an absolute requirement for Bcl2 participation in caspase-dependent apoptosis. Of the anti-apoptotic Bcl2 family members, only Bcl2 appears present at appreciable levels in the ventricular zone (Merry et al., 1994) and its targeted disruption has no apparent effect on NPC apoptosis (Veis et al., 1993). Finally, Bcl2 family members regulate apoptosis in large part by influencing cytochrome c release from mitochondria (Yang et al., 1997); however, we found no difference in mitochondrial cytochrome c levels or immunolocalization between control and AraC-treated NPCs or any effect of Bax deficiency on AraC-induced caspase 3 activation or death. Thus, a Bcl2 family-independent apoptotic pathway may regulate NPC survival in the developing nervous system.

Table 1. Apoptotic pathways in the developing mammalian nervous system

Cell type	Stimulus	p53	Bcl-X_L:Bax	Caspase 9	Caspase 3
NPC	Morphogenesis	–	–	+	+
NPC	DNA damage	+	–	+	+/–
Immature neurons	Histogenesis	–	+	+	+

+, critically involved; –, not involved; +/–, activated but not required for death per se.

Cytochrome c and caspase activation in NPCs

Our inability to detect a loss of mitochondrial cytochrome c reactivity in NPCs six hours post-AraC exposure, despite a drop in MitoTracker Red labeling and the presence of activated caspase 3, was surprising. In previous studies, we have used both cytochrome c immunolocalization and Western blot detection to demonstrate cytochrome c redistribution in several neuronal and non-neuronal apoptotic paradigms (Yin et al., 1999; Putcha et al., 1999). Cytochrome c release from mitochondria can precede changes in mitochondrial membrane potential or alternatively, be a consequence of such changes. Our findings suggest that DNA damage-induced NPC apoptosis is not triggered by cytochrome c redistribution. We focused our studies on the early events in NPC apoptosis when mitochondrial potential is decreased, but not lost. At later time points, we would expect that mitochondrial cytochrome c reactivity would be nonspecifically lost in apoptotic NPCs as a result of opening of the mitochondrial permeability transition pore. We cannot exclude cytochrome c involvement in triggering NPC apoptosis since a small, but undetectable, release of cytochrome c from mitochondria could play an

important amplifying role in apoptosis. However, cytochrome c-independent apoptotic pathways do exist and may involve caspases.

Caspase 9 expression is required for NPC apoptosis and recently, a molecular complex containing Apaf1 and caspase 9 but lacking detectable cytochrome c was isolated from cell extracts (Rodrigues and Lazebnik, 1999). Caspase 9-dependent, cytochrome c-independent apoptosis has been reported (Fujita et al., 1999) and in *C. elegans*, cytochrome c is not required for apoptosome formation or activation (Loeffler and Kroemer, 2000). Caspase 9 is typically considered an initiator caspase whose pro-apoptotic actions are mediated by effector caspases such as caspases 3, 6 and 7 (Zheng and Flavell, 2000). Caspase 3-deficient mice display a neurodevelopmental phenotype identical to that of caspase 9-deficient mice indicating that caspase 3 is downstream of caspase 9 in regulating naturally occurring neuronal programmed cell death. Caspase 3 clearly regulates the apoptotic phenotype (i.e. caspase 3-deficient cells that are committed to die do not exhibit typical apoptotic features; Keramaris et al., 2000) but caspase 3 activation is not a requirement for DNA damage-induced NPC death. In NPCs, caspase 3 activation appears critical for establishing founder cell numbers, yet unlike caspase 9, it is nonessential in DNA damage-induced death. This latter observation suggests that caspase 9 can act directly as an apoptotic effector molecule or that other effector caspases, e.g. caspase 6 or 7, may be critically involved in the NPC response to DNA damage. Additional studies are required to determine this.

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