p53 Mediates DNA Damaging Drug-induced Apoptosis through a Caspase-9-dependent Pathway in SH-SY5Y Neuroblastoma Cells[1]

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Abstract

The signaling pathway for DNA damaging drug-triggered apoptosis was examined in a chemosensitive human neuroblastoma cell line, SH-SY5Y. Doxorubicin and etoposide induce rapid and extensive apoptosis in SH-SY5Y cells. After the drug treatment, p53 protein levels increase in the nucleus, leading to the induction of its transcription targets $p21^{Waf1/Cip1}$ and MDM2. Inactivation of p53, either by the human papillomavirus type 16 E6 protein or by a dominant-negative mutant p53 (R175H), completely protects SH-SY5Y cells from drug-triggered apoptosis. Cytochrome *c* and caspase-9 function downstream of p53 in mediating the drug-triggered apoptosis in SH-SY5Y cells. In drug-treated cells, cytochrome *c* is released, and caspase-9 becomes activated. Inactivation of p53 blocks cytochrome *c* release and caspase-9 activation. Furthermore, drug-induced cell death can be prevented by expression of a dominant-negative mutant of caspase-9. These findings define a molecular pathway for mediating DNA damaging drug-induced apoptosis in the human neuroblastoma SH-SY5Y cells and suggest that inactivation of essential components of this apoptotic pathway may confer drug resistance on neuroblastoma cells.

Introduction

Triggering apoptosis has been implicated as an important mechanism underlying the cytotoxic effects of DNA damage-based chemotherapeutic agents (1). There are two major apoptotic pathways in mammalian cells (2–4). The death receptor pathway is triggered by members of the tumor necrosis factor receptor superfamily, including CD95 (Fas/Apo1), tumor necrosis factor receptor-1, DR3, and TRAIL receptors. As exemplified by the CD95 signaling pathway, binding of CD95 ligand to CD95 results in oligomerization of CD95 molecules and formation of membrane-bound signaling complexes. These complexes then recruit, via the adapter protein FADD/MORT1, several molecules of pro-caspase-8. The high local concentration of the pro-enzyme allows autoproteolytic activation of caspase-8 through its low intrinsic protease activity (5, 6). The other pathway involves mitochondria. In response to stress signals, mitochondria release cytochrome *c* and other apoptotic factors into the cytoplasm where cytochrome *c* induces formation of Apaf-1 and pro-caspase-9 complexes, which, in turn, leads to activation of caspase-9 (7–9). Once activated, both caspase-8 and caspase-9 can activate a group of effector caspases, such as caspase-3, which are responsible for the proteolytic cleavage of many intracellular proteins, resulting in the morphological and biochemical changes associated with apoptosis. Although the mitochondrial and death receptor pathways can operate independently of each other, there is cross-talk between the two pathways (4, 10). The link is provided by caspase-8-mediated cleavage of Bid, a pro-apoptotic Bcl-2 family member located in the cytoplasm. Upon cleaved by caspase-8, the truncated Bid translocates to mitochondria, where it promotes cytochrome *c* release by activation of Bax and Bak, also members of the proapoptotic Bcl-2 family (11–13).

The tumor suppressor p53 plays a pivotal role in mediating DNA damage-induced apoptosis (14, 15). The essential components of the signaling pathway that mediate the apoptotic response of p53 to DNA damage remain to be defined but probably include caspase-9 and its cofactor Apaf-1. Thymocytes and oncogene-transformed embryonic fibroblasts from caspase-9 null mice are resistant to irradiation and DNA damaging drug-induced apoptosis (16, 17), a p53-dependent process (18–20). More recently, Apaf-1 has been shown to be an essential mediator of p53-dependent apoptosis in human melanoma cells treated with the DNA damaging drug Adriamycin (doxorubicin; Ref. 21). Because cytochrome *c* is essential for Apaf-1-mediated activation of caspase-9 (7–9), these studies suggest that p53 may target the mitochondrial pathway for inducing apoptosis in response to DNA damage signals. The death receptor/caspase-8 pathway may also contribute to DNA damage-triggered apoptosis. p53 induces CD95, KILLER/DR5/TRAIL receptor 2, and PIDD, a death domain containing protein, in response to irradiation and DNA damaging drugs (22–24). In addition, caspase-8 activation has been observed in oncogene-transformed mouse embryonic fibroblasts after ionizing radiation, which appears to signal apoptosis through the Bid-mitochondrion pathway (25).

Neuroblastoma is one of the most common solid tumors in children and comprises about 8–10% of all childhood malignancies (26). Early-stage neuroblastomas (stages 1, 2, and 4s) are generally sensitive to chemotherapeutic drugs and irradiation, and high cure rates are obtained. In contrast,

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most patients with late stage tumors (stages 3 and 4), especially tumors that carry *MYCN* amplification and/or 17q gain, respond to treatment only transiently, with very poor prognosis (26, 27). Delineation of signaling pathways that mediate chemotherapeutic drug-induced apoptosis in chemosensitive neuroblastoma cells is essential to an eventual understanding of the mechanism underlying the poor response of late stage tumors to treatment.

Both CD95 and p53 have been implicated in DNA damaging drug-induced apoptosis in neuroblastoma cells (28–30). Because CD95 can trigger apoptosis either independently or downstream of p53 (22, 31, 32), it has been unclear whether they function in the same pathway in neuroblastoma cells to mediate apoptosis induced by DNA damaging drugs. We thus wanted to examine one pathway in the absence of the other. For this reason, we chose a chemosensitive neuroblastoma cell line, SH-SY5Y, in our studies. As a result of silencing through DNA methylation, SH-SY5Y cells do not express caspase-8 (33), an essential mediator of CD95-triggered apoptosis (34, 35). Here we present evidence that p53 mediates DNA damaging drug-induced apoptosis in SH-SY5Y cells through the mitochondrion/caspase-9 pathway.

Materials and Methods

Cell Culture. The human neuroblastoma SH-SY5Y cells (ATCC CRL-2266) and various cell lines derived from SH-SY5Y were grown in DMEM (Sigma) supplemented with 10% fetal bovine serum (Life Technologies, Inc.). The retroviral packaging cell line 293GPG was cultured as described (36).

Retroviral Production and Infection. The following retroviral constructs have been used to generate SH-SY5Y-derived cell lines for this study. pBabe-puro/16E6 encodes wild-type human papillomavirus type 16 E6 that can bind p53 and target it for degradation through the ubiquitin pathway (37). pBabe-puro/16E6 (73–77) codes for an internal deletion mutant of 16E6 that is unable to target p53 for degradation (38). pBabe-puro/p53 (R175H; p53DN) encodes a tumor-derived p53 mutant with arginine to histidine mutation at the residue 175, which functions as a dominant-negative mutant of p53 (39). pBabe-puro/casp9 (C287A; casp9DN) codes for a dominant-negative mutant of human caspase-9 in which the residue 287 is altered from cysteine to alanine (40). pBabe-enhanced GFP[3] was constructed by replacing the puromycin-resistant gene in pBabe-puro with a DNA fragment coding for enhanced GFP. Retroviruses were produced using the 293GPG packaging cell line as described (36). Briefly, 293GPG cells ($\sim 1 \times 10^7$) were plated in a 10-cm dish, incubated for 24 h, and then transfected with 5 μg of a retroviral plasmid, using the LipofectAmine Plus protocol (Life Technologies, Inc.). Forty-eight h after transfection, the retrovirus-containing medium was filtered through a 0.45 μm filter (Millipore) and supplemented with 4 μg/ml Polybrene (Sigma). For retroviral infections, SH-SY5Y cells ($\sim 2 \times 10^6$) were seeded in a 10-cm dish and incubated overnight. The cultured medium was then replaced by the retrovirus-containing medium. After 48 h, the viral supernatant was removed, and fresh culture medium containing 0.75 μg/ml puromycin was added. The cells were cultured in the selection medium for 3 days, and puromycin-resistant cells were pooled. The percentage of retrovirally infected cells ranged between 60 and 90%, as estimated in parallel infections using retroviruses expressing GFP. Expression of relevant genes was confirmed by functional and/or immunoblot analysis.

Immunoblot Analysis. Exponentially growing cells at 70–80% confluence were either untreated or treated with increasing concentrations of doxorubicin (Ben Venue Laboratories) or etoposide (Sigma). The cells were harvested at various time points after the drug treatment and washed once with ice-cold PBS. Cell pellets were suspended in SDS sample buffer and boiled for 10 min. After centrifugation for 10 min, the samples were subjected to 12% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was then probed with antibodies and visualized by ECL (Amersham Life Science). The following primary antibodies were used: a rabbit polyclonal antibody for p53 at 1:500 dilution (FL-393; Santa Cruz), a mouse monoclonal antibody for $p21^{Waf1/Cip1}$ at 1:250 dilution (clone SX118; PharMingen), a mouse monoclonal antibody against MDM2 at 1:100 dilution (Ab-2; Oncogene Research Products), a mouse monoclonal antibody for the T7-Tag at 1:10,000 dilution (Novagen), a mouse monoclonal antibody against human caspase-9 at 1:500 dilution (clone 96–2-22; Upstate), and a mouse monoclonal antibody for α-tubulin at 1:2,000 dilution (clone B-5-1-2; Sigma). Horseradish peroxidase-conjugated goat anti-rabbit or antimouse (ICN) antibodies were used as secondary antibodies.

Cell Death and DNA Fragmentation Assays. Twenty-four h after treatment with doxorubicin or etoposide, adherent and floating cells were pooled, collected by centrifugation, and washed once with ice-cold PBS. Cell death was determined by trypan blue dye (0.2% in PBS) staining. Six hundred to 800 cells were counted for each sample. Apoptotic DNA fragments, devoid of most high molecular genomic DNA, were isolated and analyzed as described (41).

Immunofluorescence Analysis. Immunofluorescent staining of proteins was performed using standard techniques. Briefly, cells were grown on coverslips and treated with DNA damaging drugs. At various time points after the drug treatment, cells were washed with PBS, fixed for 20 min in 4% paraformaldehyde in PBS, and permeabilized with 0.3% Triton X-100 in PBS for 5 min. The cells were blocked with 5% milk in PBS for 1 h, incubated with a primary antibody in blocking buffer for 1 h at room temperature, washed in PBS, and then incubated with the appropriate secondary antibody. Primary antibodies were used at 1:400 for p53 (FL-393; Santa Cruz) and 1:800 for cytochrome *c* (clone 6H2.B4; PharMingen). FITC-conjugated goat antirabbit IgG (1:600) and goat antimouse IgG (1:800; Molecular Probes) were used as secondary antibodies. DAPI (Sigma) was used for nuclear staining. Cells were incubated in 300 nM DAPI in PBS for 5 min at room temperature. Cells were visualized using a Nikon Eclipse E800 microscope with Image-Pro Plus software for image analysis.

[3] The abbreviations used are: GFP, green fluorescent protein; DAPI, 4′,6-diamidino-2-phenylindole; LOH, loss of heterozygosity.

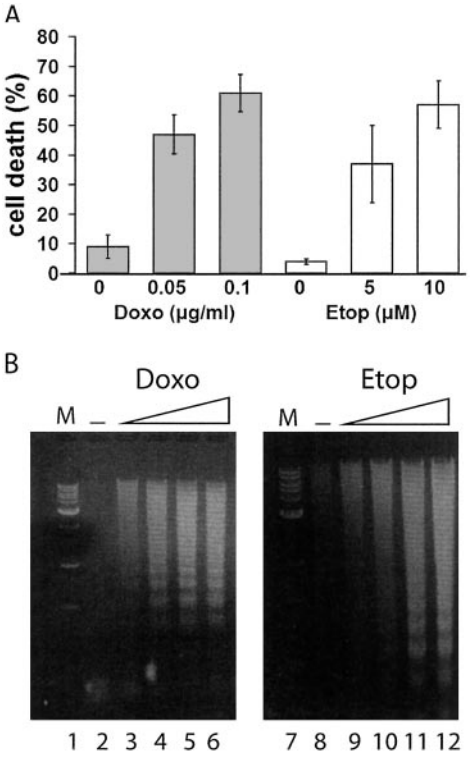

Fig. 1. Inducing apoptosis in SH-SY5Y cells by DNA damaging drugs doxorubicin and etoposide. *A*, cell death was determined by trypan blue dye staining 24 h after treatment with the indicated concentrations of doxorubicin (*Doxo*) and etoposide (*Etop*). Each *column* represents the average obtained from three independent experiments; *bars,* SD. *B*, apoptotic DNA fragments were isolated from cells that were either untreated (−) or treated for 24 h with increasing concentrations of doxorubicin (0.05, 0.1, 0.5, and 1.0 μg/ml) or etoposide (5, 10, 20, and 40 μM) and subjected to 1.2% agarose gel electrophoresis. *M,* DNA molecular weight marker.

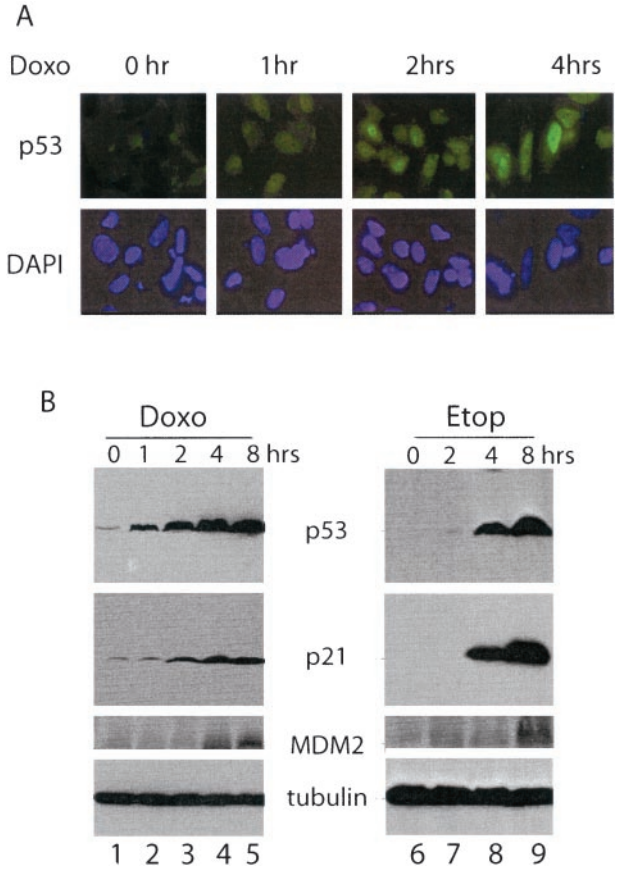

Fig. 2. Functional p53 signaling pathway in SH-SY5Y cells. *A*, immunofluorescent analysis of p53 protein levels in cells at the indicated time points following doxorubicin (0.5 μg/ml) treatment. *0 hr,* untreated cells. Cells were fixed and immunostained with a polyclonal antibody against p53. Nuclei were visualized with DAPI. ×100. *B*, immunoblot analysis of protein levels of p53, $p21^{Waf1/Cip1}$, and MDM2 in cells at the indicated time points after doxorubicin (0.5 μg/ml) or etoposide (20 μM) treatment. α-Tubulin levels are shown as loading control.

Results

DNA Damaging Drugs Induce Rapid Apoptosis in SH-SY5Y Cells through a Caspase-8-independent Pathway. SH-SY5Y neuroblastoma cells were treated with either doxorubicin or etoposide, two chemotherapeutic agents commonly used in the treatment of neuroblastoma patients (26). The cytotoxic effects of both drugs largely result from their ability to induce DNA damage by inhibiting topoisomerase II activity (42–44). As shown in Fig. 1*A,* SH-SY5Y cells were highly sensitive to the two drugs, and more than half of cell populations lost viability within 24 h of treatment with either 0.1 μg/ml of doxorubicin or 10 μM etoposide. The drugs apparently triggered cell death by apoptosis, as indicated by the characteristic fragmentation of genomic DNA (Fig. 1*B*), one of the hallmarks of apoptosis. These results demonstrate that SH-SY5Y cells retain a functional signaling pathway for DNA damaging drug-induced apoptosis. We also examined caspase-8 levels in the SH-SY5Y cell line used in this study, and consistent with a recent report (33), no caspase-8 expression was detected by immunoblot (data not shown). We therefore conclude that the drug-induced apoptosis of SH-SY5Y cells is independent of caspase-8.

p53 Signaling Pathway Is Intact in SH-SY5Y Cells. We next examined functional status of p53 in SH-SY5Y cells. Wild-type p53 protein has a short half-life and is normally maintained at low, often undetectable levels in cells (45). In response to DNA damage or other forms of genotoxic stress, p53 is stabilized, leading to a rapid increase in the p53 protein level and its activity. Activated p53 in turn induces expression of its target genes, such as $p21^{Waf1/Cip1}$ and MDM2 (46, 47). Consistent with its wild-type status, immunofluorescent staining revealed low basal levels of p53 in nuclei of untreated SH-SY5Y cells (Fig. 2*A, 0 h*). After doxorubicin treatment, there was a time-dependent increase in nuclear staining of p53 protein (Fig. 2*A, 1–4 h*). This result was corroborated by immunoblot analysis of cell samples collected before and at various time points after the drug treatment. As shown in Fig. 2*B,* both doxorubicin and etoposide triggered a time-dependent increase of p53 protein, followed by the induction of $p21^{Waf1/Cip1}$ and MDM2. Together, these results indicate that the p53 signaling pathway is functional in SH-SY5Y cells.

p53 Is Required for DNA Damaging Drug-induced Apoptosis in SH-SY5Y Cells. Given that p53 is functional in SH-SY5Y cells and the well-known role that p53 plays in

regulating apoptosis, we examined the contribution of p53 to DNA damaging drug-induced apoptosis in SH-SY5Y cells by abrogating endogenous p53 activity. Retrovirus-mediated gene transfer was used to introduce into SH-SY5Y cells either the human papillomavirus type 16 *E6* gene or the gene coding for a tumor-derived p53 mutant, p53 (R175H). 16E6 promotes p53 degradation through the ubiquitin pathway (37), and p53 (R175H) functions as a dominant-negative mutant (p53DN; Ref. 39). For comparison, we also generated SH-SY5Y cell lines expressing either GFP or an internal deletion mutant 16E6 (73–77) that is unable to bind p53 and target it for degradation (38). These cell lines were first characterized by immunofluorescent staining. In contrast to the parental SH-SY5Y cells (Fig. 3*A, left panel*), no increase in nuclear staining of p53 was observed in SH-SY5Y cells expressing 16E6 after doxorubicin treatment (Fig. 3*A, middle panel*), suggesting that expression of 16E6 resulted in degradation of endogenous p53. Expression of p53DN was confirmed by very strong nuclear staining of p53 in the absence of the drug treatment (Fig. 3*A, right panel*).

We next examined the functional status of endogenous p53 in these cell lines by immunoblot analysis. In agreement with the results of immunofluorescent studies, p53 protein was barely detectable in 16E6-expressing cells after the drug treatment, and no induction of $p21^{Waf1/Cip1}$ was observed (Fig. 3*B, left panel, Lanes 1–5*; *right panel, Lanes 15–18*). The function of endogenous p53 was also significantly compromised in cells expressing p53DN, as judged by the very low level, if any, of $p21^{Waf1/Cip1}$ induction in response to the two drugs (Fig. 3*C, left panel, Lanes 5–8*; *right panel, Lanes 9–12*). In contrast, SH-SY5Y cells expressing either 16E6 (73–77) or GFP showed normal p53 responses and the induction of $p21^{Waf1/Cip1}$ after the drug treatment (Fig. 3, *B, 16E6 (73–77)* and *C, GFP*).

These cell lines allowed us to specifically evaluate the role of p53 in DNA damaging drug-induced apoptosis in SH-SY5Y cells. As expected, SH-SY5Y cells expressing either GFP or 16E6 (73–77) remained highly sensitive to doxorubicin and etoposide (Fig. 4*A, GFP, Lanes 1, 5,* and *9*; *16E6 (73–77), Lanes 3, 7,* and *11*), with levels of cell death comparable with those of parental SH-SY5Y cells under the same condition (Fig. 1*A*). These cells were dying by apoptosis, as indicated by the fragmentation of genomic DNA [Fig. 4, *B, 16E6 (73–77),* and *C, GFP*). In contrast, SH-SY5Y cells expressing either 16E6 or p53DN displayed remarkable resistance to both drugs (Fig. 4*A, 16E6, Lanes 4, 8,* and *12*; *p53DN, Lanes 2, 6,* and *10*) and failed to undergo apoptosis after the drug treatment (Fig. 4, *B, 16E6,* and *C, p53DN*). On the basis of these findings, we concluded that the function of p53 is required for DNA damaging drug-induced apoptosis in SH-SY5Y cells.

p53 Targets the Mitochondrion/Caspase-9 Pathway for Mediating DNA Damaging Drug-induced Apoptosis in SH-SY5Y Cells. Caspase-9 has been shown to play a major role in p53-dependent apoptosis (16, 17). Activation of caspase-9 requires cytochrome *c* (9), which is released from mitochondria to the cytoplasm during apoptosis (7). We therefore asked whether the mitochondrion/caspase-9 pathway was targeted by p53 in DNA damaging drug-induced apoptosis in SH-SY5Y cells. We first examined the location of cytochrome *c* in SH-SY5Y cells before and after the drug treatment by immunofluorescent staining. In untreated cells, immunofluorescent staining revealed a punctate distribution of cytochrome *c* in the cytoplasm (Fig. 5*A*), a typical cytochrome *c* staining pattern found in normal cells (11, 48). After

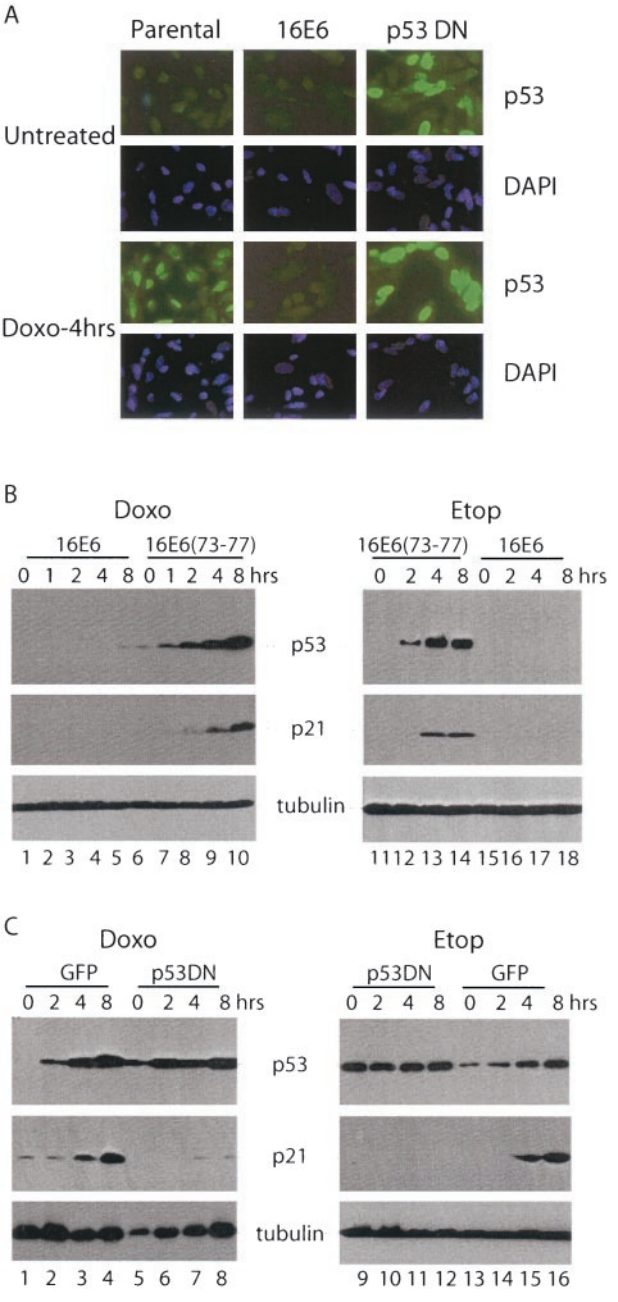

Fig. 3. Inactivation of endogenous p53 by 16E6 and a dominant-negative mutant p53 (*p53DN*). *A,* immunostaining of p53 protein in parental, 16E6-, and p53DN-expressing cells that were either untreated or treated for 4 h with doxorubicin (*Doxo,* 0.5 μg/ml). Nuclei were stained with DAPI. ×60. *B* and *C*, immunoblot analysis of protein levels of p53 and $p21^{Waf1/Cip1}$ in cells expressing 16E6, 16E6 (73–77), GFP, or p53DN at the indicated time points after doxorubicin (*Doxo,* 0.5 μg/ml) or etoposide (*Etop,* 20 μM) treatment. α-Tubulin levels are shown as loading control.

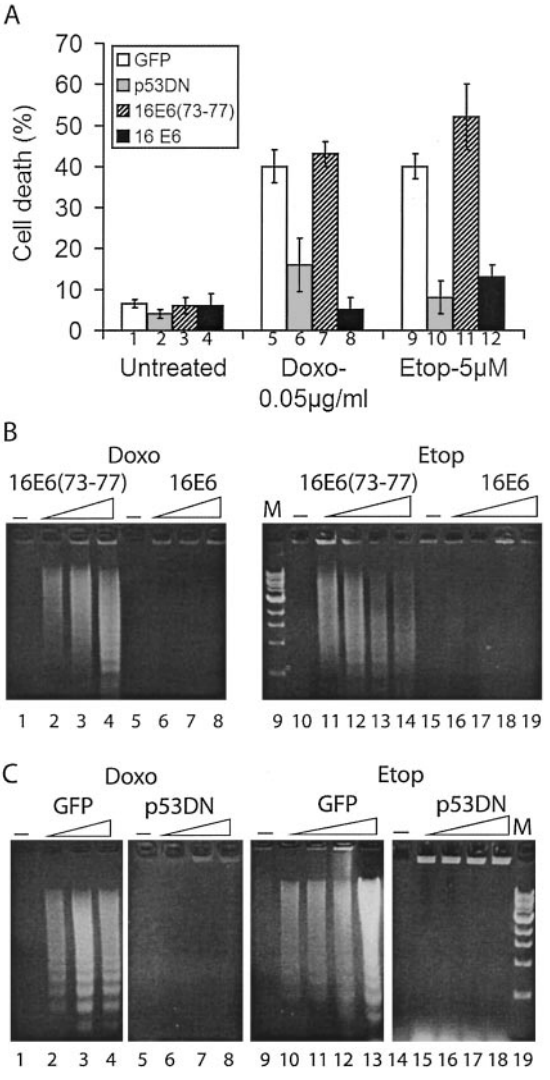

Fig. 4. Requirement for p53 in DNA damaging drug-induced apoptosis in SH-SY5Y cells. *A*, cell death was determined by trypan blue dye staining 24 h after treatment with the indicated concentrations of doxorubicin (*Doxo*) and etoposide (*Etop*). Each *column* represents the average obtained from three independent experiments; *bars,* SD. *B* and *C*, apoptotic DNA fragments were isolated from indicated cells that were either untreated (−) or treated for 24 h with increasing concentrations of doxorubicin (0.05, 0.1, and 0.5 μg/ml) or etoposide (5, 10, 20, and 40 μM) and subjected to 1.2% agarose gel electrophoresis. *M,* DNA molecular weight marker.

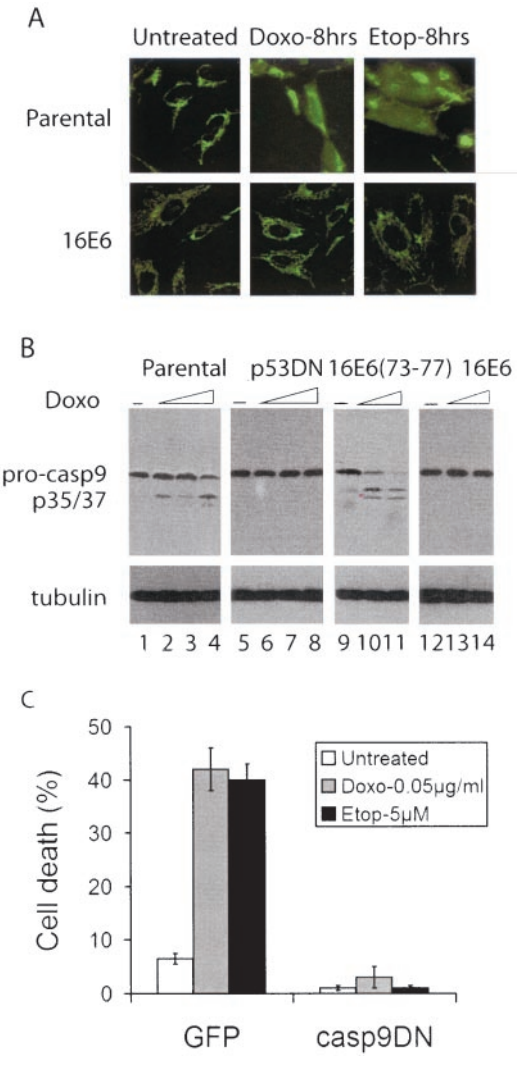

Fig. 5. Requirement for caspase-9 in DNA damaging drug-induced apoptosis in SH-SY5Y cells. *A*, immunofluorescent analysis of cytochrome *c* release in the parental and 16E6-expressing SH-SY5Y cells that were either untreated or treated with doxorubicin (*Doxo,* 0.05 μg/ml) or etoposide (*Etop,* 5 μM) for 8 h. ×60. *B*, immunoblot analysis of caspase-9 activation in the indicated cells that were either untreated (−) or treated for 24 h with increasing concentrations of doxorubicin (0.05, 0.1, and 0.5 μg/ml). Caspase-9 activation was indicated by the appearance of processed products, p35/37. α-Tubulin levels are shown as loading control. *C*, cell death was determined by trypan blue dye staining 24 h after treatment with the indicated concentrations of doxorubicin and etoposide. Each *column* represents the average obtained from three independent experiments; *bars,* SD.

the drug treatment, the punctate staining of cytochrome *c* became diffused (Fig. 5*A*), indicating the release of cytochrome *c* from mitochondria. In SH-SY5Y cells expressing 16E6, however, no cytochrome *c* release was observed after the drug treatment (Fig. 5*A*), indicating that cytochrome *c* release was a downstream event of drug-triggered p53 activation.

Cytochrome *c* release in general results in caspase-9 activation (3, 4). Indeed, caspase-9 became activated in SH-SY5Y cells treated with doxorubicin, as indicated by the appearance of its processed forms, p35/37 (Fig. 5*B, Lanes 1–4*). Caspase-9 activation was also observed in cells expressing either 16E6 (73–77) (Fig. 5*B, Lanes 9–11*) or GFP (data not shown) after the drug treatment. In contrast, no caspase-9 activation was observed in drug-treated SH-SY5Y cells expressing either 16E6 (Fig. 5*B, Lanes 12–14*) or p53DN (Fig. 5*B, Lanes 5–8*), demonstrating that wild-type p53 activity is required for DNA damaging drug-triggered caspase-9 activation in SH-SY5Y cells.

To investigate further the role of caspase-9 in DNA damaging drug-induced apoptosis in SH-SY5Y cells, we used a well-characterized caspase-9 dominant-negative mutant

caspase-9 (C287A; casp9 DN; Ref. 40). SH-SY5Y cells were infected with recombinant retroviruses coding for T7-tagged casp9 DN, and puromycin-resistant cells were pooled. Expression of casp9 DN was confirmed by immunoblot analysis (data not shown). As shown in Fig. 5*C,* whereas cells expressing GFP remained highly sensitive to doxorubicin and etoposide, casp9 DN expression completely protected SH-SY5Y cells from the drug-induced cell death. Thus, activation of caspase-9 is essential for DNA damaging drug-induced apoptosis in SH-SY5Y cells.

Discussion

Our findings demonstrate an essential role for p53 and caspase-9 in DNA damaging drug-induced apoptosis in the chemosensitive SH-SY5Y neuroblastoma cells. In this cell line, p53-mediated apoptosis occurs in the absence of a functional CD95/caspase-8 pathway. After the drug treatment, p53 protein levels increase in the nucleus, leading to the induction of its transcription targets $p21^{Waf1/Cip1}$ and MDM2. Inactivation of p53, either by the papillomavirus E6 protein or by a dominant-negative mutant p53 (R175H), completely protects SH-SY5Y cells from drug-triggered apoptosis. In drug-treated cells, cytochrome *c* is released, and caspase-9 becomes activated. Inactivation of p53 blocks cytochrome *c* release and caspase-9 activation. Furthermore, the drug-induced cell death can be prevented by expression of a dominant-negative mutant of caspase-9. These findings define a sequence of biochemical events that take place during the drug-triggered apoptosis in SH-SY5Y cells. In response to DNA damage signals, p53 protein levels and its biochemical activity are rapidly increased. This is followed by the release of cytochrome *c* from mitochondria to the cytoplasm, which in turn leads to caspase-9 activation and apoptosis.

Unlike the majority of other human cancers in which p53 is commonly inactivated by mutations or other mechanisms (14, 49), neuroblastoma cells usually carry wild-type p53 (50–55). Although cytoplasmic sequestration of p53 has been proposed as a mechanism for inactivation of the function of p53 in neuroblastoma cells (56), a number of studies reveal that the p53 signaling pathway is functional in various neuroblastoma cell lines examined (57–59). Our study further demonstrates an essential role for p53 in determining the sensitivity of SH-SY5Y neuroblastoma cells to chemotherapeutic drugs. Although we only examined the SH-SY5Y cell line, which lacks *MYCN* amplification, similar results have been obtained from studies with *MYCN* amplified cell lines. By examining two *MYCN* amplified neuroblastoma cell lines that were derived from the same patient before and after cytotoxic therapy, Tweddle *et al.* (30) report that the cell line derived before cytotoxic therapy carries wild-type p53, but the cell line derived 5 months later at relapse has lost p53 function as a result of deletion in one copy of the gene and a missense mutation in the other. In comparison with the wild-type p53 cell line, the one with p53 mutations is far more resistant to irradiation and chemotherapeutic agents (30). In another study, inactivation of p53 by the papillomavirus 16E6 protein has been shown to confer multidrug resistance in two sensitive neuroblastoma cell lines (one with and the other without *MYCN* amplification; Ref. 29). However, it appears unlikely that the p53 signaling pathway is the only one to mediate DNA damage-induced apoptosis in neuroblastoma cells, as suggested by a recent study showing no apparent correlation between resistance to irradiation and p53 status (60). Other pathways, such as CD95 (28), may also play a role in the apoptotic process in certain cellular contexts.

The mechanism by which p53 triggers cytochrome *c* release from mitochondria in response to DNA damaging drugs in SH-SY5Y cells remains to be defined. The absence of caspase-8 expression in these cells suggests that Bid is probably not involved. The protein products of several p53-inducible genes are capable of directly mediating mitochondrial cytochrome *c* release, including Bax, Noxa, and PUMA (61–65). Although Bax has been shown to play a role in DNA damaging drug-induced apoptosis (66), it may not function downstream of p53 in our system. No significant up-regulation of Bax was observed in SH-SY5Y cells after the drug treatment (data not shown). The possible involvement of other known targets of p53 is currently under investigation.

Caspase-9 is a major mediator of p53-dependent apoptosis, based on loss of thymocyte radiosensitivity and oncogene-dependent apoptosis in embryonic fibroblasts from caspase-9 null mice (16, 17, 67). Our study demonstrates that caspase-9 is also essential for the apoptotic function of p53 in SH-SY5Y neuroblastoma cells. It is intriguing that two frequent genetic abnormalities, 1p LOH and 17q gain (26, 27), which are associated with the development and aggressiveness of neuroblastoma could potentially down-regulate caspase-9 activity in these tumor cells. The human *caspase-9* gene is located at chromosome 1p34–36.1 (68), a region subject to LOH. A recent analysis shows that although one allele of the *capase-9* gene is deleted or translocated to another chromosome in all of the 17 neuroblastoma cell lines examined, the remaining allele(s) express functional caspase-9 (60). However, it remains possible that deletion of one allele may result in lower levels of caspase-9 expression in neuroblastoma cell lines and tumors with 1p LOH. Gain of the chromosome 17q distal region is strongly linked to advanced-stage neuroblastomas (69). One candidate gene for the 17q effect is *Survivin,* which is mapped to chromosome 17q25, well within the gained 17q region (70). Survivin is a cell cycle-regulated apoptosis inhibitor preferentially expressed in tumors (71). Although the details of the mechanism are unclear, Survivin appears to block apoptosis by inhibiting caspase-9 (72). Several recent studies provide convincing evidence that high levels of Survivin expression in neuroblastoma tumors strongly correlate with poor prognosis (73–75), implicating an important role for caspase-9 activity in conferring a favorable therapeutic index to neuroblastoma tumors during treatment. Thus, in defining a molecular pathway for mediating DNA damaging drug-induced apoptosis in a chemosensitive neuroblastoma cell line, our findings may aid in a molecular understanding of possible defects in signal transduction and regulation of this pathway in chemoresistant neuroblastoma cells.

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