Up-Regulation of the Proapoptotic Mediators Bax and Bak after Adenovirus-mediated p53 Gene Transfer in Lung Cancer Cells


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ABSTRACT

Overexpression of wild-type p53 in cancer cells by adenovirus-mediated p53 gene transfer can result in the induction of apoptosis. To identify the potential mediators of this p53-induced apoptosis, we examined apoptotic protein levels in human lung cancer cells after Adp53 gene transfer. We observed up-regulation of Bax and Bak protein levels 18–36 h after transduction with Adp53 in H1299, H358, and H322 lung cancer cells. Contrary to expected observations, no changes in Bcl-2 and Bcl-XL protein levels were observed. Morphological cell changes and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling staining showed evidence of apoptosis in all cell lines 48 h after transduction with Adp53. These results indicate that the induction of apoptosis by adenovirus-mediated p53 transfer may be mediated by the induction of proapoptotic mechanisms rather than suppression of antiapoptotic mechanisms.

INTRODUCTION

Adenovirus-mediated wild-type p53 gene transfer can lead to the regression of tumor both in vitro and in vivo (1). The antineoplastic mechanism of Adp53 may be attributable in part to the induction of apoptosis by wild-type p53 overexpression. Little is known, however, about the mechanism leading to apoptosis after adenovirus-mediated wild-type p53 gene transfer. Several studies have emphasized the Bcl-2 family of genes as important regulators of apoptotic cell death (2, 3). Bcl-2 and Bcl-XL are antiapoptotic members of this family, which function cytoprotectively by inhibiting the apoptosis pathway. Conversely, the Bcl-2 homologues Bax and Bak are proapoptotic regulators of apoptosis (4, 5). Several studies have suggested that p53 may regulate apoptosis through interaction with the Bcl-2 family (6, 7). Wild-type p53 has been reported to up-regulate Bax expression through direct transcriptional activation of the bax promoter with concomitant down-regulation of Bcl-2 (8). In addition, the induction of Bax by p53 was found to modulate up to 50% of p53-dependent apoptosis in an established tumor model (9). The extent and significance of these reactions in cancer cells after adenoviral-mediated p53 gene transfer has not been studied. We therefore evaluated the effects of adenovirus-mediated overexpression of wild-type p53 on these known mediators of apoptosis.

MATERIALS AND METHODS

Cell Culture. H358 and H1299 are non-small cell lung cancer cell lines with both copies of p53 deleted, and H322 is a non-small cell lung cancer cell with mutated p53 (gifts of A. Gazdar and J. Minna). Cells were maintained in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FCS and incubated at 37°C in a 5% CO2 incubator.

Adenovirus Production. The construction and properties of the replication-defective adenovirus, Adp53, have been reported elsewhere (10). The E1-deleted vector dl312 (kindly provided by T. Shenk, Princeton, NJ) was used as a control vector. Adenovirus was prepared as described previously (11). Viral titer was determined by UV-spectrophotometric analysis (viral particles/ml) and by plaque assay (pfu/ml). Adenovirus preparations were free of replication-competent adenovirus.

Gene Delivery. The MOI used in the in vitro transfection studies of all cell lines was based on cell counts of untreated plates. The MOI was chosen to result in ~70–80% transduction based on experiments using the Ad5/CMV/β-gal vector (MOI of 5 pfu for the H1299 cell line, 70 pfu for the H358 cell line, and 50 pfu for the H322 cell line).

Western Blot Analysis. Cells and cell lysates were collected at 0, 12, 18, 24, and 36 h after transduction with Adp53. Total cell lysates were prepared by lysing monolayered cells in dishes with SDS-PAGE sample buffer. Each lane was loaded

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4 The abbreviations used are: Adp53, adenoviral p53; MOI, multiplicity of infection; pfu, plaque-forming unit(s); TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling.
Apoptosis after p53 Gene Replacement

**RESULTS**

**Adp53 Infection Results in Overexpression of p53 Protein.** Expression of p53 protein in H1299, H358, and H322 cells was measured at 0, 12, 18, 24, and 36 h after Adp53 transduction by Western blot analysis. Control (mock-infected) cells and dl312-transduced (control vector) cells expressed no measurable p53 protein in H1299 and H358 cells (p53 null), whereas H322 cells (p53 mutant) revealed p53 expression in control and treated cells (Fig. 1). p53 protein was observed at the 12-h time point after transduction with Adp53. High expression at multiple phosphorylation states was observed at 24 and 36 h.

**Adp53 Transduction Results in the Induction of Apoptosis.** Apoptotic changes in cell morphology, including cell shrinkage and floating cells, were observed in cells treated with Adp53. This induction of apoptosis was quantitated at 48 h after transduction with Adp53. By flow cytometry, there was an increase in the subdiploid population of cells, consistent with those cells undergoing DNA fragmentation (Fig. 2A). This corresponded to an increase in cells labeled by the TUNEL assay (Fig. 2B). These data are consistent with increases in apoptotic cell death.

**Overexpression of p53 Results in the Induction of Pro-apoptotic Bax and Bak Proteins.** Bax protein levels were detectable in control and dl312-transduced cells. Transduction with Adp53 resulted in increased levels of Bax protein in all cell lines. This was especially evident 24–36 h after transduction (Fig. 3A). In H1299, H358, and H322 cells, peak induction of Bax occurred at 36 h, which was measured as a 6-, 4-, and 6-fold increase, respectively. Similarly, Bak protein expression was detectable by Western blot analysis in control and dl312-infected cells. After transduction with Adp53, a significant increase in Bak protein levels was also observed compared with controls, with peak levels present at 24–36 h (Fig. 3B). At 36 h, there was a 7-, 5-, and 11-fold induction of Bak in H1299, H358, and H322 cells, respectively. This induction of proapoptotic mediators occurred after initial overexpression of p53 but prior to the changes in cell morphology indicative of terminal apoptosis.

**Adp53 Transduction Did Not Affect Bcl-2 or Bcl-XL Expression.** No significant changes in the levels of Bcl-XL or Bcl-2 proteins were observed by Western blotting in Adp53-transduced versus control or dl312-transduced cells (Fig. 4).

**DISCUSSION**

Adenovirus-mediated wild-type p53 gene replacement induces apoptosis and increases the sensitivity of cancer cells to chemotherapy and radiation-induced apoptosis (10, 12). Apoptosis in this context appears to be related to p53-dependent mechanisms; however, the process by which p53 mediates apoptosis is incompletely understood. Miyashita and Reed (8) have demonstrated a p53 consensus binding site in the promoter region of the proapoptotic Bax gene and observed an increase in Bax mRNA and protein expression after induction of p53. However, the observed induction of p53-dependent apoptosis in Bax-knockout mice clearly indicates that other pathways or proteins may also be involved (12). Bak, a Bcl-2 homologue, is expressed in a variety of tissues and has been demonstrated to induce apoptosis independently of Bax expression (4, 13). Our findings of accumulation of Bak protein, in addition to Bax, in cells with adenovirus-mediated overexpression of wild-type p53 may be an additional mechanism by which p53 can induce apoptosis. We are unaware of this observation being reported previously.

Another mechanism by which p53 has been postulated to induce apoptosis is by the down-regulation of Bcl-2. Miyashita et al. (14) have reported a down-regulation of Bcl-2 in response...
Fig. 2  Induction of apoptosis by Adp53 gene transfer. Cells were trypsinized and fixed at 48 h after treatment with the control vector dl312 or media (control) or Adp53. A, flow cytometry analysis by propidium iodide exclusion, demonstrating the subdiploid cell population. B, flow cytometry analysis of TUNEL labeling. Transduction with Adp53 resulted in an increase in the subdiploid cell population and TUNEL-labeled cells consistent with increases in apoptotic cell death.

Fig. 3  Western blot analysis of Bax and Bak expression after transduction with Adp53. Nontransduced cells, dl312-transduced cells, and Adp53-transduced cells were collected and evaluated by Western blot analysis using polyclonal antibody against Bax (A) and monoclonal antibody against Bak (B). Fifty μg of protein were analyzed by SDS-PAGE and visualized by Western blotting using the ECL chemiluminescence system. Protein loading is illustrated by the Coomassie-stained protein shown below the Western blot of each cell line. Bax was detected as a Mr 21,000 protein, and Bak was detected as a Mr 25,000 protein as expected.

Fig. 4  Western blot analysis of Bcl-2 and Bcl-XL expression after transduction with Adp53. Control nontransduced cells, dl312-transduced cells, and Adp53-transduced cells were collected and evaluated by Western blot analysis using monoclonal antibodies against Bcl-2 (A) and Bcl-XL (B). Fifty μg of protein were analyzed by SDS-PAGE and visualized by Western blotting using the ECL chemiluminescence system. Protein loading by the Coomassie stain is shown below the Western blot of each cell line.
to p53 expression, creating a cellular microenvironment favorable to the initiation of p53-dependent apoptosis. A more recent report has demonstrated an increase in Bcl-XL expression after wild-type p53 transfer in the human colorectal cancer cell line HT29, explaining the lack of p53-induced apoptosis in these cells (15). In the present study, adenovirus-mediated overexpression of wild-type p53 did not result in significant changes in Bcl-2 or Bcl-XL protein levels. This may be attributable to the high levels of p53 expression after Adp53 gene transfer or simply because of differences in response between lung cancer cells and other cancer cells studied previously.

Much of the cytotoxicity of chemotherapy and radiation therapy is attributable to the induction of apoptosis, and we have observed that concomitant administration of Adp53 augments sensitivity to cisplatin and radiation (16, 17). Interestingly, Bax expression has been reported to increase cellular sensitivity to chemotherapy and radiation therapy, whereas other investigators observed that Bax deficiency correlated with drug resistance and attenuated p53-dependent apoptosis (18–20). Induction of proapoptotic mediators such as Bax and Bak may therefore be one of the mechanisms mediating the observed increase in the sensitivity of cancer cells to chemotherapy and radiation therapy after Adp53 gene transfer.

Apoptotic cell death is postulated to occur by a cascade of events that suggest temporal activation of upstream and downstream mediators. In this study, we observed overexpression of p53 at 12 h, followed by peak up-regulation of Bax and Bak at 24–36 h after transduction with Adp53. Changes in cell morphology indicative of apoptosis occurred later, between 36 and 48 h. These observations support the temporal induction of proapoptotic mediators after the p53 death signal, ultimately culminating in apoptotic cell death.

In summary, we have demonstrated that adenovirus-mediated overexpression of wild-type p53 induces the proapoptotic Bax and Bak proteins. Levels of the antiapoptotic proteins Bcl-2 and Bcl-XL were unaffected. These results suggest that the cytotoxic effects of Adp53 may be secondary to induction of proapoptotic mechanisms rather than suppression of antiapoptotic mechanisms. Strategies to enhance the apoptotic pathway driven by Bax and Bak may augment the therapeutic effects of adeno-viral-mediated p53 gene transfer.

REFERENCES


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