p53 Transcription-Dependent and -Independent Regulation of Cerebellar Neural Precursor Cell Apoptosis

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Abstract

Regulation of cerebellar neural precursor cell (NPC) death is important for both normal brain development and prevention of brain tumor formation. The tumor suppressor p53 is an important regulator of NPC apoptosis, but the precise mechanism of p53-regulated cerebellar NPC death remains largely unknown. Here, by using primary cerebellar NPCs and a mouse cerebellar NPC line, we compared the molecular regulation of cerebellar NPC death produced by staurosporine (STS), a broad-spectrum kinase inhibitor, with that caused by genotoxic agents. We found that both STS- and genotoxin-induced cerebellar NPC death were markedly inhibited by p53 or Bax deficiency. Genotoxic-induced cerebellar NPC death required new protein synthesis and PUMA, a p53 transcriptionally regulated BH3-only molecule. In contrast, STS caused cerebellar NPC death without requiring new protein synthesis or PUMA expression. In addition, genotoxic agents increased nuclear p53 immunoreactivity, whereas STS produced rapid cytoplasmic p53 accumulation. Interestingly, STS-induced death of cerebellar granule neurons was p53-independent, indicating a differentiation-dependent feature of neuronal apoptotic regulation. These results suggest that STS-induced cerebellar NPC death requires a direct effect of p53 on cytoplasmic apoptotic mediators, whereas genotoxin-induced death requires p53-dependent gene transcription of PUMA. Thus, p53 has multiple death promoting mechanisms in cerebellar NPCs.

Key Words: Bax, Cell death, Genotoxic injury, PUMA, Staurosporine.

INTRODUCTION

Neural precursor cells (NPCs) consist of multipotent neural stem cells and lineage-restricted neural progenitor cells (1). During development, cerebellar NPCs reside in the external granule layer (EGL) of the cerebellum and migrate inward to differentiate into granule neurons (2). Cerebellar NPCs are believed to be the cells of origin for medulloblastoma, the most common malignant brain tumor of childhood (3).

NPC fate is regulated by proliferation, differentiation, and death. Therefore, it is essential to define the molecular regulation of cerebellar NPC apoptosis. On receipt of an intrinsic death stimulus, proapoptotic multidomain Bcl-2 family members Bax and/or Bak are activated, increase the permeability of mitochondria, and promote the release of mitochondrial cytochrome c into the cytosol (4). BH3-domain only (BH3-only) Bcl-2 subfamily molecules such as Noxa and PUMA directly or indirectly activate proapoptotic multidomain Bcl-2 subfamily molecules Bax or Bak (5). Cytochrome c, together with Apaf-1 and procaspase-9, forms the apoptosome, which in an energy-dependent fashion activates caspase-9 and triggers the activation of downstream effector caspases (4). Our previous studies have shown that genotoxic injury induces embryonic telencephalonic NPC death through the intrinsic pathway and requires Bax/Bak, Apaf-1, and caspase-9 (6, 7).

Another important regulator of genotoxin-induced NPC death is the tumor suppressor p53. The p53 gene is the most commonly mutated gene in human cancers and has been described as the “guardian of the genome” (8). As a transcription factor, p53 is implicated in both cell-cycle arrest and apoptosis regulation in different cell types or under different stress stimuli (9, 10). p53 may act to induce expression of genes mediating cell cycle arrest (e.g. p21) or to activate transcription of target genes (e.g. noxa, puma) to promote apoptosis (11). Recently, p53 has also been found to regulate transcription-independent induction of apoptosis by directly interacting with Bcl-2 family members (12). In NPCs, p53 has been shown to mediate ionizing radiation-induced cerebellar NPC death in vivo (13) and cytosine arabinoside (AraC)-induced telencephalic NPC death both in vivo and in vitro (14, 15). However, whether p53 is required for staurosporine (STS)-induced cerebellar NPC death is unknown. STS is a broad-spectrum protein kinase inhibitor and its analog, UCN-01, is under clinical trials for antitumor activity (16). In the present study, we compared the molecular regulation of cerebellar NPC death produced by genotoxic agents versus that caused by STS. We report here that both Bax and p53 are critical regulators of both genotoxin- and STS-induced cerebellar NPC death. However, unlike genotoxin-induced death, STS-induced cerebellar NPC death is independent of p53-dependent gene transcription.
MATERIALS AND METHODS

Chemicals

AraC, bleomycin, STS, and cycloheximide (CHX), were all purchased from Sigma (St. Louis, MO). BOC-aspartyl(Ome)-fluoromethyl ketone (BAF) was purchased from MP Biomedicals (Aurora, OH).

Mice

Generation of bax-/- (17), bak-/- (18), noxa-/-, and puma-/- (19) mice have been described previously. p33+/- mice were purchased from Taconic (Germantown, NY). Endogenous and disrupted genes were detected by polymerase chain reaction analysis of tail DNA extracts as described previously (20). The day the pups were born was counted as day 0. Mice were cared for in accordance with the guidelines of the NIH Guide for the Care and Use of Laboratory Animals. All animal protocols were approved by Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Cell Cultures

Cerebellar NPCs were harvested from the cerebellum of postnatal day 6 to 7 mice. The isolation process was similar to the isolation of telencephalic NPCs, as we previously published (15). Cells were placed in uncoated flasks (Corning, Inc., Corning, NY) at 37°C in humidified 5% CO2/95% air atmosphere and glial cells, postmitotic neurons, and other adherent cell populations were allowed to attach to the bottom of the flask. Twenty-four hours later, floating cells, consisting almost exclusively of NPCs, were transferred to poly-L-lysine/laminin-coated flasks. Cerebellar NPCs formed adherent monolayer cultures and were allowed to grow. Fresh media was added to cultures every 3 days. Cell suspensions were prepared by trypsinization as stated previously and plated onto uncoated 48-well plates at a density of 30,000/well. Cultures were then incubated overnight before being used in experiments. During drug treatment, cell culture medium was switched to high modified DMEM without horse serum or FCS.

Cell Viability and In Vitro Caspase Cleavage Assays

As previously described (21, 22), cells were washed once with Locke’s buffer and then incubated at 37°C for 30 minutes in Locke’s buffer containing 5 μM calcein AM (Molecular Probes, Eugene, OR). Calcein-AM conversion was read using a fluorescence plate reader (excitation 488 nm, emission 530 nm). Cells used for in vitro caspase-3 cleavage assays were lysed in the 48-well plate followed by the addition of buffer containing 10 μM DEVD-7-amino-4-methylcoumarin (AMC) (Biomol, Plymouth Meeting, PA). The plate was incubated for 30 minutes at 37°C in the dark and the generation of the fluorescent AMC cleavage product was measured at 360 nm excitation, 460 nm emissions, using a fluorescence plate reader. Both calcine-AM conversion and DEVD-AMC cleavage was expressed relative to untreated controls.

Western Blot

Cells were washed and collected from plates in phosphate-buffered saline (PBS), resuspended with 2 x sample buffer, and boiled for 5 minutes. Proteins were then resolved in a 12% SDS-PAGE gel and transferred to a nitrocellulose membrane. After transfer, blots were used for detection of p21 (Santa Cruz Biotechnology, Santa Cruz, CA) or PUMA (Prosci Inc., San Diego, CA). Total protein levels were normalized to actin (Sigma).

Immunocytochemistry and Immunohistochemistry

For immunocytochemical detection, cells were fixed in 4% paraformaldehyde for 20 minutes at 4°C, washed with PBS 3 times and then incubated for 30 minutes in PBS-Blocking Buffer (PBS-BB; PBS containing 1% BSA, 0.2% powdered milk, and 0.3% Triton X-100). Primary antibodies were diluted in PBS-BB (without Triton X-100) at the indicated dilutions and applied overnight at 4°C. Primary antibodies used were antinestin (1:20,000; Rat-401 mouse monoclonal antibody; Developmental Studies Hydrorida Bank, University of Iowa, Iowa City, IA), antiglial fibrillary acidic protein (GFAP) (1:10,000; Z0334 rabbit polyclonal antiserum; Dako Corp., Carpenteria, CA), antineuron-specific nuclear protein (NeuN) (1:10,000; Z0334 rabbit polyclonal antiserum; Dako Corp., Carpenteria, CA), and anti-p53 (1:5000; Ncl-p53-Cm5P rabbit polyclonal antiserum; Novocastra Laboratories, New-castle upon Tyne, UK). After washes with PBS, plates were incubated with a horseradish peroxidase-conjugated donkey anti-rabbit or anti-mouse secondary antibodies (Jackson

The C17.2 cell line was cultured in high-modified DMEM (Gibco) containing 1% penicillin/streptomycin, 1% L-glutamine (all from Sigma), 5% horse serum, and 10% fetal calf serum (FCS) (all from Gibco). Fresh media was added to cultures every 2 days. Cell suspensions were prepared by trypsinization and plated onto uncoated 48-well plates at a density of 30,000/well. Cultures were then incubated overnight before being used in experiments. During drug treatment, cell culture medium was switched to high modified DMEM without horse serum or FCS.
Immunoreactivity was performed as previously described (6). Mouse brains were isolated 6 hours after injection of either AraC (25 mg/kg subcutaneously) or vehicle described (6). Mouse brains were isolated 6 hours after injection of either AraC (25 mg/kg subcutaneously) or vehicle described (6). Mouse brains were isolated 6 hours after injection of either AraC (25 mg/kg subcutaneously) or vehicle described (6).

Immunohistochemistry was performed as previously described (6). Mouse brains were isolated 6 hours after injection of either AraC (25 mg/kg subcutaneously) or vehicle described (6). All experiments were repeated at least 3 times except stated otherwise. Representative data are shown. Significant effects of treatment were analyzed by one-way analysis of variance. Genotype-specific effects of treatment were analyzed by two-way analysis of variance. Post hoc analysis was conducted using the Bonferroni test. A level of p < 0.05 was considered significant.

RESULTS

Both Genotoxic Injury and Staurosporine Induce Concentration-Dependent Increases in Cell Death and Caspase-3 Enzymatic Activity in Cerebellar Neural Precursor Cells

To study the molecular regulation of cerebellar NPC apoptosis, we first established FGF-2 expanded primary cerebellar NPC cultures from mice at postnatal day 6 to 7 in which more than 90% of the cells express nestin, a neural precursor marker (data not shown). The cultures were almost completely negative (<1% of cells) for NeuN immunoreactivity, a marker of mature neurons, and showed only a low level of GFAP expression (<5% of cells), a marker of astrocytes (data not shown). These results indicate that FGF-2 expands and maintains cerebellar NPCs in an undifferentiated state in vitro.

FGF-2 expanded cerebellar NPCs were treated with genotoxic agents, including AraC, a nucleoside analog or bleomycin sulfate, an inducer of double-strand DNA breaks or with STS. AraC (Fig. 1A), bleomycin (Fig. 1B), and STS (Fig. 1C) all induced cerebellar NPC death in a concentration-dependent manner. AraC (Fig. 1D), bleomycin (Fig. 1E), and STS (Fig. 1F) also caused concentration-dependent caspase-3 activation measured by in vitro enzymatic cleavage of DEVD-7-amino-4-methylcoumarin, a fluorogenic caspase-3 like substrate. In comparison with genotoxic injury, STS produced more rapid caspase-3 activation and cell death (Fig. 1 and data not shown). These results indicate that both STS and genotoxic injury induce cerebellar NPC apoptosis.

Both Genotoxic- and Staurosporine-Induced Cerebellar Neural Precursor Cell Apoptosis Require Bax

We further determined whether genotoxic injury and STS induced cerebellar NPC apoptosis through the intrinsic death pathway, which requires Bax and/or Bak, a multi-BH domain, proapoptotic Bcl-2 family members. Cerebellar NPCs were prepared from Bax-deficient, Bax heterozygous, and wild-type littermate mice. Compared with cerebellar NPCs prepared from wild-type or heterozygous mice, Bax-deficient cerebellar NPCs exhibited significantly less death induced by bleomycin (Fig. 2A) or STS (Fig. 2B). In contrast, cerebellar NPC death was not attenuated by the targeted deletion of Bak (Fig. 2C, D). Overall, these data indicate that, not Bak, is the major proapoptotic, multidomain Bcl-2 molecule regulating cerebellar NPC death and that both genotoxic injury and STS produce cerebellar NPC death through the Bax-dependent intrinsic pathway.

p53 Mediates Both Genotoxic- and Staurosporine-Induced Cerebellar Neural Precursor Cell Death

To study whether p53 is required for both genotoxic- and STS-induced cerebellar NPC death, we compared p53-deficient, p53 heterozygous, and wild-type cerebellar NPCs after exposure to different concentrations of either bleomycin (48 hours) or STS (6 hours). p53 deficiency decreased both bleomycin- (Fig. 3A) and STS- (Fig. 3B)-induced cerebellar NPC death. These findings indicate that p53 is required for both genotoxic- and STS-induced cerebellar NPC death.

To further determine whether p53-mediated, STS-induced cerebellar NPC death is cell differentiation-dependent, CGNs, the postmitotic neurons derived from cerebellar NPCs, were treated with 0.1 μM STS for 24 hours. p53 deficiency failed to protect CGNs from STS-induced death (Fig. 3C), suggesting that p53-dependent, STS-induced death is a differentiation-dependent feature of cerebellar NPCs.

PUMA is Required for Genotoxic- but not Staurosporine-Induced Cerebellar Neural Precursor Cell Death

After genotoxic injury, p53 transcriptionally upregulates expression of the downstream proapoptotic BH3 only molecules Noxa and PUMA (11). We previously reported that genotoxic-induced telencephalic NPC death is regulated by PUMA both in vivo and in vitro (24). To test the
contribution of PUMA to genotoxin- and STS-induced cerebellar NPC death, we examined cerebellar NPCs in vitro. Similar to the previous experiments, PUMA-deficient or wild-type cerebellar NPCs were exposed to 0.03 U/mL bleomycin for 48 hours or 1 μM STS for 6 hours. PUMA deficiency significantly decreased bleomycin-induced cerebellar NPC death but not STS-induced cerebellar NPC death (Fig. 4A). To determine if a separate p53 regulated BH3-only molecule, Noxa, is required for STS-induced cerebellar NPC death, Noxa-deficient cerebellar NPCs and wild-type cells were exposed to bleomycin or STS. Noxa deficiency failed to inhibit either bleomycin- or STS-induced cerebellar NPC death (data not shown). Overall, our results indicate that genotoxin-induced cerebellar NPC death requires p53 transcriptionally regulated PUMA expression, whereas STS-induced cerebellar NPC death does not require either PUMA or Noxa expression.

To confirm the neuroprotective effects of PUMA deficiency in vivo, postnatal day 7 mice were injected with AraC. In wild-type cerebellum, AraC produced a marked increase in cleaved caspase-3 immunoreactivity in the EGL. AraC-treated PUMA-deficient mice showed only limited activated caspase-3 immunoreactivity in the EGL (Fig. 4B). Compared with wild-type littersmates, PUMA deficiency decreased cleaved caspase-3 immunoreactivity after AraC injection. In addition, markedly increased nuclear p53 immunoreactivity, detected by a p53-specific antibody was also found in the EGL of the PUMA-deficient mice after AraC injection. These results confirm that PUMA is a key regulator of genotoxin-induced cerebellar NPC death in vivo and that after genotoxic injury, deficiency of PUMA leads to p53 nuclear accumulation in NPCs located in the EGL as a result of downstream inhibition of cerebellar NPC apoptosis.

Genotoxic Injury Causes p53 Transcription-Dependent Death, Whereas Staurosporine Produces p53 Transcription-Independent Death

Our data showed that the p53 transcriptionally regulated molecule, PUMA, is required for genotoxin-induced, but not STS-induced, cerebellar NPC death. Thus, we hypothesized that p53 causes transcriptional-dependent death upon genotoxic injury, whereas p53 causes transcription-independent death after STS treatment. CHX, an inhibitor of protein synthesis (25), was used to determine whether new macromolecular synthesis is required for genotoxin-induced or STS-induced activation of caspase-3 in cerebellar NPCs. The effect of CHX was compared with treatment with BAF, a broad-spectrum caspase inhibitor. CHX decreased bleomycin-induced caspase-3 activation (Fig. 5A), but not STS-induced caspase-3 activation (Fig. 5B). As expected, BAF inhibited caspase-3 activity produced by both stimuli. These results indicate that genotoxin-induced caspase-3 activation requires new protein synthesis, whereas STS-induced caspase-3 activation does not.
To further test the hypothesis that genotoxic injury induces p53 transcription-dependent death and STS-induced death depends on p53 transcription-independent function, p53-regulated genes were analyzed as indicators of p53 transcriptional function. p21Waf1/Cip1/Sdi1 is a p53 transcriptionally regulated gene and inhibits different phases of the cell cycle by inhibiting cyclin/cyclin-dependent kinase complex (26). The BH3-only molecule PUMA is also upregulated by p53 and can induce apoptosis upon genotoxic injury (19, 27). Therefore, p21 and PUMA were chosen as markers of p53 transcriptional function. Primary cerebellar NPCs were treated with AraC (3 \(\text{K}\)M; 6 or 12 hours), bleomycin (0.03 U/mL; 6 or 12 hours), or with STS (0.3 \(\text{K}\)M; 3 or 6 hours), and levels of p21 and PUMA proteins were measured by Western blot. Compared with untreated control, levels of p21 and PUMA did not change after exposure to STS, whereas levels of p21 and PUMA increased significantly after genotoxic injury (Fig. 5C and data not shown).

C17.2 Cells Recapitulate Cerebellar Neural Precursor Cell Apoptotic Death Pathways

To further characterize the molecular regulation of p53 in cerebellar NPC death, we used a cerebellar precursor cell line, C17.2, which was generated from the mouse cerebellar EGL by retroviral vector-mediated transduction of avian myc oncogene (28). C17.2 cells transplanted into the developing mouse cerebellum integrate in a nontumorigenic fashion and differentiate into neurons and glia (29). Our immunocytochemical analysis of C17.2 cells in vitro showed that more than 99% of the cells express the NPC marker nestin and were almost completely negative for NeuN and GFAP immunoreactivity (data not shown).

C17.2 cells undergo concentration-dependent caspase-3 activation and cell death after AraC, bleomycin, or STS treatment, similar to that of cerebellar NPCs (data not shown). Also similar to primary cerebellar NPCs, CHX decreased caspase-3 activation induced by bleomycin (Fig. 6A) but not STS (Fig. 6B) in C17.2 cells. Exposure of C17.2 cells to 3 \(\mu\)M AraC or 0.03 U/mL bleomycin for 6 hours increased p21 and PUMA protein expression levels, whereas levels of p21 and PUMA were unaffected by exposure to STS (Fig. 6C). These results suggest that, like primary cerebellar NPCs, genotoxic injury causes p53 transcription-dependent and STS induces p53 transcription-independent caspase-3 activation and death in C17.2 cells.

Genotoxic Injury Causes Nuclear p53 Accumulation, Whereas Staurosporine Produces Cytosolic p53 Accumulation

In response to genotoxic injury and other stresses, posttranslational modification of p53 leads to its nuclear accumulation and p53 binds to DNA to induce downstream gene transcription (30). The subcellular localization of p53 in C17.2 cells after genotoxic injury or STS was determined by immunocytochemical analysis. Untreated C17.2 cells showed relatively weak nuclear p53 immunoreactivity (5.0 \(\pm\) 2.5% of nucleated cells, \(n = 3\) wells) (Fig. 7A). After exposure to AraC (3 \(\mu\)M) or bleomycin (0.03 U/mL) for 6 hours, there was a marked increase in the percentage of cells displaying nuclear p53 immunoreactivity (13.3 \(\pm\) 5.7%, \(n = 3\) wells; 15.5 \(\pm\) 2.9%, \(n = 3\) wells, respectively;
DISCUSSION

In this study, we found that p53 has dual death-promoting actions in cerebellar NPCs that are differentially activated by specific death stimuli. Both genotoxic- and STS-induced cerebellar NPC death require p53. Genotoxic injury, but not STS, upregulates expression of PUMA, which is required for genotoxin-induced, but not STS-induced, cerebellar NPC death. In addition, caspase-3 activation caused by genotoxic injury requires new protein synthesis, whereas caspase-3 activation produced by STS does not. Finally, STS promoted p53 cytosolic accumulation, whereas genotoxic injury increased nuclear p53 immunoreactivity. Taken together, our experiments indicate that genotoxin-induced cerebellar NPC death requires p53-dependent gene transcription, whereas STS-induced cerebellar NPC death requires p53 but is independent of p53-dependent gene transcription.

Proapoptotic p53 activity in the absence of gene transcription or protein translation has been known for over 10 years (31, 32). However, the molecular mechanism for this effect has only recently been investigated and is incompletely understood. Several reports indicate that after genotoxic injury, a small fraction of p53 translocates to
mitochondria in addition to its nuclear accumulation (33, 34). Targeting p53 to mitochondria in p53-deficient cells has been shown to directly trigger apoptosis (34). Other groups have reported that after genotoxic injury, p53 may directly trigger Bax activation (35), and this process involves PUMA (36). In our study, STS triggered cytoplasmic p53 accumulation and p53 transcription-independent cell death in which PUMA expression was not required. Thus, we clearly define a p53 transcription-independent death pathway in cerebellar NPCs that can be activated by STS.

p53-induced apoptotic cell death is cell-type and stimulus specific. In the nervous system, p53 mediates...
neuronal cell death on a variety of injuries, including genotoxic stress, hypoxia, trophic factor insufficiency, and excitotoxicity (37, 38). STS-induced hippocampal neuron death has been reported to be p53-independent (39). We found that p53 deficiency protected cerebellar NPCs but not CGNs from STS-induced death. This differential dependence of NPCs and postmitotic neurons on p53 may be explained by the higher basal expression of p53 in NPCs relative to postmitotic neurons (40), which can result in a rapid cytosolic accumulation of p53 after STS treatment. In contrast, postmitotic neurons may have insufficient levels of p53 to induce p53 transcription-independent cell death after STS treatment. Alternatively, differential expression of Bel-2 family members in NPCs versus postmitotic neurons may affect the ability of cytosolic p53 to trigger apoptosis. For example, levels of the important antiapoptotic protein Bel-X\textsubscript{l} are low in NPCs relative to postmitotic neurons and this may predispose NPCs to the death-promoting effects of cytosolic p53 (41).

Proapoptotic, multidomain Bcl-2 molecules Bax and Bak have been found to critically regulate the intrinsic apoptotic death pathway. In unstressed cells, Bax and Bak exist in inactive conformations (42). After apoptotic stimuli, proapoptotic BH3-only molecules such as Noxa and PUMA directly or indirectly activate Bax or Bak (5). Upon activation, neuronal cell death on a variety of injuries, including genotoxic stress, hypoxia, trophic factor insufficiency, and excitotoxicity (37, 38). STS-induced hippocampal neuron death has been reported to be p53-independent (39). We found that p53 deficiency protected cerebellar NPCs but not CGNs from STS-induced death. This differential dependence of NPCs and postmitotic neurons on p53 may be explained by the higher basal expression of p53 in NPCs relative to postmitotic neurons (40), which can result in a rapid cytosolic accumulation of p53 after STS treatment. In contrast, postmitotic neurons may have insufficient levels of p53 to induce p53 transcription-independent cell death after STS treatment. Alternatively, differential expression of Bel-2 family members in NPCs versus postmitotic neurons may affect the ability of cytosolic p53 to trigger apoptosis. For example, levels of the important antiapoptotic protein Bel-X\textsubscript{l} are low in NPCs relative to postmitotic neurons and this may predispose NPCs to the death-promoting effects of cytosolic p53 (41).

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