Abstract: Background. In many tumors, the p53 gene has been mutated or deleted. p53 null mutant mice are prone to development of a variety of neoplasms at an early age. In head and neck cancer, p53 mutations are detected in most cases. p53 has been shown to induce growth arrest, differentiation, and death when overexpressed in cancer cell lines. p53 responds to DNA damage by arresting the cell cycle in G1 or G2 phase until repair can be completed. If DNA damage is severe, p53 may trigger programmed cell death by means of proapoptotic genes such as bax. Studies have suggested that p53 target genes must be intact for proper functioning of the tumor suppressor.

Methods. We stably expressed transcriptionally active p53 in head and neck squamous cell carcinoma (SCC) lines in which the endogenous gene was inactivated by mutation. We performed proliferation, cell death, cell cycle, and gene expression analysis in control clones and those treated with the DNA damaging agent etoposide.

Results. These clones proliferated slowly with accumulation of cells at the G1/S phase boundary but did not undergo growth arrest or apoptosis. Coexpression of the proapoptotic gene bax (a known target of p53) failed to induce apoptosis in these clones. However, p53 expression sensitized these cells to DNA damage–induced apoptosis by means of inhibition of bcl2 protein levels.

Conclusion. We concluded that the p53 apoptotic response to DNA damage was dependent on bcl2 but not bax in head and neck SCC lines.

Keywords: tumor suppressor; bcl2; cyclin-dependent kinase inhibitor; cell cycle; double strand break repair

The p53 gene is believed to be the most frequently mutated tumor suppressor in human cancer.1 p53 has been shown to suppress malignant transformation and block tumorigenesis. In many tumors, both p53 alleles have been mutated or deleted. Mutations in the p53 gene have been linked to Li-Fraumeni syndrome, an inherited cluster of cancers including those of soft tissue, bone, breast, brain, and bladder.2 p53 null mutant mice are prone to development of a variety of neoplasms by 6 months of age.3 These tumors were predominantly lymphomas but also included a number of sarcomas. In surgical specimens of head and neck cancer, p53 mutations were detected in 79% of cases.4 A higher incidence of p53 mutation has been detected in invasive
This study also detected sequential mutations of different exons that suggested accumulation of alterations during neoplastic transformation. Differences in codon mutations of the same exon between dysplastic lesions and cancer pointed to independent clonal development.

The p53 gene was localized to chromosome 17p13 spanning 20 kb, consisting of 11 exons and encoding a 2.9-kb mRNA. The p53 protein contains 393 amino acids, is localized to the nucleus, and is expressed in all cells. p53 is phosphorylated on several serine residues that may affect its nuclear localization. Most mutations in the p53 gene are missense mutations occurring in the highly conserved central domain of the protein.6 p53 has an acidic amino terminus containing the transcriptional activation domain, and the carboxyl terminus harbors nonspecific DNA binding, oligomerization, and nuclear localization motifs.

p53 has been shown to induce growth arrest when overexpressed in a number of cancer cell lines.7 p53 activation has also been shown to promote cell differentiation and death.8 p53 responds to DNA damage by arresting the cell cycle in G1 or G2 phase until repair can be completed. Cells lacking p53 or expressing a mutant form of the protein fail to arrest after DNA damage. p53 interacts with a variety of oncoproteins, many of which are of viral origin such as SV40 large T antigen and human papillomavirus E6 protein. SV40 large T antigen prevents p53 binding to DNA, whereas the E6 interaction promotes degradation by the ubiquitin-dependent proteasome pathway. Mdm2 induces rapid degradation of p53 by ubiquitination and subsequent proteolysis.9

p53 activates target genes that result in DNA damage repair, growth arrest, and apoptosis.10 Among these are growth arrest and DNA damage genes (GADD45), p21WAF1/Cip1, and bax. In cells in which the p21WAF1/Cip1 gene was disrupted, p53 was unable to induce growth arrest after DNA damage.11 These studies suggest that p53 target genes must be intact for proper functioning of the tumor suppressor. If DNA damage is severe, p53 may trigger programmed cell death by means of proapoptotic genes such as bax. Different p53 mutants have been shown to be defective in one or more transactivation, growth arrest, or apoptotic functions.12,13

Introduction of p53 into head and neck cancer cell lines using adenoviral vectors reportedly induced growth arrest and apoptosis14 and prevented establishment of tumors in animal models15 but had limited success in clinical trials. The reasons for these conflicting results may have been related to delivery of the adenovirus, number of cells transduced, p53 expression levels in transduced cells, or cellular resistance to antiproliferative and apoptotic effects of the tumor suppressor. To investigate this latter possibility, we examined the effects of reintroducing wild-type p53 expression to three head and neck squamous cell carcinoma (SCC) lines containing well-characterized mutations in this gene.16 We demonstrate that p53 did not induce growth arrest or cell death in these lines but increased levels of DNA damage–induced apoptosis that was dependent on bcl2 but not bax expression.

MATERIALS AND METHODS

Cell Culture and Stable Transfection. The human head and neck SCC lines used in this study were purchased from the American Type Culture Collection (Rockville, MD) and have been described previously.17 Cells were cultured in Dulbecco’s modified Eagle medium (DMEM), 10% fetal bovine serum, 40 μg/mL gentamicin at 37°C in a humidified atmosphere of 5% CO2. SCC9, SCC15, and SCC25 cells were transfected with 5 μg expression vectors for p53, bax, bcl2, or neomycin resistance plasmid alone using lipofectamine reagent according to manufacturer’s recommendations (Life Technologies, Carlsbad, CA). Cells were selected in 400 μg/mL G418 for 14 days. Resistant clones were picked for expansion and characterization.

Transient Transfection and Reporter Gene Analysis. Triplicate cultures of cells were transiently transfected with 5 μg p21WAF1/Cip1 promoter/reporter vector (kindly provided by Dr. Leonard Freedman) along with 2 μg p53 or blank expression plasmids using lipofectamine according to the manufacturer’s recommendations (Invitrogen, Carlsbad, CA). One microgram β-galactosidase expression plasmid was used to normalize for transfection efficiency. Cells were harvested and reporter gene activity determined using a commercially available kit (Roche, Bedford, MA). Luciferase activity was normalized to β-galactosidase levels for each sample.

Cell Death and Proliferation Assays. The terminal-transferase dUTP nick-end labeling (TUNEL) assay for in situ cell death detection was performed using a commercially available kit (Roche, Indianapolis, IN).
Some cultures were treated with 10 μg/mL etoposide to induce DNA strand breaks or 0.1% dimethyl sulfoxide (DMSO) vehicle for 24 hours. Cells were fixed in 4% paraformaldehyde (pH 7.4) and permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate for 2 minutes on ice. A mouse immunoglobulin M (IgM) anti-human Fas antibody (Molecular Biology Laboratories, Woburn, MA) that induces apoptosis in sensitive cell lines was used as the positive control. An isotype-matched control antibody was used as the negative control. After washing with phosphate-buffered saline (PBS), cells were incubated with fluorescein isothiocyanate (FITC)-conjugated Annexin V and terminal deoxynucleotidyl transferase (TdT) for 1 hour. After washing in PBS, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated Annexin V and terminal deoxynucleotidyl transferase (TdT) for 60 minutes at 37°C according to manufacturer's recommendations (Roche Molecular Biochemicals, Indianapolis, IN). After washing three times in PBS, apoptotic cells were visualized by fluorescence microscopy. The annexin-V assay to detect early apoptotic changes was performed using a commercially available kit (Roche Molecular Biochemicals). Annexin-V conjugated to fluorescein was incubated with the cells in N-2-hydroxyethylpiperazine-N’-R-ethanesulfuric acid (HEPES) binding buffer for 1 hour. After washing in PBS, apoptotic cells were visualized by fluorescence microscopy. Proliferation was analyzed by plating 5 × 10^4 cells in triplicate cultures for up to 6 days. Cells were trypsinized and counted with a hemocytometer at 2-day intervals. To determine G1 to S phase progression, bromodeoxyuridine (BrdU) incorporation analysis was performed. Cells were incubated with 10 μM BrdU for 1 hour, washed in PBS, and fixed in 70% ethanol containing 50 mM glycine (pH 2) for 20 minutes at −20°C. After washing in PBS, the cells were incubated with mouse anti-BrdU primary antibody for 30 minutes at 37°C according to manufacturer’s recommendations (Roche Molecular Biochemicals). The cells were washed in PBS and incubated with anti-mouse IgG secondary antibody conjugated to fluorescein. After extensive washing in PBS, the number of BrdU-positive cells were visualized by fluorescence microscopy and expressed as a percentage of the total cells counted in 10 random high-power fields. Statistical analysis was performed by t test.

**Western Blot.** Seventy-five micrograms total cellular protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% resolving gels under denaturating and reducing conditions. Separated proteins were electroblotted to polyvinylidene difluoride (PVDF) membranes according to manufacturer's recommendations (Roche Molecular Biochemicals). Blots were incubated with antibodies to human p53, bax, bcl2, bak, bid, p53 upregulated modulator of apoptosis (PUMA), GADD45, epidermal growth factor receptor (EGFR), c-met, cdk4, cyclin A, β-actin (Santa Cruz Biotechnology, Santa Cruz, CA), or p21WAF1/Cip1 (Transduction Laboratories, San Jose, CA) for 16 hours at 4°C. After washing in Tris-buffered saline containing 0.1% Tween 20 (TBST, pH 7.4), blots were incubated for 30 minutes at room temperature with anti-IgG secondary antibody conjugated to horseradish peroxidase. After extensive washing in TBST, bands were visualized by the enhanced chemiluminescence method (Roche Molecular Biochemicals). For cell fractionation studies, cells were lysed in hypotonic Tris buffer containing 0.5% Nonidet P-40 for 5 minutes at 4°C. The nuclei were obtained by centrifugation at 1000g for 10 minutes to obtain the mitochondrial fraction, which was washed extensively in lysis buffer. The remaining supernatant constituted the cytoplasmic fraction. All fractions were dissolved in 1× Laemmli buffer and subjected to Western blot as previously described.

**RESULTS**

The limited success of p53 gene therapy in head and neck cancer clinical trials may have been related to cellular resistance to antiproliferative and apoptotic effects of the tumor suppressor. To determine whether wild-type p53 was transcriptionally active in head and neck cancer cells, we cotransfected the p53 expression vector with a p21WAF1/Cip1 promoter–reporter construct into three lines (SCC9, SCC15, SCC25). These three head and neck cancer cell lines were previously shown to have inactivating mutations in the p53 gene. As shown in Figure 1A, the p53 vector induced p21WAF1/Cip1 promoter activity threefold compared with cultures transfected with blank expression plasmid (p < .0007). This level of induction was similar in all three cell lines. We also examined induction of the endogenous target gene GADD45 in response to wild-type p53 expression in SCC25 cells. As shown in Figure 1B, p53 stable expression produced fourfold induction in GADD45 protein levels. These results...
indicate that wild-type p53 is transcriptionally active in head and neck SCC lines containing mutant p53 proteins.

In previous studies, introduction of p53 into head and neck cancer cell lines induced growth arrest and apoptosis. When we stably selected p53-expressing clones from three SCC lines, we observed that the cells proliferated at only 60% of the control rate (p < .01; Figure 2A). However, we did not observe morphologic or molecular evidence of growth arrest, apoptosis, or terminal differentiation in these clones (data not shown and Figure 3). To determine whether reduced cellular proliferation correlated with inhibition of cell cycle progression, we performed BrdU incorporation analysis. As shown in Figure 2B, the percentage of BrdU-positive cells in p53-expressing clones was reduced by 50% compared with control cells (p < .0006). These results suggest that p53 expression decreased the number of cells entering S phase from G1. This correlated with reduced expression of the late S phase marker cyclin A (Figure 2C). Expression of the cyclin-dependent kinase cdk4 was also reduced. However, expression of the cyclin-dependent kinase inhibitor p21WAF1/Cip1 (a known p53 target gene) was strongly induced in these clones, which correlated with p53 protein levels. p53-mediated induction of p21WAF1/Cip1 gene expression correlated with increased p21WAF1/Cip1 promoter activity shown in Figure 1A. To examine possible mechanisms for the reduced proliferation of p53-expressing clones, we examined levels of growth factor receptors in these cells. Expression of EGFR and c-met (receptor for hepatocyte growth factor (HGF)/scatter factor) was reduced by more than 90% in p53-expressing clones (Figure 2C). These results suggest that p53 functions to inhibit cellular proliferation at the G1/S phase boundary by reducing growth factor receptor expression in SCC lines.

Among the p53 target genes that function to induce apoptosis after DNA damage is the proapoptotic bcl-2 family protein bax. Bax expression in the head and neck SCC lines used in this study was not detectable by Western blot (Figure 3A, top panel). We hypothesized that lack of bax expression may be responsible for the inability of wild-type p53 to induce apoptosis in SCC lines. We, therefore, created stable clones expressing bax, p53, or p53 and bax together. Expression of bax and p53 in these clones is shown in Figure 3A (top panel). To determine that bax protein was functionally active in these cells, we treated SCC25 stable clones with 10 μg/mL etoposide to induce a DNA damage apoptotic response followed by fractionation of these cells into nuclear, mitochondrial, and cytoplasmic components. As shown in Figure 3A (lower panel), bax protein was predominantly localized to the cytoplasm with low level expression in the mitochondrial fraction in vehicle-treated cells. However, etoposide treatment caused marked relocalization of bax protein to the mitochondrial fraction, consistent with its role in the mitochondrial cell death pathway. Bax expression was not detected in vector-transfected clones. Despite high p53 and bax expression, we did not observe increased numbers of apoptotic cells in bax or...
p53/bax clones as demonstrated by TUNEL and annexin assays (Figure 3B). By contrast, treatment with anti-Fas antibody resulted in 80% apoptotic SCC25 cells after 24 hours (positive control). We concluded that neither p53 nor bax mediated spontaneous apoptosis in head and neck SCC lines. To determine whether p53 or bax expression increased the susceptibility of SCC lines to DNA damage–induced apoptosis, we treated p53, bax, and p53/bax clones with 10 μg/mL etoposide or vehicle for 24 hours. As shown in Figure 3C, etoposide treatment resulted in a 50-fold increase in the number of apoptotic cells in control cultures. Bax expression did not result in significantly more apoptotic cells after etoposide treatment. However, etoposide treatment resulted in 70% apoptotic cells in p53- and p53/bax-expressing clones compared with 50% in control clones after 24 hours as measured by cell death assays ($p < .05$). These results indicate that bax expression alone does not significantly increase DNA damage–induced apoptosis in head and neck SCC lines. However, p53 sensitizes these cells to DNA damage, resulting in increased programmed cell death.

Previously, we demonstrated that head and neck SCC lines express bcl2, bak, and bid but not bax and bad.17 To determine whether these other bcl2 family members expressed by head and neck SCC lines could be responsible for p53-mediated DNA damage apoptosis, we examined expression of bcl2, bak, bid, and PUMA in p53, bax, and p53/bax stable clones. As shown in Figure 4A (top panel), p53 inhibited bcl2 expression by twofold to threefold in both SCC25 and SCC9 stable clones. p53 expression had no effect on bak and bid expression.
FIGURE 3. p53 and bax fail to induce apoptosis in squamous cell carcinoma (SCC) lines. (A) p53, bax, or both proteins (p53/bax) were expressed in SCC25 and SCC9 cells. Control cells were transfected with neomycin resistance vector alone. p53 and bax expression was determined by Western blot using the indicated antibodies (top panel). To determine whether bax was functionally responsive to apoptotic stimuli in SCC lines, cell lysates from vector and bax stable clones treated with etoposide or vehicle were separated into nuclear (N), mitochondrial (M), and cytoplasmic (C) fractions. These fractions were subjected to Western blotting using anti-bax antibody (bottom panel). These experiments were performed three times using independently isolated protein extracts. Representative blots are shown. (B) The clones just described (vector, p53, bax, p53/bax) were subjected to cell death assays to determine numbers of apoptotic cells as described in “Materials and Methods.” Anti-Fas–treated cells were used as the positive control. The percentage of TUNEL- (top panel) and annexin- (bottom panel) positive cells was determined by counting using fluorescence microscopy. (C) p53 but not bax sensitizes SCC25 and SCC9 cells to DNA damage–induced apoptosis. The clones described above (vector, p53, bax, p53/bax) were subjected to TUNEL and annexin assays after treatment with etoposide (+E) or vehicle to determine numbers of apoptotic cells as described in “Materials and Methods.” The percentage of TUNEL- (top panel) and annexin- (bottom panel) positive cells was determined by counting using fluorescence microscopy. These experiments were performed three times with similar results. Error bars represent standard error of the mean. Asterisks indicate statistically significant differences (p < .05).
levels, nor did bax affect expression of any bcl2-related protein. PUMA expression in these lines was below the limit of detection for Western blot. To determine whether bcl2 was functionally responsive to apoptotic stimuli in SCC lines, cell lysates from vector and bcl2 stable clones treated with etoposide or vehicle were separated into nuclear (N), mitochondrial (M), and cytoplasmic (C) fractions. These fractions were subjected to Western blotting using anti-bcl2 antibody (bottom panel). These experiments were performed three times using independently isolated protein extracts. Representative blots are shown.

**FIGURE 4.** p53 sensitizes head and neck squamous cell carcinoma (SCC) lines to DNA damage–induced cell death by inhibiting bcl2 expression. (A) Expression of additional bcl2 family members (bak, bid, bcl2) and the p53 target gene PUMA was determined by Western blot in vector, p53, bax, and p53/bax transfected SCC25 and SCC9 clones (top panel). Bcl2 and/or p53 expression vectors were stably transfected into SCC25 and SCC9 cells. Bcl2 and p53 expression in a representative SCC25 clone is shown (middle panel). To determine whether bcl2 was functionally responsive to apoptotic stimuli in SCC lines, cell lysates from vector and bcl2 stable clones treated with etoposide or vehicle were separated into nuclear (N), mitochondrial (M), and cytoplasmic (C) fractions. These fractions were subjected to Western blotting using anti-bcl2 antibody (bottom panel). These experiments were performed three times using independently isolated protein extracts. Representative blots are shown. (B) Bcl2 overexpression blocks p53-mediated increases in cell death after DNA damage. The clones described above (vector, p53, bcl2, p53/bcl2) were subjected to TUNEL and annexin assays after treatment with etoposide (+E) or vehicle to determine numbers of apoptotic cells as described in "Materials and Methods." The percentage of TUNEL- (top panel) and annexin- (bottom panel) positive cells was determined by counting using fluorescence microscopy. These experiments were performed three times with similar results. Error bars represent SEM. Asterisks indicate statistically significant differences (p < .05).
assays, p53 expression increased the number of apoptotic cells in response to etoposide as shown previously. However, bcl2 expression decreased the number of apoptotic cells in response to DNA damage (48% apoptotic cells in control vs 35% in bcl2 overexpressing cells; p < .05). Bcl2 also inhibited the p53-mediated DNA damage response (64% apoptotic cells in p53-expressing clones vs 50% in p53/bcl2 cells; p < .05). These results indicate that the p53-mediated increase in apoptosis resulting from DNA damage is mediated in part through inhibition of bcl2 expression in head and neck SCC lines.

DISCUSSION

In this study, p53 expression failed to induce growth arrest or apoptosis in head and neck cancer cell lines in which the endogenous gene was inactivated by mutation. Wild-type p53 was transcriptionally active and inhibited cellular proliferation by slowing the G1 to S phase transition. This inhibition was accompanied by changes in cell cycle regulatory protein expression, notably that of the cyclin-dependent kinase inhibitor p21WAF1/Cip1, a well-characterized p53 target gene.10,11 Changes in upstream signaling pathways included a dramatic decrease in EGFR expression. We previously demonstrated that EGFR inhibition decreases proliferation of head and neck SCC lines.20 Interestingly, the EGFR promoter has been shown to contain a consensus p53 binding site.21 It is not known whether this site is functional in head and neck SCC lines or whether it mediates the inhibitory effect of p53 on EGFR expression. Also it is worth noting that activation of an AP-1 site in the p21WAF1/Cip1 promoter decreased expression of the cyclin-dependent kinase inhibitor,22 suggesting that inhibition of upstream growth factor receptor signaling by p53 may be an important additional mechanism for inducing p21WAF1/Cip1 expression.

Some tumor-derived p53 mutations can abrogate specific functions of the protein. The 175P mutation can induce growth arrest in certain cells but lacks apoptotic function.12 Cell cycle arrest caused by overexpression of this mutant occurred primarily in G1 phase. Our data using wild-type p53 also indicated inhibition of the G1 to S phase transition. The 175P mutant also retained transcriptional activation function and the ability to induce target gene expression. Subsequently, a number of p53 mutants were characterized, some of which demonstrated normal cell cycle arrest and apoptotic functions, whereas others lacked both activities. Temperature-sensitive p53 mutants were also characterized that retained cell cycle arrest but were defective in transactivation and apoptotic functions. It has been proposed that lower levels of p53 expression dictate growth arrest, whereas higher levels promote apoptosis.23 However, we do not favor this reason for the lack of apoptosis in p53 transfected head and neck SCC lines given that levels of the tumor suppressor were up to 10-fold higher in these clones than control cells (Figure 2). Some cancer cell lines have been shown to be resistant to the apoptotic effects of p53.24 It was proposed that p21WAF1/Cip1 protected these cells from apoptosis but could not entirely account for the differential response to p53. These studies indicate the complex nature of p53 regulation of cell cycle and apoptosis in human cancer cells.

If DNA damage is severe, p53 may trigger programmed cell death by means of proapoptotic genes such as bax. Bax expression was undetectable by Western blot and not induced by p53 in the head and neck SCC lines used in this study (Figure 3). We hypothesized that bax deficiency in these lines may prevent p53-mediated induction of apoptosis. However, transfection of bax alone or in conjunction with p53 failed to induce apoptosis in the lines used in this study. p53 sensitized the cells to etoposide-induced DNA damage as seen in the increased numbers of apoptotic cells after drug treatment. However, bax expression did not produce enhanced apoptotic response to DNA damage alone or in conjunction with p53. Instead, p53 expression inhibited bcl2 protein levels in head and neck SCC lines. Forced overexpression of bcl2 inhibited etoposide-induced apoptosis and also the p53-mediated increase in apoptotic cells in response to DNA damage. We previously demonstrated that death receptor–mediated apoptosis (extrinsic pathway) in head and neck SCC lines was not dependent on bcl2 expression.25 The results of this study indicate that bcl2 has an important role in mediating apoptosis by means of the intrinsic pathway, specifically regulation of the p53-mediated response to DNA damage.

In summary, p53 overexpression results in cell cycle inhibition but not growth arrest and apoptosis in head and neck SCC lines. p53 also sensitizes these cells to DNA damage–induced apoptosis by means of inhibition of bcl2 protein levels, which is independent of bax expression. Future studies will examine the mechanisms for
failure of wild-type p53 to induce apoptosis in head and neck SCC lines.

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