Cytotoxic Effects of Ad5CMV-p53 Expression in Two Human Nasopharyngeal Carcinoma Cell Lines

Jian-Hua Li, Peixiang Li, Henry Klamut, and Fei-Fei Liu

Departments of Radiation Oncology [F-F. L.] and Research [J-H. L., P. L., H. K., F-F. L.], Princess Margaret Hospital/Ontario Cancer Institute, and Departments of Radiation Oncology [F-F. L.] and Medical Biophysics [P. L., H. K., F-F. L.], University of Toronto, Toronto, Ontario, M5G 2M9 Canada

ABSTRACT

Nasopharyngeal carcinoma (NPC) is a malignant disease of the head/neck region with a 5-year survival level of approximately 65%. To explore novel therapeutic strategies in the management of this disease, the potential of Ad5CMV-p53-mediated gene transfer to NPC cells was investigated in vitro. Two NPC cell lines, CNE-1 and CNE-2Z, were infected with either Ad5CMV-p53 or AdSCMV-β-galactosidase and evaluated for transduction efficiency and cytotoxicity. At a multiplicity of infection of 50 plaque-lactosidase and 3-galactosidase expression were detected in almost 65%. To explore novel therapeutic strategies, they demonstrated significantly lower in vitro. 

INTRODUCTION

NPC is a malignant disease of the head/neck region that is endemic in Southeast Asia and has an annual incidence of up to 25-50/105. It affects predominantly a young population, with the median incidence age in the fourth decade (1). Currently, the only proven curative modality is ionizing radiation, but the 5-year overall survival is still only around 65% (1, 2). This is clearly a disease site whereby novel experimental therapeutic strategies need to be developed. Recent reports have demonstrated that expression of a wild-type p53 gene from an adenovirus vector results in significant toxicity in a number of human tumor cell lines and xenografts (3-10). Hence, this prompted us to examine the potential of this therapeutic strategy for NPC.

There is currently controversy with regard to p53 status in human NPC. The majority of reports indicate that the p53 gene is not mutated in the primary tumor but is more likely to be mutated in metastases and derived cell lines (11-15). However, two recent reports that examined p53 expression using immunohistochemical techniques indicate that the majority of human NPCs overexpress the p53 protein (16, 17). The etiology of NPC remains to be elucidated, but there is the almost constant association with EBV, whose protein products have been demonstrated to bind p53 (18-20). The functional significance of this protein binding is not clear but might in part account for the conflicting observations with regard to p53 in human NPC. Introduction of a recombinant human wild-type p53 gene using the adenovirus route seems to be most toxic in tumors with null or mutated p53 (3-7, 9, 10), but in some cases, has also been shown to be cytotoxic in tumors expressing wild-type p53 (5-7).

One of the major difficulties in the clinical application of gene therapy in cancer treatment is efficient delivery of the vector-gene product to the tumor site. The nasopharynx can potentially be amenable to the introduction of exogenous gene products through either an aerosol route or direct infiltration. The reality of these theoretical advantages remains to be proven with conflicting observations with regard to p53 in human NPC.
MATERIALS AND METHODS

Cells and Culture Conditions. The NPC cell lines CNE-1 (21) and CNE-2Z (22) were obtained from the Cancer Institute/Chinese Academy of Medical Sciences in China. Both cell lines have identical mutations in the p53 gene within exon 8, resulting in a change in the nucleotide sequence from AGA to ACA at codon 280 (12, 13). The adenovirus was propagated in the human embryonic kidney cell line 293 (American Type Culture Collection); purity of adenovirus was tested using the Ad5CMV-3-galactosidase (0.1–50 plaque-forming unit/cell) in medium containing 2% FBS. One h later, the serum concentration was increased to 10%, and cells were incubated for an additional 48 h. Cells were washed with PBS, fixed with 2% formaldehyde and 0.2% glutaraldehyde for 5 min at 4°C, and stained with β-galactosidase. Blue staining in cells indicated expression of β-galactosidase; infection efficiency was evaluated by the ratio of the number of positively and negatively stained cells.

Western Blot Analysis of p53, WAF1/CIP1, bcl-2, and bax Proteins. Cells (5 × 10^5) were plated in T-25 flasks (Falcon, Franklin Lakes, NJ) and infected with Ad5CMV-p53/Ad5CMV-β-galactosidase ranging from 1–50 plaque-forming unit/cell as described above. At each time point after infection (4, 8, 24, and 48 h), cells were harvested on ice, washed three times with PBS, and lysed in a lysis buffer (0.1 M Tris (pH 8.0), 1% SDS, 10 mM EDTA, and 2 mM DTT). The cell lysate was homogenized by 10 cycles of aspiration/expiration using a 1-ml syringe with a 21-gauge needle, then boiled for 2 min, and centrifuged at 14,000 rpm for 2 min (Eppendorf centrifuge; Brinkmann Instruments, Inc.). The protein concentration in the supernatant was determined using a bicinecinonic acid protein assay kit (Pierce, Rockford, IL). Samples containing 30 μg of protein were denatured by boiling (for 5 min) in loading buffer (20% glycerol, 5% β-mercaptoethanol, 4% SDS, 125 mM Tris (pH 6.8), and 0.1 mg of bromphenol blue) and then electrophoresed on a 10% SDS-PAGE for 120 min at 100 V (Bio-Rad, Richmond, CA). The protein was transferred onto a nitrocellulose membrane using a trans-blot semi-dry cell (Bio-Rad). The membrane was blocked using PBST (0.1% Tween 20 in PBS) containing 5% low-fat milk for 60 min at room temperature and probed with the primary antibodies as follows: 1 μg/ml mAb for p53 (Ab-6; Oncogene Science); 1 μg/ml mAb for WAF1/CIP1 (Ab-1; Oncogene Science); 1 μg/ml mAb for bcl-2 (Ab 124; DAKO, Glostrup, Denmark); 1 μg/ml mAb for bax (Ab 4F11; Immunotech, France) in PBST with 5% low-fat milk. The blot was then washed with PBST and incubated with horseradish peroxidase conjugated to a secondary antibody. The specific complexes were detected by the chemiluminescence reagent (DuPont New England Nuclear, Boston, MA), and relative levels of p53, WAF1/CIP1, bcl-2, and bax proteins were evaluated using a computerized densitometer scanner (Molecular Dynamics, Sunnyvale, CA).

Effect of Ad5CMV-p53 on Cell Growth. To evaluate the effect of the adenovirus vector on cell growth, 1 × 10^5 cells were seeded onto 6-well plates (Nunc, Inc.). After 24 h, cultures were infected with either Ad5CMV-p53 or Ad5CMV-β-galactosidase at various MOI (1, 10, 25, and 50 plaque-forming unit) in 0.5 ml of medium containing 2% FBS at 37°C for 1 h. Subsequently, 3.5 ml of medium with 10% FBS were added to each well, and the infected cultures were incubated for 3 h. Cells were then trypsinized and plated onto 96-well plates (Nunc, Inc.) at cell densities of 1.5 × 10^3/well for CNE-1 and CNE-2Z cells and
3 × 10^3/well for GM38 fibroblasts. The effect of the adenovirus vectors on cell growth was assessed using a MTT (Sigma) assay (29). In brief, MTT was dissolved in PBS at 5 mg/ml, and a sterile filtered 20-μl aliquot of MTT stock solution was added to each well and incubated at 37°C for 3 h. Acid-isopropanol with 0.04 N HCl was added to each well and mixed thoroughly to dissolve the blue MTT formazan crystals. The plates were read on a Bio-Rad 3350 microplate reader at a wavelength of 570 nm.

Serially increasing cell numbers from 10^6.4 to 10^7 cells/dish. The dishes were then incubated in a 37°C/5% CO₂ incubator for 7–10 days. Staining was conducted using methylene blue in 50% ethanol, and the number of colonies in each stained dish was subsequently counted.

Clonogenic survival was assayed at the same time by reseeding the cells onto 100-mm dishes at a density of 10^2–10^4 cells/dish. The dishes were then incubated in a 37°C/5% CO₂ incubator for 7–10 days. Staining was conducted using methylene blue in 50% ethanol, and the number of colonies in each stained dish was subsequently counted.

**DNA Fragmentation Analysis.** After infection with Ad5CMV-p53 or Ad5CMV-β-galactosidase at various time intervals, cells were harvested and washed with PBS. The cell pellets were resuspended in 200 μl of 5 M guanidium containing 0.1 M 2-mercaptoethanol and 100 μl of 7.5 M ammonium acetate, followed by 600 μl of 100% ethanol. The samples were then placed on ice for at least 2 h. DNA was precipitated by centrifugation at 14,000 rpm for 30 min (4°C) in the Eppendorf centrifuge and washed twice using 75% ethanol. DNA extracts were dissolved in Tris-EDTA buffer (10 mM Tris, 1 mM EDTA (pH 8.0)) and subjected to electrophoresis on a 2% agarose gel; DNA bands were visualized using EB staining.

**Results**

**Ad5CMV-p53 and Ad5CMV-β-galactosidase Vectors.** To ensure that the adenovirus vector was incapable of replication in nonpermissive cells, consecutive infection tests were performed by treating HeLa cells with the purified adenovirus vector. The second and the third sets of HeLa cell cultures were infected by the first and second sets of infected cell extracts, respectively. No cytopathic effect was observed in the second and third rounds of tested HeLa cell cultures. PCR analysis indicated that the purified Ad5CMV-p53 contained p53 cDNA but was devoid of E1 sequences, and no viral DNA was detected after the first round of Ad5CMV-p53 infection. In contrast, the wild-type adenovirus-infected group, viral DNA replication was detected in all three rounds of HeLa cell cultures, and PCR also detected the E1 sequences (data not shown). Plaque assay

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**Fig. 1** X-galactosidase staining for β-galactosidase expression in Ad5CMV-β-galactosidase infection in CNE-1, CNE-2Z, and GM38 cells. Cells were exposed to either 50 pfu/cell of Ad5CMV-β-galactosidase or medium alone for 48 h. A, CNE-1 control; B, CNE-1 + Ad5CMV-β-galactosidase (50 pfu/cell); C, CNE-2Z control; D, CNE-2Z + Ad5CMV-β-galactosidase (50 pfu/cell); E, GM38 control; F, GM38 + Ad5CMV-β-galactosidase (50 pfu/cell).
demonstrated that the range of titers of the purified viral vectors was between $10^9$–$10^{10}$ pfu/ml.

**Ad5CMV-β-galactosidase-mediated Expression of β-galactosidase.** To examine the infection efficiency and transgene expression of the adenoavirus vector in CNE-1, CNE-2Z, and GM38 cells, β-galactosidase activity was assessed in cells infected with Ad5CMV-β-galactosidase. As demonstrated in Fig. 1, all three cell lines stained positively; the proportion of stained cells correlated with MOI in the range of 0.1–50 pfu/cell of Ad5CMV-β-galactosidase (data not shown). However, the proportion of stained cells differed between cell lines infected with the same dose of Ad5CMV-β-galactosidase. Positive staining was detected in both CNE-1 and CNE-2Z cells at 0.1 pfu/cell of Ad5CMV-β-galactosidase infection. At 25 pfu/cell of Ad5CMV-β-galactosidase, more than 90% of CNE-1 cells demonstrated positive X-galactosidase staining. However, 50 pfu/cell of Ad5CMV-β-galactosidase were required to infect CNE-2Z cells to the same level. In contrast, positively stained GM38 cells were first observed at a MOI of Ad5CMV-β-galactosidase of 1 pfu/cell and reached 50% at 50 pfu/cell of Ad5CMV-β-galactosidase. The intensity of X-galactosidase staining was greater in NPC cells as compared to GM38 fibroblasts, with CNE-1 cells displaying the most intense staining (Fig. 1; Table 1). No positively stained cells were observed in mock-infected cultures.

**Ad5CMV-p53-mediated p53 Expression.** To investigate the extent that Ad5CMV-p53 can mediate expression of a p53 minigene in infected cells, each cell line was exposed to a series of MOI of Ad5CMV-p53 or Ad5CMV-β-galactosidase for 4, 8, 24, and 48 h, and p53 protein expression was analyzed.
Fig. 3  Effect of Ad5CMV-p53 on cell growth. Cells were infected with Ad5CMV-p53 at MOIs of 1, 10, 25, and 50 pfu/cell or Ad5CMV-β-galactosidase at 50 pfu/cell. Culture medium was used as mock infections. Cells were seeded in quadruplicate in 96-well plates. Cell proliferation was analyzed by using the MTT assay on each day after infection, until day 5. Each data point represents the mean ± SE from three separate experiments. A, CNE-1 cells; B, CNE-2Z cells; C, GM38 cells, in which the Y axis has been magnified to facilitate data interpretation.

by Western blotting. As shown in Fig. 2A, all three cell lines have low levels of endogenous p53 expression, and there was a dose- and time-dependent pattern of recombinant p53 expression. Increasing levels of recombinant p53 expression were observed in all cell lines by 8 h after infection with 50 pfu/cell of Ad5CMV-p53. Recombinant p53 expression peaked at 24 h in NPC cells, with more than 800-fold induction in CNE-1 and 200-fold induction in CNE-2Z cells. In contrast, a 100-fold induction in recombinant p53 expression was observed in GM38 fibroblasts at the 48-h time point, although note that only 50% of GM38 cells were apparently infected with Ad5CMV-p53 (Fig. 1). Cells infected with Ad5CMV-β-galactosidase (50 pfu/cell) did not demonstrate p53 induction.

Effect of Ad5CMV-p53 on WAF1/CIP1 Expression. To investigate whether the virus-encoded p53 protein was biologically active, we evaluated the induction of WAF1/CIP1 expression. Western blot analysis demonstrated low basal levels of WAF1/CIP1 expression in both NPC cell lines (Fig. 2A). A dose-dependent increase in WAF1/CIP1 expression was observed after exposure to Ad5CMV-p53; 1 pfu/cell of Ad5CMV-p53 was sufficient to induce a significant increase in WAF1/CIP1 expression in both NPC cell lines (30- and 11-fold increase in CNE-1 and CNE-2Z cells, respectively). WAF1/CIP1 expression peaked at 24 h postinfection in both NPC cell lines and declined by 48 h. Interestingly, by 48 h, WAF1/CIP1 expression in cells infected at 50 pfu/cell was lower for both NPC cell lines than when infected with 10 pfu/cell.

Effect of Ad5CMV-p53 on bcl-2 and bax Expression. In an attempt to obtain some understanding of the mechanism of cytotoxicity of Ad5CMV-p53 infection on NPC cells, expression of gene products that may mediate apoptosis, downstream of p53, were analyzed using Western blotting. Specifically, we studied bcl-2 and bax expression (Fig. 2B). Both NPC cell lines demonstrated minimally detectable levels of bcl-2 and bax at baseline, but infection with either Ad5CMV-β-galactosidase (50 pfu/cell) or Ad5CMV-p53 (up to 50 pfu/cell) had no observable effect on expression of either gene product.

Effect of Ad5CMV-p53 on NPC Cell Growth. The cytotoxic effects of Ad5CMV-p53 on NPC cells were assessed using MTT and clonogenic assays. As shown in Fig. 3, infection of NPC cells with Ad5CMV-p53 from 1–50 pfu/cell resulted in a dose-dependent inhibition of cell growth in both NPC cell lines. When exposed to 50 pfu/cell of Ad5CMV-p53, complete inhibition of cell growth was observed in the CNE-1 cells, and 90% inhibition was observed in CNE-2Z cells at 5 days postinfection (Fig. 3A and B). In contrast, GM38 fibroblasts were resistant to the cytotoxic effects of Ad5CMV-p53 because more than 70% of cells continued to proliferate 5 days after treatment with 50 pfu/cell of Ad5CMV-p53 (Fig. 3C). Cells treated with Ad5CMV-β-galactosidase (50 pfu/cell) displayed little effect on growth (Fig. 3).

Fig. 4 demonstrates that Ad5CMV-p53 infection results in a significant reduction in clonogenicity in NPC cells. The probability of survival for CNE-1 and CNE-2Z cells was 0.35 and 11% relative to that of control (uninfected) cells after exposure to 25 pfu/cell of Ad5CMV-p53. Exposure of GM38 fibroblasts to the same dose of Ad5CMV-p53 resulted in only a slight decline in survival (Fig. 4 and Table 1). Despite the fact that the end points of these two assay systems are different (the MTT assesses biochemical viability, and the clonogenic assay determines the potential of viable cells to form colonies), their results are consistent in a dose-dependent manner for all three cell lines.

DNA Fragmentation Analysis. To confirm that the cells have undergone apoptosis after Ad5CMV-p53 treatment, DNA fragmentation analysis was performed by agarose gel electrophoresis. The analysis demonstrated that DNA fragments (180–200 bp) were present in both NPC cell lines (Fig. 5). The DNA ladder was evident by 24 h postinfection for CNE-1 cells and 48 h in CNE-2Z cells after treatment with 50 pfu/cell of Ad5CMV-p53. No fragmented DNA was observed in mock-
infected controls, cells treated with Ad5CMV-β-galactosidase, or GM38 fibroblasts (data not shown).

**Morphological Analysis of Apoptosis.** Morphological changes characteristic of apoptosis were investigated using AO-EB fluorescence staining after Ad5CMV-p53 or Ad5CMV-β-galactosidase treatment. The hallmarks of apoptosis, including chromatin condensation, loss of the nuclear envelope, and cellular fragmentation into apoptotic bodies were observed in both NPC cells 2 days postinfection with Ad5CMV-p53 at 50 pfu/cell (Fig. 6, E and F). No morphological changes were observed in mock-infected NPC cells or cells treated with Ad5CMV-β-galactosidase (Fig. 6, A–D).

**DISCUSSION**

In this study, we demonstrated that Ad5CMV-p53 was effectively cytotoxic against two human NPC cell lines. Infection with Ad5CMV-p53 resulted in high levels of recombinant p53 expression (and WAF1/CIP1), leading to cell death mediated through an apoptotic pathway. These data are similar to those of other groups that have tested this strategy in a myriad of human cancer cell lines, including melanoma (8), head/neck squamous cell (5, 7), breast (6, 10), ovarian (4), colorectal (3), and prostate cancers (9). The consistent observation is that recombinant p53 expression is increased after infection, and apoptosis seems to be responsible for the cytotoxicity. However, our data do raise some novel observations with regard to specific aspects of this gene therapy strategy.

The precise mechanisms of Ad5CMV-p53-mediated cytotoxicity are not well understood. It is known that p53 is involved in regulation of apoptosis and that an increase in its level can propel cells down an apoptotic pathway (31). It is logical that this technique of reconstituting normal p53 function in cancer cells with mutated p53 would be effective, and this has been demonstrated in a number of situations (4–7, 9, 10). Interestingly, however, this technique also seems to be effective against human cancer cell lines or tumors that express wild-type p53, although this seems to be less effective than when compared to cells or tumors with null or mutated p53 (5–7). Why this strategy is cytotoxic to cells expressing wild-type p53 is not clear, but this underscores the complexity of the mechanisms involved in Ad5CMV-p53-mediated cytotoxicity.

Our data illustrated that even in two human cancer cell lines with the same histological origin (NPC) and with identical p53 mutations (12), infection with Ad5CMV-p53 resulted in a 30-fold difference in cytotoxicity (Fig. 4 and Table 1). We have tested the sensitivity of these two NPC cell lines after exposure to three cytotoxic modalities: ionizing radiation, heat, and Cisplatinum, and found no significant differences between them, with the exception that CNE-1 seems to be slightly more sensitive to each cytotoxic maneuver as compared to CNE-2Z cells.4 We have preliminary data suggesting that differences in infection efficiency between the two NPC cell lines may account for the variation in cytotoxicity, although the reason for the difference in incorporation efficiency currently eludes us.
Fig. 6 Morphological analysis of apoptosis. NPC cells infected with 50 pfu/cell Ad5CMV-p53 or Ad5CMV-β-galactosidase were stained by AO-EB and then examined at 48 h postinfection under fluorescent microscopy for morphological changes indicative of apoptosis. A, CNE-1 control; B, CNE-2Z control; C, CNE-1 + Ad5CMV-β-galactosidase (50 pfu/cell); D, CNE-2Z + Ad5CMV-β-galactosidase (50 pfu/cell); E, CNE-1 + Ad5CMV-p53 (50 pfu/cell); F, CNE-2Z + Ad5CMV-p53 (50 pfu/cell).

Our study demonstrated that Ad5CMV-p53 infection in NPC cell lines resulted in an increase in recombinant p53 expression, which in turn induced WAF1/CIP1 production. The one group that has published data on WAF1/CIP1 expression after Ad5CMV-p53 infection did not conduct any kinetics experiments (6). We demonstrate that there is a time- and dose-dependent response in WAF1/CIP1 expression after introduction of the Ad5CMV-p53 gene, but by 48 h postinfection with Ad5CMV-p53 (50 pfu/cell), there was a significant decline in WAF1/CIP1 levels, particularly in the more sensitive CNE-1 cells. We do not have a clear explanation for this observation. However, it was evident that by 48 h, the majority of CNE-1 cells have lifted off the dish, DNA fragmentation was at its highest intensity (Fig. 5), and <1% of the cells displayed clonogenic potential (Fig. 4). In other words, most of the cells have died, so that presumably the machinery required to manufacture WAF1/CIP1 was no longer functioning and may therefore account for the decline in WAF1/CIP1 levels.

In an attempt to obtain some understanding of the complex mechanisms of Ad5CMV-p53-mediated cytotoxicity in the two NPC cell lines, we also evaluated the relative expression of bcl-2 and bax. These two gene products belong to the bcl-2 family, which has been implicated as mediators of p53-induced apoptosis, in that the ratio of bcl-2:bax can apparently influence the likelihood of cells to undergo apoptosis (32). Excess bax will promote apoptosis; excess bcl-2 can inhibit apoptosis. Our results demonstrated that Ad5CMV-p53 infection did not alter either bcl-2 or bax expression, indicating that neither protein seems to be an important mediator of apoptosis for the NPC cell lines in this study.

A number of groups have noted a differential sensitivity with this strategy in that there seems to be preferential cytotoxicity to cancer cells, with relative sparing of normal cells (6, 7, 10). Our data demonstrated that there was up to 200-fold greater resistance (Table 1 and Fig. 4) when the cells were treated with Ad5CMV-p53 at 25 pfu/cell between the NPC cells and the GM38 fibroblasts. The mechanisms behind the sparing of cytotoxicity of normal cells are also not known, but we suggest that viral incorporation efficiency may in part account for this difference. When we evaluated β-galactosidase expression by X-galactosidase staining (Fig. 1 and Table 1), it was evident that the least staining was observed in the GM38 fibroblasts; the most intense staining occurred with the CNE-1 cells, with slightly less intense staining seen in the CNE-2Z cells. Viral incorporation efficiency, however, likely does not explain the entire picture because there is a 200-fold difference in cytotoxicity between the GM38 and CNE-1 cells, but there is not that degree of difference in X-galactosidase staining intensity between the cells. Seth et al. have estimated the number of adenoviral receptors using 35S labeling and observed undetectable activity in human donor bone marrow cells as opposed to very high receptor numbers in several human breast cancer cell lines (10). The same group, in another report, evaluated β-galactosidase activity using a calorimetric assay but found no correlation between β-galactosidase activity and sensitivity to Ad5CMV-p53 infection (6). Clayman et al. (7) demonstrated that F138 human fibroblasts infected with Ad5CMV-p53 did not demonstrate growth inhibition despite recombinant p53 expression (assayed using Western blot), but their blot suggested that recombi-
nant p53 protein level in F138 fibroblasts was less than that of the human squamous cell carcinoma cell line Tu-138. This suggests the possibility that viral incorporation efficiency may be lower in the fibroblasts compared to the cancer cell line (7).

The relevance of this therapeutic strategy to treatment of NPC in human patients remains to be determined. As stated earlier, p53 gene mutations seem to be relatively rare in human NPC (11-15), but immunohistochemical evaluation suggests that p53 is overexpressed in more than 50% of NPCs (16, 17). What is clear, however, is the ubiquitous association of EBV with NPC (33). It is uncertain whether EBV-encoded proteins (BZLF1; EBNA-5) can bind to p53 in vitro (18-20), although the functional significance of this association is currently unclear. The NPC cell lines that we have studied in this current work have shed their EBV, hence, verification of the potential clinical application of this strategy would require the demonstration, in progressively more complex experimental models, that NPC tumors with retained EBV are sensitive to the cytotoxic effects of this therapeutic maneuver.

REFERENCES

Cytotoxic effects of Ad5CMV-p53 expression in two human nasopharyngeal carcinoma cell lines.

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