Selective Sensitization to DNA-damaging Agents in a Human Rhabdomyosarcoma Cell Line with Inducible Wild-Type p53 Overexpression

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ABSTRACT

Drug-induced cytotoxicity or apoptosis may be influenced by the expression of the p53 tumor suppressor gene and by the specific oncogene expressed, which may dictate the threshold at which a cytotoxic response may be induced. The objective of the study was to elucidate how DNA-damaging agents with different mechanisms of action were sensitized in the context of expression of the Pax3/FKHR fusion protein, a transformation event unique to alveolar rhabdomyosarcomas (ARMSs), and wild-type p53 (wt53). A wt53 cDNA was subcloned into the pGREP-2/EBV vector with dexamethasone-inducible overexpression and transfected into Rh30 ARMS that express Pax3/FKHR and a mutant p53 phenotype. Following dexamethasone induction of wt53 overexpression in a derived clone (Cl.27), growth was slowed, and cells accumulated in G1. Functional wt53 activity was demonstrated by selective transactivation of p50-2, a wt53 chloramphenicol acetyltransferase reporter construct, and by up-regulated expression of endogenous p21wtf. Data demonstrated p53-dependent sensitization (≥4-fold) to bleomycin, actinomycin D, and 5-fluorouracil and considerably less p53-dependence (≤2-fold) for doxorubicin, topotecan, etoposide, and cispbatin in Cl.27 compared to an equivalent clone containing the pGRE5-EBV vector alone (VC#3). Data demonstrate that ARMS cells show a selective sensitization to DNA-damaging agents when wt53 is overexpressed. The cytotoxicity of agents that are not potentiated substantially must, therefore, depend upon p53-independent factors that relate to the mechanism of drug action.

INTRODUCTION

The function of the p53 tumor suppressor gene in normal cells is not well understood, although it is known to be involved in the control of cell growth. Inactivation of p53 by mutation or deletion results in genomic instability (1, 2), which can lead to gene amplification, possibly caused by changes in cell cycle progression. The wt53 protein is a transcriptional activator of target genes, functions as a G1 cell cycle checkpoint, and becomes elevated in response to DNA-damaging agents due to stabilization of the protein (3, 4). Analyses have indicated that only one or a small number of unrepair DNA breaks may be sufficient to cause growth arrest (5, 6). Following transcriptional activation of the Waf1 gene, up-regulated expression of p21wtf initiates inhibition of cyclin-cyclin-dependent kinase interactions, thereby inducing G1 arrest (7, 8). If DNA damage is not repaired, prolonged arrest resembling senescence can be induced in normal diploid fibroblasts (9), or alternatively, apoptosis is induced (9) by the action of effector proteins downstream of p53, the response depends upon the cell type and its physiological state. However, the precise mechanism by which p53 functions in apoptosis is unknown. In neoplastic cells, the diversity in cellular responses to DNA-damaging agents in the presence of wt53 may be influenced by the deregulated expression of oncogenes, the transforming potential of the oncogene, and hence the specific oncogene expressed. Thus, sensitivity to drugs under these conditions may depend upon an oncogenic mutation that lowers the threshold at which cell injury triggers apoptosis and the cellular context of its expression. There is also increasing evidence that chemotherapeutic agents may induce apoptosis or kill cells by p53-independent mechanisms (10-14), including involvement of S or G2 phase arrest (11, 13), or the induction of a retinoblastoma phosphatase activity (14), suggesting that G1 checkpoint control is not linked in all instances to the apoptotic machinery of the cell.

Based upon the importance of a wt53 gene in maintaining normal regulation of myogenesis (15, 16) and the potential for sensitization to drug-induced apoptosis, the wt53 gene was transfected into a human ARMS3 cell line Rh30 expressing mp53 to determine whether the relative resistance of Rh30 to DNA-damaging agents may be overcome. We have focused on the inducible overexpression of wt53 in Rh30 that expresses the Pax3/FKHR fusion protein as a consequence of a specific t(2;13) translocation unique to this histiotype (17). Rh30 expresses two p53 alleles, as determined from dideoxynucleotide sequencing of genomic DNA, one wt and the second with a

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3 The abbreviations used are: ARMS, alveolar rhabdomyosarcoma; wt, wild type; mp53, mutant p53; Fura, 5-fluorouracil; VP-16, etoposide; dexamethasone; CAT, chloramphenicol acetyltransferase.
Drug Sensitization and wtp53 Overexpression

C→T point mutation at codon 273, and hence can tolerate the presence of a wtp53 gene, although the phenotype expressed is of mp53. Thus, Rh30 demonstrates expression of high levels of mp53, low levels of p21<sup>Waf1</sup>, and is less sensitive to chemotherapeutic agents than other ARMS cell lines that express wtp53 alone. The purpose of the study was to elucidate how DNA-damaging agents with different mechanisms of action are potentiated in the presence of overexpressed wtp53 in the context of expression of the Pax3/FKHR fusion protein. Data demonstrate p53-dependent potentiation (≥4-fold) of bleomycin, actinomycin D, and FUra cytotoxicity in the presence of induced wtp53 expression and less p53 dependence (≤2-fold) for doxorubicin, topotecan, VP-16, and cisplatin.

**MATERIALS AND METHODS**

**Cell Culture.** The Rh30 human ARMS cell line that expresses the Pax3/FKHR fusion protein secondary to the t(2;13) translocation was established from the bone marrow of a patient with metastatic disease (17). There is no apparent amplification of Myc or MDM2 genes, and normal p21<sup>Ras</sup> is expressed as determined by PCR-sequencing of H-, K-, and N-Ras genes (18, 19). Rh30 expresses two p53 alleles as determined by dideoxynucleotide sequencing of genomic DNA (20), one wt and the second with a C→T (arg→cys) point mutation at codon 273. Cells were cultured in RPMI 1640 (BioWhittaker) supplemented with 10% FBS and 2 mm glutamine in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C. The doubling time was 30 h. To establish a wtp53-expressing clone, a wtp53 cDNA was transfected into the BamHI site of the dex-inducible pGRE5-2/EBV vector, which contains five synthetic glucocorticoid response elements in the promoter region (21). Because the vector replicates episomally within cells, expression of the subcloned gene may be influenced by the level of antibiotic selection pressure used and also by the concentration of dex.

Following transfection using lipofectin (Life Technologies, Inc.) according to the manufacturer’s directions and subsequent plating at cloning density, individual clones were selected for resistance to 400 units/ml hygromycin. Several wtp53-transfected clones and similarly derived vector control clones were selected for further study. The growth of Rh30 and individual clones was determined over a period of 4 days, both in the absence and in the presence of 5 μM dex. Dexamethasone-treated cultures were subjected to flow cytometric analysis and compared to cells grown under normal conditions as described previously (22). Hygromycin-selected clones were maintained at 200 units/ml of the antibiotic.

**Western Analyses.** To determine the functional activity of transfected wtp53, the expression of p21<sup>Waf1</sup> was examined. Cell extracts were prepared, Western analyses were conducted using 12.5% Ready-Gels (Bio-Rad), and p21<sup>Waf1</sup> was detected using the primary mouse anti-human p21<sup>Waf1</sup> monoclonal antibody 6B6 (PharMingen), a secondary sheep anti-mouse antibody conjugated to horseradish peroxidase (immunoglobulin-HRP; Amersham Corp.), and ECL reagents as described previously (23).

**Transient Transfections.** As an additional measure of functional p53 activity, the ability of p53 to enhance the expression of p50–2, a wtp53 CAT reporter construct, was determined as reported previously (23). This reporter gene consists of two copies of a 50-bp wtp53 response element from the murine muscle-specific creatine phosphokinase promoter inserted upstream of the adenovirus major late TATA box and terminal deoxynucleotidyltransferase initiator element; pCAT-basic (Promega) and pCMV-CAT (a gift from Dr. Cliona Rooney, SJCRH) were used as negative and positive controls, respectively, and all transfections were conducted simultaneously with pSV-βgal (Promega Corp.) to control for transfection efficiency.

**Drug Sensitivity.** Rh30 cells transfected with either the pGRE5–2/EBV vector (VC#3) or pGRE5–2/EBV-wtp53 (CI#27) were plated at a density of 100,000 cells/well in six-well plates (Falcon). Following overnight attachment, cells were simultaneously treated with dex (5 μM) and various concentrations of chemotherapeutic agents (bleomycin, actinomycin D, doxorubicin, cisplatin, topotecan, VP-16, FuRa, and vincristine), which were pharmaceutical preparations except for topotecan (SmithKline Beecham) and FuRa (Sigma Chemical Co.). Drug treatment was for a period of three doubling times (5 and 7 days for VC#3 and CI#27, respectively) at 37°C. For harvesting, cells were initially washed twice in 1 ml 0.9% saline followed by the addition of 1 ml hypotonic HEPES solution for 5 min. Nuclei were obtained following treatment with 120 μl of lysis solution (5% ethylhexadecyldimethylammonium bromide and 3% glacial acetic acid); 500 μl were removed and placed in 15 ml 0.9% saline and counted using a Coulter particle counter. Data for drug-treated cells were expressed as a percentage of the untreated control. To verify the similarity among responses of VC clones or wtp53-transfected clones, three clones from each condition were compared for their sensitivity to bleomycin both in the absence and presence of dex (5 μM).

**Apoptosis.** The presence of apoptotic cells in drug-treated cultures was evaluated either by gel electrophoresis of isolated DNA to analyze the extent of nucleosomal ladder formation as described previously (23) or by light microscopy.
Table 1 Flow cytometric analysis of Rh30 and clones

<table>
<thead>
<tr>
<th></th>
<th>Cl.#27</th>
<th>VC#3</th>
<th>Rh30</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>S</td>
<td>G2-M</td>
<td>G1</td>
</tr>
<tr>
<td>0 h</td>
<td>48</td>
<td>42</td>
<td>10</td>
</tr>
<tr>
<td>24 h</td>
<td>61</td>
<td>23</td>
<td>16</td>
</tr>
<tr>
<td>48 h</td>
<td>38</td>
<td>47</td>
<td>15</td>
</tr>
<tr>
<td>72 h</td>
<td>34</td>
<td>53</td>
<td>13</td>
</tr>
<tr>
<td>+Dex</td>
<td>40</td>
<td>47</td>
<td>13</td>
</tr>
</tbody>
</table>

Fig. 1 Expression of p21^waf1 in VC#3 and Cl.#27 cells either in the absence (−) or presence (+) of dex (5 μM) for 4 days. Cell extracts were analyzed by Western analysis as described in “Materials and Methods.”

Table 2 Classification of DNA-damaging agents

<table>
<thead>
<tr>
<th>Drug Class</th>
<th>Agent</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cross-link DNA</td>
<td>Cisplatin</td>
<td>Formation of DNA-interstrand, -intrastrand, and -protein cross-links. The former is considered to initiate cytotoxicity.</td>
</tr>
<tr>
<td>Formation of free radicals</td>
<td>Bleomycin</td>
<td>Generation of free radicals causes DNA DSBs. *</td>
</tr>
<tr>
<td>Top inhibitor/intercalator</td>
<td>Doxorubicin</td>
<td>Cytotoxicity is via free radical formation or topII-dependent DNA damage → DSBs.</td>
</tr>
<tr>
<td></td>
<td>Actinomycin D</td>
<td>Dual topII inhibitor; inhibits RNA synthesis.</td>
</tr>
<tr>
<td>TopI inhibitor</td>
<td>Topotecan</td>
<td>Forms reversible cleavable complexes and DNA SSBs. Forms DNA DSBs upon collision of a replication fork with a topI-cleavable complex.</td>
</tr>
<tr>
<td>TopII inhibitor</td>
<td>VP-16</td>
<td>Inhibits TS. Causes DNA SSBs and DSBs via depletion of dTTP pools and misincorporation of FUrA or Ura bases in DNA.</td>
</tr>
<tr>
<td>Antimetabolite</td>
<td>FUrA</td>
<td>Mitotic spindle poison.</td>
</tr>
<tr>
<td>Non-DNA-damaging agent</td>
<td>Vincristine</td>
<td></td>
</tr>
</tbody>
</table>

* DSB, double-strand break; SSB, single-strand break; top, topoisomerase.

using chamber slides (Nunc) and staining with H&E according to standard procedures.

RESULTS

Demonstration of Functional wtp53 Activity. The growth of Rh30, VC#3, and Cl.#27 is shown in Fig. 1. Growth over a period of 4 days was determined both in the absence and presence of dex (5 μM). Rh30, VC#3, and Cl.#27 demonstrated similar growth kinetics in the absence of dex, with doubling times of 30–35 h. dex exerted no influence on the growth of Rh30 or VC#3, but consistent with overexpression of wtp53, proliferation of Cl.#27 was reduced, thereby increasing the doubling time to 52 h.

The distribution of cells within different phases of the cell cycle at the onset and following dex (5 μM) treatment was determined by flow cytometric analysis (Table 1). In Cl.#27, the
Fig. 4  Sensitivity of VC#3 and CI#27 to various concentrations of seven DNA-damaging agents and to the mitotic spindle poison vincristine. Experiments were conducted as described in "Materials and Methods." Results are the means of three determinations at each drug concentration from representative experiments; bars, SD. Results for drug-treated cultures are expressed as a percentage of the untreated controls.
respectively, when compared to the sensitivity of VC#3 cells. ELM),

The functional activity of transfected wtp53 was examined in Cl.#27 and VC#3 cells by analysis of the expression of p21^{Waf1}. Levels of p21^{Waf1} were determined by Western analysis for up to 4 days in the absence or presence of dex induction (Fig. 2). In Cl.#27 only, p21^{Waf1} was induced and expressed at high levels for at least 4 days following dex treatment. Data indicated tight regulation of the functional activity of the wtp53 gene subclassed into pGRE5-2/EBV and prolonged expression following dex induction.

To additionally verify functional wtp53 activity in Cl.#27 following dex treatment, the ability of p53 to enhance the expression of p50-2 was significantly elevated in Cl.#27 after dex induction and also in Cl.#27 (-dcx). However, transactivation of p50-2 was induced and expressed at high levels for at least 4 days following dex treatment. Data indicated tight regulation of the functional activity of the wtp53 gene subclassed into pGRE5-2/EBV and prolonged expression following dex induction.

**Table 3** Cellular sensitivity to DNA-damaging agents

<table>
<thead>
<tr>
<th>Agent</th>
<th>IC_{50} (nM)</th>
<th>VC#3</th>
<th>Cl.#27</th>
<th>Fold</th>
<th>n^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bleomycin</td>
<td>345</td>
<td>43</td>
<td>8.0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Actinomycin</td>
<td>0.10</td>
<td>0.02</td>
<td>5.0</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>FUra</td>
<td>4.3</td>
<td>1.0</td>
<td>4.3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>6.3</td>
<td>3.1</td>
<td>2.0</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Topotecan</td>
<td>5.5</td>
<td>3.0</td>
<td>1.8</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>VP-16</td>
<td>109.5</td>
<td>61.0</td>
<td>1.8</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Cisplatin</td>
<td>450</td>
<td>330</td>
<td>1.4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Vincristine</td>
<td>0.3</td>
<td>0.3</td>
<td>1.0</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

^a n, number of separate experiments from which the IC_{50}s were derived.

The functional activity of transfected wtp53 was examined in Cl.#27 and VC#3 cells by analysis of the expression of p21^{Waf1}. Levels of p21^{Waf1} were determined by Western analysis for up to 4 days in the absence or presence of dex induction (Fig. 2). In Cl.#27 only, p21^{Waf1} was induced and expressed at high levels for at least 4 days following dex treatment. Data indicated tight regulation of the functional activity of the wtp53 gene subclassed into pGRE5-2/EBV and prolonged expression following dex induction.

To additionally verify functional wtp53 activity in Cl.#27 following dex treatment, the ability of p53 to enhance the expression of p50-2, a wtp53 CAT reporter construct, was obtained at 72 h after treatment with bleomycin (0.01-10,000 μU) in all cell lines, as indicated by nuclear chromatin condensation, nuclear membrane blebbing, and the formation of apoptotic bodies (data not shown).

**DISCUSSION**

Relatively little information is available concerning the susceptibility of human tumor cell populations to drug-induced cytotoxicity in the presence of wtp53, although the responses reported have been diverse. One of the earliest reports of the role of wtp53 in sensitizing cells to cytotoxic agents and ionizing radiation was in the context of E1A and T24 H-Ras-transformed mouse embryo fibroblasts (MEFs), originally derived from normal or p53(-/-) transgenic mice (24). Under these conditions, general sensitization to the cytotoxic effects of doxorubicin, VP-16, FUra, and ionizing radiation occurred in the presence of a wtp53 gene. wtp53 dependency for VP-16 toxicity (25) and for adenosine deaminase deficiency causing the abnormal accumulation of dATP (26) was determined in thymocytes. Sensitization of glioblastoma cells (27) or spheroid cultures of the non-small cell lung cancer cell line H358 (28) to cisplatin was also representative of clones either overexpressing wtp53 or containing the vector alone, additional clones were examined for their relative sensitivities to bleomycin either in the absence or presence of dex (5 μM; Table 4). It is evident that the sensitivities to bleomycin of VC and wtp53-transfected clones in the absence of dex were in the same range, and dex exerted no influence on bleomycin sensitivity in VC clones. However, an 8-fold or greater sensitization occurred in the wtp53-transfected clones only after wtp53 induction by dex.

**DNA Damage.** Isolated DNA was examined by gel electrophoresis for the formation of nucleosomal ladders following treatment with actinomycin D, doxorubicin, topotecan, and vincristine for three doubling times at drug concentrations that were cytotoxic (Fig. 5). No endonucleosomal DNA cleavage was observed under any drug treatment condition either in VC#3 or in Cl.#27. The formation of nucleosomal ladders was also not observed in parental Rh30 cells after drug treatment (data not shown). However, morphological evidence of apoptosis was obtained at 72 h after treatment with bleomycin (0.01-10,000 μU) in all cell lines, as indicated by nuclear chromatin condensation, nuclear membrane blebbing, and the formation of apoptotic bodies (data not shown).
Fig. 5. Agarose gel electrophoresis of purified DNA from Cl.#27 and VC#3 cells following exposure to cytotoxic concentrations of actinomycin D (0.5 and 10 nM), doxorubicin (50 and 750 nM), topotecan (50 nM and 1 μM), and vincristine (0.3 and 100 nM), respectively, for a period of three doublings. No endonucleosomal DNA cleavage was detected.

toxicity in colon carcinoma cells (31). In addition, sensitization of MCF-7 breast cancer cells to cisplatin was determined in the presence of disrupted p53 function (32).

Consistent with p53 function in Rh30 ARMS cells overexpressing wtp53, reduced growth rate and accumulation of cells in G1 was observed. Of particular interest in the present study was a demonstrated selective sensitization to DNA-damaging agents after wtp53 overexpression under these conditions. Cl.#27 was sensitized to the greatest extent to agents that formed free radicals (bleomycin), damaged DNA indirectly due to perturbation in deoxyribonucleoside triphosphate pools (FUra), or inhibited RNA synthesis (actinomycin D). Of interest was that agents that were pure inhibitors of either topoisomerase I (topotecan) or topoisomerase II (VP-16) were not potentiated to the greatest extent in the presence of overexpressed wtp53. In addition, although endonucleosomal DNA cleavage was not observed after drug treatment, morphological evidence of apoptosis was observed, consistent with observations made in other systems (33). Also consistent with these results were the data reported by Yang et al. (34), who observed a greater differential in IC_{50} for FUra (4-fold) in WTd human colon carcinoma cells with inducible overexpression of wtp53, in comparison with results obtained with topotecan (2.1–2.9) or ionizing radiation (1.5–1.8) that initiates DNA damage via free radical formation, similar to the mechanism of action of bleomycin.

The complexity in the interpretation of data derived in different model systems may be influenced by the oncogene expressed in specific cell types. For example, deregulated expression of c-Myc by amplification is common (35), suggesting that overexpression is important in the oncogenesis of certain histiotypes. Its function is complex, because it can induce either cellular proliferation or apoptosis, dependent upon the presence of other cellular factors (33). Overexpression of c-Myc has enhanced drug sensitivity in leukemic cells, inhibitable by expression of mp53 (36). In contrast, transfection of oncogenic Ras has shown significantly less ability to induce spontaneous apoptosis in immortalized rat fibroblasts (37). In addition, it has been less efficient than c-Myc in promoting radiation-induced apoptosis in rat embryo cells (38) or in sensitizing rat embryo fibroblasts to cisplatin cytotoxicity (39). In our model system, the Pax3/FKHR fusion protein, an oncogene unique to ARMS, may have weaker transforming potential than Ras or c-Myc oncogenes, because it can coexist with other oncogenes (40, 41).

Thus, for oncogenes with strong transforming potential, as in the case of E1A1124-H-Ras-transformed MEFs (24), the threshold for induction of apoptosis may be lowered to such an extent that general sensitization to multiple agents that damage DNA may be achieved in the presence of a wtp53 gene. This may be in contrast to the diversity in cellular responses that would be anticipated in the absence of wtp53 based upon the individual mechanism(s) of drug action.

Thus, other factors that are independent of the expression of tumor suppressor genes or oncogenes may be involved in influencing the threshold of drug-induced cytotoxicity. Apopto-
sis occurs under a variety of circumstances, only some of which require p53. Gartenhaus et al. (10) found in lymphocyte cell lines transformed with T-cell leukemia virus type I that, irrespective of the p53 status of the cell lines, induction of p21\textsuperscript{waf1} expression and apoptosis was induced by doxorubicin. The phase of the cell cycle at which cells arrest after treatment with drugs or ionizing radiation may also significantly influence the treatment outcome, and this can also be independent of p53. Two human colon carcinoma cell lines expressing mp53 with similar levels of topoisomerase I and kinetics of DNA synthesis inhibition demonstrated differential sensitivity to the topoisomerase I inhibitor CPT-11 (11). Sensitive SW620 cells were found to be irreversibly blocked in S phase, whereas the more resistant KM12 cell line arrested in G\textsubscript{2}. Han et al. (13) determined that the human promyelocytic cell line HL60, which is deficient in p53, undergoes apoptosis following X-irradiation, a decision which is made at a G\textsubscript{2} checkpoint. Furthermore, induction of a retinoblastoma phosphatase activity by drugs including VP-16 has accompanied p53-independent G\textsubscript{1} arrest and apoptosis in other model systems (14). Taken together, results suggest that p53 expression or overexpression can sensitize cells to DNA-damaging agents that may be influenced by the specific oncogene expressed and also by the mechanism of drug action, which may be p53 independent and hence can override the influence of wt53 expression.

REFERENCES

Selective sensitization to DNA-damaging agents in a human rhabdomyosarcoma cell line with inducible wild-type p53 overexpression.


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