Wild-Type p53 Can Induce p21 and Apoptosis in Neuroblastoma Cells But the DNA Damage-induced G₁ Checkpoint Function Is Attenuated

Pamela P. McKenzie, Sylvie M. Guichard, David S. Middlemas, Richard A. Ashmun, Mary K. Danks, and Linda C. Harris

INTRODUCTION

p53 protein has been described as the “guardian of the genome” because it has the ability to induce apoptosis when either DNA is damaged or cell growth is deregulated (1). In this way, p53 acts as a tumor suppressor by activating an apoptotic pathway in cells that may be accumulating mutations or proliferating abnormally. The mechanism by which p53-dependent apoptosis occurs is not completely understood, but it appears to be mediated by the ability of p53 to bind DNA and act both as a positive and negative regulator of transcription (2). After DNA damage, p53 activates transcription of the cdk2 inhibitor p21, which mediates a growth-inhibitory function (3). Cells then arrest in the G₁ phase of the cell cycle until either DNA damage has been repaired or apoptosis is induced (1, 3). As a consequence, cells that express a functional wtp53 and have the ability to induce p53-dependent apoptosis are generally more sensitive to the action of DNA-damaging agents (4–6).

The p53 gene is the most frequently mutated gene in human cancer (7), and p53-deficient mice have a high frequency of spontaneous tumor formation (8), consistent with the function of p53 as a tumor suppressor. The mutation frequency of p53 varies among tumor histotypes, ranging from 0 to 5% in NBs (9) to 75–80% in colon carcinomas (7); however, even if the gene itself is not mutated, the function of wtp53 protein or another protein in a p53-activated cascade may be attenuated. One of the best characterized mechanisms by which wtp53 function can be inhibited is by the binding of p53 protein to MDM2 (10, 11). More recently, cytoplasmic sequestration of wtp53 protein has also been suggested as a mechanism by which its function can be attenuated (12, 13).

NBs are pediatric tumors derived from neural crest cells of the sympathetic nervous system (14). They generally respond well to initial chemotherapeutic regimens and are therefore classed as a chemosensitive tumor type (15). Consequently, patients that present with local or regional disease have an excellent prognosis with a survival rate of 80–100% (14). As described above, NBs usually contain a wtp53 gene (9), an observation consistent with their chemosensitive phenotype (15). However, it is presently controversial as to whether p53 is functional in this tumor type. Moll et al. (13) have reported that p53 protein is sequestered in the cytoplasm of NBs and that the DNA damage-induced G₁ arrest phenotype is attenuated. In contrast, Goldman et al. (16) have reported both nuclear and...
cytoplasmic p53 localization and an intact G1 arrest after DNA damage. Using highly sensitive immunofluorescence immuno-

histochemistry and equitoxic doses of IR, we have demonstrated that p53 is present in the nucleus of NB cells and that it is transcriptionally active. In addition, although the ability of these cells to induce p53-mediated apoptosis is functional, the p53-

mediated G1 arrest pathway is attenuated.

MATERIALS AND METHODS

Cell Lines. The NB cell lines SJNB-1 and SJNB-4 were derived at SJCRH; cell lines NB-1643 and NB-1691 were obtained from the Pediatric Oncology Group cell bank (17). The Rh30 rhabdomyosarcoma cell line, obtained from Dr. Peter Houghton, was also derived from a SJCRH patient tumor. ML-1 myeloid leukemia cells were obtained from Dr. Gerard Zambetti (SJCRH). These cell lines were cultured in RPMI 1640 (Bio-

Whitaker, Walkersville, MD) supplemented with 10% FCS (Hy-

crone, Logan, UT) and incubated at 37°C in a humidified atmosphere of 5% CO2, 95% air. Nonessential amino acids (Life Technologies, Inc., Grand Island, NY) were added to medium used to grow ML-1 cells. The stably transfected NB-1643 cells, designated NB-1643p53TDN-1 (p53TDN-1) were maintained in the presence of 250 μg/ml Geneticin (Life Technologies, Inc.). SK-N-SH cells and IMR32 cells, obtained from the American Type Culture Collection (Rockville, MD), were cultured in DMEM medium (BioWhitaker) also containing 10% FCS (Hy-

crone) and incubated at 10% CO2, 90% air.

Antibodies. The p53 monoclonal antibodies used in the indirect immunofluorescence studies were DO7 and BP53.12. These antibodies were obtained from PharMingen (San Diego, CA) and Oncogene Research Products (Cambridge, MA), respectively. The isotype-matched control antibodies were IgG2a and IgG2b, respectively (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Primary antibodies used in the Western blot analyses were p21 (C-19), p53 DO1-HRP-conju-
gated antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and a monoclonal anti-β-tubulin antibody from Sigma Chemical Co. (St. Louis, MO).

Adenoviral Vector and Transduction. The p53 adenovir-

al vector Ad.p53 was obtained from Genetic Therapy, Inc., a Novartis Company (Gaithersburg, MD). Cells were transduced (multiplicity of infection 10) for 2 h in medium containing 2% FCS (Hyclone), after which time complete medium (10% se-

rum) was added. The virus was removed, and fresh medium was added at 24 h.

Indirect Immunofluorescence Stain. These studies were performed as described previously (18) with minor mod-

ifications. Briefly, cells were grown on glass chamber well

slides (Nunc, Naperville, IL) and fixed with either freshly pre-

pared 1% paraformaldehyde at room temperature or acetone:

methanol (1:1) at 20°C for 2 min. All subsequent procedures

were carried out at room temperature. When cells had been

fixed, nuclear membranes were permeabilized with 0.25% Tri-

ton in PBS for 20 min. Cells were washed three times in PBS

and then incubated with 10% swine serum for 20 min to prevent

nonspecific binding of antibodies. Incubation with the primary

antibody or an isotype-matched control was for 2 h in a humid-

ified chamber. Cells were then washed with PBS for 10 min,

changing the wash solution five times during this period. The cells were then incubated with a FITC-conjugated donkey anti-

mouse IgG from Jackson ImmunoResearch Laboratories, Inc.

for 1 h. After washing in PBS for 10 min, cells were incubated with 30 μM Hoechst 33342 for 3 min to stain the DNA. The

slides were air dried and mounted.

Fluorescence Image Cytometry. To eliminate investi-

gator bias, fields of cells to be analyzed for p53 content were

chosen solely on the basis of Hoechst (DNA) fluorescence.

DNA fluorescence was used to confirm that nuclei were intact

and in a single focal plane. DNA images of cells meeting these

criteria were acquired and stored for analysis as described

previously (18). The microscope was then reconfigured to

visualize p53 FITC fluorescence. Images of FITC fluorescence

were acquired using 5-s exposures and stored for subsequent

analysis. Cells were analyzed on a Zeiss Axiosvert 135TV mi-

roscope connected to a Silicon Graphics Crimson workstation

as described previously (18). Photomicrographs were printed

using linear contrast. The nonspecific electronic background

fluorescence introduced by the camera to the microscopic field

was subtracted prior to printing.

Preparations of Soluble Cellular Proteins. Extracts of

soluble cellular protein were prepared by resuspending cell

pellets in extract buffer [50 mM Tris-HCl (pH 8.0), 0.3 mM NaCl, 1 mM EDTA, 0.5 mM DTT, and 0.1% NP40] containing freshly

added protease inhibitors (10 μg/ml each of antipain, aprotinin, and leupeptin, 1 mM sodium orthovanadate, and 2 mM phenyl-
methylsulfonyl fluoride). Cells were disrupted by three cycles of

freezing on dry ice for 5 min, followed by thawing 37°C for 1

min. These preparations were then centrifuged at 14,000 rpm at

4°C for 10 min, and the pellets were discarded. Protein concen-

trations of all extracts of soluble cellular proteins were deter-

mined by the Bradford method (19) using the Bio-Rad protein
determination kit (Bio-Rad Laboratories, Richmond, CA).

Western Analysis. Cell extracts were electrophoresed in

12.5% SDS-polyacrylamide gels using a Bio-Rad Mini Protein

II system, and proteins were electroblotted to polyvinylidine difluoride membranes (Immobilon-P; Millipore, Bedford, MA) using a Bio-Rad Mini electroblotter. Membranes were blocked in Blotto [5% nonfat dried milk in TBS-T: 20 mM Tris-HCl (pH 7.6), 137 mM NaCl, and 0.1% Tween 20] for 30 min at room

temperature, followed by incubation with a primary antibody
diluted in Blotto (0.5 μg/ml) for 1 h. After three washes in TBS-T, membranes were incubated, when appropriate, with the secondary antibody (HRP-conjugated donkey antirabbit or rabbit IgG from Amersham, Arlington Heights, IL) for another hour. The final three washes with TBS-T consisted of two

washes for 15 min and one wash for 30 min, after which Enhanced ChemiLuminescence (ECL; Amersham) detection re-

agents were added, as described by the manufacturer. Immuno-

reactive proteins were visualized by exposure to X-ray film

(Kodak BioMax MR; Eastman Kodak Co., Rochester, NY) for

varying time intervals dependent on protein abundance.

Cell Cycle Analysis. Subconfluent NB cells and ML-1

cells were irradiated with either an IC80 or 10-Gy dose of IR

24 h after plating. IC80s are defined as the dose of irradiation

that inhibited cell growth by 80% over three cell doublings.

After IR, cells were harvested after one cell doubling and resuspended at 1 × 106 cells/ml in propidium iodide staining

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solution (0.05 mg/ml propidium iodide, 0.1% sodium citrate, 0.1% and Triton X-100). Immediately prior to analysis, cells were treated with RNase at a final concentration of 14 μg/ml (Calbiochem, San Diego, CA) for 30 min at room temperature and filtered through 40 μm nylon mesh. Fluorescence was measured using a Becton Dickinson FACScan flow cytometer (Becton Dickinson Immunocytometry, San Jose, CA) using laser excitation at 488 nm. The percentages of cells in different phases of the cell cycle were determined using the ModFit computer program (Verity Software House, Topsham, ME).

**Cell Death Assays.** Cell death in these studies was measured by trypan blue exclusion assay and TUNEL assay. For the trypan blue exclusion assay, trypan blue solution (Sigma) was added to an aliquot of cells at a final concentration of 0.2%. Cells were then counted using a hemocytometer. For the TUNEL assay, ten million cells/sample were isolated after IR as described above and washed with PBS. The cells were centrifuged, resuspended in 0.5 ml PBS, and then added dropwise into 1% paraformaldehyde while vortexing. After incubating on ice for 15 min, the cell suspension was centrifuged, washed with cold PBS, and centrifuged again. Ice-cold ethanol (1 ml) was added dropwise to cell pellet while mixing gently, and suspended cells were stored at −20°C until staining.

For staining, each sample was divided equally, washed twice with cold PBS, and resuspended in 50 μl of reaction media [5× TdT buffer, and CoCl₂, digoxigenin-11-dUTP (1:10); Boehringer Mannheim Corp., Indianapolis, IN] made with or without the TdT enzyme (Boehringer Mannheim). Cells were thoroughly mixed and placed in a 37°C water bath for 1 h, after which time ice-cold PBS was added, and cells were centrifuged. The cell pellet was washed with cold PBS and resuspended in a titered excess of anti-digoxigenin-FITC monoclonal antibody (Boehringer Mannheim), mixed thoroughly, and incubated at room temperature for 30 min in the dark. Ice-cold PBS containing 2 mM azide and BSA (0.35%) was added to cell suspension, and cells were centrifuged and washed once with ice-cold 0.1% Triton X-100/PBS and then resuspended in 1 ml of PBS/azide/BSA containing 62.5 μg/ml propidium iodide. To each sample, 10 μl of concentrated RNase (5 mg/ml) were added, cells were vortexed, incubated for 30 min at room temperature, and returned to ice. Samples were filtered through a 40 μm nylon mesh and analyzed by flow cytometry for DNA content from propidium iodide fluorescence and DNA fragmentation (from FITC-labeled dUTP incorporation) for matched samples either with or without TdT enzyme. Jurkat cells treated with etoposide were used as a positive control for apoptosis.

**RESULTS**

**Characterization of NB Cell Lines.** The p53 status of all of the cell lines used in this study have been published previously and are summarized in Table 1. SJNB-1, NB-1643, NB-1691, IMR32, and SK-N-SH cells all contain a wtp53 gene sequence (13, 20, 21). The p53 expressed by SJNB-4 has a mutation at codon 176 (TGC to TTC; Ref. 20). NB-1691 cells contain a mutation at codon 178 (TGC to TCG; Ref. 20). NB-1643 cells contain a 5′ nucleotide deletion at position 301 (G to A), which affects the translation of the protein. SJNB-4 and Rh30 cell lines and the amplified MDM2 cell line (Rh30) are heterozygous for a mutation in codon 273 in the p53 gene, consistent with constitutive overexpression of MDM2 protein, as demonstrated by Western blot analysis (20). NB-1643 cells stably transfected with a TDN p53 cDNA (22) contained within the pcDNA3 expression vector to generate a cell line in which endogenous p53 protein function has been inhibited. This TDN-p53 cDNA has mutations at positions 14 (Leu to Gln), 19 (Phe to Ser), and 281 (Asp to Gly), which inhibit the transactivation and DNA binding functions of p53 (2). A NB-1643 clone stably expressing TDN-p53 was isolated and designated NB-1643p53TDN-1 (p53TDN). The myeloid leukemia (ML-1) cell line used in this study as a control for p53-induced G₁ arrest after IR has a wtp53 (23). The rhabdomyosarcoma cell line (Rh30) is heterozygous for a mutation in codon 273 in the p53 coding sequence, resulting in an Arg to Cys amino acid substitution (20). The Rh30 cells are used in this study as a control for G₁ arrest after adenoviral transduction of wtp53.

This panel of lines was characterized with respect to their growth rates and sensitivity to exposure to IR. The growth rate of the cells was measured to determine the time for the cell number to double (Table 1). Dose-response curves for exposure to IR were then performed as described in “Materials and Methods” to calculate equitoxic doses of radiation (IC₈₀) for each cell line. These IC₈₀ doses are summarized in Table 1 and represent the dose of IR required to inhibit cell growth by 80% over three cell doublings.

**Table 1 Characterization of cell lines and IC₈₀ doses of IR**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC₈₀ dose IR (Gy)</th>
<th>Cell doubling time (h)</th>
<th>p53 status</th>
</tr>
</thead>
<tbody>
<tr>
<td>SJNB-4</td>
<td>6.40</td>
<td>60</td>
<td>mp53</td>
</tr>
<tr>
<td>SJNB-1</td>
<td>1.85</td>
<td>55</td>
<td>wt</td>
</tr>
<tr>
<td>SK-N-SH</td>
<td>2.60</td>
<td>34</td>
<td>wt</td>
</tr>
<tr>
<td>IMR32</td>
<td>1.90</td>
<td>29</td>
<td>wt</td>
</tr>
<tr>
<td>NB-1691</td>
<td>7.80</td>
<td>46</td>
<td>wt (amp MDM2)</td>
</tr>
<tr>
<td>NB-1643p53TDN-1</td>
<td>1.95</td>
<td>64</td>
<td>wt + TDN</td>
</tr>
<tr>
<td>ML-1</td>
<td>1.50</td>
<td>24</td>
<td>wt</td>
</tr>
<tr>
<td>Rh30</td>
<td>7.25</td>
<td>37</td>
<td>wt</td>
</tr>
</tbody>
</table>

This panel of lines was characterized with respect to their growth rates and sensitivity to exposure to IR. The growth rate of the cells was measured to determine the time for the cell number to double (Table 1). Dose-response curves for exposure to IR were then performed as described in “Materials and Methods” to calculate equitoxic doses of radiation (IC₈₀) for each cell line. These IC₈₀ doses are summarized in Table 1 and represent the dose of IR required to inhibit cell growth by 80% over three cell doublings.

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**Localization of p53 Protein in NB Cells.** Immunofluorescence assays using DO7 and BP35.12 antibodies were carried out in the NB cell lines described in Table 1. As described previously (25), the DO7 antibody detects only nuclear p53, whereas the BP35.12 antibody detects both cytoplasmic and nuclear p53 protein. Photomicrographs of representative cells
for each cell line are shown after staining with DO7 antibody, and results demonstrate nuclear p53 expression in all cell lines (Fig. 1). Using a second antibody (BP53.12), we demonstrated that p53 is both nuclear and cytoplasmic, irrespective of p53 genotype, in these NB cells (data not shown). Immunofluorescence assays using NB-1643 cells and antibody BP53.12 have been published previously (25).

**Analysis of p53 DNA Damage Response.** The cell lines were each exposed to both an equitoxic IC$_{80}$ and a 10-Gy dose of IR. Cell pellets were harvested at 4 h for whole-cell soluble protein extracts and at one-cell doubling for cell cycle analysis. A one-cell doubling time point was chosen to best visualize radiation-induced G$_1$ arrest while minimizing the number of cells reentering the cell cycle or undergoing cell death. The amount of cellular p53 protein increased after DNA damage in all NB cell lines containing wtp53 (Fig. 2). Results published previously (25) have demonstrated that p53 induction occurs in both the cytoplasm and nucleus of NB-1643 cells. There was a
concomitant increase in expression of the cdk inhibitor, p21 (Fig. 2), and Bax (data not shown), indicative of functional p53 transcriptional activity. In contrast to these results and consistent with inactivation of the p53 pathway, no increase in p53 protein was observed in SJNB-4 (mp53) or p53TDN-1 cell lines after IR with either an IC_{80} or a 10-Gy dose of radiation. In addition, there was no increase in expression of p21 in these two cell lines, characteristic of a nonfunctional p53 phenotype (Fig. 2).

Both p53 and p21 protein levels were high in nontreated NB-1691 cells with no detectable increase in p21 protein levels after IR (Fig. 2).

It has been shown that wtp53 is required for radiation-induced G_{1} arrest (23); therefore, cell cycle analysis was performed, and the results are presented in Fig. 3 as relative change in the proportion of cells in G_{1} and S phases of the cell cycle after exposure to IR. For these studies, a relative change ranging from 90 to 110% in the number of cells accumulating in G_{1} is considered to be insignificant, whereas an increase of 111–140% in cells in G_{1} is classified as a moderate or attenuated response. A relative change >140% was considered to be a significant G_{1} arrest. Results shown in Fig. 3A demonstrate that after both a 10-Gy and IC_{80} dose of IR, there was a high proportion of ML-1 cells that accumulated in the G_{1} phase of the cell cycle (148 and 189% of resting levels, respectively). There was a concomitant reduction of cells in S phase (45 and 20% of resting levels; Fig. 3B), characteristic of functional wtp53 (23).

In contrast, only NB-1643 and IMR32 cells (wtp53) exhibited a moderate G_{1} arrest (121 and 122% of resting levels, respectively) after an IC_{80} dose of IR. The other NB cell lines demonstrated changes in G_{1} of only 53–113% of resting levels after exposure to both IC_{80} and 10-Gy doses of IR. Although not all cell lines exhibited a G_{1} arrest, a reduction in the number of cells...
in S phase was observed in many NB cell lines accompanied only by an increase in an accumulation of cells in G2 (data not shown). This radiation-induced G2 arrest was more significant in cells after exposure to the high dose of IR (10-Gy; 1.4–5.5-fold greater than controls). Although wt-p53 has been shown to be capable of mediating a G2 arrest (26, 27), IR can also induce a G2 arrest independent of p53 status (28). Therefore, this report will focus on p53-mediated changes in G1.

**Exogenous Expression of Wild-Type p53 after Ad.p53 Transduction.** The DNA damage-mediated G1 arrest phenotype of NB cells may be attenuated either because insufficient p53 is being induced (i.e., because of a defect in the signaling pathway leading from DNA damage to p53 induction) or because of a defect in the downstream pathway from p53 leading to cell cycle arrest. To test whether the cells were capable of responding to an induction of p53, we transduced NB cells with an adenovirus containing the p53 cDNA (Ad.p53) to overexpress exogenous wt-p53 independent of the DNA damage signal transduction pathway. Cells were harvested at one cell doubling after Ad.p53 transduction and processed for cell cycle and Western analysis as described above. Western blot analyses indicated increased expression of p53 protein in all NB cell lines transduced with Ad.p53, including those cell lines with mp53 (SJNB-4) and amplified MDM2 (NB-1691; Fig. 4). The transcriptional activity of exogenous p53 expression was demonstrated by the observation that p21 protein expression was induced in all cell lines tested (Fig. 4). ML-1 cells were not included in these studies because they are of hematopoietic origin and therefore cannot be efficiently transduced with adenoviral vectors (29). In addition, the effects of exogenous p53 on the ability of NB-1643 cells to G1 arrest could be analyzed without the use of p53TDN-1 cells.

Despite the increase in wt-p53 expression in NBs after transduction with Ad.p53 and the subsequent increase in p21 protein, SJNB-4, SK-N-SH, IMR-32, and NB-1691 showed no significant G1 arrest, and NB-1643 and SJNB-1 cells exhibited only a moderate G1 arrest (both at 132% of resting levels; Fig. 5A). However, this moderate Ad.p53-mediated G1 arrest in NB-1643 and SJNB-1 cells was significantly less than that observed in the mp53 Rh30 rhabdomyosarcoma control cell line (172% of resting levels; Fig. 5A). All NB cell lines showed a reduction in the number of cells in S phase after exposure to Ad.p53 (Fig. 5B).

**Analysis of Irradiation-mediated Cell Death in NB Cells.** p53 functions in the regulation of apoptosis; therefore, we investigated the extent of cell death in ML-1, NB-1643, and p53TDN-1 cell lines. The trypan blue exclusion test and TUNEL assay were used as a means of measuring cell death in these cell lines after a 10-Gy dose of IR as described in “Materials and Methods.” The proportion of cells taking up the trypan blue dye

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**Table 2** Measurement of cell death in irradiated cells by trypan blue exclusion.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>ML-1</th>
<th>NB-1643</th>
<th>p53TDN-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>IR (Gy)</td>
<td>0</td>
<td>1%</td>
<td>19%</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>38%</td>
<td>73%</td>
</tr>
</tbody>
</table>
Fig. 6 IR-induced apoptosis in NB cells. Populations of ML-1, NB-1643, and p53TDN-1 cells were exposed to a 10-Gy dose of IR and then harvested at one cell doubling. Cells were prepared in TUNEL analyses as described in “Materials and Methods.” Results showing the distribution of TUNEL-FITC stained cells after treatment with terminal deoxynucleotidyl transferase enzyme are presented. Y-axis, propidium iodide staining intensity (DNA Content); X-axis, terminal deoxynucleotidyl transferase-FITC staining intensity. A shift to the right in the population of cells staining positive using the FITC-labeled antidigoxigenin monoclonal antibody is indicative of cells undergoing apoptosis (arrows). The percentage of cells undergoing apoptosis is indicated in each panel.

reflected the number of dead or dying cells, whereas the TUNEL assay detects whether cell death is by apoptosis. On the basis of trypan blue assay findings, there were a higher number of dead cells after the 10-Gy dose of IR than untreated cells for all of these cell lines. However, a larger percentage of NB-1643 cells were dead or dying (73%) compared with the percentage of ML-1 cells (38%) and p53TDN-1 cells (27%) at a one-cell doubling time after exposure to IR (Table 2). These findings correspond to the cell cycle data that suggest that ML-1 cells are undergoing a more pronounced radiation-induced cell cycle arrest (Fig. 3) and less cell death. In addition, TUNEL assay data suggested that a greater portion of the wtp53 NB-1643 cells were undergoing cell death by apoptosis (67%) compared with the control ML-1 cells (7%) and the p53TDN-1 cell line (37%) in which p53 is nonfunctional (Fig. 6). NB-1643 cells were also shown to induce Bax expression after IR (data not shown), indicative of p53 functioning in the apoptotic pathway.

DISCUSSION

p53 mutations are rare in NBs (9), and therefore, it has been suggested that in this tumor type, wtp53 function is attenuated by cytoplasmic sequestration of the protein (13). Currently, the subcellular localization of p53 protein within NB cells is controversial. Moll et al. (13) have suggested that p53 is localized only in the cytoplasm; however, we have demonstrated previously that the 1801 antibody used in those studies is not specific for p53 and produces the same staining pattern in Saos2 cells that have their p53 gene deleted (25). A report by Goldman et al. (16) demonstrated that both nuclear and cytoplasmic p53 are expressed in NB cells, as determined by fractionated Western analysis. Furthermore, Isaacs et al. (24) have suggested that localization is dependent upon the type of NB cell analyzed, i.e., neuroblastatic or nonneuronal.

After careful analysis of several commonly used p53 monoclonal antibodies that recognize different p53 protein conformations (25), we chose BP53.12 and DO7 for our studies. Consistent with the predetermined characteristics of these antibodies, BP53.12 detected both cytoplasmic and nuclear p53 protein in all NB cell lines (data not shown); and DO7 detected only nuclear protein but generated a readily detectable, intense immunofluorescence signal. Results with this antibody (Fig. 1) confirm that nuclear p53 protein is expressed in NB cell lines independent of their p53 status, and although cytoplasmic p53 is also expressed, this characteristic is not unique to NB cells (25). Two of the cell lines used in this study, SK-N-SH and IMR32, were reported previously to have p53 localized exclusively to the cytoplasm (13). We attribute the differences between the two studies to be a consequence of the antibodies used.

The ability of NB cells to activate a p53-mediated G1 arrest after DNA damage is another controversial issue. Moll et al. (13) evaluated four NB cell lines containing a wtp53 gene sequence for their ability to arrest after exposure to a very low 0.5-Gy dose of IR and concluded that NB cells have a defect in their G1 checkpoint function. In contrast, Goldman et al. (16) evaluated two different wtp53 containing lines and after exposure to 4-Gy IR, concluded that the p53-associated G1 arrest in NB cells was not attenuated. In our studies, we evaluated wtp53 induction and subsequent G1 arrest after exposure to equitoxic doses of IR (IC50; Table 1) in order that cell lines which demonstrated varying radiosensitivities could be directly compared at biologically equivalent doses. ML-1 cells were used as a positive control because this cell line has been characterized previously to display the G1 arrest function of wtp53 (23). Despite functional wtp53 transactivation of p21 in NB cells containing a wtp53 gene, induction of an IR-mediated G1 arrest was attenuated (Fig. 3) compared with that in ML-1 cells. The failure of the SJNB-4 and p53TDN-1 cell lines to G1 arrest is consistent with the mutant p53 genotype and nonfunctional p53 status of these cell lines and correlates with the inability of these two cell lines to transcriptionally activate p21. The inability of
NB-1691 cells to arrest in response to DNA damage is consistent with the observation that NB-1691 cells overexpress the MDM2 protein (20), which can negate wt p53 transactivation activity (30).

We also evaluated IR-induced G1 arrest after exposure to a uniform high dose of IR. Although this dose resulted in a higher level of p53 induction in SJNB-1, SK-N-SH, and NB-1643 cells (Fig. 2), an enhanced effect on G1 arrest was observed in only SJNB-1. Because none of the cell lines generated the same degree of IR-mediated G1 arrest compared with ML-1 cells, we conclude that although wt p53 is transcriptionally active in NB, their wt p53-mediated G1 arrest function is attenuated. Similar results were attained by Moll et al. (13) with respect to a lack of G1 arrest in the SK-N-SH and IMR32 cell lines. The reasons for the different results obtained by Goldman et al. (16) are unclear, except for the fact that the lines chosen by this group were different and may display an alternate phenotype. It is possible that a subset of NB cell lines can induce a G1 arrest after DNA damage.

The attenuated DNA-damage induced G1 arrest observed in the majority of NB cell lines could be either mediated by a defect in the upstream signaling pathway leading from DNA damage to insufficient accumulation of cellular p53 or in the downstream pathway induced by p53 activation. To investigate whether additional expression of p53 could mediate a G1 arrest, we used an adenoviral vector (Ad.p53) to overexpress exogenous wt p53 in the NB cell lines. Western analysis demonstrated adenoviral production of p53 that could induce p21 expression in all cell lines (Fig. 4) but could not mediate a significant G1 arrest (Fig. 5). The increase in p21 protein in NB-1691 cells after transduction with Ad.p53 was not observed after IR, as the function of the DNA damage-induced p53 was inhibited by overexpression of MDM2 (20). NB-1643 and SJNB-1 cells exhibited a moderate arrest consistent with the results observed after IR exposure (Figs. 3 and 5). These results demonstrated that additional exogenous p53 expression could not enhance the G1 arrest phenotype and that the defect in NB cells appears to be downstream from p53 expression.

DNA damage, such as IR, induces endogenous p53 expression, which in turn transactivates p21, a cdk inhibitor responsible for inhibiting cyclin D1/cdk4/6 and cyclin E/cdk2 kinase activity (3, 31, 32). p21 induction in NB cells did not facilitate a G1 arrest, suggesting that p21 may be nonfunctional in this cell type. Mutations in p21 could potentially result in persistent cdk activity, Rb hyperphosphorylation, and progression through the cell cycle (33). Although p21 mutations are rare in human tumors (34–36), those that have been identified appear to be deficient in inhibiting cdk activity and inducing G1 arrest (36, 37). Alternative possibilities that may account for attenuated G1 arrest include amplification of cyclin D1, cdk4 or cdk6, and mutations in pRb. These types of alterations have been reported in NBs, although the frequency is low (38).

The ability of wt p53 to induce a G1 cell cycle arrest in NBs may be attenuated; however, those cells expressing a wt p53 gene are generally more radiosensitive (Table 1). Expression of wt p53 has been shown to be associated with sensitivity to IR and chemotherapeutic agents (4–6, 39–41), whereas expression of mutant p53 is associated with resistance (42). In agreement with these observations, the NB cell lines expressing wt p53 were generally more radiosensitive than those cells with inactive p53. The IR IC50 values of cells with a wt p53 gene status ranged from 1.25 to 2.6 Gy, whereas the values for the NB lines with inactive p53 were higher and ranged from 1.95–7.8 Gy (Table 1). NB-1643 cells that had been engineered to express TDN-p53 (p53TDN-1) and thus inactivate endogenous wt p53 function were slightly more resistant (1.6-fold) to IR compared with the parental NB-1643 line (Table 1), suggesting that wt p53 expression plays a role in radiation-induced cell death. Indeed, the ability of wt p53 to induce apoptosis, at least in NB-1643 cells, appears normal in that inhibition of p53 function in this cell line (p53TDN-1) resulted in a decrease in the percentage of cells undergoing apoptosis (Fig. 6; Table 2). In addition, less cell death and apoptosis was evident in the ML-1 cells (Fig. 6; Table 2), consistent with the greater degree of G1 arrest after exposure to IR in this cell line (Fig. 3).

Expression of nonfunctional p21 in NBs could potentially contribute to an apoptotic phenotype, because it has been demonstrated previously that inhibition of p21 function can enhance drug sensitivity (43, 44). In addition, embryonic fibroblasts isolated from p21−/− mice do not G1 arrest in response to DNA damage, yet p21−/− thymocytes readily undergo apoptosis to a greater extent than that observed in p53−/− mice (45). The reason for the effect of p21 on apoptosis is probably similar to that observed in cells that have lost pRb function, where inappropriate cell proliferation signals in the absence of an arrest promote cell death (46).

In conclusion, we have performed a comprehensive study that helps to resolve the controversy that presently exists in this research area. Data generated confirm that wt p53 is expressed in the nucleus of NB cells and that it is transcriptionally active. However, after DNA damage, the ability of this cell type to undergo p53-mediated G1 arrest is attenuated, whereas p53-mediated apoptosis seems unaffected.

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REFERENCES


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