Tumor-targeted Gene Therapy for Nasopharyngeal Carcinoma

Jian-Hua Li, Marie Chia, Wei Shi, D. Ngo, Craig A. Strathdee, Dolly Huang, Henry Klamut, and Fei-Fei Liu

ABSTRACT

The unique feature of human nasopharyngeal carcinoma (NPC) is its almost universal association with the EBV, which is expressed in a latent form exclusively in cancer cells, and not in the surrounding tissues. We have exploited this differential by constructing a novel replication-deficient adenovirus vector (ad5oriP) in which transgene expression is under the transcriptional regulation of the family of repeats domain of the origin of replication (oriP) of EBV. When EBNA1, one of the latent gene products of EBV, binds to the family of repeats sequence, this activates transcription of downstream genes. Vector constructs were made using the β-galactosidase and luciferase reporter genes (ad5oriPβgal and ad5oriPluc) or the p53 tumor suppressor gene (ad5oriPp53). 5-Bromo-4-chloro-3-iodo-β-D-galactopyranoside staining demonstrated extensive expression only in EBV-positive NPC cells, specifically in response to the presence of EBNA1. The relative expression sequence between EBV-positive and -negative cell lines is approximately 1000-fold. This selective expression was corroborated in EBV-positive and -negative tumor models, along with an absence of transgene expression in the host liver. Significant cytototoxicity was achieved using the advoriPp53 therapeutic gene only in EBV-positive NPC cells, which was enhanced with the addition of ionizing radiation. Cytotoxicity was mediated primarily by induction of apoptosis. These results demonstrate that the oriP sequence can achieve high levels of gene expression targeted specifically to EBV-positive NPC cells in the context of the adv vector. This has now provided the tumor-specific expression system from which additional interventions can be evaluated in future treatment strategies for patients with nasopharyngeal cancers.

INTRODUCTION

One of the major challenges in human cancer gene therapy is achievement of tumor-specific expression and cytotoxicity. Our laboratory has investigated the potential of adv-mediated p53 gene therapy in several human NPC models (1–5). It was evident that tumor-specific expression was important, given the significant cytotoxicity observed in nasopharyngeal fibroblasts, which served as the “normal tissue” comparator (2). A unique feature of NPC is its almost universal association with the EBV (6, 7), which is expressed exclusively in the malignant tissues, and not in the surrounding normal tissues. The EBV is also monoclonal (8), thereby providing an exploitable opportunity for tumor-specific targeting.

The EBV genome is a linear double-stranded DNA (9), which exists in NPC cells in a state of persistent latent infection. In NPC, characterized by type 2 latency (7), EBV-encoded nuclear proteins (e.g., EBNA1) and small nonpolyadenylated nuclear RNAs (EBERs 1 and 2) are uniformly expressed, and latent membrane protein-1 is detected in 65% of instances (10). EBNA1 maintains the EBV genome in its episomal state, where it exists as multiple circular DNA molecules (11). There are three EBNA1-binding sites within the EBV genome; the most important site, which has the highest affinity, consists of 20 tandem 30-bp repeats, otherwise known as the FR (9). This FR sequence is located within the oriP of the EBV genome. Among its many known functions such as facilitating matrix attachment and mitotic segregation (12, 13), of key interest to our research was the binding of EBNA1 to the FR region to transcriptionally activate other EBV genes (14).

Based on this observation and work by others (15, 16), we designed a novel replication-deficient (ΔE1) adv vector in which the transcriptional cassette of the oriP-FR elements along with a basal CMV promoter was cloned in juxtaposition with either reporter (β-gal or luciferase) or therapeutic (p53) genes (3). We demonstrate that EBV-specific expression is successfully achieved both in vitro and in vivo. Significant cytotoxicity is also observed, enhanced by the addition of ionizing radiation (XRT), and mediated primarily through apoptosis.

MATERIALS AND METHODS

Cells and Culture Conditions. Details on the experimental procedures have been described in previous publications (1–5). Briefly, the NPC cell line C666-1 was provided by Dr. D. Huang, and the presence of EBV has been consistently demonstrated in this cell line (3, 17). C666-1 cells were maintained in RPMI 1640 supplemented with 10% FBS (Wisent Inc). The EBV-negative NPC CNE-2Z cell line was obtained from the Cancer Institute/Chinese Academy of Medical Sciences (18). KS1 is a primary fibroblast strain that we obtained from the nasopharynx of a patient with NPC (2). HT1080 human fibrosarcoma cells served as the in vivo model for an EBV-negative xenograft tumor (19). The human fetal renal 293 cells and HeLa cells were used for the adv studies. All cell lines were maintained in α-MEM plus 10% FBS, and the experiments were conducted when the cells were in an exponential growth phase.

Construction of the Recombinant Adenovirus. An 897-bp SalI-HindIII fragment containing the EBV oriP-FR region and basal CMV IE promoter was excised from plasmid pEIG3 (by author C. A. S.) and cloned into the SalI/HindIII sites of the pΔE1sp1A shuttle plasmid (Microbix) to create pE1sp1A/oriP (Fig. 1). The oriP-CMV promoter consists of the 621-bp FR region containing 20 palindromic EBNA1-binding sites (20) within the 1.9-kb oriP in the EBV genome (12, 20–23) and was cloned upstream of the 70-bp minimal promoter of the human CMV IE gene regulatory region (24). Plasmids pE1sp1A/oriP-luciferase, pEsp1A/oriP-β-gal, and pEsp1A/oriP-p53 were constructed by inserting BamHI/HindIII fragments containing luciferase, nuclear-localizing β-gal, or human wild-type p53 coding regions into BamHI and HindIII sites downstream of the oriP-CMV promoter in pΔE1sp1A/oriP. To generate recombinant adenoviruses, pEsp1A/oriP-luciferase, pEsp1A/oriP-β-gal, or pEsp1A/oriP-p53 were cotransfected with pJM17 (Microbix) in 293 cells by calcium phosphate precipitation. Individual adv plaques were expanded in 293 cells and purified using cesium chloride gradient ultracentrifugation (25). Viral titers were determined by the plaque-forming assay; the final titer of the purified viral vectors ranged between 10^9 and 10^10 plaque-forming units (pfu/ml) (1, 2). Ad5CMV.p53, in which the human wild-type p53 gene was expressed from...
as compared with untreated cells. Western blot analyses and luciferase assays effects were observed in the second and third rounds of tested HeLa cell cultures E1sp1A shuttle plasmid. Reporter (pHEX6300 cells A, plasmid in 293 packaging cells resulted in generation of the respective novel ad5.oriP vectors. Reporter sequences, along with the CMV IE promoter, were cloned into the SalI/HindIII sites of the ΔE1sp1A shuttle plasmid. Reporter (β-galactosidase or Luciferase) or therapeutic (Wild-type p53) constructs were inserted into the BamHI/HindIII cut sites downstream of the oriP-CMV promoter in pΔE1sp1A oriP. Homologous recombination with the pM17 plasmid in 293 packaging cells resulted in generation of the respective novel ad5.oriP vectors. the CMV IE enhancer promoter, was kindly provided by Dr. F. Graham (Hamilton, Canada; Refs. 1–5).

All large-scale preparations were tested for the absence of replication-competent (wild-type) adenovirus as described previously (1, 2). Consecutive infection tests were performed by treating HeLa cells with the purified adv vectors (ad5oriP.βgal, and ad5oriP.luciferase). No cytopathic effects were observed in the second and third rounds of tested HeLa cell cultures as compared with untreated cells. Western blot analyses and luciferase assays indicated that the respective p53, β-gal, and luciferase proteins were detected in the EBV-positive C666-1 cells.

Infection and Irradiation of Cells in Vitro. C666-1 cells were planted in culture flasks or wells (3 × 10⁶ cells/T-25 flask; 1 × 10⁶ cells/T-80 flask; 2 × 10⁶ cells/well in 96-well plates) in RPMI 1640 containing 10% FBS. After 3 days, the cells were infected with either ad5CMV.p53 or ad5oriP.p53 in culture medium containing 2% FBS at 37°C for 1 h; ad5oriP.luciferase served as the adenovirus (negative) control. Infected cultures were incubated for 24 h in RPMI 1640 plus 10% FBS. When indicated, they were then irradiated at room temperature using a 137Cs unit (Gamma-cell 40 Exactor; Nordion International Inc. Canada) at 6 Gy (dose rate of 1.1 Gy/min). Cells were harvested at different time points for protein extraction, morphological analysis of apoptosis, or the MTT assay.

Stable Transfectants of EBNA1-expressing CNE-2Z Cells. The pHERO6300 plasmid containing the EBNA1 sequence and control plasmid pHEX6300 were obtained from author C. A. S. CNE-2Z cells were plated in 24-well plates and transfected with 1.0 µg of plasmid DNA using Lipo-ffectAMINE 2000 reagent (Life Technologies, Inc.) according to manufacturer’s protocol. After 24 h, the cells were passaged 1:10 into fresh growth medium. The next day, selection medium containing Zeocin (Invitrogen) was added. Drug-resistant colonies were examined for EBNA1 expression using immunohistochemical staining (EBNA Ab-1; Oncogene, Cambridge, MA).

Detection of Ad5oriP.βgal Expression by X-gal Staining. To evaluate adv infection efficiency and transgene expression, target cells were infected with ad5oriP.βgal, and β-gal activity was detected using X-gal staining (1). Cells were seeded onto 24-well culture plates, and after one doubling (3 days for C666-1 and 1 day for CNE-2Z cells), they were exposed to various concentrations of ad5oriP.βgal (1–25 pfu/cell) in culture medium containing 2% FBS. One h later, the serum concentration was increased to 10% FBS, and cells were then incubated for 48 h. Cells were washed with PBS °, fixed with 2% formaldehyde/0.2% glutaraldehyde for 5 min at 4°C, and stained with X-gal staining solution after washing with PBS °/0.02% NP40 buffer. Infection efficiency was scored as the percentage of positively (blue) staining cells, as described previously (1).

Quantitative Evaluation of Ad5oriP.luciferase or Ad5CMV.βgal Expression. To evaluate transgene expression from ad5oriP.luciferase or ad5CMV.βgal in KS1 and NPC cells, luciferase and β-gal activity were measured using the Dual-Light Reporter Gene Assay Kit (Tropix, Inc., Bedford, MA). Cells were seeded in 6-well culture plates. After one doubling, cells were coincubated with serial concentrations of ad5oriP.luciferase or ad5CMV.βgal simultaneously at 2–25 pfu/cell for 1 h. Cultures were incubated in growth medium for 2 days. Cell lysates were analyzed for luciferase and β-gal activities using the Dual-Light Reporter Gene Assay Kit and a Luminometer (Lumat LB9507; EG & G Berthold) according to the manufacturers’ protocols.

Western Blot Analysis of p53 Protein. Cells treated with ad5oriP.p53 or ad5oriP.βgal with or without XRT were harvested on ice at selected time points. Cell extracts were prepared in lysis buffer [0.1% Triton-X-100, 0.1% SDS, 10 mM EDTA, and 2 mM DTT], and protein concentrations were determined using the BCA protein assay (Pierce). Immunoblotting was carried

Fig. 1. Cloning strategy for construction of the novel ad5.oriP vectors. The oriP-FR sequences, along with the CMV IE promoter, were cloned into the SalI/HindIII sites of the ΔE1sp1A shuttle plasmid. Reporter (β-galactosidase or Luciferase) or therapeutic (Wild-type p53) constructs were inserted into the BamHI/HindIII cut sites downstream of the oriP-CMV promoter in pΔE1sp1A oriP. Homologous recombination with the pM17 plasmid in 293 packaging cells resulted in generation of the respective novel ad5.oriP vectors.

Fig. 2. X-gal staining for β-gal expression after ad5oriP.βgal infection in CNE-2Z/pHEX6300 (control plasmid), KS1, C666-1, and CNE-2Z/pHERO6300 (stable transfectants expressing EBNA1). Cells were exposed to ad5oriP.βgal for 48 h and then stained using X-gal. A, CNE-2Z/pHEX6300 cells + ad5oriP.βgal (10 pfu/cell); B, KS1 + ad5oriP.βgal (10 pfu/cell); C, C666-1 + ad5oriP.βgal (10 pfu/cell); D, CNE-2Z/EBNA1 + ad5oriP.βgal (10 pfu/cell).
out as described previously (1–5). Briefly, samples containing equal amounts of protein (20 μg) were loaded onto 8–16% SDS-PAGE gels, electrophoresed for 120 min at 125 V using a mini-gel protein electrophoresis cell (Helixx Technologies, Ontario, Canada), and transferred onto nitrocellulose membranes for 120 min at 125 V using a mini-gel protein electrophoresis cell (Helixx Technologies, Ontario, Canada). Nonspecific binding sites were blocked with 5% low-fat milk in PBS for 60 min at room temperature and washed extensively with PBST (PBS containing 0.05% Tween-20). Blots were incubated for 60 min at room temperature with an appropriate dilution of the primary antibody (1:1000), which was then washed with PBST and incubated with horseradish peroxidase-conjugated secondary antibody. The specific complexes were detected using chemiluminescence (DuPont, Boston, MA).

**RESULTS**

**Effect of Ad5oriP.p53 on Cell Viability.** To evaluate the effect of ad5oriP.p53 treatment on viability, cells were seeded in 96-well plates and allowed to attach. X-gal staining was performed overnight at 37°C as described previously (3, 5). In brief, MTT (Sigma Chemical Co.) was dissolved in PBS at 5 mg/ml and added to each well, and the plates were incubated at 37°C for 3 h. The percentage of blue-stained cells was scored under light microscopy. As we observed previously (1–3), >90% of all tested cells demonstrated positive X-gal staining when infected with 10 pfu/cell ad5CMV.βgal (data not shown). When the same cells were treated with ad5oriP.βgal, extensive staining was observed in EBV-positive C666-1 cells (Fig. 2C), only rare blue-stained cells were observed in the EBV-negative CNE-2Z cells (transfected with control plasmid) system (Fig. 2A), and no blue-stained cells were observed in KS1 fibroblasts (Fig. 2B). To demonstrate that the oriP-based promoter was responding to EBNA1, stable transfectants of C2-Z cells expressing recombinant EBNA1 were also evaluated. As shown in Fig. 2D, extensive staining was observed in the CNE-2Z/EBNA1 transfectants but was rarely seen (1 in 104 cells) in the CNE-2Z cells stably transfected with control plasmid (data not shown).

**Selectively High Levels of Transgene Expression in EBV-positive C666-1 Cells.** Levels of reporter gene expression provided by the oriP promoter were compared with the CMV promoter after coinfection of cells (3, 5, 17) were evaluated as the EBV-positive tumor model. HT1080 cells (5 × 104), and C666-1 cells (1 × 105) were implanted s.c. in SCID mice. Once the tumor dimension reached approximately 5–10 mm, the animals received intratumoral injection of either ad5oriP.βgal or ad5CMV.βgal (total dose of 2 × 107 pfu). Forty-eight h later, the animals were sacrificed, and their tumors and livers were removed. Sequential 10-μm sections were cut at five depths throughout either the liver or the injected tumor perpendicular to the needle tract. X-gal staining was performed overnight at 37°C as described previously to evaluate β-gal expression (27). Sections were also counterstained using H&E to determine cellular morphology.

**RESULTS**

**Ad5oriP.βgal-mediated Expression of β-Gal.** To determine the infection efficiency and transgene expression provided by the novel EBNA1-regulated adv vectors, EBV-negative cells (CNE-2Z and KS1) and EBV-positive cells (C666-1) were infected with either ad5CMV.βgal or ad5oriP.βgal. β-gal activity was assessed using X-gal staining 48 h after infection, and the number of positively stained cells was scored under light microscopy. As we observed previously (1–3), >90% of all tested cells demonstrated positive X-gal staining when infected with 10 pfu/cell ad5CMV.βgal (data not shown). When the same cells were treated with ad5oriP.βgal, extensive staining was observed in EBV-positive C666-1 cells (Fig. 2C), only rare blue-stained cells were observed in the EBV-negative CNE-2Z cells (transfected with control plasmid) system (Fig. 2A), and no blue-stained cells were observed in KS1 fibroblasts (Fig. 2B). To demonstrate that the oriP-based promoter was responding to EBNA1, stable transfectants of C2-Z cells expressing recombinant EBNA1 were also evaluated. As shown in Fig. 2D, extensive staining was observed in the CNE-2Z/EBNA1 transfectants but was rarely seen (1 in 104 cells) in the CNE-2Z cells stably transfected with control plasmid (data not shown).

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tion with ad5CMV.βgal and ad5oriP.luciferase in KS1, CNE-2Z, and C666-1 cells. As shown in Fig. 3, both luciferase and β-gal activities increased significantly with increasing MOIs (2–25 pfu/cell) for all three cell lines. Luciferase activities were 1000-fold higher in the EBV-positive C666-1 cells as compared with the EBV-negative KS1 fibroblasts and 350-fold higher than that in the CNE-2Z cells when cells were treated with ad5oriP.luciferase. In contrast, only a 2–10-fold difference was observed in β-gal activity among the three cell lines, likely reflecting variations in viral incorporation efficiencies, as reported previously by our group (2).

Ad5oriP.p53 Effectively Mediates p53 Expression in C666-1 Cells. To investigate whether ad5oriP.p53 can mediate efficient expression of the p53 gene in EBV-positive NPC cells, C666-1 cells were exposed to increasing concentrations of ad5oriP.p53 or ad5oriP.βgal for 24 h and then subjected to XRT (6 Gy). Cell extracts were prepared 1 day later, and p53 expression levels were examined using Western blotting. As shown in Fig. 4, low levels of endogenous p53 could be detected in mock-infected C666-1 cells, with no influence after XRT. Infection with ad5oriP.βgal induced p53 expression slightly, with no further increase after XRT. However, the highest levels of p53 expression were observed for all doses of ad5.oriP.p53, which was again unaffected by XRT administration.

Ad5oriP.p53 Reduced Viability of EBV-positive C666-1 Cells. The cytotoxic effect of ad5oriP.p53 on NPC cells was assessed using the MTT assay and compared with the effect of ad5CMV.p53. As shown in Fig. 5, A and B, infection of NPC cells with the ad5CMV.p53 at MOIs of 2–50 pfu/cell resulted in a dose-dependent reduction in cell survival in both EBV-negative CNE-2Z (Fig. 5A) and EBV-positive C666-1 (Fig. 5B) cells. In contrast, ad5oriP.p53 caused significant reduction of C666-1 cell viability (Fig. 5B), with minimal impact on CNE-2Z cells (Fig. 5A). Treatment of C666-1 cells with ad5oriP.p53 or ad5CMV.p53 achieved a similar extent of toxicity, suggesting that the two promoters provided equivalent levels of transgene expression. With the addition of 6 Gy of XRT, survival of the C666-1 cells was further reduced to 0.4% (Fig. 5B), suggesting a more-than-additive interaction between the two cytotoxic modalities of XRT and p53 gene therapy. This is consistent with previous observations reported by our laboratory (2, 3). Ad5oriP.luciferase treatment at this dose range had minimal effects on C666-1 cells (Fig. 5B).

Ad5oriP.p53 Induces Apoptosis in EBV-positive C666-1 Cells. The morphological changes and destruction of the structural organization of the nucleus indicative of apoptosis were investigated by AO-EB fluorescence staining after ad5oriP.p53 or ad5oriP.luciferase treatment of C666-1 cells (Fig. 6 and Table 1). As shown in Table 1, when C666-1 cells were treated under control conditions or with XRT alone or ad5oriP.luciferase with or without XRT, <6% of C666-1 cells underwent apoptosis (Fig. 6, A –D). However, ad5oriP.p53 infection (25 pfu/cell) significantly increased the proportion of apoptotic cells to 27% (Fig. 6E). Combined treatment with XRT (6 Gy) further increased the percentage of C666-1 cells undergoing apoptosis to 48% (Fig. 6F).

Xenograft Models Corroborate Selective Expression in EBV-positive C666-1 Tumors. To determine whether the in vitro data of selective expression of the oriP-driven adv vector can be corroborated in a more complex in vivo model, EBV-positive and -negative tumors were treated with either ad5oriP.βgal or ad5CMV.βgal. As shown in Fig. 7A, intratumoral injection of ad5CMV.βgal resulted in significant X-gal staining in both the EBV-negative HT1080 and the EBV-positive C666-1 tumors. In contrast, ad5oriP.βgal was expressed only in the EBV-positive C666-1 tumor. Systemic (liver) expression of the virus was observed only with the ad5CMV.βgal-treated HT1080 mouse (Fig. 7B). No X-gal staining was observed in the livers of the

![Fig. 5. Effect of ad5oriP.p53 on viability of CNE-2Z and C666-1 cells. Cells were infected with ad5oriP.p53 or ad5CMV.p53 at MOIs of 0, 2, 5, 10, 25, or 50 pfu/cell for 24 h. C666-1 cells were additionally infected with ad5oriP.p53, ad5CMV.p53, or ad5oriP.luciferase at the above-mentioned viral doses, with or without the addition of a single exposure to 6 Gy of XRT 24 h after infection. Mock infections were performed by exposing the cells to medium alone. The MTT assay was conducted on day 5 after XRT. Each data point represents the mean ± SE from three independent experiments. A, CNE-2Z cells; R, C666-1 cells.](Image 321x118 to 547x740)
mice bearing either the C666-1 tumor treated with ad5CMV.βgal or tumors injected with ad5oriP.βgal.

**DISCUSSION**

This is the first report documenting successful tumor-specific gene expression for EBV-associated human NPC. Transfection studies demonstrate that in the context of the adv genome, the oriP promoter responds specifically to the presence of EBNA1 (Fig. 2D). Human NPC is unique in that the EBV is expressed exclusively in the cancer cells, and not in the surrounding normal tissues (10). Among the three histological subtypes of NPC (28), the most common histology, undifferentiated carcinoma of nasopharyngeal type, is universally associated with the EBV genome (10) and is endemic to certain geographic regions, such as Southeast Asia (28). EBV is monoclonal in NPC cells (8), thereby providing a unique exploitable tumor-specific target.

EBV exists in NPC cells in a state of latent infection (7). Uniform expression of EBNA1 is essential for maintenance of the EBV genome in its episomal state (9). Binding of EBNA1 to FR sequences in the EBV genome enhances transcriptional activity of downstream genes. This property was first harnessed for therapeutic applications by Judde et al. (16). In their study, the FR sequence was placed upstream of the thymidine kinase promoter. Transfection of these plasmids resulted in selective expression and cytotoxicity in EBV-positive B-lymphoma cells.

Previous studies of adv-mediated p53 gene transfer (ad5CMV.p53) in our laboratory (1–5) demonstrated very high transduction efficiencies in NPC cells using adv vectors (>90% infectivity at 2 pfu/cell).

In these studies, the CMV IE enhancer promoter resulted in significant levels of transgene expression in both NPC cells and normal nasopharyngeal KS1 fibroblasts (2), underscoring the need for selective NPC-specific expression. In the present study, adv vectors were constructed in which the CMV enhancer promoter was replaced with an oriP.CMV promoter. This promoter provides 350-fold higher levels of β-gal reporter gene expression in EBV-positive NPC cells compared with EBV-negative NPC cells (Figs. 2, A and C, and 3). This 350-fold differential expression is far greater than the minimally higher viral incorporation efficiency of the C666-1 cells compared with the CNE-2Z cells. The latter NPC cell line was originally EBV positive, but a very small proportion (i.e., <1%) may still harbor the EBV genome (29). This may account for the 350-fold differential in reporter gene expression between the two NPC cell lines, as compared with the 1000-fold difference observed between C666-1 cells and EBV-negative KS1 fibroblasts (Figs. 2, B and C, and 3).

### Table 1

<table>
<thead>
<tr>
<th>Condition</th>
<th>% Apoptotic cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.0 ± 0.7</td>
</tr>
<tr>
<td>Control + 6 Gy</td>
<td>2.7 ± 1.1</td>
</tr>
<tr>
<td>Ad5oriP.luciferase (25 pfu/cell)</td>
<td>3.0 ± 1.0</td>
</tr>
<tr>
<td>Ad5oriP.luciferase (25 pfu/cell) + 6 Gy</td>
<td>5.5 ± 0.8</td>
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<tr>
<td>Ad5oriP.p53 (25 pfu/cell)</td>
<td>27.5 ± 4.0</td>
</tr>
<tr>
<td>Ad5oriP.p53 (25 pfu/cell) + 6 Gy</td>
<td>48.2 ± 6.6</td>
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</tbody>
</table>

C666-1 cells were infected with either Ad5oriP.luciferase or Ad5oriP.p53 for 24 h and then irradiated with either 0 or 6 Gy. The proportion of cells displaying morphological features of apoptosis was counted 3 days after XRT. This experiment has been conducted twice, and the data represent the mean and SD from 10 microscopic fields.

Fig. 6. C666-1 cells were irradiated with single exposures of 0 or 6 Gy 24 h after infection with 25 pfu/cell of ad5oriP.p53 or ad5oriP.luciferase. Cells were then stained with AO-EB and examined under fluorescence microscopy at 72 h post-XRT for morphological changes indicative of apoptosis. A, control; B, 6 Gy; C, ad5oriP.luciferase (10 pfu/cell); D, ad5oriP.luciferase (10 pfu/cell) + 6 Gy; E, ad5oriP.p53 (10 pfu/cell); F, ad5oriP.p53 (10 pfu/cell) + 6 Gy.
fold differential is even more noteworthy given that the oriP.CMV promoter is not completely silent in KS1 fibroblasts. The low levels of luciferase activity detected in these fibroblasts likely reflect basal expression from the minimal CMV IE promoter.

Human CMV can replicate in several different tissue types such as endothelial tissue, smooth muscle tissue, fibroblasts, and macrophages, but not in lymphocytes, neutrophils, or certain embryonal cells (30). CMV replication is dependent on the expression of major IE genes (e.g., IE1 p72 and IE2 p86), which are regulated by a variety of positive [e.g., nuclear factor κB and activator protein 1 (24, 31)] and negative [e.g., YY1 and Gfi-1 (32, 33)] cis-acting sequence elements. Many of these elements are contained within the CMV enhancer region between −65 and −550 bp upstream of the transcription start site. The 70-bp minimal CMV IE promoter contains a TATA box, several activating transcription factor- and SP-1-binding sites, and the IE2 protein-binding site (cis repression sequence) at the cap site (24). In the absence of the enhancer, the minimal CMV promoter exhibits only low levels of activity, likely with some degree of variability from one cell type to another. Although no minimal promoter will be completely silent in all cell types, it is reassuring that the ad5oriP.p53 produced minimal cytotoxicity in the CNE-2Z cells (Fig. 5A).

It was also heartening to observe that the in vitro data could be replicated in tumor models because there is the occasional report of reduced efficiency of the CMV promoter in vivo (34). In the C666-1 tumor model, X-gal staining was similar between the ad5oriP.βgal-
treated and the adSCMV βgal-treated cells, which was consistent with the
in vitro data, demonstrating approximately equivalent reporter gene expression and cytotoxicity between the respective adSCMV and ad5 oriP vectors in the C666-1 cells (Figs. 3 and 5B). It is known that with extensive intratumoral expression of the adv vector, transgene expression can be observed in the host liver (Fig. 7B and Ref. 35), which underscores the need to limit expression to the target tumor tissues. To this end, we have also been successful, given the absence of X-gal staining in the liver of tumor-bearing mice treated with adSoriP βgal.

We and others have consistently demonstrated that the therapeutic effects of p53 gene therapy have been largely mediated through the induction of apoptosis (1–3, 36, 37), with enhanced cytotoxicity when combined with XRT (2, 3, 38–40). The detailed mechanism of p53-induced cytotoxicity remains to be completely elucidated. p53 functions as a transcription factor, and members of the bcl-2 family of proteins, (specifically, bax) have been postulated to be mediators of p53-induced apoptosis (41). Their role in NPC, however, remains unclear because neither bcl-2 nor bax expression changes after treatment with adSCMV p53 (2). Given the significant induction of p53 expression with such therapies (1–3), we rationalize that this cytotoxicity may be a dose-dependent phenomenon, whereby the sudden expression with such therapies (1–3) would likely be exceedingly low in any one patient. Nevertheless, this is an important safety issue that must be evaluated when this strategy is applied clinically.

In conclusion, we have demonstrated for the first time a tumor-specific targeting strategy for EBV-positive human NPC. This oriP-based adv vector provides a platform from which further developments can be devised that will ultimately improve outcome for patients with NPC.

REFERENCES


Tumor-targeted Gene Therapy for Nasopharyngeal Carcinoma
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