Erschienen in: Molecular Therapy ; 12 (2005), 6. - S. 1083-1090
https://dx.doi.org/10.1016/j.ymthe.2005.06.443

# Oncolytic Adenovirus Expressing a p53 Variant Resistant to Degradation by HPV E6 Protein Exhibits Potent and Selective Replication in Cervical Cancer

Daniëlle A. M. Heideman,[1,*] Renske D. M. Steenbergen,[1] Jaco van der Torre,[2] Martin Scheffner,[3] Ramon Alemany,[4] Winald R. Gerritsen,[2] Chris J. L. M. Meijer,[1] Peter J. F. Snijders,[1] and Victor W. van Beusechem[2]

[1]Department of Pathology and [2]Division of Gene Therapy, Department of Medical Oncology, VU University Medical Center, 1007 MB Amsterdam, The Netherlands
[3]Laboratory of Biochemistry, Department of Biology, University of Konstanz, Constance, Germany
[4]Translational Research Laboratory, Institut Catala d'Oncologia, Barcelona, Spain

*To whom correspondence and reprint requests should be addressed at the Department of Pathology, VU University Medical Center, P.O. Box 7057, 1007 MB Amsterdam, The Netherlands. Fax: +31 20 444 2964. E-mail: dam.heideman@vumc.nl.

**Rationale in the development of novel treatment strategies for HPV-associated cancers is targeting on the basis of the presence of HPV in (pre)malignant cells. Here, we designed a new conditionally replicating adenovirus (CRAd) for selective and effective oncolytic replication in HPV-containing cells. As the backbone, we used the CRAd AdCB016, which replicates selectively in cells expressing HPV E6 and E7 proteins. To enhance its oncolytic potency, we armed AdCB016 with p53 variant mp53(268N), which is resistant to HPV E6-mediated degradation. The new CRAd AdCB016-mp53(268N) was analyzed for its lytic replication properties in cervical carcinoma cell lines, HPV-immortalized keratinocyte cell lines representing dysplastic cells, and primary human keratinocytes. AdCB016-mp53(268N) exhibited 10- to 1000-fold greater efficacy than AdCB016 on high-risk HPV-positive cervical carcinoma cells and HPV-immortalized keratinocytes. Importantly, infection with AdCB016-mp53(268N) did not affect primary nonmalignant human keratinocytes. This favorable efficacy and safety profile was confirmed in organotypic raft cultures. Our findings suggest that AdCB016-mp53(268N) is a promising new agent for treatment of HPV-associated human cancers.**

**Key Words: virotherapy, cervical cancer, organotypic raft culture, CRAd, AdCB016, AdΔ24, HPV, p53, primary keratinocyte, CIN**

## Introduction

Cervical cancer is the second leading cause of cancer death among women worldwide. Infection with high-risk human papillomaviruses (hr-HPV), particularly the hr-HPV types 16 (HPV16) and 18 (HPV18), is causally linked to the development of cervical cancer [1,2]. It is now well established that persistent expression of viral oncogenes E6 and E7, which interact with the master cell cycle regulatory proteins p53 and pRb, respectively, drive the oncogenic property of hr-HPV [2–5]. Comprehensive screening programs enhance early detection of cervical cancer, and premalignant as well as early malignant stages of disease are often well treatable with LLETZ (large loop excision of the transformation zone) and a combination of surgery and/or radiotherapy [6]. However, morbidity and recurrence rates following therapy are not negligible, and, importantly, treatment of more advanced stages of cervical cancer is often unsatisfactory. Therefore, new therapeutic approaches, either in the adjuvant setting or not, for both advanced and early disease are definitely needed. Virotherapy with selectively lytic viruses could perhaps contribute to a rational treatment strategy for CIN (cervical intraepithelial neoplasia) lesions and cervical cancer. The uterine cervix is readily accessible to an intralesional administration approach without invasive techniques and systemic administration of truly tumor-selective oncolytic viruses could be considered for patients with metastatic disease.

Conditionally replicating adenoviruses (CRAds) represent one class of virotherapy agents (reviewed in [7–9]). CRAds are designed to take advantage of tumor-specific changes allowing preferential replication in tumor cells. Regarding treatment of CIN and cervical cancer, selective targeting can be achieved by taking advantage of the

presence of high-risk HPV in the cells. Such a type of CRAd is represented by AdCB016 [10]. AdCB016 exhibits preferential replication in HPV E6/E7-expressing keratinocytes compared to normal keratinocytes due to two deletions in its E1A protein, i.e., in the CR2 domain to impede sequestration of cellular pRb from E2F [11,12] and in the CR1 domain to also abolish E1A binding to p300 acetyl transferase, which can be functionally complemented by HPV oncoproteins [10]. AdCB016 is thus designed to benefit from the presence of hr-HPV in cells, which makes it a particularly useful agent for treatment of CIN and cervical cancer. However, AdCB016 was found to be far less effective in killing cancer cells than its parent AdΔ24, which comprises only the CR2 mutation [10], calling for efficacy enhancement to reach its full potential.

The anticancer potency of a CRAd depends on the efficiency at which the virus lyses tumor cells and disseminates throughout a tumor. Previously, we found that exogenous expression of the tumor suppressor protein p53 expedited the lysis of CRAd-infected cancer cells, allowing a more rapid spread of the virus [13]. Exogenous p53 augmented the oncolytic potency of the CRAd AdΔ24 against a variety of cancer cell lines *in vitro* and xenografts *in vivo* [13–15]. However, in HPV-associated cancers, p53 is targeted for ubiquitination by HPV E6 protein [5]. Therefore, in these cancers progeny release and lateral spread of p53-expressing CRAds might still be hampered as a result of efficient HPV E6-promoted degradation of exogenous p53. Indeed, p53 enhanced oncolytic potency of AdΔ24 against HPV-positive HeLa cells only marginally [13]. Fortunately, a p53 variant that is not recognized and ubiquitinated by HPV16 and HPV18 E6 was identified. This variant, mp53(268N), was derived from mouse p53 by substituting a single aspartate residue with the corresponding asparagine residue from position 268 in human p53 [16]. We hypothesized that CRAds could be made into more effective lytic agents for HPV-associated cancers by expressing degradation-resistant mp53(268N) instead of wild-type p53. In the present study, we tested this hypothesis by evaluating the anticancer activity of AdCB016-mp53(268N) in comparison to its precursors AdΔ24, AdCB016, and p53-expressing AdΔ24 on cervical cancer cell lines, HPV-immortalized keratinocyte cell lines representing dysplastic cells in CIN lesions, and normal primary human keratinocytes in monolayer cultures, as well as on organotypic raft cultures.

## Results and Discussion

### HPV-Positive Cervical Cancer Cells Exhibit Resistance to p53-mediated Enhancement of AdΔ24-Induced Oncolysis

We previously found that the CRAd AdΔ24 expressing p53 exhibited enhanced oncolytic capacity compared to its parent without p53 against most cancer cell lines of various tissue origins. However, only moderate improvement was seen on HPV-positive HeLa cells [13]. To discover if resistance to p53-mediated oncolysis enhancement is a general characteristic of HPV-containing cervical cancers, we evaluated sensitivity to AdΔ24 and AdΔ24-p53 on three different HPV-positive cervical cancer cell lines. We used the colorimetric WST-1 cell viability assay (Fig. 1) and the crystal violet assay (Fig. 3) to compare the oncolytic activities of the two CRAds. The data of both assays correlated accurately. Indeed, in HPV-positive cell lines, AdΔ24-p53 caused less oncolysis enhancement compared to AdΔ24 than was seen in HPV-negative cancer cell lines (i.e., 2- to 4-fold versus 12-fold enhancement at 50% cell viability; Fig. 1), similar to what we observed before [13]. Since HPV-positive cervical cancer cells express E6 capable of inducing p53 degradation [5,17], we reasoned that in these cells the efficacy of oncolysis enhancement by exogenous p53 might have been inhibited by E6-mediated degradation.

### A p53 Variant Resistant to HPV E6-Promoted Degradation Enhances the Oncolytic Potency of AdΔ24 in HPV-Positive Cervical Cancer Cell Lines

It was found before that p53 variant mp53(268N) cannot be targeted for degradation by HPV16 and HPV18 E6 [16]. It is thus to be expected that mp53(268N) has higher activity than p53 in the face of oncogenic HPV E6 expression. To verify this, we compared the transactivation capacities of p53 and mp53(268N) in hr-HPV-positive cervical cancer cell lines and in pCMV-16E6-transfected p53-null SaOs-2 cancer cells using p53-responsive reporter constructs. Fig. 2 shows that p53 and mp53(268N) transactivate equally well in HPV-negative SaOs-2 cells, while mp53(268N) exhibited up to 30-fold higher p53-specific transactivation capacity than wild-type p53 in HPV16-positive SiHa and CaSki cells, HPV18-positive HeLa cells, and HPV16 E6-expressing SaOs-2 cells, with transactivation activity of p53 in CaSki cells not even exceeding background level. On the basis of these results, we decided to replace the wild-type p53 gene in AdΔ24-p53 with the mp53(268N) variant. We compared the oncolytic potency of the new CRAd AdΔ24-mp53(268N) to AdΔ24-p53 and AdΔ24 in three human HPV-positive cervical cancer cell lines by crystal violet assay (Fig. 3). As controls, we included the HPV-negative cervical cancer cell line C33A and breast cancer cell line MDA-MB-231, which is very sensitive to the oncolysis-enhancing effect of wild-type p53. AdΔ24-mp53(268N) exhibited 10 times more effective lytic replication than AdΔ24-p53 on HPV-positive cell lines, while both CRAds were equally effective against HPV-negative cell lines. Compared to AdΔ24, AdΔ24-mp53(268N) was up to 100 times more effective against HPV-positive and HPV-negative cancer cell lines. Hence, prevention of E6-

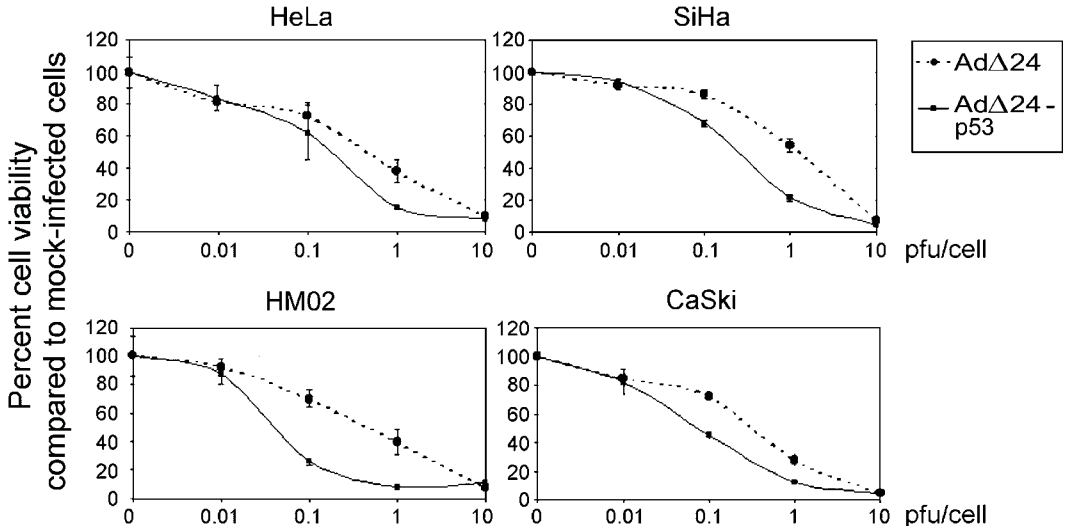


**FIG. 1.** Exogenous expression of human p53 marginally augments oncolytic replication of AdΔ24 in HPV-positive cervical cancer cells. HeLa, SiHa, and CaSki HPV-positive cervical cancer cell lines, and HPV-negative control cell line HM02, known to be susceptible to augmentation of CRAd-induced oncolysis by enforced p53 expression, were infected with AdΔ24 or AdΔ24-p53 at the indicated m.o.i. and cultured for 11 days. The cell viability was determined by WST-1 conversion assay and compared with the viability of mock-infected control cultures. Data shown are the mean results ± SD of a representative experiment performed in triplicate.

dependent degradation of exogenous p53 overcame relative resistance to p53-mediated oncolysis enhancement in HPV-positive cancer cells. Importantly, mp53(268N) is insensitive to degradation by E6 of both HPV types 16 and 18. Since the latter HPV types count for the majority of HPV infections causing cervical cancers [1], mp53(268N)-based CRAds might be applicable in the treatment of the majority of cervical cancers. Moreover, it has been shown that HPV16 is also the most important hr-HPV type involved in the carcinogenesis of tumors of oropharyngeal and anogenital sites [18,19]. Consequently, mp53(268N)-based CRAds might be useful for the majority of HPV-related diseases. Furthermore, it remains of interest to study the stability of mp53(268N) in the presence of E6 oncoproteins from other HPV types that can be considered oncogenic. This may extend the therapeutic potential of mp53(268N)-based CRAds to cancers associated with HPV types other than 16 and 18 and to tumors carrying multiple HPV types [2].

### The CRAd AdCB016 Exhibits Selective Replication in CIN and Cervical Cancer Cells and Its Oncolytic Potency Is Enhanced by mp53(268N) Expression

AdΔ24 is defective in sequestering cellular pRb from E2F due to a deletion in the pRb-binding CR2 domain of its E1A protein [11,12]. This dictates selective replication in cells with lost G1/S-phase checkpoint control. While being very effective in killing cancer cells, AdΔ24-based CRAds have also been observed to replicate to some extent in nonmalignant cells [10,11,14]. Indeed, we also found lytic replication by AdΔ24 in normal primary keratinocytes (Fig. 4A). This is probably caused by E2F being temporarily released from pRb during normal regulated cell cycle progression. The double-mutant CRAd AdCB016, however, previously demonstrated stringent selectivity of replication in HPV E6/E7-expressing cells versus normal keratinocytes [10]. We confirmed that this CRAd is cytotoxic to normal keratinocytes only at very high dose (Fig. 4A), but unfortunately, also found that this CRAd has severely attenuated oncolytic efficacy (Fig. 4B).

Aiming to merge HPV E6/E7-selective AdCB016 replication with high oncolytic capacity mediated by mp53(268N) expression, we constructed the new CRAd AdCB016-mp53(268N). The oncolytic potency of AdCB016-mp53(268N) was compared to that of AdΔ24 and of its parent AdCB016 during multiple replication cycles on a panel of human cervical cancer cell lines, HPV-immortalized keratinocyte cell lines, and primary keratinocytes. In all HPV-positive cancer cell lines, AdCB016-mp53(268N) caused a massive increase in oncolysis efficiency compared to its parent lacking p53 (result on SiHa shown in Fig. 4B). Semiquantitative assessment of oncolytic potency enhancement by mp53(268N) expression showed 10- to 100-fold enhancement for HeLa, 100-fold for CaSki, and 1000-fold for SiHa cells. Consequently, AdCB016-mp53(268N) reached a level of oncolytic potency similar to that of AdΔ24. Most importantly, like AdCB016 and in contrast to AdΔ24, AdCB016-mp53(268N) hardly replicated in primary keratinocytes (Fig. 4A). Thus, while exogenous mp53(268N) expression augmented oncolytic potency, it did not compromise AdCB016 selectivity. Knowing that HPV infection with accompanied elevated expression of E6/E7 is present not

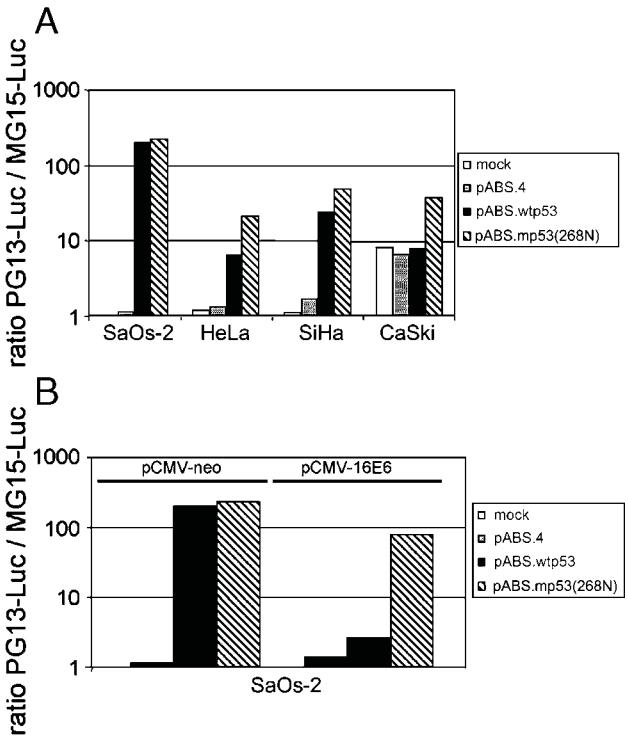


**FIG. 2.** Transcriptional activity of mp53(268N) is not inhibited by HPV E6. (A) Functional activity of mp53(268N) in endogenously HPV16 or HPV18 E6-expressing cells. SaOs-2 (HPV-negative), SiHa (HPV16), CaSki (HPV16), and HeLa (HPV18) cells were cotransfected with p53 or mp53(268N) expression plasmids or empty vector (pABS.4) and PG13-Luc or control MG15-Luc reporter plasmids. After 24 h of culture, luciferase expression was measured in cell lysates. (B) Functional activity of mp53(268N) in the context of exogenous HPV16 E6 expression. SaOs-2 cells were cotransfected with mixtures consisting of a p53 or mp53(268N) expression plasmid or empty vector (pABS.4), pCMV-16E6 or control pCMV-neo, and PG13-Luc or control MG15-Luc. Twenty-four hours after transfection, luciferase expression was measured in cell lysates. The data are expressed as the relative luciferase expression in PG13-Luc-transfected cells compared with MG15-Luc-transfected cells. Shown are data of a representative experiment performed in triplicate; SDs of the triplicate RLU measurements were less than 5%.

only in almost all cervical cancer cells, but also in dysplastic cells in high-grade CIN lesions [20], we additionally evaluated the potential utility of AdCB016-mp53(268N) for treatment of premalignant lesions. For this, we used four different hr-HPV-immortalized keratinocyte lines representing dysplastic cells in CIN lesions. Interestingly, AdCB016-mp53(268) also replicated with increased efficacy of 10- to 100-fold compared to AdCB016 in all four HPV-immortalized keratinocyte lines (result on FK18B shown in Fig. 4C). Thus, AdCB016-mp53(268N) with selective replication in HPV-positive cells may be used not only for treatment of cancer but for local treatment of premalignant lesions as well. Taken together, among the CRAds analyzed, AdCB016-mp53(268N) displayed the best selectivity-efficacy index for HPV-containing premalignant and cancer cells. In general, expression of mp53(268N) more strongly augmented the efficacy of AdCB016 than that of AdΔ24. This might be explained by the dysfunctional p300-binding domain of E1A in AdCB016. Although HPV E7 also binds p300, this could perhaps leave more p300 available to activate p53.

### AdCB016-mp53(268N) Preferentially and Effectively Replicates in Cervical Cancer Organotypic Raft Cultures

Finally, selectivity and efficacy of AdCB016-mp53(268N) replication were investigated in organotypic raft cultures. A proper evaluation of replication selectivity of oncolytic viruses requires an organotypic model that represents the architectural and physiological conditions in the stratified epithelium of the cervix, containing both proliferating and differentiated cells or cervical cancer tissue [10]. Therefore, we included studies on organotypic cultures of primary human keratinocytes and cervical cancer cells for a most accurate comparison of the different CRAd variants. We infected primary human foreskin keratinocytes and SiHa cancer cells with AdΔ24, AdΔ24-mp53(268N), AdCB016, or AdCB016-mp53(268N) before transferring them onto a dermal equivalent and allowing them to differentiate at the air-medium interface for 11 days. We then harvested the rafts and sectioned them for histological analysis. Fig. 5 shows the histology and hexon staining of control raft cultures and cultures infected with the various CRAds. As can be seen, AdCB016 hardly affected keratinocytes nor cancer cells, whereas AdΔ24 and AdΔ24-mp53(268N) caused widespread cytotoxicity due to viral replication in both normal and cancer rafts. Also in rafts made of a mixture of infected and uninfected cells containing only 10% or even 1% AdΔ24- or AdΔ24-mp53(268N)-infected keratinocytes, we observed widespread cell killing and virus replication (data not shown). These data confirmed that AdCB016 was ineffective against cervical cancer cells and AdΔ24-based CRAds were not sufficiently selective for cancer cells, at least in the applied experimental setting. In contrast, AdCB016-mp53(268N) selectively and effectively replicated in and thereby killed SiHa cells in organotypic raft culture, while leaving normal epithelium unaffected. Histology of rafts made of AdCB016-mp53(268N)-infected keratinocytes closely resembled normal epithelium. Of note, even under the conditions used by us, under which we infected keratinocytes before stratification of the epithelium had occurred to ensure infection of basal proliferating cells, AdCB016-mp53(268N) did not replicate in the multilayer epithelium. Some limited cytopathic effects might be seen and a few (<2%) cells stained positive for hexon in the rafts made of normal keratinocytes. Because these effects were confined to the basal stratum, they probably reflect abortive viral infection in cells used to establish the raft cultures. These results suggest that AdCB016-mp53-(268N) will not replicate under physiological conditions under which more likely mainly differentiated parabasal

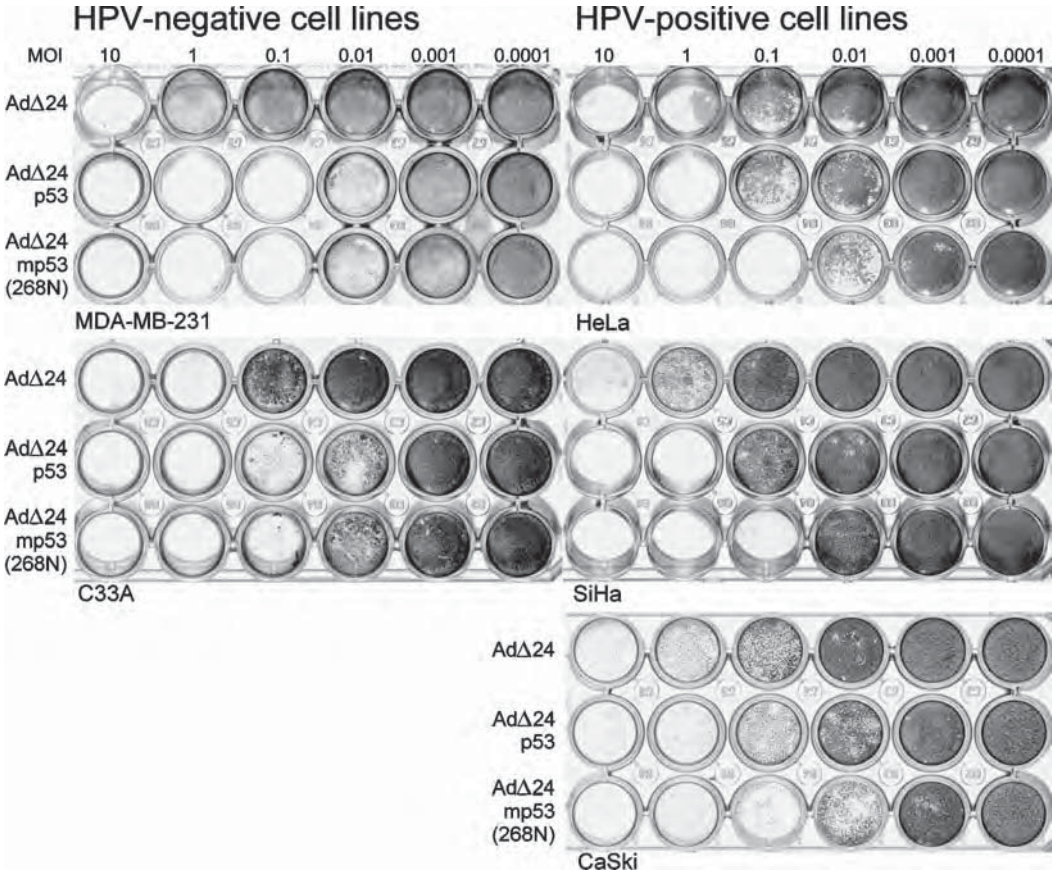


**FIG. 3.** AdΔ24-mp53(268N) exhibits enhanced oncolytic potency on human cervical cancer cells. HeLa, SiHa, CaSki, C33A, and MDA-MB-231 cells were infected with the indicated CRAds at the indicated m.o.i. and cultured *in vitro* to allow lateral spread of viral progeny through the cell monolayer. After 14 days of culture, the remaining viable cells were stained with crystal violet. Data are representative examples of three independent experiments performed on each cell line.

cells will be infected and proliferating basal cells can only be reached via microlesions. Our data thus strongly suggest the safety and efficacy of AdCB016-mp53-(268N)-based virotherapy for cervical cancer.

### Therapeutic Utility of Virotherapy for CIN and Cervical Cancer

Worldwide, cervical cancer is a major health problem, especially in developing countries, indicating the urgent need for development of new treatment or prophylactic modalities. The causal role of hr-HPVs in CIN and cervical cancer offers possibilities for preventive vaccination strategies. Indeed, the first trials using virus-like particles specific for HPV16 or a mixture of HPV16 and HPV18 showed promising results [21,22]. However, long-term follow-up studies are needed to determine if such prophylactic HPV vaccination strategies will ultimately protect against cervical cancer. Moreover, efforts have to be made to generate preventive vaccines against the other HPV types that are classified as oncogenic. Until this is realized, alternative strategies have to be followed to treat CIN and cervical cancer. In this regard, therapeutic vaccination efforts failed to be successful so far, which warrants alternative treatment strategies. Hence, investments in development of virotherapy for CIN and cervical cancer are worthwhile, especially since CRAds may be designed for use in therapeutic vaccination as well. Therapeutic genes, such as genes encoding activators of the immune system, might be incorporated in a CRAd to strengthen its anti-tumor efficacy further [23]. This immune strategy may importantly combine the oncolytic activity and tumor antigen-releasing effect of CRAds with the immune response-inducing activity of immunomodulatory proteins. In addition, the stringent replication profile of AdCB016-mp53(268N) may allow potential systemic administration to patients with metastatic disease. Nevertheless, to improve safety further for use in systemic treatment strategies, specific tumor cell targeting motifs could be incorporated in the virus to limit sequestering of administered CRAd by nontarget cells. Finally, the best chance of completely curing cancer

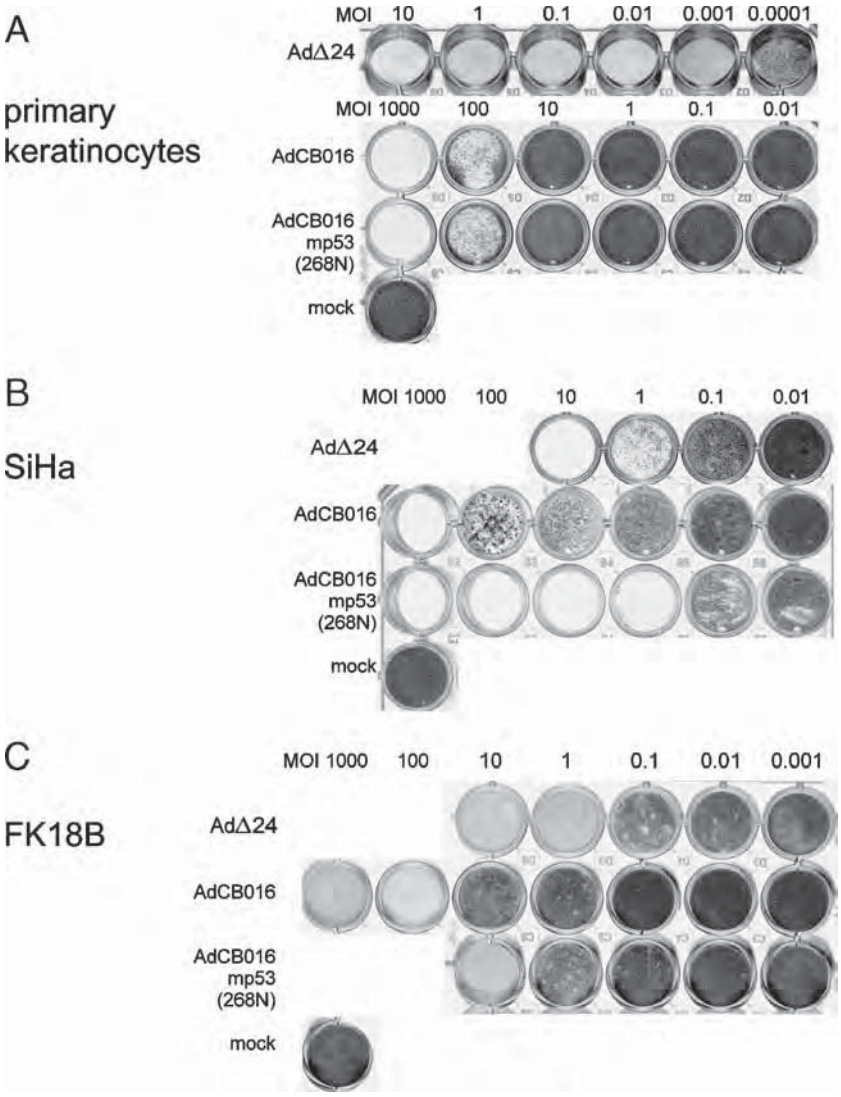


**FIG. 4.** AdCB016-mp53(268N) exhibits selective replication and enhanced oncolytic potency on human cervical cancer cells. Primary keratinocytes, HPV-immortalized keratinocyte cell lines, and cervical carcinoma cell lines were infected with indicated CRAds at indicated m.o.i. and cultured *in vitro* to allow the lateral spread of viral progeny through the cell monolayer. After 2–3 weeks of culture, adherent cells were stained with crystal violet. (A) Representative example of primary keratinocytes at 13 and 17 days p.i. for AdΔ24- and AdCB016-based CRAds, respectively. (B) Representative example of SiHa cervical cancer cell line at 21 days p.i. (C) Representative example of FK18B (HPV18-immortalized) keratinocyte cell line at 21 days p.i.

patients lies with attacking malignant cells through a combination of different agents with distinct mechanisms of action. Clearly, further research will be needed to evaluate carefully the efficacy of AdCB016-mp53(268N) as a single agent or in an adjuvant setting for treatment of cervical cancer and/or CIN lesions.

In conclusion, we have shown that expression of a functional p53 variant that cannot be degraded by HPV E6 enhances the oncolytic potency of CRAd AdCB016 on HPV-positive CIN and cervical cancer cell lines, while retaining its selectivity. AdCB016-mp53(268N) is a promising candidate to contribute to a more optimized treatment of HPV-associated cancers and precursor lesions. As not only cervical cancers but also many other cancers of anogenital and oropharyngeal sites contain hr-HPV, enhancement of CRAd potency through expression of functional mutant mp53(268N) in AdCB016 may have wider applicability for more effective and selective treatment of many human cancers.

## Materials and Methods

***Cell lines.*** MDA-MB-231 breast cancer cells and HeLa, SiHa, and CaSki cervical cancer cells were obtained from the American Type Culture Collection (Manassas, VA, USA). C33A cervical cancer cells, HM02 gastric cancer cells, and SaOs-2 osteosarcoma cells were kind gifts from Dr. A. T. Das (Academic Medical Center, Amsterdam, The Netherlands), Dr. A. van der Ende (Academic Medical Center, Amsterdam), and Dr. F. van Valen (Westfalische Wilhelms-Universität, Munster, Germany), respectively. HeLa, SiHa, and CaSki are hr-HPV positive, containing HPV18, 16, and 16, respectively, whereas C33A, HM02, MDA-MB-231, and SaOs-2 are HPV negative. All cell lines were maintained in F12-supplemented DMEM with 10% FCS and antibiotics, except for C33A, which was cultured in DMEM with 10% FCS, MEM minimal essential amino acids, and antibiotics (all from Life Technologies, Breda, The Netherlands).

Primary keratinocytes were obtained from human foreskin essentially as described before [24]. HPV16-immortalized (FK16A and FK16B) and

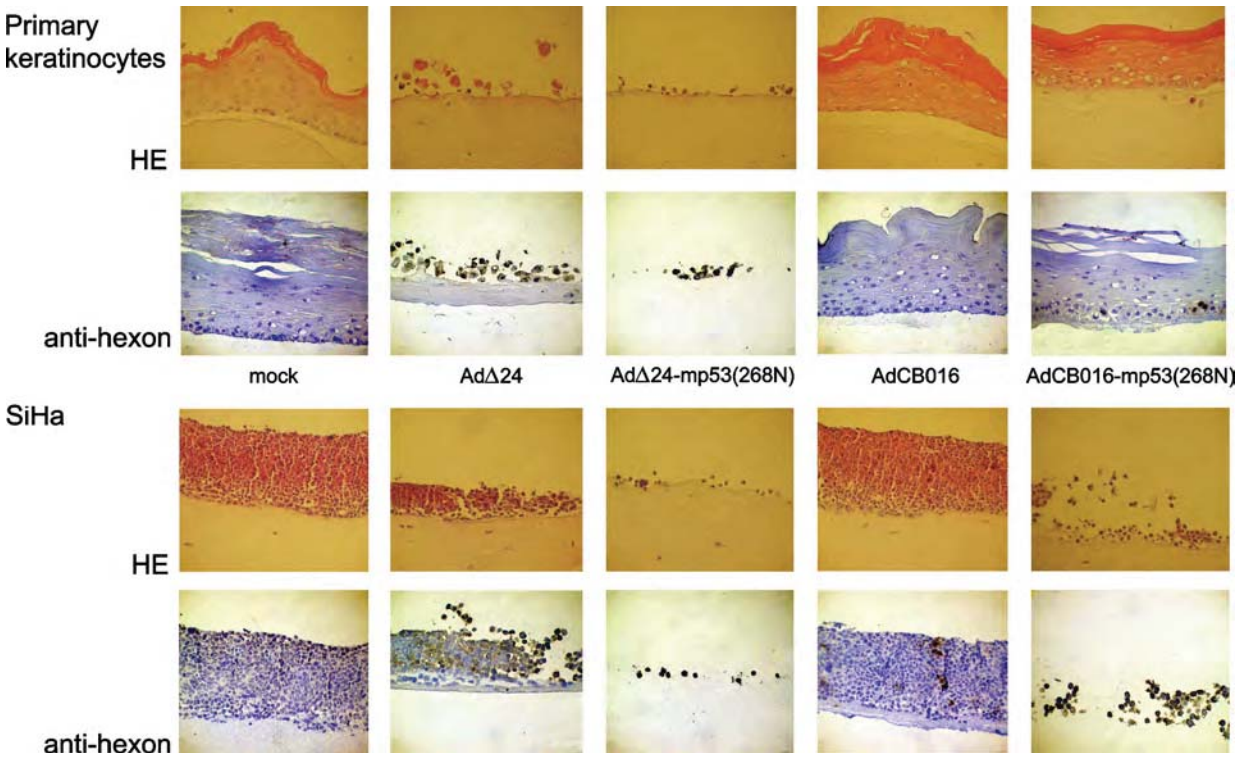


**FIG. 5.** Effects of CRAd infection in raft cultures of normal primary keratinocytes and SiHa cervical carcinoma cells. Primary keratinocytes and SiHa cells were infected with the indicated adenoviruses at an m.o.i. of 1 prior to being transferred to dermal equivalents (see Materials and Methods) and allowed to stratify for 11 days. Data shown are representative hematoxylin-eosin and hexon stainings of one of the two independent experiments performed in duplicate. Original magnification ×120.

HPV18-immortalized (FK18A and FK18B) keratinocyte cell lines were established as described before [20,24]. These keratinocyte cell lines represent dysplastic cells in CIN lesions [20]. Primary keratinocytes and keratinocyte cell lines were grown in serum-free KGM (Life Technologies) supplemented with bovine pituitary extract (50 μg/ml), epidermal growth factor (5 ng/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM) (Life Technologies).

***Recombinant adenoviruses.*** CRAds AdΔ24, AdCB016, and AdΔ24-p53 have been described previously [10,13]. To construct adenoviruses with an expression cassette for mp53(268N) in place of the E3 region, the mp53(268N) cDNA was released from pRc/CMV.mp53(268N) [16] by digestion with *Hin*dIII and *Xba*I and used to replace the human p53 gene in pABS.4-p53 [13]. The resulting construct, pABS.4-mp53(268N), was digested with *Pac*I, and the 3.1-kb fragment encompassing the SV40 early (SVE) promoter-driven mp53(268N) expression cassette and kanamycin resistance gene was inserted into *Pac*I-digested pBHG11 (Microbix Biosystems, Toronto, Canada). A clone with an insert in the orientation that places the SVE-mp53(268N) cassette on the adenovirus L strand was isolated, and the kanamycin resistance gene was removed by digestion with *Swa*I followed by self-ligation, yielding pBHG11-mp53(268N)-L. Expression of mp53(268N) protein from plasmid expression cassettes was confirmed by p53 reporter assay and Western blotting.

CRAds were made by homologous recombination in 911 cells [25] between the pXC1 (Microbix Biosystems) derivative pXC1-Δ24, which carries a 24-bp deletion corresponding to amino acids 122-129 in the CR2 domain of E1A necessary for binding to the Rb protein [12], or pXC1-CB016, which carries the Δ24 mutation and a deletion spanning amino acids 27 to 80 in the CR1 domain of E1A [10], and pBHG11-mp53(268N)-L. This way, AdΔ24-mp53(268N) with the E1A CR2 mutation and AdCB016-mp53(268N) with E1A CR1 and CR2 mutations were made.

Viruses were plaque purified on 911 cells and propagated on A549 cells, and the E1AΔ24 and/or E1ACB016 mutations and SVE-mp53(268N) insertion were confirmed by PCR on the final products. Functional mp53(268N) expression was confirmed by p53 reporter assay and Western blotting. Functional PFU titers were determined side by side by limiting-dilution plaque titration on 293 cells according to standard techniques. In all experiments, infections were normalized on the basis of PFU titers.

***Colorimetric WST-1 cell viability assay.*** Cells were seeded at $10^4$ cells/well in 96-well plates and cultured overnight. The next day, they were infected with AdΔ24 or AdΔ24-p53 at various m.o.i. for 1 h at 37°C. The cells were subsequently cultured at 37°C with 50% medium changes every 3–4 days. At day 11, the culture medium was removed and replaced by 100 μl of 10% WST-1 (Roche Diagnostics, Mannheim, Germany) diluted in culture medium. Depending on the cell type and density, the formation of the formazan dye was allowed to proceed for 60–90 min at 37°C, and the $A_{450}$ was measured on a Bio-Rad (Hercules, CA, USA) Model 550 microplate reader. WST-1 conversion was expressed as a percentage of the conversion by uninfected control cells, after subtraction of background values of WST-1 incubated in the absence of cells.

***Assay for oncolytic activity of CRAds on cancer cells.*** Cells were seeded at $5 \times 10^4$ cells/well in 24-well plates and cultured overnight. The next day, they were infected with CRAds at various m.o.i. for 1 h at 37°C. The cells were subsequently cultured at 37°C with 50% medium changes every 3–4 days. Depending on the inherent replication rate of the viruses on each cell line, cultures were maintained for 2–3 weeks. The culture medium was then removed, and the adherent cells were stained using crystal violet dye as described [13].

***p53 reporter assay.*** To confirm functional p53 expression from plasmid constructs, these were cotransfected into SaOs-2, SiHa, CaSki, or HeLa cells together with either the p53-dependent reporter plasmid PG13-Luc [26] or the negative control construct MG15-Luc [26] using Lipofectamine Plus (Life Technologies) according to the method described by the manufacturer. PG13-Luc carries the luciferase gene driven by a p53-dependent promoter, MG15-Luc carries mutated p53-binding elements. Cells were seeded at $5 \times 10^4$/well in 24-well plates and transfected the next day, and after 24 h of culture at 37°C, the culture medium was replaced by Reporter Lysis buffer (Promega, Madison, WI, USA). Culture plates were subsequently subjected to three freeze/thaw cycles. Chemiluminescence was measured with a Lumat LB 9507 luminometer (EG&G Berthold, Bad Wildbad, Germany). The relative luciferase expression in PG13-Luc-transfected cells compared with MG15-Luc-transfected cells was used as a measure for functional p53 expression.

To confirm resistance of mp53(268N) to E6-mediated degradation, pABS.4-p53 or pABS.4-mp53(268N) was cotransfected into SaOS-2 cells together with pCMV-16E6 or control vector pCMV-neo [27] and with the p53-dependent reporter plasmid PG13-Luc or control MG15-Luc and luciferase activity was measured as above.

***Organotypic culture on collagen rafts.*** Organotypic raft cultures of foreskin keratinocytes or cervical carcinoma cells were prepared essentially as described [23,24]. Primary keratinocytes isolated from two different donors were used. The dermal equivalent contained Swiss 3T3 J2 fibroblasts (a gift from Elaine Fuchs, University of Chicago, Chicago, IL, USA) and raft culture medium contained DMEM:Ham's F-12 (3:1) supplemented with 10% FCS (Life Technologies), hydrocortisone (0.4 μg/ml), 0.1 nM cholera toxin, transferrin (5 μg/ml; Sigma, Saint Louis, MO, USA), insulin (5 μg/ml; Sigma), and human epidermal growth factor (0.5 ng/ml; Life Technologies). For adenoviral infection prior to raft preparation, cells were seeded at $10^6$ cells/well in 6-well plates and cultured overnight. The next day, they were infected with CRAds for 4 h at 1 PFU/cell in serum-free KGM at 37°C and 5% $CO_2$ and transferred onto dermal equivalents thereafter. In addition, also mixtures of infected and uninfected cells at 1:9 and 1:99 ratio were plated. Furthermore, a small aliquot of each cell suspension (approx $10^5$ cells/well) was plated in a 24-well culture plate for simultaneous monolayer evaluation of adenoviral replication and oncolysis. Raft cultures were allowed to differentiate for 11 days. The cultures were then harvested, fixed in 10% buffered formalin, and embedded in paraffin. Four-micrometer sections were stained with hematoxylin and eosin for histological examination. Expression of adenoviral hexon protein was analyzed by immunohistochemistry on 4-μm-thick sections using polyclonal antibody AB1056 (Chemicon International, Temecula, CA, USA), biotinylated rabbit-anti-goat antibody (Zymed Laboratories, Inc., Sanbio bv, Uden, The Netherlands), streptavidin-horseradish peroxidase conjugate (DakoCytomation, Glostrup, Denmark), and chromagen diaminobenzidine, as described before [15]. Slides were counterstained with hematoxylin. Two independent raft experiments in duplicate were performed.

## ACKNOWLEDGMENTS

*We thank Sander Spiekstra, Melanie Breetveld, and Sue Gibbs (Department of Dermatology, VU University Medical Center) for their help in obtaining primary keratinocytes. We thank Jeroen Mastenbroek (Division of Gene Therapy, Department of Medical Oncology, VU University Medical Center) and Wil Dekker (Department of Pathology, VU University Medical Center) for expert technical assistance. We thank Erik Hooijberg (Department of Pathology, VU University Medical Center) and Ruud Brakenhoff (Department of Otolaryngology/Head and Neck Surgery, VU University Medical Center) for helpful discussions. R.D.M.S. and V.W.v.B. are supported by research fellowships of the Royal Netherlands Academy of Arts and Sciences.*

## REFERENCES

1. Walboomers, J. M., *et al.* (1999). Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J. Pathol.* **189:** 12–19.
2. Munoz, N., *et al.* (2003). Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N. Engl. J. Med.* **348:** 518–527.
3. von Knebel Doeberitz, M., Oltersdorf, T., Schwarz, E., and Gissmann, L. (1988). Correlation of modified human papilloma virus early gene expression with altered growth properties in C4-1 cervical carcinoma cells. *Cancer Res.* **48:** 3780–3786.
4. Dyson, N., Howley, P. M., Münger, K., and Harlow, E. (1989). The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science* **243:** 934–937.
5. Scheffner, M., Werness, B. A., Huibregtse, J. M., Levine, A. J., and Howley, P. M. (1990). The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell* **63:** 1129–1136.
6. Prendiville, W. (2003). LLETZ: theoretical rationale, practical aspects, clinical experience, optimizing the technique. In *Colposcopy: Management Options* (W. Prendiville, J. Ritter, S. Statti, L. Twiggs Eds.). Saunders/Elsevier, London.
7. Heise, C., and Kirn, D. H. (2000). Replication-selective adenoviruses as oncolytic agents. *J. Clin. Invest.* **105:** 847–851.
8. Alemany, R., Balague, C., and Curiel, D. T. (2000). Replicative adenoviruses for cancer therapy. *Nat. Biotechnol.* **18:** 723–727.
9. Oosterhoff, D., and van Beusechem, V. W. (2004). Conditionally replicating adenoviruses as anticancer agents and ways to improve their efficacy. *J. Exp. Ther. Oncol.* **4:** 37–57.
10. Balague, C., Noya, F., Alemany, R., Chow, L. T., and Curiel, D. T. (2001). Human papillomavirus E6E7-mediated adenovirus cell killing: selectivity of mutant adenovirus replication in organotypic cultures of human keratinocytes. *J. Virol.* **75:** 7602–7611.
11. Heise, C., *et al.* (2000). An adenovirus E1A mutant that demonstrates potent and selective systemic anti-tumoral efficacy. *Nat. Med.* **6:** 1134–1139.
12. Fueyo, J., *et al.* (2000). A mutant oncolytic adenovirus targeting the Rb pathway produces anti-glioma effect in vivo. *Oncogene* **19:** 2–12.
13. van Beusechem, V. W., van den Doel, P. B., Grill, J., Pinedo, H. M., and Gerritsen, W. R. (2002). Conditionally replicative adenovirus expressing p53 exhibits enhanced oncolytic potency. *Cancer Res.* **62:** 6165–6171.
14. Geoerger, B., *et al.* (2004). Oncolytic activity of p53-expressing conditionally replicative adenovirus AdDelta24-p53 against human malignant glioma. *Cancer Res.* **64:** 5753–5759.
15. Geoerger, B., *et al.* (2005). Expression of p53, or targeting towards EGFR enhances the oncolytic potency of conditionally replicative adenovirus against neuroblastoma. *J. Gene Med.* **7:** 584–594.
16. Hengstermann, A., Linares, L. K., Ciechanover, A., Whitaker, N. J., and Scheffner, M. (2001). Complete switch from Mdm2 to human papillomavirus E6-mediated degradation of p53 in cervical cancer cells. *Proc. Natl. Acad. Sci. USA* **98:** 1218–1223.
17. Scheffner, M., Munger, K., Byrne, J. C., and Howley, P. M. (1991). The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. *Proc. Natl. Acad. Sci. USA* **88:** 5523–5527.
18. Ferreux, E., *et al.* (2003). Evidence for at least three alternative mechanisms targeting the p16INK4A/cyclin D/Rb pathway in penile carcinoma, one of which is mediated by high-risk human papillomavirus. *J. Pathol.* **201:** 109–118.
19. van Houten, V. M., *et al.* (2001). Biological evidence that human papillomaviruses are etiologically involved in a subgroup of head and neck squamous cell carcinomas. *Int. J. Cancer* **93:** 232–235.
20. Steenbergen, R. D., *et al.* (1998). Viral E6–E7 transcription in the basal layer of organotypic cultures without apparent p21cip1 protein precedes immortalization of human papillomavirus type 16- and 18-transfected human keratinocytes. *J. Virol.* **72:** 749–757.
21. Koutsky, L. A., *et al.* (2002). A controlled trial of a human papillomavirus type 16 vaccine. *N. Engl. J. Med.* **347:** 1645–1651.
22. Harper, D. M., *et al.* (2004). Efficacy of a bivalent L1 virus-like particle vaccine in prevention of infection with human papillomavirus types 16 and 18 in young women: a randomised controlled trial. *Lancet* **364:** 1757–1765.
23. Huang, X. F., *et al.* (2003). A broadly applicable, personalized heat shock protein-mediated oncolytic tumor vaccine. *Cancer Res.* **63:** 7321–7329.
24. Steenbergen, R. D., *et al.* (1996). Transition of human papillomavirus type 16 and 18 transfected human foreskin keratinocytes towards immortality: activation of telomerase and allele losses at 3p, 10p, 11q and/or 18q. *Oncogene* **13:** 1249–1257.
25. Fallaux, F. J., *et al.* (1996). Characterization of 911: a new helper cell line for the titration and propagation of early region 1-deleted adenoviral vectors. *Hum. Gene Ther.* **7:** 215–222.
26. el-Deiry, W. S., *et al.* (1993). WAF1, a potential mediator of p53 tumor suppression. *Cell* **75:** 817–825.
27. Kessis, T. D., *et al.* (1993). Human papillomavirus 16 E6 expression disrupts the p53-mediated cellular response to DNA damage. *Proc. Natl. Acad. Sci. USA* **90:** 3988–3992.