Normal p53 Status and Function Despite the Development of Drug Resistance in Human Breast Cancer Cells

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Abstract

Loss of or mutations in p53 protein have been shown to decrease both radio- and chemosensitivity. The present study assessed the p53 gene status, ability to arrest in G1, of the cell cycle, the functionality of the p53 transduction pathway, and apoptosis following treatment with radiation in a series of drug-resistant human breast cancer cells to determine whether p53 alterations occur during the development of drug resistance. We used 13 sublines derived from MCF-7, ZR75B, and T47D cells, which were resistant to doxorubicin, paclitaxel, vinblastine, cisplatin, etoposide, and amsacrine. Eleven of 12 drug-resistant sublines retained the parental p53 gene status, as determined by sequence analysis and functional yeast assay; only one subline was found to have acquired a mutation in the p53 gene. The MCF-7 TH subline was found to both acquire mutated p53 and to have major changes in p53 protein expression and function. In 12 other drug-resistant sublines, the G1 checkpoint was conserved or only slightly impaired. A normal accumulation of p53, p21Cip1/Waf1, and Mdm2 proteins and hypophosphorylation of Rb protein occurred in response to radiation with only small differences noted in the kinetics of p53 and p21Cip1/Waf1 induction. Increased susceptibility to apoptosis was found in the ZR75B drug-resistant sublines, whereas no evidence for apoptosis was observed in the ZR75B, MCF-7, and T47D parental MCF-7 and T47D drug-resistant sublines. This effect could not be explained by alterations in bcl-2 or bax expression. Our results demonstrate that alterations in: (a) p53 gene status; (b) ability to arrest in G1; (c) induction of p53 protein and p53-dependent genes; and (d) decreased activation of apoptosis is not a requirement for the onset of drug resistance. The function of p53 appears to be dissociated from drug resistance in our model system.

Introduction

Human breast cancer cell lines that are resistant to chemotherapeutic agents display a wide variety of alterations. Drug resistance mechanisms, including overexpression of drug efflux pumps such as P-glycoprotein or multidrug resistance-related protein, or changes in topoisomerase II or glutathione S-transferase have been proposed (1–3). But alterations not obviously associated with drug resistance also occur. For instance, we have observed a frequent up-regulation of growth factors and growth factor receptors in drug-resistant human breast cancer sublines, with concurrently decreased growth rates. How these cells acquire a slower growth rate is unknown. Several checkpoints in cell cycle progression control growth in response to diverse positive and negative regulatory signals (4). For example, ionizing radiation or agents that physically damage DNA slow growth by inducing delays in the G1-S and G2-M transitions of the cell cycle (5). The available evidence suggests that cell cycle arrest is necessary for repair of DNA damage (5). If this fails, cells with unrepair DNA are directed into an apoptotic pathway (6).

DNA damage results in induction of endogenous wild-type p53 protein and the activation of a cascade in which p53-dependent genes, such as p21Cip1/Waf1 and mdm2, are transcriptionally activated (7–9). The p21Cip1/Waf1 protein appears to be a potent inhibitor of cyclin-dependent kinases (10). This inhibition suppresses the phosphorylation of Rb by cyclin/cyclin-dependent kinase complexes (8). Hypophosphorylated Rb is bound to the transcription factor E2F1 during G1, but upon phosphorylation, E2F1 is released and activates the transcription of genes necessary for transit into S phase (11). The activation of p21Cip1/Waf1, therefore, leads to G1 arrest. Mdm2 protein appears to inhibit the transcriptional activity of wild-type p53, and its induction by p53 acts as an autoregulatory feedback loop (9). Mutations in the p53 gene cluster predominantly within the DNA-binding domain of p53, leading to defective activation (12).

p53 protein can function as a genetic switch capable of activating G1 arrest, resulting in DNA repair or apoptosis (13). Apoptosis is a highly characteristic suicide program in which loss of membrane integrity is typically preceded by chromatin condensation and internucleosomal cleavage of genomic DNA (14). Apoptosis induced by DNA damage requires p53, whereas p53-independent apoptosis has been described for stimuli that mimic physiological cell-deletion signals (15, 16). Recently, it was shown that cells with mutated p53 gene display perturbed G1 arrest or apoptosis (7, 17). In addition, defects in apoptosis caused by inactivated p53 protein appears to reduce the sensitivity to DNA-damaging agents, and it has been suggested that this represents a mechanism whereby tumor cells may acquire drug resistance (18–21). A defective activation of G1 arrest and apoptosis may contribute to genomic instability by loss of a mechanism that allows the cells time to repair DNA and to eliminate cells with genetic damage (5). Bcl-2 protein is known to play a role in promoting cell survival and inhibition of apoptosis. It has been reported that Bcl-2 blocks cell

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2 K. Wosikowski, unpublished data.
Table 1: Biological features of drug-resistant human breast cancer cells.

<table>
<thead>
<tr>
<th>Cell line*</th>
<th>IC50</th>
<th>Relative resistanceb</th>
<th>Cell doubling</th>
<th>mdh-1 mRNA expression</th>
<th>Functional p53 gene statusc</th>
<th>p53 sequenced</th>
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<tr>
<td></td>
<td>(ng/ ml)</td>
<td></td>
<td>(h)</td>
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<tr>
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<td>182</td>
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* Cell lines were selected in the compound and maintained at a concentration in ng/ml indicated by their names. MCF-7 TH and AdVp were selected in doxorubicin. Ad, doxorubicin; Tx, paclitaxel; CDDP, cisplatin; VP-16, etoposide; Vb, vinblastine; ND, not determined.

b Relative resistance refers to the fold increase in IC50 of the drug-resistant subline compared to the IC50 of the parental cell line for the compound selected.

c p53 gene status as determined by the functional yeast assay (28).

d p53 gene status as determined by sequence analysis (described in "Materials and Methods").

In this paper, we used 13 drug-resistant sublines derived from MCF-7, ZR75B, and T47D human breast cancer cell lines. Table 1 shows the IC50 for the compounds the cells were selected in as well as their resistance relative to parental cells. A number of drug-resistant cell lines express the P-glycoprotein drug efflux pump, including MCF-7 TH, MCF-7 Tx200, and all of the resistant ZR75B sublines. The resistant T47D sublines did not express P-glycoprotein, as determined by Northern blot analysis. Also depicted in the table are the doubling times of the parental and drug-resistant sublines. The growth rates of all the resistant sublines are decreased compared to the parental.

To determine whether p53 had undergone any mutations in the course of the drug selections, the p53 gene status was initially determined by a functional assay in yeast (28). In this method, the p53 PCR product is expressed in Saccharomyces cerevisiae and tested for its ability to activate transcription. The results of the functional yeast assays are shown in Table 1. Four of the five drug-resistant sublines analyzed in this assay had conserved p53 gene status. Subsequently, sequence analysis of MCF-7 and ZR75B parental and drug-resistant sublines extended this observation to a total of 10 of 11 drug-resistant sublines that displayed wild-type p53 gene status. Both the T47D parental cell line (known to have mutated p53) and its Ad12 drug resistance subline examined in the functional yeast assay were determined to have mutated p53. Thus, no changes from the parental form of p53 were observed during selection in the majority of the drug-resistant sublines. Only the MCF-7 TH subline was found to have mutated p53. Sequence analysis revealed a deletion of 21 bp, beginning at exon 5.
To define the ability of the drug-resistant sublines to arrest in G₁ of the cell cycle, the cells were exposed to 5 Gy of ionizing radiation and analyzed for cell cycle distribution by FACS analysis. Shown in Fig. 1 are the G₁-arrested cells, calculated by subtracting the percentage of cells in G₁ after 0.4 μg/ml nocodazole treatment alone from the percentage of cells in G₁ phase 17 h after 5 Gy radiation in the presence of nocodazole and dividing by the percentage of cells distributed in G₁ without treatment or irradiation. The mitotic inhibitor nocodazole was added to ensure that cells already distributed throughout the cell cycle would not reenter G₁. Some of the drug-resistant sublines (MCF-7 TH, ZR75B, Vb10, Tx10, and Tx40) displayed a significant reduction in the number of G₁-arrested cells after irradiation compared to the parental cell lines. However, it should be noted that the ability to arrest in G₁ remains present in the ZR75B Vb10, Tx10, and Tx40 sublines, in contrast to the MCF-7 TH cells in which no G₁ checkpoint was observed after treatment with γ rays. The failure of MCF-7 TH cells to arrest in G₁ after irradiation is comparable to the results of T47D cells, which have mutated p53. The negative values reflect the fact that radiation followed by incubation in nocodazole leads to a further decrease in G₁ distribution than nocodazole treatment alone, probably because of radiation-mediated G₂ arrest occurring in the absence of G₁ arrest. The ability to arrest in G₁ is unchanged and low in the drug-resistant T47D sublines, as expected for cells with mutated p53. We also analyzed the cell cycle distribution of MCF-7 Tx200 and MCF-7 mAMSA sublines (data not shown). However, these results were difficult to interpret since the amount of cells in G₁ 17 h after nocodazole treatment alone were not markedly reduced compared to the control. Since these sublines had decreased growth rates, one possible explanation is that a relatively high percentage did not cycle out of G₁, even without irradiation.

The protein of the p53 gene is a DNA-binding protein that can regulate transcription in the nucleus. To determine whether correct intracellular localization after radiation of the p53 protein exists in the MCF-7 cells, we performed immunocytochemistry on these cells and used the T47D cells as a positive control. This was important since cytoplasmic p53 staining has been reported in some MCF-7 cell lines, raising questions about the functionality of p53 in these cells (29). As shown in Fig. 2, immunocytochemistry showed a small amount of perinuclear staining of p53 protein in exponentially growing MCF-7 cells. Irradiation resulted in nuclear p53 staining in an increased number of cells, a response pattern typical for wild-type p53 (5). Nuclear p53 was observed as soon as 2 h and persisted up to 24 h after radiation. Nuclear p53 staining was apparent in all control and irradiated T47D cells, typical for mutant p53-expressing cells.

The ability of the cells to induce p53 and downstream effectors, including p21Waf1/Cip1, Mdm2, and hypophosphorylation of Rb following radiation, was assessed by analyzing the cells for induction of these proteins at 4 and 24 h after radiation. As shown in Fig. 3, MCF-7 AdVp cells were most like the parental cells, with comparable levels and patterns of p53, p21Waf1/Cip1, Mdm2 protein induction, and Rb hypophosphorylation. For MCF-7 Tx200, MCF-7 CDDP, and MCF-7 VP-16 cells, protein levels of p53, p21Waf1/Cip1, and Mdm2 tended to be lower, both before and after induction by radiation, but only minor differences in the time course of induction and hypophosphorylation of

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1 The abbreviations used are: FACS, fluorescence-activated cell sorting; mAMSA, amscarine, 4′-(9-acridinylamino)methanesulfonyl-m-anisidine.
Rb were observed. Fig. 3 also includes the actin levels of these samples to confirm regular protein loading.

In contrast to the MCF-7 parental cells, an elevated level of endogenous p53 was detected in the MCF-7 TH subline with no protein induction after radiation, as shown in Fig. 3. Also, the difference in molecular weight of the p53 protein was readily detectable, which resulted from the 21-bp deletion in exon 5. No p21<sup><s>Cr/Cot</s></sup> was detected in the MCF-7 TH cells. Very low Mdm2 protein levels and no induction after radiation was observed. In addition, no Rb protein could be detected. Together with the loss of G<sub>1</sub> arrest, these observations are consistent with results observed in other studies using mutant p53 cell lines (30).

Between the ZR75B cell line and its drug-resistant sublines, no major differences were detected in p53 level and time course of induction (Fig. 4). In the ZR75B parental line and the ZR75B Ad24, ZR75B Tx10, and ZR75B Tx40 sublines, induction of p21<sup>Cip1/Waf1</sup> was observed 4 h after radiation, with an additional increase at 24 h after treatment. For the ZR75B Ad120 subline, p21<sup>Cip1/Waf1</sup> induction was only observed 24 h after treatment.

Since it has been suggested that the involvement of p53 in the apoptotic response represents a mechanism of drug resistance (20, 21), we investigated whether induction of apoptosis was altered in the drug-resistant breast cancer cell lines. Apoptosis as measured by DNA laddering was not observed in the MCF-7 parental and drug-resistant sublines when treated with 12 Gy of γ-irradiation and analyzed 24 h later, a finding consistent with previous reports of the absence of early apoptosis in MCF-7 cells (31, 32). Also, no DNA laddering characteristic for apoptosis was observed in the ZR75B and T47D parental lines or in the T47D Ad12 subline. Whereas in the ZR75B-resistant sublines, DNA fragmentation was apparent 24 h after exposure to ionizing radiation, as shown in Fig. 5. The degraded DNA was present in oligomers that were multiples of approximately 180 to 200 bp, suggesting intranucleosomal cleavage.

One potential explanation for the increased apoptosis in the ZR75B drug-resistant sublines following irradiation would be a collateral increase in sensitivity to radiation, which has been described in drug-resistant cells (33, 34). To explore this, we performed a survival experiment following
5 Gy of radiation, the dose given in the apoptosis experiments. As shown in Fig. 6, the drug-resistant ZR75B sublines were actually more resistant to radiation than the parental cells.

Since expression of bcl-2 and a high ratio to bax have been associated with a protective effect on apoptosis (26, 27), we analyzed bcl-2 and bax expression in the drug-resistant breast cancer cells. As shown in Fig. 7, decreases in bcl-2 mRNA and protein levels were apparent in the doxorubicin-resistant MCF-7 TH, MCF-7 AdVp, and ZR75B Ad120 cells and the cisplatin- and mAMSA-resistant MCF-7 sublines. No bcl-2 expression was found in the MCF-7 VP-16 subline or the T47D parental and resistant sublines. Small increases (less than 2-fold) in bax expression were observed in the drug-resistant sublines compared to the parentals. Only MCF-7 CDDP cells demonstrated a small decrease in bax expression (less than 2-fold as determined by densitometry).

**Discussion**

In this report, we studied 13 drug-resistant human breast cancer sublines derived from MCF-7, ZR75B, and T47D cells selected by exposure to increasing concentrations of cytotoxic compounds including doxorubicin, vinblastine, paclitaxel, cisplatin, etoposide, and mAMSA. Doxorubicin, cisplatin, etoposide, and mAMSA are known to be DNA-damaging agents, whereas vinblastine and paclitaxel inter-
The p53 gene status of 15 parental and drug-resistant sublines was determined by sequence analysis and/or functional yeast assay, and one acquired mutation was found. When p53 protein and its signaling pathway was induced by irradiation, we found no major differences in protein function or its transduction pathway in 12 of 13 sublines but observed increased susceptibility to apoptosis in 3 drug-resistant sublines. Irradiation was used because differences in sensitivity of the sublines to cytotoxic compounds, due to drug transport or other possible mechanisms, would make it difficult to compare the effect of cytotoxic compounds on p53 induction and effectors.

Cells with intact response to DNA damage accumulate p53 protein in the nucleus and arrest in G1, after damage by ionizing radiation or chemotherapeutic compounds (5). p53 has a central role in this pathway in that it induces expression of p21Waf1/Cip1, which inhibits the activity of cyclin-associated kinases that normally phosphorylate Rb (8, 10). This in turn arrests the cells to prevent progression through G1. Also, p53 has been found to be required for radiation-induced apoptosis (15, 16). Recently, several reports suggested that the expression of p53 may influence the radio- and chemosensitivity of the cell. Absence of p53 expression increased cellular resistance to DNA damage by cytotoxic compounds or γ-irradiation (20, 21). Also, mutations in the p53 gene were associated with decreased sensitivity to DNA-damaging agents, implying that tumor cells acquired drug and radiation resistance through mutations in p53 (17, 18). Lowe et al. (21) demonstrated that radiation resistance and relapse in p53-expressing tumors in mice were correlated with acquired mutations in the p53 gene (21). In clinical settings, mutations in p53 have been associated with chemoresistance in B-cell chronic lymphocytic leukemia and in breast cancer (36, 37). However, it has not been elucidated whether development of drug resistance is accompanied by alterations in p53 gene status or protein function as a possible mechanism of drug resistance.

Of the 11 drug-resistant sublines in which the p53 gene status was determined by sequence analysis, one acquired mutation was found. Furthermore, normal patterns of induction of p53 and p53-dependent genes were observed in all except one cell line, suggesting that 10 of the 11 sublines conserved their parental p53 protein function. These results are in agreement with the report by Brown et al. (38) in which they found no changes in p53 gene status in cisplatin-resistant ovarian cell lines. However, they found elevated levels of p53 protein in 3 of 6 drug-resistant sublines, whereas our results show either no or minimally decreased levels of p53 in the majority of the drug-resistant sublines.

All of the drug-resistant MCF-7 and ZR75B sublines, except the MCF-7 TH cells, arrested in G1 after irradiation. The MCF-7 TH subline showed no evidence of G1 arrest, similar to the result obtained in the mutant p53-expressing T47D cells. In some of the resistant sublines, the ability to arrest in G1 was decreased. The p53, p21Waf1/Cip1, and Mdm2 proteins accumulation and Rb hypophosphorylation were normally increased in the drug-resistant sublines, except in MCF-7 TH, suggesting no alterations in the p53 transduction pathway. Only differences in the kinetics of induction and the basal level of these proteins were altered. Whether the slightly decreased ability to arrest in G1 contributes to the drug-resistant phenotype in these cells is hard to distinguish at this point. It has been hypothesized that an abrogated G1, checkpoint contributes to a genetically more unstable cell line since it has less time available to repair DNA damage (5). Especially in cells that are continuously exposed to drug, this could be important to allow the cells to more rapidly acquire mutations that are beneficial in a cytotoxic environment. For example, lack of G1 arrest mediated by loss of wild-type p53 gene has been shown to result in increased frequency of gene amplification in cells (39), an event occurring in some multidrug-resistant cells. In our drug-resistant sublines, only MCF-7 TH and MCF-7 Tx200 display gene-amplified P-glycoprotein overexpression. In contrast, in a selected set of gene-amplified sublines, several alterations in p53 and its signaling pathway were observed. Another mechanism of resistance that has been proposed is a decreased ability to activate the cell death program (17, 18, 20, 21). p53 is involved in this form of resistance because it is required for apoptosis induced by DNA dam-

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age (16). However, we could not find any evidence of decreased susceptibility to apoptosis in our parental and drug-resistant sublines. The wild-type p53 containing MCF-7 and the mutant p53 containing T47D parental and drug-resistant sublines did not activate programmed cell death after treatment with radiation. Other groups also have had difficulty detecting apoptosis in MCF-7 cells. Oberhammer et al. (32) found no evidence for apoptosis in MCF-7 cells that were serum depleted, whereas Scolova et al. (31) could detect DNA laddering only in MCF-7 cells treated with high doses of etoposide for 4 days or longer. No apoptotic response was observed in the ZR75B parental cell line. Interestingly, the drug-resistant ZR75B sublines were sensitized to apoptosis. We demonstrated that this was not explained by collateral sensitivity to radiation of cells resistant to cytotoxic compounds (33, 34). Also, absence of the protective role of bcl-2 or its ratio to bax could not account for the apoptotic response of the ZR75B drug-resistant sublines since only one of the sublines showed a decrease in bcl-2 expression or ratio to bax. The reason for the increased susceptibility to apoptosis of the drug-resistant ZR75B sublines is, therefore, unclear. A possible explanation is the interplay of other genes considered to be important in apoptosis (40).

Taken together, our results suggest that alterations in p53 gene status or protein function are not critical for the development of multidrug resistance in human breast cancer cells. Mechanisms like overexpression of P-glycoprotein, multidrug resistance-related protein, or alterations in topoisomerase II or glutathione S-transferase are observed more rapidly in cells selected for resistance to cytotoxic compounds (1–3). However, this does not exclude the fact that cells with acquired p53 gene mutations are more resistant to chemotheraphy and radiation than wild-type p53-containing cells, as observed by other groups (17–21).

Materials and Methods

Cell Culture Conditions. All cells were grown as monolayer cultures at 37°C with 5% CO2 and maintained by regular passage in Eagle's Improved MEM (Biofluids, Rockville, MD) supplemented with 10% FCS (GIBCO, Grand Island, NY), 2 mM glutamine, 15 mM HEPES, and 25 µg/ml gentamicin (growth medium). The drug-resistant sublines were generated by stepwise selection or as otherwise mentioned in the text. Cells were cultured for 3 to 5 days out of drug before the start of the experiments.

Growth Response, Cytotoxicity, and Survival Assay. For cell growth analysis, 2000 cells/well were seeded in 96-well plates. On days 1, 3, 5, and 7, cells were fixed with 10% trichloroacetic acid and stained with 0.4% sulforhodamine B (Sigma Chemical Co., St. Louis, MO) in 1% acetic acid, which binds to basic amino acid residues in the fixed cells (41). Unbound dye was washed from the plates, and the bound dye was extracted for absorbance determination at 540 nm, using an Elisa microplate reader (Bio-Rad Laboratories, Hercules, CA). The cell numbers were determined in quadruplicate and expressed as percentage growth compared to the amount of cells counted on day 1. The doubling times were calculated from these graphs.

Cytotoxicity studies were performed similarly; 2000 cells/well were plated and treated with increasing concentrations of drug 24 h after plating. After 4 days in culture, the cells were fixed. Untreated control wells were assigned a value of 100%, and the IC50 was defined as the dose of drug required to inhibit the absorbance measured at 540 nm to 50% of the control value. For the survival assay, 2000 cells/well were plated in 96-well plates and exposed to increasing doses of radiation 24 h after plating. The cells were then further incubated for 4 days and fixed with 10% trichloroacetic acid as described above. Untreated control wells were assigned a value of 100%. Data represent the mean values ± SD (n = 8).

RNA Extraction and Northern Blot Analysis. Total RNA was extracted from cells by homogenizing in guanidine isothiocyanate buffer, followed by centrifugation over a CsCl cushion (42). The integrity of the RNA was verified by ethidium bromide staining after separation in a formaldehyde gel. For Northern analysis, 8 µg of total RNA were electrophoretically separated in a 1% agarose-6% formaldehyde gel and transferred onto a nylon membrane (Gene Screen Plus, DuPont, NEN, Boston, MA). Equal loading, blotting, and quality of RNA samples were verified by staining the membrane with methylene blue (43). Subsequently, the blots were hybridized with 32P-labeled mdr-1 riboprobe and exposed to a film.

Analysis of p53 Gene Status. For sequence analysis of p53, reverse transcription of 1 µg of total RNA using random primers was performed using Moloney murine leukemia virus reverse transcriptase (Bio-Rad). PCR amplifications were performed as described before (44) using 1.8 mM MgCl2, annealing temperature of 55°C for 35 cycles, and primers (BioServe Biotechnologies, Laurel, MD) upstream of exon 5 and downstream of exon 9. Primer sequences were: p53fw1, 5′-TTGTAGTGGATGGTGGCTGATGG-3′; and p53bw1, 5′-GCCTCATTCGCTTCGGAAC-3′. PCR-amplified cDNA was purified with PCR Select-III spin columns (5 Prime-3 Prime, Inc., Boulder, CO) and directly sequenced without any intermediate cloning step. Amplified template DNA was sequenced with the Taq DyeDeoxyT terminator Cycle Sequencing kit following the manufacturer's instructions (Applied Biosystems, Inc., Foster City, CA).

Three new primers were used to sequence, in addition to the above described primers: p53fw2, 5′-GCCCTCTCAGCATCATATCC-3′; p53bw2, 5′-TGGTCTGGGCTGCTGG-3′; and p53bw3, 5′-CCCTCCACTCCTCCGATACAATCGTACAG-3′. The reaction products were purified with Centri-Sep spin purification columns (Princeton Separations, Adelphiea, NJ), electrophoresed on 48-cm/4.75% polyacrylamide/urea gels, and analyzed by an automated DNA sequencing system (Model 373A; Applied Biosystems, Inc.). The sequences were aligned to the wild-type p53 sequence using the software program MacVector (Kodak International Biotechnology, Inc., New Haven, CT). The data on the p53 gene status determined by functional yeast assay were generously provided by Drs. Steven Friend and Michelle Gadd (MGH Cancer Center, Charlestown, MA; Ref. 28). The p53 gene sequencing of MCF-7 TH cells was performed by OncorMed (Gaithersburg, MD).

Cell Cycle Analysis. Cells were plated in growth media at 105 cells/100-mm Petri dishes and 2 days later were exposed to 5 Gy of ionizing radiation. Seventeen hours after treatment, cell cultures were washed twice with PBS and trypsinized. Subsequently, the cells were centrifuged and resuspended in 500 µl stain solution (0.1 mg/ml propidium iodide and 0.6% NP40 in PBS). Five hundred µl of 2 mg/ml RNase in PBS were added, and the samples were incubated for 30 min at room temperature with gentle shaking. Cell nuclei were disaggregated by passage through an 18-gauge needle and syringe. Prior to FACS analysis, the samples...
were passed through nylon mesh filters. FACS analysis was performed on a FACSort fluorescence-activated cell sorter (Becton Dickinson, Franklin Lakes, NJ). Excitation was at 488 nm using a 15-mW argon laser, and fluorescence was detected using a 585-nm filter. Cell cycle distribution was determined by DNA content, as assayed by propidium iodide staining. The percentage of cells in each phase of the cell cycle was determined using the Cellfit software provided by the manufacturer. Data are represented as the means ± SD for three experiments.

**Immunocytochemistry.** Cells were grown in tissue culture chamber slides for 48 h and treated with 5 Gy radiation; after the indicated time periods, cells were washed with PBS and fixed in methanol:acetone (1:1). The slides were stained for p53 using primary antibody at a concentration of 1 μg/ml (Ab-2; Oncogene Science, Manhasset, NY) in PBS with 0.2% BSA. After washing in PBS containing 0.05% Tween 20, the p53 protein was visualized using the avidin-biotin complex immunoperoxidase system (Vector Laboratories, Burlingame, CA) and diaminobenzidine tetrahydrochloride, according to the manufacturer's recommendations.

**Western Blot Analysis.** Cells were plated 2 days before exposure to 5 Gy of ionizing radiation. Four and 24 h later, treated and untreated cells were lysed and harvested in Laemmli lysis buffer [6.25 mm Tris-HCl (pH 6.8), 2 mm EDTA, 15% sucrose, 10% glycerol, 3% SDS, and 0.7 mM mercaptoethanol] or TNES buffer [50 mm Tris (pH 7.5), 1% NP40, 2 mm EDTA, 100 mm NaCl, 20 μg/ml leupeptin, 20 μg/ml aprotinin, and 1 mm phenylmethylsulfonyl fluoride]. Protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad), and a 10–100 μg protein sample was used for Western blot analysis. Proteins were transferred to Immobilon membranes (Millipore, Bedford, MA). Membranes were blocked for 1 h in 10% dried milk in TTBS [20 mm Tris (pH 7.6), 0.05% Tween 20, and 0.9% NaCl], probed for 1 h with the primary antibodies to detect p53 (Ab-2; Oncogene Science, Manhasset, NY), p21^Cip1/Waf1 (Ab-1; Oncogene Science), Mdm2 (Ab-1; Oncogene Science), Rb (clone G3; 2-45; Pharmingen, San Diego, CA), Bcl-2 (clone 124; Dako, Carpentaria, CA), and actin (Ab-1; Oncogene Science) as internal control. Subsequently, membranes were probed with horseradish peroxidase-labeled secondary antibodies (Amersham Corp., Arlington Heights, IL). Detection was performed using chemiluminescence, according to the manufacturer’s recommendations (DuPont NEN, Boston, MA).

**DNA Laddering Analysis.** Fragmentation of cellular DNA was measured 24 h after treatment as described (31). Adherent and nonadherent cells were pooled, washed in PBS, and resuspended in ice-cold buffer containing 5 mm Tris-HCl (pH 7.4), 20 mm EDTA, and 0.5% Triton X-100. Cells were lysed on ice for 2 h and then centrifuged at 3000 g for 30 min. The supernatants were treated with 0.5 mg/ml RNase A for 60 min at 56°C, followed by proteinase K (0.4 mg/ml) in the presence of 1% SDS for another 60 min at 56°C. The supernatant was extracted with phenol and chloroform, and DNA was recovered by ethanol precipitation. Samples were resuspended in 20 μl Tris-EDTA, and 10 μl were used for electrophoresis on 1.8% agarose gels.

**PCR Analysis.** Reverse transcription of 1 μg of total RNA using specific 3’ primers was performed using Moloney murine leukemia virus reverse transcriptase (Bio-Rad) as described previously (44). The cDNA was then amplified for 30 cycles. For bcl-2, the optimal annealing temperature was 60°C, and the magnesium concentration was adjusted to 2.0 mm. The bcl-2 forward primer was 5’-GGTGGCACCTGTTGGTTCCACCTG-3’; the bcl-2 backward primer was 5’-GGTCACTGTGCGCCCAGTAGG-3’ (45). For α-bax, the optimal annealing temperature was 55°C, and the magnesium concentration was adjusted to 2.5 mm. The bax forward primer was 5’-TGCTCTAGGTTTCTATTCCAGG-3’; the bax backward primer was 5’-TGGCAAGT-GAAAAGGGCCA-3’. All of the resulting cDNA products were used to determine bcl-2 expression or was diluted 1:64 to determine bax expression. The products were separated on a 2% NuSieve (FNC Bio Products, Rockland, ME) 1% agarose gel and quantitated by densitometry of the ethidium bromide-stained gel.

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**References**

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