Conditionally Replicative Adenovirus Expressing p53 Exhibits Enhanced Oncolytic Potency

Victor W. van Beusechem, Petra B. van den Doel, Jacques Grill, Herbert M. Pinedo, and Winald R. Gerritsen

Division of Gene Therapy, Department of Medical Oncology, VU University Medical Center, 1081 HV Amsterdam, the Netherlands [V. W. v. B., P. B. v. d. D., J. G., H. M. P., W. R. G.J, and Department of Pediatrics and Laboratory of Pharmacotoxicology and Pharmacogenetics, Instituut Gustave Roussy, 94 805 Villejuif, France [J. G.]

ABSTRACT

Conditionally replicative adenoviruses (CRAds) hold promise as anticancer agents. Their potency depends on their replication efficiency in cancer cells and their capacity to destroy these cells by oncolysis. In this regard, a critical determinant is the capacity of CRAds to induce cell death at late stages of infection to release their progeny. One of the cell death pathways that are exploited by adenoviruses involves the tumor suppressor protein p53. Unfortunately, many cancer cells have a nonfunctional p53 pathway and thus do not effectively support CRAd-induced cell death. We hypothesized that restoration of the p53-dependent cell death pathway in cancer cells would promote CRAd-induced cell lysis. Exogenous expression of p53 in human cancer cells during adenovirus replication accelerated cell death by several days and augmented early virus progeny release. The p53-enhanced oncolysis occurred independent of E1A binding to pRb and independent of E3 functions. On the basis of these findings, we constructed a new CRAd, AdΔ24-p53. This virus expressed functional p53 while replicating in cancer cells. Most importantly, AdΔ24-p53 exhibited enhanced oncolytic potency on 80% of tested human cancer cell lines of various tissue origins and with different p53 status. CRAd potency was increased up to >100-fold by p53 expression. We conclude that CRAds expressing p53 are promising new agents for more effective treatment of many human cancers.

INTRODUCTION

CRAds represent a novel class of anticancer agents (reviewed in Refs. 1, 2). CRAds are designed to selectively replicate in tumor cells and to destroy these cells by inducing lysis. The release of viral progeny from lysed tumor cells offers the potential to amplify CRAds in situ and to achieve lateral spread to neighboring cells in a solid tumor, thus expanding the oncolytic effect. The restriction of CRAd replication to tumor cells dictates the safety of the agent. Tumor-selective replication is achieved through either of two alternative strategies. In the first strategy, a tumor-specific promoter controls the expression of an essential early adenovirus gene (3, 4). The second strategy involves the introduction of mutations in viral genes to abrogate the interaction of the encoded proteins with cellular proteins, necessary to complete the viral life cycle in normal cells but not in tumor cells (5–7). Currently, CRAd-based cancer treatments are already being evaluated in clinical trials (see, for example, Refs. 8–10).

The anticancer potency of a CRAd depends on the efficiency at which the virus disseminates throughout a tumor and kills tumor cells via replication. A critical step determining the rate of virus replication is the release of newly formed virus from an infected cell through the induction of lysis at late stages of infection. It has been suggested that host cells are lysed as a consequence of suppression of cellular protein production (11). However, other important mechanisms used by adenoviruses to accomplish cell death involve induction of apoptosis (reviewed in Refs. 12, 13). In different cell lines, p53-dependent as well as p53-independent apoptosis has been documented after adenovirus infection, and these processes were shown to involve adenovirus E1A and E4orf4 proteins. In addition, the E3-11.6kDa adenovirus death protein was shown to mediate efficient cell lysis at late stages of infection by a currently unresolved mechanism (14). To prevent premature death of a host cell, which would compromise virus progeny production, apoptosis needs to be suppressed during the early stages of viral replication. In this respect, important adenovirus-encoded regulators of apoptosis include the E1B-19kDa and -55kDa proteins and the E4-34kDa protein. The E1B-55kDa and E4-34kDa proteins cooperate to suppress apoptosis at least in part by forming a complex with p53 and inhibiting p53-mediated transactivation as well as promoting p53 degradation (15–17). The E1B-19kDa protein interacts with proapoptotic members of the bcl-2 family to inhibit the caspase-9-dependent apoptosis pathway (18).

Interestingly, replicating adenovirus has been shown to kill cells expressing functional p53 more rapidly than cells deficient in p53 (19, 20). In addition, the formation of a complex between p53 and the E1B-55kDa protein was found to be essential for this rapid induction of cell death by adenovirus (20). It was thus concluded that adenoviruses exploit multiple cell death processes, of which a p53- and E1B-55kDa protein-dependent pathway is the most rapid one. However, the gene encoding p53 is nonfunctional through deletion or mutation in ~50% of human cancers (21). In addition, in cancer cells that express wild-type p53, the protein may also be functionally compromised by, for example, loss of p14ARF, amplification of MDM2, or viral protein-promoted degradation (22–25). Thus, in many, and perhaps all, human tumors, CRAd progeny release and lateral spread might be hampered as a result of functional p53 deficiency. We hypothesized, therefore, that CRAds could be made into more effective oncolytic agents by expressing exogenous p53 protein in infected cancer cells. This should accelerate oncolysis and virus release, leading to faster lateral spread and more effective tumor regression.

In the present study, we tested this hypothesis by expressing p53 during adenovirus replication in tumor cell lines with differing p53 status. Enforced expression of p53 induced more rapid cell death and earlier virus release in p53-deficient as well as p53 wild-type cancer cells. On the basis of these findings, we constructed the p53-expressing CRAd, AdΔ24-p53. This virus killed human cancer cells of various tissue origins and p53 status more effectively than did its parental control virus without the p53 insert. We conclude that CRAds expressing p53 are promising agents for cancer treatment.

MATERIALS AND METHODS

Cell Lines. A549 and NCI-H460 lung carcinoma cells, MCF-7 and MDA-MB-231 breast carcinoma cells, HT-29 and COLO205 colon carcinoma cells, U373MG astrocytoma cells, HeLa cervix carcinoma cells, A431 vulva squa-
nous carcinoma cells, HepG2 hepatoma cells and SK-OV-3, OVCAR-3, and A2780 ovary carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). SF763 astrocytoma cells were provided by the UCSF Neurosurgery Tissue Bank (San Francisco, CA), SaOs-2 and MNG-HOS osteosarcoma cells, SW1398 colon carcinoma cells, U118MG glioma cells, UM-SCC-11B and UM-SCC-22A head and neck squamous carcinoma cells, MKN28 and MKN45 gastric carcinoma cells, MG-63 osteosarcoma cells, T24 bladder carcinoma cells, and PC-3 prostate carcinoma cells were kind gifts of Dr. F. van Valen (Westfalsche Wilhelms-Universität, Münster, Germany); Dr. E. Boven (VUMC, Amsterdam, the Netherlands); Dr. J. T. Douglas (Gene Therapy Center, UAB, Birmingham, AL); Dr. R. Brak- enhoff (VUMC, Amsterdam, the Netherlands); Dr. M. Tsujii (Osaka University School of Medicine, Osaka, Japan); Dr. C. W. Lowik (LUMC, Leiden, the Netherlands); Dr. H. van der Poel (Netherlands Cancer Institute, Amsterdam, the Netherlands), and Dr. L. Blok (Erasmus University Rotterdam, the Netherlands), respectively. All cell lines were maintained in F12-supplemented DMEM with 10% FCS and antibiotics, except for MKN28, MKN45, and NCI-H460, which were cultured in RPMI 1640 with 10% FCS and antibiotics (all from Life Technologies, Inc., Paisley, United Kingdom). Available literature information on the p53 genetic status of the cell lines is given in Table 2.

Recombinant Adenoviruses. The replication-deficient adenovirus vector AdGFP that expresses cytoplasmalgaevirus immediate early promoter-driven enhanced green fluorescent protein has been described previously (26). Adwtp53 (27) was made by homologous recombination in 293 cells (American Type Culture Collection) between ClaI-digested Ad5dl324 and the linearized plasmid pAdHumPwt.SVE, which comprises the Ad5 left-hand arm with a human p53 expression cassette in the E1-region. The p53 gene is driven by a SV40 promoter and includes intron 4. Wild-type Ad5 was kindly provided by Dr. R. C. Hoeben (LUMC, Leiden, the Netherlands). AdE1–Luc (a kind gift of Dr. R. Brakenhoff, VUMC, Amsterdam, the Netherlands) was derived from Ad5 through replacement of the p19k open reading frame in the E3 region by the firefly luciferase gene. Luciferase expression is therefore under the control of the endogenous E3 promoter.

To construct adenoviruses with an expression cassette for p53 in the E3 region, the SVE-p53 expression cassette was released from pAdHumPwt.SVE by digestion with KpnI and XhoI (partial). The 2.6-kb fragment was inserted into KpnI/XhoI-digested pABS.4 (Microbix Biosystems, Toronto, Canada). The resulting construct, pABS.4-p53, was digested with PstI, and the 4.0-kb fragment carrying the SVE-p53 cassette and kanamycin resistance gene was inserted into PstI-digested pBHG11 (Microbix Biosystems). A clone with an insert in the orientation that places the SVE-p53 cassette on the adenovirus L-strand was isolated, and the kanamycin resistance gene was removed by digestion with Ssul followed by self-ligation, yielding pHBGI1-p53-L. Expression of p53 protein from plasmid expression cassettes was confirmed by p53 reporter assay.

CRAds were made by homologous recombination in 293 cells between the pXCI (Microbix Biosystems) derivative pXCI–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 (Ref. 7; a kind gift of Dr. R. Alemeney, Gene Therapy Center, UAB, Birmingham, AL), with pHBG11 or pBHG11–p53-L. This way, Ad2A4, with the E1A CR2 mutation, and its p53-expressing derivative, Ad2A4-p53, were made.

Viruses were plaque purified, propagated on 293 cells for replication-deficient vectors or on A549 cells for replicative viruses, and purified by CsCl gradient according to standard techniques. The E1Δ24 mutation and SVE-p53 insertion were confirmed by PCR on the final products. Particle titers of all adenoviruses were determined by absorbance measurements at 260 nm. The functional PFU titers were determined by limiting-dilution plaque titration on 293 cells according to standard techniques. Particle/PFU ratios were 4 for AdGFP, 33 for Adwtp53, 8 for Ad5, 2 for AdE1–Luc, 42 for Ad2A4, and 22 for Ad2A4-p53. In all experiments, infections were normalized on the basis of PFU titers.

Replication of Dual Adenovirus Mixtures on Cancer Cells. Cells were seeded at 5 × 10⁴ cells/well in 24-well plates and cultured overnight. The next day, dual-virus mixtures with equal PFU titers were prepared of AdE1–Luc with AdGFP or AdE1–Luc with Adwtp53. The mixtures were used to infect SaOs-2 cells at a MOI of 50 PFU/cell for each virus 1 h at 37°C. A549, NCI-H460, MCF-7, and U373MG were infected at 100 PFU/cell, and OVCAR-3 and HT29 cells were infected at 500 PFU/cell of each virus for 1 h at 37°C. MOIs were chosen on the basis of pilot experiments with AdGFP to enable efficient infection of each cell line, such that many cells would contain both mixed viruses. The cells were then washed once with medium and subsequently cultured at 37°C for up to 14 days. At several time points, culture medium was harvested and cleared by centrifugation. The cell-free supernatant was used to measure the titers of released AdE1–Luc virus. The cells were subjected to WST-1 assay to measure their viability. Nonadherent cells collected by centrifugation and adherent cells scraped from the culture plate were combined, resuspended in culture medium, and subjected to three freeze/thaw cycles. The lysate was cleared by centrifugation and used to measure the AdE1–Luc virus titer inside the cells.

Colorimetric WST-1 Cell Viability Assay. The culture medium was removed and replaced by 200 μl of 10% WST-1 (Roche Diagnostics, Mannheim, Germany) in culture medium. Depending on the cell type and density, the formation of the formazan dye was allowed to proceed for 30–60 min at 37°C. Subsequently, 100 μl of WST-1 medium was transferred to a 96-well ELISA plate (Greiner, Frickenhausen, Germany) and the A570 was measured on a Bio-Rad (Hercules, CA) 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.

Titration of AdE1–Luc Virus Produced in Cell Cultures. Cleared culture supernatant or cell lysate was serially diluted in culture medium and used to infect A549 cells seeded at 10⁴ cells/well in 96-well plates 24 h before infection. A control titration of AdE1–Luc virus with known PFU titer was included. After 20–24 h, the culture medium was replaced by Luciferase Chemiluminescent Assay System Reporter Lysis Buffer (Promega, Madison, WI), and the culture plates were subjected to a single freeze/thaw cycle. Chemiluminescence was measured with a Lumat LB 9507 luminometer (EG&G Berthold, Bad Wildbad, Germany) during the 10 s immediately after addition of the bioluminescence assay reagent. The A570 range of the serial dilution was used to calculate the luciferase infectious unit titer. The assay was linear over three to four orders of magnitude, with a threshold of 10⁴ PFU.

Assay for Oncolytic Activity of CRAds on Cancer Cells. Cells were seeded at 5 × 10⁴ cells/well in 24-well plates and cultured overnight. The next day, they were infected with AdΔ2Δ or AdΔ2Δ-p53 at the indicated MOI or with dual-virus mixtures consisting of 50 PFU/cell AdGFP or Adwtp53 with 0.01, 0.1, 1, or 10 PFU/cell Ad5 or AdΔ2Δ for 1 h at 37°C. The cells were subsequently cultured at 37°C with 50% medium changes every 4–5 days. Depending on the inherent replication rate of AdΔ2Δ on each cell line, cultures were maintained for 1–3 weeks. The culture medium was then removed, and the adherent cells were washed with PBS (10.9 mm NaHPO4, 1.8 mm Na2HPO4, 8.2 g/l NaCl), fixed for 10 min at room temperature in a 4% (v/v) formaldehyde in PBS, and stained using 10 g/l crystal violet dye in 70% (v/v) ethanol for 70 min at room temperature. After several washes with water, the culture plates were air-dried and scanned on a Bio-Rad GS-690 imaging densitometer.

Western Analysis. Cells were seeded in 6-well plates at a density of 5 × 10⁵ cells/well. The next day, the cells were infected with AdΔ2Δ or AdΔ2Δ-p53 at 100 PFU/cell for 1 h, washed once with medium, and incubated with fresh medium at 37°C and 5% CO2. The cells were harvested and lysed in 200 μl of a mixture of 140 mm NaCl, 0.2 1, and 0.2 triethanolamine, 2 g/l deoxycholate, 1 mm phenylmethylsulfonil fluoride, and 50 μg/ml antipain by three freeze/thaw cycles at 24, 48, 72, and 96 h after infection. The cytopathic effect of the adenovirus precluded analysis at later time points. Lysates were collected by centrifugation and adherent cells scraped from the culture plate. The AdE1–Luc virus. The cells were air-dried and scanned on a Bio-Rad GS-690 imaging densitometer.
Cells Accelerates Virus Release.

The relative luciferase expression in PG13-Luc-transfected cells compared with MG15-Luc-transfected cells was used as a measure for functional p53 expression. Ratios between 0.5 and 2.0 were considered to represent a p53-deficient status, ratios of 2–10 as impaired p53 activity, and ratios >10 were scored as representing a functional p53 status. To confirm functional p53 expression from plasmid constructs, these were cotransfected into SaOs-2 cells together with PG13-Luc and assayed as above.

To assess p53-specific transactivation as a result of adenovirus infection and exogenous p53 introduction, A549 and SaOs-2 cells were seeded and transfected with PG13-Luc as above. The next day, the cells were infected with AdΔ24 or AdΔ24-p53 for 1 h at 100 PFU/cell. Cells were cultured for 2 days, and luciferase activity was measured as above. p53-dependent transactivation was expressed as the luciferase activity in relative light units, after subtraction of the background expression in cells transfected with an irrelevant control plasmid.

Statistical Analysis. Statistical significance between experimental groups, i.e., virus titters and percentage of virus release in the presence versus absence of p53 expression (Table 1) and p53-enhanced oncolysis of cell lines with p53 functional versus p53-deficient status (Table 2), was tested by two-tailed nonparametric Mann–Whitney test.

RESULTS

Expression of Functional p53 in Adenovirus-infected Cancer Cells Enhances Oncolysis. Human cancer cells were infected with a dual-virus system consisting of equal amounts of the replication-competent adenovirus AdE1+Luc, which expresses the firefly luciferase marker gene, and the replication-defective vector Adwtpt53, which expresses human wild-type p53. This dual-virus system creates a situation in which p53 is expressed in the context of a replicating adenovirus. As a negative control, Adwtpt53 was replaced by the irrelevant control vector AdGFP. In addition to control cultures, cells were infected with AdGFP or Adwtpt53 only to investigate the effect of cell cycle arrest or cell death promotion by p53 per se. Four human cancer cell lines with different p53 statuses were infected, and cell viability was monitored over a 2-week period (Fig. 1A). Adwtpt53 infection alone affected SaOs-2 and OVCAR-3 cells during the first few days after infection, but had no significant effect on the viability of A549 and U373MG cells. Moreover, all Adwtpt53-infected cultures fully recovered during the course of the experiment. Thus, p53 expression alone did not produce effective killing of these cancer cell lines. In contrast, AdE1+Luc/Adwtpt53 dual infection killed the cancer cells effectively. Most importantly, comparison with AdE1+Luc/AdGFP-infected cultures showed that p53 expression accelerated the killing of all four tested cell lines by at least 2 days. Strikingly, to be at all sensitive to adenovirus-induced cell death, OVCAR-3 cells required wild-type p53 expression. Hence, the combination of adenovirus replication and wild-type p53 expression caused the fastest and most effective cell death.

Expression of Functional p53 in Adenovirus-infected Cancer Cells Accelerates Virus Release. To investigate whether enhanced oncolysis leads to earlier release of infectious virus progeny, the AdE1+Luc virus titers were determined in the culture medium of dual-virus-infected cells (Fig. 1B). Introduction of functional p53 by Adwtpt53 infection enhanced the virus titers in the medium shortly after infection (with a range of 3–20-fold). In the case of SaOs-2 cells, where adenovirus replication was slow, this was at the expense of a decrease in the total amount of virus produced. On the other hand, p53 expression enhanced the virus output from U373MG cells and did not influence virus production by the other two cell lines. In the presence of p53, progeny virus release by all four cell lines was completed within 1 week, whereas this took 10–14 days in control cultures.

The faster increase of virus titer in the culture medium of infected cells could be the result of accelerated virus production and/or earlier virus release. To assess the cause for the observed titer differences early after infection, the AdE1+Luc virus titer was measured in cell lysates as well as in culture media of seven different cancer cell lines 3 days after infection (Table 1). The intracellular virus progeny production was not significantly affected by p53 expression (from a 5-fold decrease to a 7-fold increase in different cell lines; P = 0.80). In contrast, p53 expression reproducibly increased the AdE1+Luc titer in the medium by an average of 46-fold (P = 0.007). In cells co-infected with Adwtpt53, a larger proportion of the total AdE1+Luc virus progeny were released within 3 days (P = 0.004). Thus, accelerated lysis of cancer cells attributable to exogenous p53 expression led to enhanced release of infectious virus progeny.

Expression of p53 Enhances the Oncolytic Potency of AdΔ24 CRAd. On the basis of the observations described above, we decided to construct conditionally replicative adenoviruses expressing p53. As the type of CRAd, we chose AdΔ24 (7), which carries a mutation encoding a deletion of eight amino acids in the pRb-binding CR2 domain of E1A. Furthermore, AdΔ24 lacks the entire E3 coding region. Because E1A and E3 functions may influence cell death processes, we first investigated whether p53-enhanced oncolysis required wild-type E1A and E3 functions. To this end, SaOs-2 cancer
Luc virus titers in these fractions were determined. Harvested separately, and the AdE1/H11001 virus mixtures. After 3 days of culture, the cell-free culture medium and the cells were AdGFP together with wild-type Ad5 (E1A/H11002). Ad5 and Ad24 or Adp53 wild-type A549 cancer cells infected with Ad/H9004 E3 protein expression appeared essential for p53-augmented oncolysis 10-fold. Thus, neither pRb binding by adenovirus E1A protein nor p53 expression was functional in p53-deficient cancer cells at regulatory levels comparable to those found in CRAd-infected wild-type p53 cells. Interestingly, AdΔΔ24-p53 appeared to replicate faster than AdΔΔ24 in both cell lines. In AdΔΔ24-p53-infected cells, fiber expression was detectable sooner and p53 expression declined more rapidly. Functional activity of the introduced p53 was confirmed by specific transactivation of the reporter plasmid PG13-Luc, which contains a luciferase gene linked to a p53-dependent promoter (28; Fig. 3f). Luciferase activity in PG13-Luc-transfected SaOs-2 cells remained low after infection with AdΔΔ24, but rose markedly after infection with AdΔΔ24-p53, indicating that the expression of p53 caused transactivation of p53-dependent genes.

### Table 1 Exogenous p53 enhances the early release of infectious adenovirus progeny from human cancer cells

<table>
<thead>
<tr>
<th>Cell line (p53 status)</th>
<th>Co-infection with AdE1+Luc</th>
<th>AdE1+Luc titer 3 days after infection</th>
<th>Progeny released (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inside cells</td>
<td>In medium</td>
<td></td>
</tr>
<tr>
<td>A549 wild type</td>
<td>AdGFP</td>
<td>6199 ± 1528</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>NCI-H460 wild type</td>
<td>AdGFP</td>
<td>19794 ± 1645</td>
<td>1061 ± 87</td>
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<tr>
<td>MCF-7 wild type</td>
<td>AdGFP</td>
<td>482 ± 69</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>SaOs-2 (null)</td>
<td>AdGFP</td>
<td>1178 ± 119</td>
<td>194 ± 30</td>
</tr>
<tr>
<td>SaOs-2 (mut R248Q)</td>
<td>AdGFP</td>
<td>25607 ± 4813</td>
<td>12 ± 6</td>
</tr>
<tr>
<td>HT-29 (null)</td>
<td>AdGFP</td>
<td>2444 ± 4184</td>
<td>279 ± 55</td>
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<tr>
<td>HT-29 (mut R237H)</td>
<td>AdGFP</td>
<td>159 ± 55</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>U373MG AdGFP</td>
<td>AdGFP</td>
<td>39 ± 4</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>U373MG Adwtp53</td>
<td>AdGFP</td>
<td>718 ± 56</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>SaOs-2 (mut R237H)</td>
<td>AdGFP</td>
<td>397 ± 77</td>
<td>13 ± 3</td>
</tr>
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</table>

* Data are mean (×10⁶) infectious units ± SD of five cultures.

### Discussion

We hypothesized that CRAds could be made into more effective oncolytic agents by enhancing adenovirus-induced cell death in infected cancer cells. Recently, this concept was supported by the finding that induction of cell death in adenovirus-infected cancer cells after completion of virus assembly enhanced progeny virus release. In contrast, induction of apoptosis during adenovirus replication compromised the virus progeny production (31). Thus, careful timing of apoptosis induction was crucial. Because CRAd replication on a population of tumor cells tends to be asynchronous, we reasoned that precise timing is difficult to achieve unless induction of cell death could be linked to the CRAd replication cycle. We envisaged that this could perhaps be accomplished by exploiting the inherent capacity of adenoviruses to regulate apoptosis; we therefore chose to construct a CRAd in which the central coordinator of apoptosis, p53, was constitutively expressed. For proper regulation of apoptosis during CRAd replication, we relied on the natural adenovirus-encoded regulators of the p53-dependent apoptosis pathway. In addition, two recent findings suggested that even if early p53 expression were not antagonized completely by CRAd-encoded proteins, this would not compromise virus production. The first finding was that expression of p53 or a chimeric p53 variant with full transcriptional activity but

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**Fig. 2. Exogenous p53 expression enhances oncolysis by adenoviruses with mutant E1A and deleted E3 genes. SaOs-2 cells were infected with AdGFP or Adwtp53 at 50 PFU/cell alone or in combination with AdΔΔ24 at the indicated MOI.**
lacking the binding domain for the E1B-55kDa protein did not affect adenovirus replication in cancer cells (32). The second finding was that an adenovirus encoding a mutant E1B-55kDa protein with no ability to bind and degrade p53 could replicate efficiently in p53 wild-type cancer cells (33). We thus expected that enforced expression of p53 during CRAd replication would restore the ability of cancer cells with dysfunctional p53 to undergo programmed cell death, without loss of CRAd progeny production.

Because the formation of a complex between p53 and the E1B-55kDa protein was previously found essential for a rapid induction of cell death by adenovirus (20) and because other adenovirus proteins such as E3-adenovirus death protein were perhaps also involved, we tested our hypothesis by expressing p53 during replication of a recombinant adenovirus that comprises an almost complete Ad5 genome, except for the E3-gp19k gene, which was replaced by the luciferase marker gene. Reproducibly, p53 expression accelerated cell lysis by several days and enhanced early progeny virus release. These effects appeared independent of the p53 genetic background of the cell. Comparisons between adenoviruses with or without deletions in the E1A and E3 regions showed that p53-enhanced oncolysis occurred independent of E1A pRb binding and E3 functions. On the basis of these observations, we constructed the novel p53-expressing CRAd, AdΔ24-p53. This adenovirus is conditionally replicative because of a deletion in the pRb-binding CR2 domain of the E1A protein and lacks the entire E3 region, but has intact E1B and E4 regions.

AdΔ24-p53 replicated efficiently in human cancer cells and expressed functional p53 protein. Most importantly, AdΔ24-p53 exhibited enhanced oncolytic potency compared with its parent control AdΔ24 on the majority (21 of 26) of tested cancer cell lines. Although, as expected, the oncolytic potency was enhanced most profoundly on p53-deficient cells, there was no significant correlation with p53 status. Beforehand, we had anticipated that exogenous p53 expression would be ineffective in two types of cancer cells: (a) cells with enhanced p53 degradation and (b) cells expressing dominant-negative p53 mutants. However, on these cell types AdΔ24-p53 also clearly exhibited enhanced oncolytic potency.

Cancer cells expressing wild-type p53 are often resistant to p53 gene therapy with replication-defective adenovirus vectors (34). This is explained by efficient degradation of p53 in these cells. Most of the p53 wild-type cells included in our study were p14ARF-deficient and were thus expected to efficiently degrade exogenous p53 through binding to human MDM2 (22, 23). In addition, HeLa cells express human papilloma virus-E6 capable of inactivating p53 (24, 35, 36). Nevertheless, in most of these p53 wild-type cell lines, p53 introduction enhanced CRAd-induced oncolysis. This suggested that exogenous p53 levels were high enough to overcome sequestration. Alternatively, binding of CRAd-encoded proteins to exogenous p53 may have prevented MDM2- or E6-mediated degradation.

Mutant p53 proteins in cancer cells sometimes interfere in a dominant-negative fashion with the apoptosis promoting action, but not with the antiproliferative effect, of exogenous wild-type p53 (34, 37). Relative amounts of wild-type and mutant p53 proteins in a cell determine the dominant-negative effects (30, 38). However, we did not find evidence for such interference with p53-mediated oncolysis enhancement. In fact, enhanced oncolysis and accelerated virus release by wild-type p53 expression was seen in most cell lines expressing p53 missense mutations, including two cell lines with the R273H mutation for which a
clear dominant-negative effect has been observed in apoptosis assays (37). Perhaps AdΔ24-p53 expressed high enough levels of wild-type p53 in cancer cell lines expressing mutant p53 to outnumber the endogenous protein. Alternatively, the molecular process underlying p53-mediated oncolysis enhancement may be distinct from p53-specific apoptosis induction. In fact, our preliminary findings from a study into the mechanism of AdΔ24-p53-induced cell death suggest that classical p53-dependent apoptosis, as evidenced by mitochondrial membrane disruption (39, 40), is not the sole cause of rapid oncolysis (4). Further studies are warranted to unravel the process underlying p53-mediated oncolysis enhancement. Differential gene expression profiling of responsive and unresponsive cell lines infected with AdΔ24-p53 might serve as a starting point for such investigations.

A potentially complementing approach to accelerate the lateral spread of replication-competent adenoviruses by enhancing apoptosis is to delete the gene coding for the E1B-19kDa protein (41). The absence of this major inhibitor of early apoptosis was shown to enhance programmed cell death in adenovirus-infected human lung cancer cells. Moreover, E1B-19kDa-deficient adenovirus exhibited augmented virus release, increased plaque size (indicative of accelerated viral spread), and in vivo antitumor efficacy on the p53 wild-type A549 cell line (41, 42). One could thus speculate that E1B-19kDa-deleted adenoviruses promote a p53-dependent cell death pathway. The new CRAd described here is believed to restore this pathway in p53-deficient human cancers. It would therefore be interesting to investigate whether by deleting the E1B-19kDa gene from AdΔ24-p53 its oncolytic potency can be enhanced even further.

In conclusion, we have shown that expression of functional p53 enhances the oncolytic potency of a CRAd on the majority of tested cancer cell lines. Our study included a wide variety of cancer cell lines of different tissue origins and p53 genetic background, including mutations at p53 residues 248 and 273 in the DNA-binding domain, which represent the two most common p53 mutations found in human cancers (5). Hence, enhancement of CRAd potency through expression of functional p53 may have wide applicability for more effective treatment of many human cancers.

REFERENCES


Table 2 Semiquantitative assessment of enhanced oncolysis by AdΔ24-p53 in human cancer cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>p53 genetic statusa</th>
<th>p53 functional statusb</th>
<th>Enhancement of CRAd oncolytic potency by p53 expressionc</th>
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<tbody>
<tr>
<td>A2780</td>
<td>Wild type (43)</td>
<td>ND (1.5)</td>
<td>– (2)</td>
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<tr>
<td>MKN45</td>
<td>Wild type (44)</td>
<td>Functional</td>
<td>– (1.5)</td>
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<td>UM-SSC-11B</td>
<td>Unknown</td>
<td>Functional</td>
<td>+ (1.5)</td>
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<td>+ (2)</td>
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<td>MCF-7</td>
<td>Wild type (47, 48)</td>
<td>Functional</td>
<td>+ (2)</td>
</tr>
<tr>
<td>NCI-H460</td>
<td>Wild type (47, 49)</td>
<td>Impaired</td>
<td>+ (2)</td>
</tr>
<tr>
<td>HeLa</td>
<td>Wild type, HPV-E6+</td>
<td>Impaired</td>
<td>+ (2)</td>
</tr>
<tr>
<td>SF763</td>
<td>Arg158 to Leu (50)</td>
<td>Deficient</td>
<td>– (1.5)</td>
</tr>
<tr>
<td>U118MG</td>
<td>Arg213 to Gln (50, 51)</td>
<td>Impaired</td>
<td>+ (3)</td>
</tr>
<tr>
<td>OVCAR-3</td>
<td>Arg248 to Gln (47, 52)</td>
<td>Deficient</td>
<td>+ (3)</td>
</tr>
<tr>
<td>MKN28</td>
<td>Ile261 to Leu (44)</td>
<td>Deficient</td>
<td>+ (2)</td>
</tr>
<tr>
<td>COLO 205</td>
<td>Gly266 to Gln (47)</td>
<td>Deficient</td>
<td>+ (2)</td>
</tr>
<tr>
<td>U373MG</td>
<td>Arg273 to His (50, 51, 53)</td>
<td>Deficient</td>
<td>+ (2)</td>
</tr>
<tr>
<td>HT29</td>
<td>Arg273 to His (54)</td>
<td>Deficient</td>
<td>+ (2)</td>
</tr>
<tr>
<td>A431</td>
<td>Arg273 to Cys (55)</td>
<td>Deficient</td>
<td>+ (2)</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>Arg260 to Lys (47)</td>
<td>Deficient</td>
<td>+ (2)</td>
</tr>
<tr>
<td>T24</td>
<td>Tyr126 deletion or stop (56, 57)</td>
<td>Deficient</td>
<td>+ (2)</td>
</tr>
<tr>
<td>PC-3</td>
<td>Ala138 deletion or frame shift at alternative stop at 169 (47, 58, 59)</td>
<td>Deficient</td>
<td>+ (2)</td>
</tr>
<tr>
<td>UM-SSC-22A</td>
<td>Lost SA exon 9 (AG→AT)f</td>
<td>Deficient</td>
<td>+ (2)</td>
</tr>
<tr>
<td>SK-OV-3</td>
<td>His179 to Arg/no mRNA (47, 52)</td>
<td>Deficient</td>
<td>– (2)</td>
</tr>
<tr>
<td>MNG-HOS</td>
<td>No mRNA (60)</td>
<td>Deficient</td>
<td>– (2)</td>
</tr>
<tr>
<td>MG-63</td>
<td>Null (60)</td>
<td>Deficient</td>
<td>– (2)</td>
</tr>
<tr>
<td>SaOs-2</td>
<td>Null (60)</td>
<td>Deficient</td>
<td>– (2)</td>
</tr>
<tr>
<td>SW1398</td>
<td>Unknown</td>
<td>Deficient</td>
<td>– (2)</td>
</tr>
<tr>
<td>HM02</td>
<td>Unknown</td>
<td>Deficient</td>
<td>++ (1.5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+ + + (1.5)</td>
</tr>
</tbody>
</table>

a Genetic status according to version R5 of the IARC database (61) and references given for each cell line.

b Functional status was determined by measuring relative luciferase expression after PG13-Luc transfection compared with MG15-Luc transfection. Scores: ratio 0.5–2.0, p53 deficient; ratio 2–10, impaired p53 activity; and ratio >10, functional p53.

c Enhancement of oncolytic potency was determined semiquantitatively by comparative crystal violet assay. Each cell line was tested in at least two independent experiments. For representative examples, see Fig. 4. Results were scored as: –, insignificant enhancement; +, <10-fold enhancement; + +, 10–100-fold enhancement; ++ +, >100-fold enhancement. Values in parentheses indicate duration of culture in weeks.

d ND, not determined. Functional p53 transactivation could not be determined on A2780 cells because these cells exhibited high specific MG15-Luc background transactivation.

e HPV, human papillomavirus.

f Dr. R. Brakenhoff, personal communication.

4 Unpublished results.


Conditionally Replicative Adenovirus Expressing p53 Exhibits Enhanced Oncolytic Potency

Victor W. van Beusechem, Petra B. van den Doel, Jacques Grill, et al.


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