0026-895X/00/5901-135–143$3.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 59:135–143, 2000

Vol. 59, No. 1
372/875637
Printed in U.S.A.

MDM2 Mediated Nuclear Exclusion of p53 Attenuates Etoposide-Induced Apoptosis in Neuroblastoma Cells

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Received June 21, 2000; accepted October 2, 2000

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

The p53 gene in neuroblastoma tumors (NB) is rarely mutated but the protein accumulates in the cytoplasm. Because p53 can mediate the cytotoxic effects of chemotherapeutic agents, it is important to determine whether accumulation of p53 in the cytoplasm impairs p53 function. Data presented here indicate that hyperactive nuclear export of p53 suppresses etoposide-induced apoptosis but does not prevent growth arrest. We compared p53 function in a pair of NB subclones. Our data show etoposide induces complete *trans*-location of p53 to the nucleus and activation of apoptosis in the neuroblastic NB cell line SH-SY5Y (N-type), which expresses low levels of MDM2. However, in Schwann cell-like SH-EP1 cells (S-type), which have up to 10-fold higher levels of MDM2, p53 accumulates in the cytoplasm and the cells are extremely resistant to etoposide-induced apoptosis. Notably, when MDM2 expression is inhibited in S-type cells, with a phosphorothioated antisense oligonucleotide (AS5), then p53 accumulates in the nucleus and the SH-EP1 cells undergo apoptosis. Surprisingly, induction of p21 and G_1-arrest are not attenuated in S-type cells, despite the predominantly cytoplasmic location of p53. Whereas, G_1-arrest is attenuated in the SH-SY5Y cells, which have high levels of nuclear p53. Taken together, these findings suggest attenuation of G_1-arrest is related to the differentiation status of neuroblastomas and occurs downstream of p53 nuclear accumulation. These results demonstrate for the first time that hyperactive nuclear export of p53 attenuates chemotherapy-induced apoptosis in NB cells, and our findings suggest that inhibitors of MDM2 may enhance the therapeutic efficacy of etoposide by promoting apoptosis rather than *trans*-differentiation.

Neuroblastomas are embryonal tumors of the peripheral nervous system that arise from the neural crest (Jaffe, 1976). Although low stage disease has a favorable outcome, it is rare; 70% of patients have disseminated disease on presentation. There have been some advances in treatment, but only 10 to 15% of stage IV patients undergo long-term survival. Unlike the majority of pediatric malignancies, chemotherapy has not yet made a significant impact on the survival of children with NB (Souhami and Tobias, 1998). It has recently been shown that some clinically used chemotherapeutic agents induce partial differentiation, rather than death, of NB tumor cells (Santos et al., 1999). The cells change morphology but do not undergo senescence; eventually, they re-enter the cell cycle, forming drug resistant tumors (Santos et al., 1999). Why some NB cells undergo differentiation rather than apoptosis after chemotherapy remains unknown, although the p53 target $p21^{waf1/cip1}$ (hereafter written as p21) does provide a survival advantage to differentiating NB tumor cells (Poluha et al., 1996). Therefore, it is possible that p53 could influence the cellular outcome of chemotherapy.

This study was funded by the Friends of Rosie Childrens Cancer Research Fund.

The tumor suppressor p53 is a nuclear transcription factor that is activated by DNA damage. Its known targets include genes involved in growth arrest (p21 and GADD45), differentiation (p21), and apoptosis (Bax, CD95) [for review, see May and May (1999)]. In addition, p53 *trans*-activates its own negative regulator, MDM2 (Barak et al., 1993). The p53 status of neuroblastoma tumors is unusual. NB is one of a small group of early onset tumors that do not have mutations in the p53 gene, yet overexpression of p53 protein is the most universal change observed in the disease (Davidoff et al., 1992; Vogan et al., 1993; Castresana et al., 1994, Hosoi et al., 1994; Moll et al., 1995). The overexpressed p53 accumulates in the cytoplasm of NB cells and it was initially thought that p53 was excluded from the nucleus (Moll et al., 1995). However, more recent evidence has shown p53 is not anchored in the cytoplasm, but continually exported from the nucleus by an MDM2 dependent pathway (Rodriguez-Lopez et al., 1999; Lu et al., 2000). When nuclear export of p53 is inhibited using Leptomycin B or antisense MDM2, then p53 accumulates in the nucleus of NB cells (Rodriguez-Lopez et al., 1999; Smart et al., 1999; Stommel et al., 1999; Lu et al., 2000).

The important question remains: does accumulation of p53

ABBREVIATIONS: NB, neuroblastoma; DMSO, dimethyl sulfoxide; SSB, single strand breaks.

in the cytoplasm impair p53 function, or does it act as a reservoir of p53 protein, primed for action when cells are stressed? Published studies so far have focused on the response to γ-irradiation and results have been conflicting. In a study by Moll et al. (1996), the p53 response was found to be severely attenuated, whereas Goldman et al. (1996) published data suggesting the p53 signal transduction pathway was intact. An elegant study by Isaacs et al. (1998) has helped to resolve this discrepancy; they showed that different NB subtypes, which coexist in NB cell lines, undergo distinct responses to γ-irradiation. In this study, we show the neuronal differentiation status also affects the response to chemotherapy.

Neuroblastoma tumors and cell lines derived from them are not a single cell type but are composed of a range of neural crest cell types from neuroblasts to melanocytes (Machin, 1982). NB subtypes have been subcloned and shown to have distinct phenotypes and patterns of gene expression (Ciccarone et al., 1989; Ross et al., 1983). The predominant cell type (N-type) exhibits neuronal properties and is neuroblastic in appearance, having small round cell bodies and neural processes. The second most common cell type (S-type) has features of Schwann and melanocytic cells and resembles epithelial cells, being large and flattened (Ross et al., 1983). By using cloned populations of N- and S-type cells, Isaacs et al. (1998) have shown that NB subtypes have distinct responses to γ-irradiation. S-type cells up-regulate p53 and the cyclin-dependent kinase inhibitor p21 and accordingly undergo G_1-arrest. N-type cells, in contrast, do not seem to be able to mediate a biological response to irradiation damage (Isaacs et al., 1998). Isaacs et al. (1998) concluded that the differential cellular outcomes occur because p53 has access to the nuclei of S- but not N-type cells. However, the impact of p53 sequestration on radiosensitivity was not reported (Isaacs et al., 1998). Because disseminated NB is treated predominantly by chemotherapy, we sought to determine to what extent nuclear exclusion of p53 affects the efficacy of drug-treatment. Here we present evidence that etoposide can activate a p53 response in both N- and S-type cells. However, overactive nuclear export of p53 limits activation of apoptosis by etoposide in S-type NB cells.

Materials and Methods

Cell Culture. The cell lines used were a kind gift from Dr. Robert Ross (Fordham University, New York, NY) (Ciccarone et al., 1989) and were grown routinely as monolayers in a 1:1 mixture of Eagle's minimum essential medium and Ham's nutrient mixture F-12 supplemented with 15% heat-inactivated fetal calf serum. Cells were used over a maximum of 15 passages to minimize intersubline differentiation.

Cytotoxicity Assays. Etoposide (Sigma, Poole, UK) sensitivity was measured by clonogenic assay as described previously (Chresta et al., 1996). Five hundred cells were plated per 2-cm^2 dish. Cells were treated for 1 h with etoposide or vehicle control (DMSO). Results are the mean ± S.E. of three independent experiments and duplicate plates were used in each individual experiment. Apoptosis was quantified by the filter binding assay as described previously (Bertrand et al., 1991; Chresta et al., 1996). This is an assay of endonuclease activation that we have confirmed to correspond well with frequency of morphologically apoptotic NB cells counted using Hoechst 33258 staining.

Alkaline Elution. DNA single-strand breaks (SSBs) were measured by alkaline elution (pH 12.1) as described by Kohn et al. (1976). The frequency of SSBs induced by etoposide were converted to rad equivalents using a calibration graph derived from the number of SSBs produced by a known X-ray dose.

Cell Cycle. Position of cells in the cell cycle was determined by flow cytometric analysis of DNA content using propidium iodide as described previously (Chresta et al., 1996).

Immunoblotting. Whole-cell lysates were prepared and analyzed by immunoblotting as described previously (Chresta et al., 1996). Protein (20 μg/sample) was used on minigels. Antibodies: p53, D0–1; p21, EA10 (both from Calbiochem, Nottingham, UK); MDM2, SMP14 (Neomarkers, Fremont, CA).

Immunostaining. Cells were seeded onto eight-chamber slides at approximately 20% confluency (Falcon, Cowley, UK) and incubated at 37°C overnight to adhere. They were treated the next day with either etoposide or oligonucleotides as described in the figure legend. After treatment, they were rinsed once with PBS and fixed in methanol/acetone solution (1:1) for 10 min at room temperature. Fixed cells were rinsed twice with PBS then incubated with antibodies diluted in PBS (1:250) with 0.1% fetal calf serum for 1 h at room temperature. Cells were rinsed with PBS and incubated with Cy-3 conjugated secondary antibody (1:200) (Jackson/Immunoresearch, West Grove, PA) for 1 h at room temperature. Cells were then washed with PBS and DNA stained with Hoescht 33258 (1 μg/ml in PBS) for 5 min. p53 antibodies, AbDO-1 and Ab421, were obtained from Calbiochem.

Northern Blotting. Total RNA was isolated using RNAzol B as described by the manufacturers (Biogenesis, Poole, Dorset, UK). Total RNA (20 μg) was loaded per lane onto formaldehyde gels and separated overnight. Samples were prepared, electrophoresed, and blotted using standard procedures. Transcript levels were determined by phosphor-image analysis. Probes used were MDM2, *Hin*dIII fragment of the human cDNA clone FL4 [kindly provided by Dr. B. Vogelstein (Johns Hopkins Oncology Center, Baltimore, MD)]; $p21^{waf\text{-}1/cip\text{-}1}$ insert from pBABE [kindly provided by Dr. S. Picksley (University of Dundee, Scotland)].

Antisense Oligonucleotide Treatment. The 20-mer phosphorothioate oligodeoxynucleotides used in this study have previously been described by Chen et al. (1998). AS5 is an antisense to the coding region of the MDM2 and M4 is four-base pair mismatch used as a control. The sequences are as follows AS5—GATCACTCCCACCTTCAAGG— and M4—GATGACTCACACCATCATGG. Cells were transiently transfected using 7 μg/ml lipofectin (Life Technologies, Gaithersburg, MD) in 1% serum containing medium (Optimem). Cells were treated with the oligonucleotides or vehicle control (water) (200 nM) for 18 h. They were then either treated with etoposide or vehicle control (DMSO) for 4 h and cells prepared for immunoblotting, immunostaining, or apoptosis assays.

Determination of Caspase Activation. The substrate used was Ac-DEVD-AMC (Calbiochem), which was made to 5 mM stock in dimethylformamide. On the day of the assay, substrate stock was diluted in water to 500 μM and kept on ice. Cell lysate (20 μg) was diluted to equal volumes (20 μl) in Nonidet-P40 lysis buffer and 1/10th of the volume of substrate was added. Exactly 10 min later, the reaction was stopped by adding the mix into 2 ml of water. Fluorescence was read on a fluorometer at an excitation wavelength of 380 nm and emission wavelength of 460 nm.

Results

p53 *trans*-activation Activity after Etoposide-Induced DNA Damage in N- and S-Type Neuroblastoma Cells. To study the p53 DNA damage pathway in response to chemotherapy, we employed the topoisomerase II poison etoposide, which is commonly used in the treatment of NB and two subclones of the NB cell line SK-N-SH. Both cell lines possess wild-type p53 but they differ in differentiation status. SH-SY-5Y is N-type and SH-EP1 is a substrate adherent

S-type (Ciccarone et al., 1989). Alkaline elution analysis demonstrated that etoposide was approximately equally active as a DNA-damaging agent in both NB sublines. The DNA SSB frequencies in SH-EP1 and SH-SY5Y cells were 810 ± 98 and 712 ± 11 rad equivalents, respectively, after a 1-h treatment with 8 μM etoposide.

We examined the following hallmarks of the p53 response after treatment with etoposide: increase in p53 protein levels, *trans*-activation of p53-dependent genes, apoptosis, and cell cycle arrest.

Immunoblotting demonstrated that etoposide increased p53 protein levels by approximately 5-fold in both cell types (Fig. 1A). However, the absolute levels of p53 protein were significantly higher in the N-type SH-SY5Y cells because of the 7-fold difference in basal p53 protein levels (Fig. 1A). Paradoxically, higher p53 protein levels in SH-SY5Y cells did not translate into enhanced *trans*-activation activity, as can be clearly seen by comparing the histograms in Fig. 1, B and C, with Fig. 1A. The p21 transcript was up-regulated by only 3-fold in SH-SY5Y cells, compared with 8-fold in SH-EP1 cells and the Mdm2 transcript was increased by approximately 3-fold in both cell types (Fig. 1, B and C). The absolute levels of p21 and Mdm2 transcripts were approximately 3-fold higher in drug treated SH-EP1. In the case of p21, this was because of greater induction (see above), whereas, in the case of Mdm2, it was because the baseline level of Mdm2 transcript was higher. Similarly, immunoblotting showed p21 and MDM2 protein levels to be significantly higher in SH-EP1 than in SH-SY5Y cells (Fig. 1D). Densitometry analysis of three independent experiments indicated baseline levels of Mdm2 were up to 10-fold higher in SH-EP1 cells than SH-SY5Y. Furthermore, etoposide treatment consistently resulted in greater induction of Mdm2 protein in SH-EP1 cells (2.99-fold in SH-EP1 cells compared with 1.5-fold in SH-SY5Y (n = 3). Bax, which is also a p53 transcriptional target in some cell types, did not show the same differential expression and was not up-regulated by etoposide (Fig. 1D).

Cellular Outcomes of P53 Activation in S- and N-Type Cells. Activation of p53 has previously been associated with three cellular outcomes, apoptosis, cell cycle arrest, and differentiation. As shown in Fig. 2, etoposide induced remarkably different outcomes in S- and N-type cells. The N-type SH-SY5Y cells rapidly undergo apoptosis, confirmed by cleavage of poly(ADP)ribose polymerase just 4 h after etoposide treatment (Fig. 2A, inset). In contrast, even 24 h after drug treatment S-type, SH-EP1 cells showed no evidence of apoptosis (Fig. 2A). Furthermore, SH-EP1 cells were found to be >3-fold more drug-resistant in a long-term viability assay (clonogenic assay) (Fig. 2, A and B). The SH-EP1 cells responded to etoposide damage by undergoing a stable arrest in G_1-phase of the cell cycle, consistent with the high level of p21 expression in the S-type cells. (Fig. 2C). There was also morphological and biochemical evidence of differentiation in SH-EP1 cells 2 to 4 weeks after etoposide treatment (Fig. 2D). Morphologically, etoposide-treated SH-EP 1 cells became smaller with a lower cytoplasm-to-nucleus ratio and exhibited extended neuronal processes (Fig. 2D). The cells also expressed de novo the neuronal markers Bcl-2 and neurofilament 68, neither of which are expressed in S-type subclones of the parental line SK-N-SH (Fig. 2D and data not shown) (Ciccarone et al., 1989; Hanada et al., 1993). Although the biochemical evidence supports differentiation to a

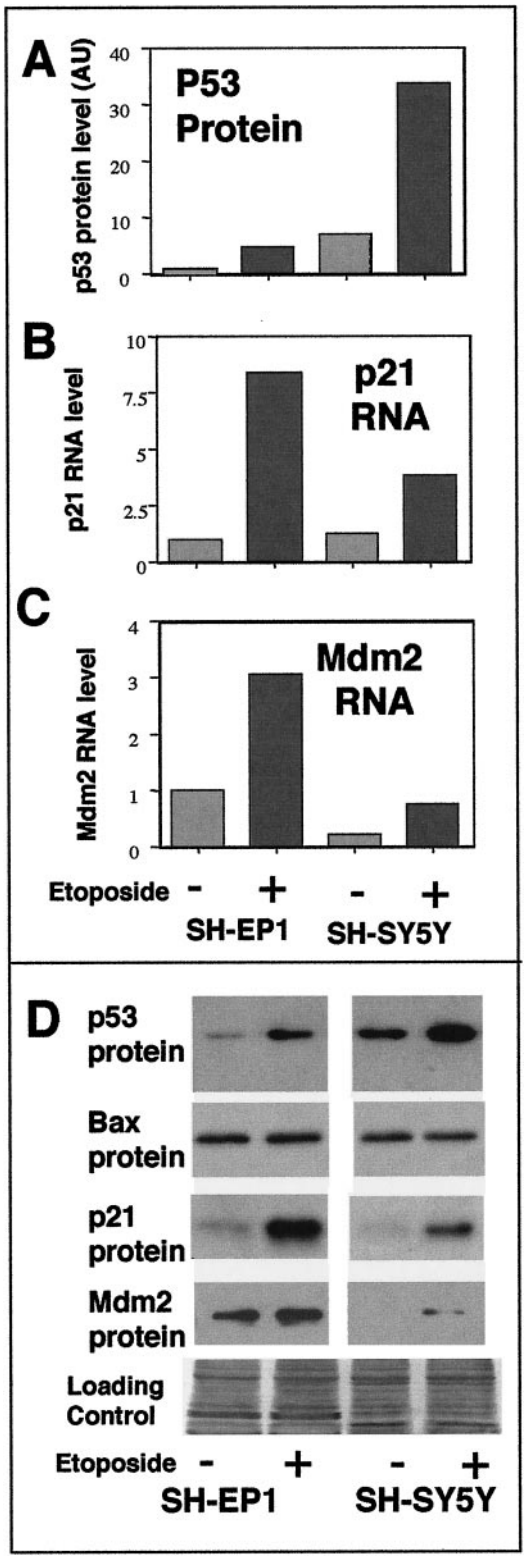

Fig. 1. p53 *trans*-Activation activity after etoposide damage in N- and S-type neuroblastoma cells. Cells were treated for 4 h with either etoposide IC_{90} (+) or vehicle control (−). IC_{90} for SH-EP1 is 38 μM; IC_{90} for SH-SY5Y is 12 μM. A, p53 protein levels analyzed by immunoblotting and quantified by scanning densitometry. B and C, histograms of p21 and Mdm2 transcript levels. RNA levels were quantified by PhosphorImager analysis and normalized to actin to correct for loading. All results were then calculated relative to the SH-EP1 control, which was made to equal 1 arbitrary unit (AU). D, Immunoblots of p53, Bax, p21, and MDM2 protein levels. The India ink-stained membrane shows that protein loading is equal.

more neuronal type, the morphological appearance of the etoposide-treated cells most closely resembles intermediate (I-type) cells (Ciccarone et al., 1989). Potentially, induction of high levels of p21 may allow SH-EP1 cells to survive while undergoing differentiation. p21-Induction has previously been shown to be essential for survival during nerve growth factor-induced differentiation of SH-SY5Y neuroblastoma cells (Poluha et al., 1996).

Subcellular Localization of p53. Attenuated p53 *trans*-activation activity in the N-type SH-SY5Y could result from cytoplasmic sequestration of the p53 protein. We therefore analyzed p53 localization in control and etoposide-treated cells. p53 subcellular localization was probed using two antibodies, the monoclonal Ab DO1, which recognize the N-terminal of p53, and Ab421, which recognizes the C-terminal of p53. In untreated NB cells, there is no evidence of p53 immunostaining in either cell line when C-terminal antibody is used (Fig. 3A and E). This finding is in agreement with the study of the parental line SK-N-SH by Ostermeyer et al. (1996), who showed the C-terminal epitope to be masked by cytoplasmic anchor proteins. However, use of DO-1, the N-terminal antibody, demonstrated intense cytoplasmic staining for p53 in untreated SH-SY5Y cells and relatively weak cytoplasmic staining in SH-EP1 (Fig. 3, C and G). This is in agreement with the 7-fold difference in expression of p53 detected by immunoblotting in untreated cells (Fig. 1, A and D).

Surprisingly, etoposide treatment of the cell lines for 4 h [equitoxic concentrations (IC_{90})] results in intense nuclear p53 staining in SH-SY5Y (Fig. 3, B and D) but not in drug resistant SH-EP1 cells (Fig. 3, F and H). Although the *trans*-activation activity of p53 is 3-fold higher in SH-EP1 cells, than in SH-SY5Y, there is only slight increase in p53 nuclear staining in SH-EP1, with the majority of the etoposide-induced p53 accumulating in the cytoplasm (Fig. 3H). Once p53 is in the nucleus it is detectable by both C- and N-terminal antibodies.

Inhibition of MDM2 Synthesis Allows Accumulation of P53 in the Nucleus of SH-EP1 Cells and Promotes Apoptosis. We and others have recently shown that MDM2

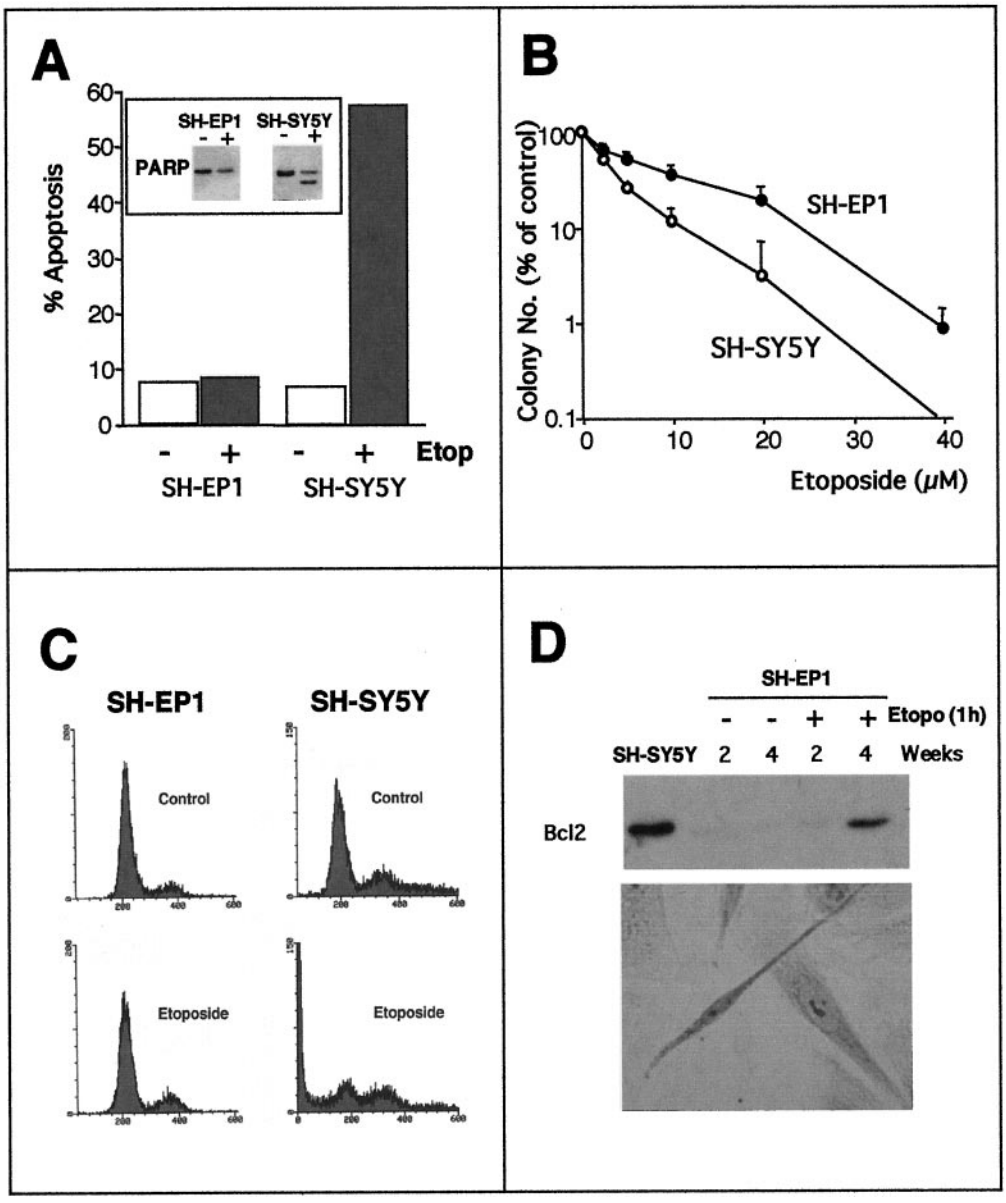

Fig. 2. Differential cellular outcomes of p53 activation in S- and N-type cells. A, apoptosis. Cells were either treated for 4 h with etoposide (IC_{90}) and apoptosis assessed qualitatively by poly(ADP)ribose cleavage (inset) or treated for 2 h with etoposide ($3 \times IC_{90}$) and apoptosis was assessed quantitatively 24 h later using the filter binding assay. IC_{90} for etoposide in SH-EP1 is 38 μM; IC_{90} in SH-SY5Y is 12 μM. B, viability. Loss of viability was measured by clonogenic assays. Cells were treated for 1 h with etoposide and analyzed 14 days later for colony formation. C, G_1 arrest. Cell cycle 48 h after a 1-h treatment with either etoposide (IC_{90}) or vehicle control. D, differentiation. Morphological interconversion of S-type cells to N-type cells by etoposide. Top, immunoblot of Bcl-2 in untreated SH-SY5Y cells and in SH-EP 1 cells 2 or 4 weeks after a 1-h treatment with etoposide IC_{90} (+) or vehicle control (−). Bottom, phase contrast microscopy of SH-EP1 cells treated for 1 h with etoposide (IC_{90}) and analyzed 2 weeks later.

is involved in the export of p53 out of the nucleus of NB cells (Rodriguez-Lopez et al., 1999; Lu et al., 2000). It was therefore hypothesized that higher levels of MDM2 in SH-EP1 were responsible for lack of p53 nuclear localization after DNA damage. To test this hypothesis we inhibited MDM2 synthesis using phosphorothioated oligonucleotides (see below). As can be seen in Fig. 3J, inhibition of MDM2 synthesis using 200 nM HDM AS5 (18 h treatment) resulted in complete relocation of p53 to the nucleus of SH-EP1 cells. This is not an artifactual response to oligonucleotide treatment because the same concentration of M4, a mismatch control oligonucleotide, did not result in p53 nuclear localization (Fig. 3I). Identical results were seen in etoposide-treated cells. Addition of etoposide (IC_{90} for 4 h) to cells that had been pretreated with M4 oligonucleotides resulted in accumulation of p53 in the cytoplasm, whereas addition of etoposide to antisense-treated cells resulted in nuclear accumulation of p53 (data not shown and Fig. 3K).

Immunoblotting demonstrated that treatment of SH-EP1 cells for 18 h with 200 nM HDM AS5 significantly decreased MDM2 protein levels and resulted in an increase in p53 protein levels (Fig. 4A). This demonstrates MDM2 is involved

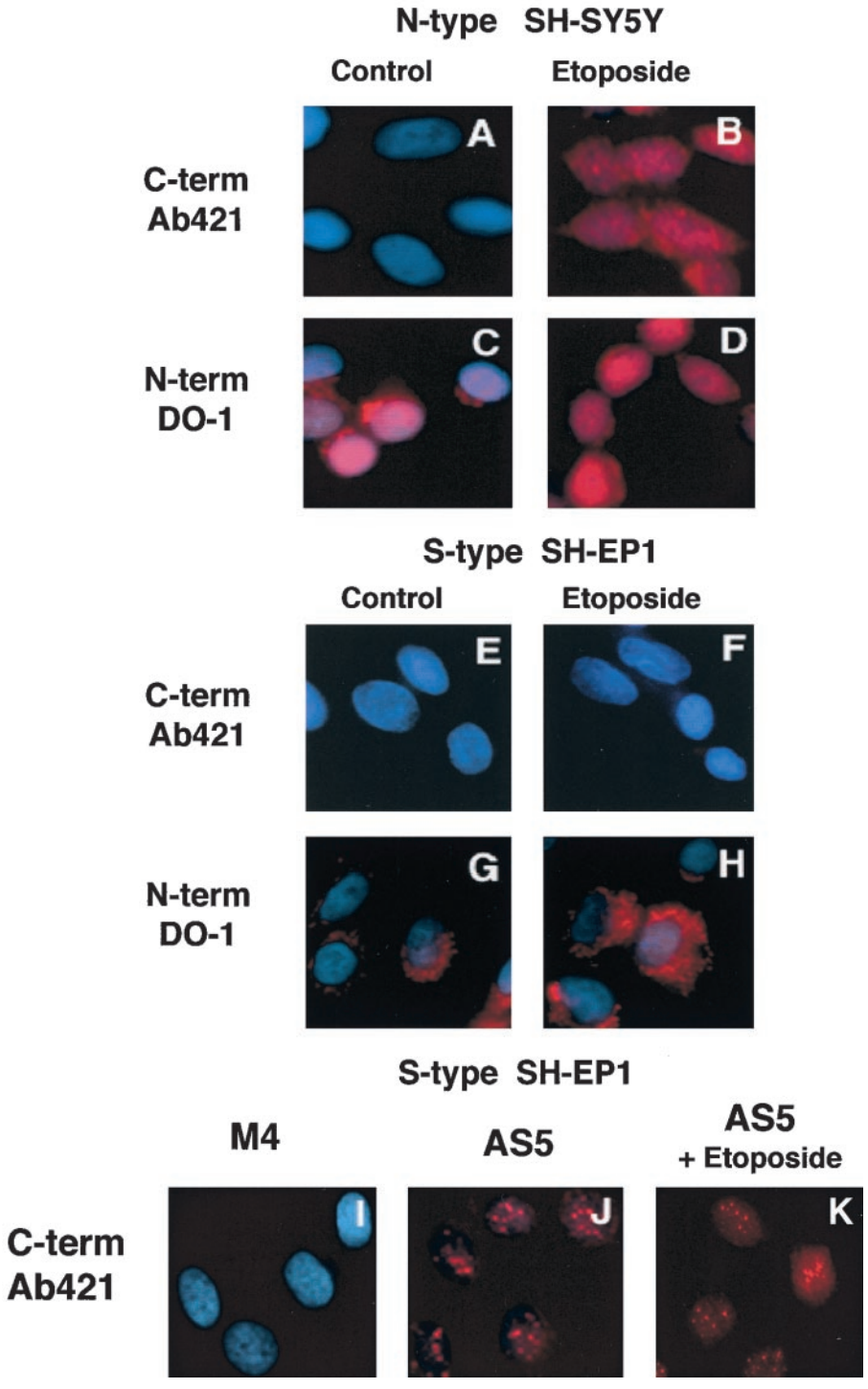

Fig. 3. Differential subcellular localization of p53 in N and S-type NB cells. p53 immunostaining (red) with C-terminal (Ab 421) and N-terminal (Ab DO1) antibodies of SH-SY5Y (N-type) cells (top four panels); SH-EP1 (S-type) cells (bottom seven panels). Untreated cells are shown in panels A, C, E, and G. Cells treated with etoposide (IC_{90} for 4 h) are shown in panels B, D, F, and H. Panels I, J, and K show cells treated for 18 h with either 200 nM mismatch control (I) or 200 nM antisense Mdm2 oligonucleotide alone (J) or antisense (18 h) followed by etoposide (4 h) (K). Nuclei are counterstained with Hoechst 33258 (blue).

in both nucleocytoplasmic shuttling and degradation of p53 in NB cells. p53 *trans*-activation activity is also increased by 4.4-fold in AS5-treated cells (Fig. 4B). Interestingly, although addition of etoposide to AS5-treated cells did not result in significant further stabilization of p53, the *trans*-activation activity increased by about 5-fold (compare AS5 and AS5 + Etoposide in Fig. 4, A and B). This suggests access to the nucleus alone may not be sufficient to activate p53, and a proportion of the nuclear p53 seems to be in a latent form until activated by etoposide.

As noted above, etoposide alone does not normally activate apoptosis in SH-EP1 cells (Fig. 2A). Because overexpression of Mdm-2 protects against p53-mediated apoptosis in response to DNA-damaging agents, we investigated whether inhibition of MDM2 synthesis would enhance etoposide-induced apoptosis in SH-EP1 cells (Kondo et al., 1995; Chen et al., 1998; Arriola et al., 1999). Depletion of MDM2 significantly enhanced etoposide-induced apoptosis in SH-EP1 cells, resulting in approximately 26% apoptosis 4 h after drug treatment, approximately 3- to 4-fold above the value for etoposide alone (Fig. 4, C and D). Treatment for 18 h with the MDM2 AS5 oligonucleotide alone also induced apoptosis to approximately 2-fold higher levels (15% apoptosis) than those seen in mismatch control-treated cells (7% apoptosis) (Fig. 4C).

Discussion

Chemotherapy is the first-line treatment for disseminated NB. In this study we have addressed the question: does accumulation of p53 in the cytoplasm of NB cells impair p53 function after drug treatment? Although the majority of studies agree that the p53 gene of neuroblastomas (NB) is rarely mutated and that p53 protein is predominantly cytoplasmic,

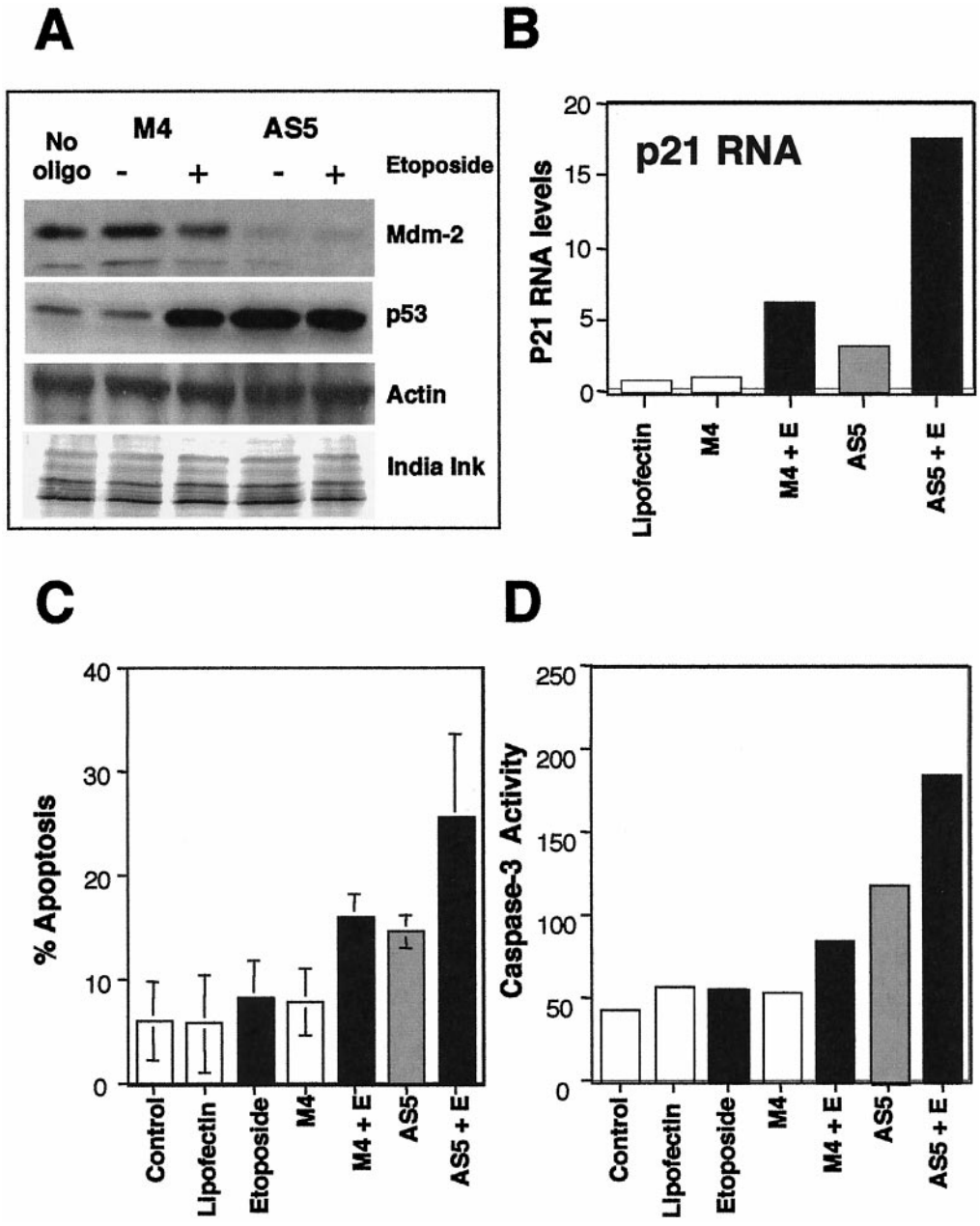

Fig. 4. Inhibition of MDM2 synthesis promotes apoptosis. SH-EP1 cells were treated for 18 h with either: lipofectin alone (no oligo), 200 nM mismatch control (M4), or 200 nM antisense Mdm2 oligonucleotide (AS5). They were then treated with either etoposide 38 μM (E) (+) or vehicle control (−) (DMSO) for 4 h. A, p53 and MDM2 protein analyzed by immunoblotting. B, p21 transcript levels analyzed by Northern blotting and quantified by PhosphorImager analysis. The p21 RNA levels were normalized to actin to correct for loading. Results were then calculated relative to the SH-EP1 control, which was made to equal 1 arbitrary unit (AU). C and D, apoptosis. Cells were treated for 4 h with etoposide (E) ($IC_{90} \times 3$) and apoptosis assessed by measuring DNA fragmentation and caspase-3 activation as described under *Materials and Methods*.

a controversy exists regarding the effect that cytoplasmic sequestration has on p53 function after DNA damage (see above). We studied three characteristic indicators of p53 function, sequence-specific *trans*-activation, G_1-arrest, and apoptosis. We present evidence that etoposide-induced p53 *trans*-activation activity is sufficient to induce a G_1-arrest in S-type NB cells, even when only a small proportion of total p53 protein accumulates in the nucleus. However, activation of apoptosis by chemotherapy seems to require high levels of nuclear p53; therefore, it is attenuated in NB subtypes with overactive nuclear export of p53.

We initially sought to determine to what extent cytoplasmic sequestration impaired p53 sequence-specific *trans*-activation activity and G_1-arrest after drug treatment. Previous studies, employing γ-irradiation, indicated that p53 *trans*-activation activity and G_1-arrest were normal in S-type cells, where DNA damage induced nuclear accumulation of p53, but impaired in N-type cells, because p53 remained in the cytoplasm (Isaacs et al., 1998). Using the topoisomerase II poison etoposide as a source of DNA damage, we similarly found induction of G_1-arrest and the expression of the p53 responsive gene p21 were greater in S- than N-type cells (Fig. 1). However, our results suggest the potency of the p53 response is related to the neuronal phenotype, not to the localization of p53. Etoposide (unlike γ-irradiation) results in accumulation of p53 in the cytoplasm of SH-EP1 cells (S-type) and in the nucleus of SH-SY5Y cells (N-type). However, *trans*-activation of p21 is still greater in SH-EP1 than in SH-SY5Y cells. Collectively these findings suggest two important points. Firstly, the cytoplasmic phenotype does not preclude p53 activity. Rather, it reflects a dynamic process in which p53 enters the nucleus, activates transcription, and then is rapidly exported to the cytoplasm (Fig. 3). Secondly, the attenuation of the p53 response in N-type cells is downstream of p53 nuclear accumulation. It is possible that p53 *trans*-activation activity is low in N-type cells because additional cofactors such as p300/CBP, which enhance DNA binding and *trans*-activation activity of p53, are not expressed (Avantaggiati et al. et al., 1997; Giaccia and Kastan et al., 1998). This suggestion is supported by a recent study by McKenzie et al. who found that direct overexpression of the downstream target p21, but not p53 itself, was able to activate G_1-arrest in SK-N-SH cells (the parental cell line of SH-EP1 and SH-SY5Y) (McKenzie et al., 2000). However, in some NB cells lines, even direct overexpression of p21 is not sufficient to activate Rb and G_1-arrest, suggesting there may be additional defects, downstream of p21 (McKenzie et al., 2000).

To exclude the possibility that an artifact of different experimental protocol resulted in the different findings with etoposide compared with γ-irradiation (Isaacs et al., 1998), we studied the localization of p53 in cells treated with the radiomimetic agent, bleomycin. We found bleomycin produced similar results to γ-irradiation, namely modest accumulation of p53 in the nucleus of S-type cells and up-regulation of the p21 protein (data not shown) (Isaacs et al., 1998). Therefore, it seems that the type of damaging agent used can influence accumulation of p53 to the nucleus.

Why does etoposide induce nuclear accumulation of p53 in N-type but not S-type cells? One potential explanation could be if the DNA damage signal were higher in N-type cells, resulting in greater inhibition of the p53/Mdm2 interaction. However, because we used equitoxic drug concentrations of etoposide (38 μM in SH-EP1 and 12 μM in SH-SY5Y), the DNA damage was actually greater in S-type cells. A more likely explanation for differences in p53 nuclear accumulation in NB subtypes is differential expression of the MDM2 protein. The MDM2 protein, which plays a key role in nuclear export of p53, is expressed at higher baseline levels in S-type cells than N-type cells and is induced to a higher degree by etoposide (2.99-fold induction in SH-EP1 compared with 1.5-fold induction in SH-SY5Y). When MDM2 synthesis is inhibited by antisense, then etoposide is able to induce nuclear accumulation of p53 (Fig. 3K). Differences in induction of MDM2 may also explain why Bleomycin (but not etoposide) is able to induce p53 nuclear accumulation in SH-EP1, because bleomycin results in only modest MDM2 protein up-regulation (1.6-fold) in this cell type (data not shown).

The cellular outcome of chemotherapy treatment was significantly different in S- and N-type cells. S-type cells up-regulate the cyclin-dependent kinase inhibitor p21 to high levels and undergo cell-cycle arrest in G_1-phase followed by differentiation (Fig. 2, C and D). The morphology of SH-EP 1 changes with drug treatment; they become smaller with a lower cytoplasm-to-nucleus ratio and have two or more extended processes. In addition, the cells begin to express markers characteristic of intermediate (I-type) and neuroblastic (N-type) cells, neurofilament 68, and Bcl-2 (Fig. 2D and data not shown) (Ciccarone et al., 1989; Hanada et al., 1993). In contrast, the N-type SHSY5Y cells undergo rapid apoptosis, with biochemical characteristics of apoptosis evident as early as 4 h after drug treatment (Fig. 2). Similar findings of activation of apoptosis in N-type cells and differentiation in S-type cells have been reported after treatment with the enediyne DNA cleaving agent neocarzinostatin (Hartsell et al., 1996). However, after treatment with neocarzinostatin, the S-type cells underwent Schwann cell-like differentiation (Hartsell et al., 1996). Although the morphological appearance of etoposide and neocarzinostatin treated cells are similar, the expression of neurofilament 68 and Bcl-2 suggest that etoposide induces neuronal, rather than Schwann cell, differentiation. Significantly, in NB cells, the initial cellular decision to enter an apoptotic or differentiation pathway does influence overall cell survival. Data from long-term clonogenic assays showed chemosensitivity (loss of cloning potential) correlated well with susceptibility to undergo apoptosis using several chemotherapeutic agents (Fig. 2 and data not shown). Although etoposide and other chemotherapeutic agents seem to induce differentiation of NB, the cells are not terminally differentiated and do not undergo senescence (Santos et al., 1999). We found the transiently arrested cells eventually form colonies in vitro and, more significantly, Santos et al. have shown transiently differentiated NB cells escape cell cycle arrest and repopulate solid tumors in vivo (Santos et al., 1999).

Why does etoposide not induce apoptosis in both cell types? Our results strongly suggest that the induction of apoptosis by a chemotherapeutic drug is related to its ability to induce high levels of nuclear p53. Etoposide alone can induce nuclear accumulation of p53 in SH-SY5Y cells, presumably by inhibiting the p53/Mdm2 interaction and preventing Mdm2-mediated nuclear export. However, in SH-EP1 cells, which have significantly higher levels of MDM2 (Fig. 1), the MDM2 feedback loop is extremely efficient and nuclear accumula-

tion of p53 is inhibited (Fig. 3). To assess the importance of nuclear accumulation of p53 in activation of apoptosis, we inhibited Mdm2 synthesis using antisense Mdm2. Under these conditions p53 accumulated in the nucleus of S-type cells and the cells underwent etoposide-induced apoptosis (Figs. 3 and 4, c and d). In addition, when we extended our studies to include other clinically used agents, we found a strong correlation between nuclear accumulation of p53 and activation of apoptosis using bleomycin, cisplatin, and paclitaxel (data not shown). A critical role for p53 nuclear accumulation in activation of apoptosis, is suggested by a recent study using leptomycin B (a small-molecule inhibitor of nuclear export) (Smart et al., 1999). Smart et al. (1999) have shown leptomycin B activates cell death in NB cells with wild-type p53 but is significantly less toxic in NB cells expressing a dominant negative p53 peptide. It is also possible that S-type cells are more resistant to apoptosis because of induction of high levels of p21 by etoposide. Poluha et al. have previously shown that p21 protects SH-SY5Y cells from apoptosis while they undergo nerve growth factor-induced differentiation (Poluha et al., 1996).

Although nuclear accumulation of p53 is concomitant with apoptosis in etoposide-treated N-type cells, there is no evidence that apoptosis occurs as a result of transcription of specific target genes. In general, the *trans*-activation activity of p53 seems to be lower in the N-type cells than in S-type cells (Fig. 1B & C). Furthermore, the proapoptosis protein Bax is not up-regulated by p53 in SH-SY5Y cells (Fig. 1A). Potentially, the extremely high levels of p53 protein in the nuclei of N-type cells may activate apoptosis in a *trans*-activation-independent manner. The mechanism of activation of apoptosis varies between cell types; in some cell types, apoptosis can occur in the presence of inhibitors of transcription and translation (Caelles et al., 1994). Recently, *trans*-repression has been reported to be involved in activation of apoptosis in cells engineered to overexpress p53 (Haupt et al., 1995; Chen et al., 1996; Ronen et al., 1996; Murphy et al., 1999). Further studies are required to identify how p53-activates apoptosis in NB cells. Nevertheless, the finding of Smart et al. that leptomycin B preferentially activates cell death in NB cells with wild-type p53 firmly supports a role for p53 in activation of apoptosis in neuroblastoma (Smart et al., 1999).

In conclusion, our results demonstrate that the cytoplasmic phenotype does not preclude p53 activity after drug treatment. Rather, it reflects a dynamic process where p53 enters the nucleus, activates transcription, and then is exported to the cytoplasm. In S-type cells, this process seems to be overactive and to limit activation of apoptosis. Our findings suggest that inhibitors of MDM2 synthesis or p53/MDM2 interaction could significantly enhance the efficacy of chemotherapy in neuroblastomas by resulting in drug-induced apoptosis in NB subtypes that usually undergo growth arrest and differentiation.

Acknowledgments

We would like to thank the following persons for gifts of probes and cells used in this study. Neuroblastoma cell lines used were a kind gift from Dr. Robert Ross (Fordham University, New York, NY); the MDM2 human cDNA clone FL4− was kindly provided by Dr. B. Vogelstein (Johns Hopkins Oncology Center, Baltimore, MD); and the $21^{waf\text{-}1/cip\text{-}1}$ cDNA was kindly provided by Dr. S. Picksley (University of Dundee, Dundee, Scotland).

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