Replication-selective oncolytic adenoviruses represent a promising anticancer approach with proven efficacy in cancer cells and tumor xenografts in vivo without cross-resistance to conventional clinical therapies (1, 2). Numerous mutants have been constructed to target tumors specifically, enabling viral gene expression and amplification at the tumor site with minimal toxicity to normal cells (1, 3). Safety has been shown in clinical trials with various adenoviral mutants in hundreds of patients (4). The majority of clinical trials evaluated mutants designed to complement the dysfunctional p53 activity frequently present in human tumors. The first clinical application of this group of biologicals was dl1520 (ONYX-015; ΔE1B55K and ΔE3B; refs. 5–7). Recently, a similar mutant, H101, was licensed for anticancer therapy in China (Shanghai Sunway Biotech; ref. 8). Whereas tumor selectivity was shown for both mutants, efficacy was only reported in combination with cytotoxic drugs (3, 8, 9). It was later shown that essential functions of the deleted E1B55K and E3B genes (such as late viral RNA transport and protection against host immune defense, respectively) contributed to the attenuated efficacy of these viruses (7, 10).

Recently, oncolytic mutants have been constructed that retain functions essential for the viral life cycle by deletion of smaller gene regions, for example, the d922-947 virus mutants with similar CR2 deletions (Δ24; refs. 11–13) and by incorporating selective promoters targeting prostate (14, 15) or the majority of solid tumors (16–19). Several of these mutants have also been tested in the clinic, and promising outcomes were reported when combined with standard chemotherapy (14, 15, 20, 21). Various mutants with the E1ACR2 deletion were highly efficacious in preclinical studies (22–24). Their potency was superior to that of dl1520 in most models even when...
additional deletions in the E3 genes were included (11, 25, 26). The majority of oncolytic adenoviral mutants evaluated in the clinic to date lack the E3B region, a deletion associated with high levels of macrophage infiltration at the injection sites in glioblastoma patients (27). We showed previously that higher levels of macrophage infiltration in xenografts infected with an E3B-deleted virus correlated with greater clearance of virus from the tissue, whereas mutants with an intact E3 region had higher levels of replication and enhanced antitumor responses (10, 28). More recently, we found that the attenuated efficacy in vivo of E3B-deleted mutants could be rescued by combining virotherapy with suboptimal doses of cytotoxic drugs (29). These findings suggested that viral efficacy could be improved through several strategies including engineering of both E1 and E3 genes and through coadministration with cytotoxic agents. To this end, we generated a set of replication-selective mutants based on the potent E1ACR2 deletion with intact E3 genes to enhance in vivo efficacy.

Although the potency of previously constructed ΔCR2 viruses was clearly higher than that of other adenoviral mutants, replication could still proceed in proliferating normal cells (11). The E1ACR2 region is responsible for binding and inactivation of pRb, thereby releasing E2F for S-phase induction. Consequently, in proliferating normal cells and in tumor cells with deregulated cell cycle control (mainly pRb and p16 alterations), the E1ACR2 region is redundant. To further improve on the selectivity by attenuating viral replication in cycling normal cells, we included a deletion of the antiapoptotic E1B19K gene that sensitizes normal tissue to death receptor-induced signaling and apoptosis in vivo, pathways that are deregulated or inactivated in the majority of cancer cells. Previously, we showed that ΔE1B19K mutants had increased therapeutic index and lower liver toxicity in vivo, whereas antitumor potency was retained (30, 31). The antiapoptotic E1B19K protein promotes viral replication and spread by blocking Bax-Bak oligomerization and mitochondrial pore formation analogous to the cellular Bcl-2 homologue (32, 33). In contrast to the E1B55K protein that mainly inhibits p53-dependent pathways, E1B19K inhibits both death receptor-induced and intrinsically induced apoptosis through p53-dependent and p53-independent mechanisms (34–38). Recently, we showed that the AdΔΔ29K mutant (E1B19K-deleted) could synergize with gemcitabine to kill pancreatic cancer cells (39). Based on these findings, we hypothesized that a mutant deleted in both E1B19K and E1ACR2 region would not only improve safety in vivo but also promote cell death in response to cytotoxic drug-induced apoptosis.

Here, we report that a replication-selective mutant (AdΔΔ) targeting alterations in pRb (ΔCR2) and apoptosis pathways (ΔE1B19K) with intact E3 region improved efficacy and selectivity both as a single agent and in combination with standard chemotherapeutics. Viral replication and oncolysis in prostate and pancreatic carcinoma cells were as potent as that of wild-type virus with significant efficacy in human prostate cancer xenografts in athymic mice. In animals with intact immune responses, higher efficacy was observed with E3-intact mutants compared with the corresponding E3B-deleted mutants. A trend towards decreased macrophage invasion was also observed in tumors infected with E3-intact mutants.

**Materials and Methods**

**Cancer and normal cells.** Human carcinoma cell lines from prostate PC3, DU145, LNCaP, and 22Rv1 (American Type Culture Collection), pancreas PT45 and Suit2, and lung H460 (Cell Services, Cancer Research UK) were cultured in DMEM supplemented with 10% FCS (Life Technologies). Normal human bronchial (NHBE) and prostate epithelial cells (PreC Lonza) were cultured according to the manufacturer’s instructions.

**Adenoviruses and mutant construction.** Adenovirus type 5 (Ad5) mutants were generated by homologous recombination as described previously (40). The complete Ad5 genome was used as the backbone in all new mutants and was derived from the pTG3602 plasmid (a generous gift from Dr. M. Methali, Transgene). The following viruses were generated: Ad5tg (wild-type Ad5), AdΔΔ29K (E1B19K-deleted), AdΔΔCR2 (E1ACR2-deleted), and AdΔΔΔ (E1B19K- and CR2-deleted). All newly generated mutants were characterized for purity, sequence determination (E1 genes), gene expression, cell-killing activity, and replication as reported previously (10, 29, 39). The nonreplicating ΔΔ312 (E1A- and E3B-deleted) and AdGFP (CMV-GFP replacing E1 region) mutants were used as controls. All viruses had a viral particle (vp) to infectious unit ratio of 10 to 40 vp/plaque-forming units (pfu).
**Cell-killing assay and synergistic interactions.** Cells were infected with viruses and/or treated with docetaxel (Sanofi Aventis) or mitoxantrone (Baxter) 24 h after seeding and assayed for viability 4 to 7 days later using the MTS assay (Promega). Dose-response curves were generated to calculate the concentration of each agent killing 50% of cells (EC50) using untreated cells or cells treated with one agent only as control; isobolographs were generated from these data as described previously (29).

**Adenovirus replication assay.** Cells were infected with viral mutants at 1 to 100 particles per cell (ppc), and 24 to 72 h later, cells and media were collected, freeze-thawed, and titrated on JH293 cells by the limiting dilution method (TCID50; ref. 29). Each assay was repeated three times, averaged, and expressed as pfu/cell ± SD. An internal Ad5 control of known activity was included in each assay.

**Immunoblot analysis.** Cells were infected with viruses, harvested after 24, 48, and 72 h, and lysed in buffer [50 mmol/L HEPES (pH 7.4), 250 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L DTT, 1 mmol/L NaF, 1% Triton X-100] containing a protease inhibitor cocktail (Roche). Total protein (10-20 μg) was separated on SDS-polyacrylamide gels under reducing conditions and transferred to polyvinylidene difluoride membranes (Millipore). Viral and cellular proteins were detected by the following antisera: rabbit anti-Ad2 E1A at 1:200 (SC-430; Santa Cruz Biotechnology), rabbit anti-hexon at 1:2,000 (AutogenBioclear), mouse anti-caspase-3 at 1:500 (Alexis), and mouse anti-β-tubulin at 1:20,000 (Sigma-Aldrich). Detection was by horseradish peroxidase–conjugated secondary antibodies (Dako), chemiluminescence reagent (Amersham/Pharmacia), and autoradiography (BioMax film; Kodak).

**Flow cytometric analysis.** Mitochondrial depolarization was analyzed in cells infected with viruses at 100 ppc for 2 h and harvested 24 to 96 h post-infection. Cells were stained with tetramethylrhodamine ethyl ester perchlorate (Molecular Probes/Invitrogen) at 60 ng/mL in PBS containing 4′,6-diamidino-2-phenylindole at 1 μg/mL and analyzed on a LSRI (Becton Dickinson). Data were expressed as percentages of decreases in mitochondrial membrane potential (ΔΨ).

**In vivo tumor growth.** Tumors were grown in one flank of C57BL athymic (ICRF nu/nu) by subcutaneous implantation of cells at 1 × 10⁶ for DU145 and 1 × 10⁷ for PC3. Murine carcinoma cells CMT-64, CMT-93, and TRAMPC (Cell Services, Cancer Research UK) were grown in immunocompetent C57BL mice at 1 × 10⁵, 5 × 10⁵, and 1 × 10⁴ cells per flank, respectively, as described previously (10, 28). Dose responses to viral mutants or docetaxel were determined by administration of virus intratumorally at 1 × 10⁶ to 1 × 10¹⁰ vp/injection × 3 at 48 h intervals and docetaxel at 5.0 to 15.0 mg/kg intraperitoneally twice on days 2 and 8 after virus injection. To determine efficacy in response to combination treatments, suboptimal doses of

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**Fig. 1.** Deletions of the E1ACR2 region and the E1B19K region improved cell-killing potency of Ad5 in several tumor cell lines. A, Ad5 wild-type (Ad5tg) and the respective mutants with the E1B19K (AdΔ19K), E1ACR2 (AdΔCR2), or both gene regions deleted (AdΔΔ) were generated. All mutants had intact E3 region. B, cell-killing efficacy determined as EC50 values in PC3, DU145, 22Rv, and LNCaP prostate, PT45 and Suit2 pancreatic, and H460 lung carcinoma cell lines with Ad5tg, AdΔ19K, AdΔCR2, and AdΔΔ viruses. Decreases in EC50 values are presented as percentages of the EC50 for wild-type Ad5tg. Average ± SE (n ≥ 3). *, P < 0.05.
each agent were administered. Tumor volumes were estimated twice weekly: volume = (length × width² × π) / 6. Treatments were initiated when tumors were 100 ± 20 μL with tumor growth and progression followed until tumors reached 1.44 cm² or until symptomatic tumor ulceration occurred (according to UK Home Office Regulations). Survival analysis was done according to the method of Kaplan-Meier (log-rank test for statistical significance). Tumor growth curves were compared using one-way ANOVA for significance.

In vivo replication and gene expression. Tumors grown as above but implanted in both flanks were each injected once with the respective viral mutant (1 × 10¹⁰ vp) with and without one intraperitoneal dose of docetaxel 24 h later, 10 or 15 mg/kg for DU145 and TRAMPC, respectively. The DU145 tumors were harvested 3, 6, and 10 days after initial virus administration and the TRAMPC tumors were harvested on days 4, 8, and 15. One tumor from each animal was processed for immunohistochemistry of E1A and hexon expression and for the TRAMPC tumors the macrophage marker CD68 as described previously (10, 28, 30). The second tumor was immediately frozen (N₂(l)), homogenized, and processed for viral genome analysis or replication.

Quantitative PCR. For quantification of viral genome amplification in tumors and monolayer cells, DNA was

![Graphs and diagrams]

**Fig. 2.** Viral replication and gene expression of the novel mutants paralleled that of the wild-type virus. A, amplification of viral genomes was determined over time for all mutants with quantitative PCR of the hexon gene in DU145, 22Rv, and LNCaP cells infected at 5 ppc and in PC3 cells at 100 ppc. Only cells were included in the DNA analysis. Average ± SE of three samples. Differences between viruses were not significant (P > 0.5). B, viral replication determined in burst assays by TCID₅₀ at 48 and 72 h after infection with the respective mutants at 100 ppc in all cell lines. Both cells and media were analyzed. C, early E1A and late hexon gene expression in DU145 cells 24 and 48 h after infection. Representative immunoblot. D, viral replication in the NHBE and PrEC normal cells determined by TCID₅₀ assay at 48 and 72 h after infection with 100 ppc. Average ± SE of three samples (B and D). *, P < 0.05.
isolated from tumor homogenates and cell lysates by extraction and purification using the QIAamp DNA Blood Mini kit (Qiagen). Hexon DNA was detected by SYBR Green and quantified as described previously (39). Viral genomes were expressed as ratio of particles in each tumor relative to total DNA, averages of three tumors per treatment group, and three replicates per cell lysate were presented as means/total DNA.

Results

**Sensitivity to the novel adenoviral mutants is cell line dependent.** To verify the identity of each novel virus (Fig. 1A), PCR analysis and E1 gene sequencing were done (Supplementary Fig. S1). Cell-killing efficacy of Ad5tg wild-type and the AdΔ19K, AdΔCR2, and AdΔΔ mutants was determined in seven carcinoma cell lines including prostate (PC3, DU145, 22Rv, and LNCaP), pancreas (PT45 and Suit2), and lung (H460). PC3 cells were the least sensitive (PC3, DU145, 22Rv, and LNCaP), pancreas (PT45 and Suit2), and lung (H460). PC3 cells were the least sensitive compared with Ad5tg (Fig. 1B). The CR2 deletion (AdΔCR2) also increased cell-killing activity compared with wild-type virus in all cell lines, except the 22Rv and LNCaP cells. Interestingly, these two cell lines were more sensitive to the combination mutant AdΔΔ, with a reduction in EC50 values of 1.1 ± 0.3, 1.2 ± 0.5, and 0.6 ± 0.2 ppc for DU145, 22Rv, and LNCaP cells, respectively. The deletion of E1B19K (AdΔ19K) enhanced cell-killing potency, except in the PC3, DU145, and Suit2 cells where activity was similar to that of Ad5tg (Fig. 1B). The CR2 deletion (AdΔCR2) also increased cell-killing activity compared with wild-type virus in all cell lines, except the 22Rv and LNCaP cells. Interestingly, these two cell lines were more sensitive to the combination mutant AdΔΔ, with a reduction in EC50 values of >50% compared with both wild-type virus and the ΔCR2 mutant (Fig. 1B). In fact, the AdΔΔ virus was also more potent than Ad5tg in H460, PT45, and Suit2 cells with a similar trend (not significant) in DU145 and the highly insensitive PC3 cells. The corresponding E3B-deleted mutants with ΔCR2 or ΔE1B19K (dΔ922-947 and dΔ337, respectively) were also at least as potent as wild-type virus in these cell lines (data not shown). The nonreplicating control viruses dΔ312 and AdGFP induced <20% cell death at doses up to 1 × 107 ppc in all cell lines. **Potent viral gene expression in the cancer cell lines.** To determine whether viral gene expression was affected by the deletions in the different mutants, cell lysates were probed for early (E1A) and late (hexon) viral proteins 24 and 48 h after infection (Fig. 2C; data not shown). The expression pattern was similar for wild-type and mutants in all cell lines.

**Attenuated replication of the novel mutants in normal cells.** To test our hypothesis that inclusion of the E1B19K deletion would increase safety of replication-selective oncolytic mutants through inhibition of viral spread in non-tumor tissue, we determined the level of replication in normal cells. All mutants replicated to a significantly lesser degree (P < 0.05) than Ad5tg in PrEC cells 48 and 72 h after infection (Fig. 2D). In NHBE cells, replication of the AdΔΔ mutant was greatly attenuated and the AdΔ19K mutant to a lesser degree, whereas replication for the ΔCR2 deletion was closer to that of Ad5tg. The attenuation of AdΔΔ compared with AdΔCR2 in normal cells was likely caused by premature induction of cell death in the absence of the E1B19K gene as shown previously for AdΔ19K in NHBE cells (39). This was reflected in the lower EC50 values for these mutants in normal cells: 10% of the Ad5tg value for E1B19K-deleted viruses compared with 40% for the single ΔCR2 mutant in NHBE cells (Supplementary Table S1). Lower EC50 values for the corresponding E3B-deleted dΔ922-947 and dΔ337 mutants (Supplementary Table S1) with parallel decreases in replication were also observed as reported previously (31).

**Novel viral mutants synergistically enhance docetaxel-induced cell killing.** Our previous observations suggest that the clinically used cytotoxic drugs docetaxel and mitoxantrone could synergistically increase cell killing when combined with Ad5 in prostate cancer cells.1 Here, we show synergistic effects on cell death when the newly generated deletion mutants were combined with docetaxel in PC3 and DU145 cells (Fig. 3A; Table 1). Synergistic interactions were observed in DU145 cells when docetaxel was combined with any of the mutants at all combinations tested, whereas, with the wild-type virus, the majority (75%) of test conditions resulted in only additive effects. In PC3 cells, synergistic cell death was greater with the AdΔΔ mutant than with the single-deleted viruses, whereas combinations with Ad5tg caused antagonistic effects (Table 1). Similar trends were seen in both DU145 and PC3 cells when the mutants were combined with mitoxantrone to a lesser degree in the LNCaP cells (data not shown). In the LNCaP and 22Rv cells that were more sensitive to virus- and drug-induced cell death than the DU145 and PC3 cells, enhanced sensitization to docetaxel was also observed with the AdΔΔ mutant compared with the single-deleted mutants (Supplementary Fig. S2). These data suggest that further improved antitumor efficacy of the potent AdΔΔ mutant can be achieved if combined

1 S. Radhakrishnan et al., unpublished data.
with low doses of cytotoxic drugs, for example, 50% cell death was achieved with 1,200- to 1,900-fold and 1- to 2-fold reductions in docetaxel and virus doses, respectively, compared with each agent alone (DU145).

**E1B19K-deleted mutants induce caspase-3 activation in 22Rv cells.** In previous studies, we found that the AdΔ19K virus could potently stimulate apoptotic cell death in combination with gemcitabine in pancreatic cancer cells (39).

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**Fig. 3.** Novel viral mutants synergistically enhanced docetaxel-induced cell killing in prostate cancer cell lines. A, DU145 and PC3 cells were treated with combinations of viral mutants and docetaxel at fixed ratios. EC50 values were calculated for each condition and isobolograms were generated to determine synergistic interactions on cell death. The straight line represents the theoretical line for additive effects and each data point represents the respective combination ratio. B, caspase-3 activation in response to infection with viral mutants at 100 ppc after 24 and 48 h in 22Rv1 and DU145 cells. C, mitochondrial depolarization (ΔΨ; tetramethylrhodamine ethyl ester perchlorate staining) to determine proportion of live 22Rv1 and DU145 cells 24 to 96 h after infection at 10 ppc. Average ± SD (n = 3).
Surprisingly, both AdΔ19K and AdΔΔ mutants induced procaspase-3 cleavage in 22Rv cells 48 h after infection even in the absence of cytotoxic drugs (Fig. 3B, right). In combination with docetaxel, caspase-3 activation was only observed when the 22Rv cells were simultaneously infected with the AdΔΔ mutant (Supplementary Fig. S3). At the low doses used in this study, neither docetaxel alone nor in combination with Ad5tg-induced procaspase cleavage. Caspase 3 activation was not observed in other prostate and pancreatic cell lines at these time points as shown for DU145 cells (Fig. 3B, left). Apoptotic death was further shown to contribute to the enhanced cell-killing activity by the E1B19K-deleted mutants through increased mitochondrial depolarization 48 to 96 h after infection in the 22Rv cells but not in the DU145 cells (Fig. 3C).

Combinations of the double-deleted mutant (AdΔΔ) and docetaxel inhibit growth of DU145 and PC3 tumor xenografts in vivo. The potent response to combination treatments in cultured cells was verified in vivo in two human xenograft models. Subcutaneous tumors of DU145 and PC3 cells were treated with suboptimal doses of each agent (Fig. 4). Both wild-type and AdΔΔ viruses could potentially inhibit tumor growth and prolong survival in combination with low doses of docetaxel in DU145 cells. Time to tumor progression was significantly ($P < 0.09$) prolonged from 72 to 111 days for AdΔΔ in combination with docetaxel (5 mg/kg) compared with a median survival of 44 days for docetaxel alone ($P < 0.02$). The response to the combination treatment was greater than additive because this low dose of docetaxel had no efficacy in this model (Fig. 4A). In fact, the combination treatment resulted in a higher proportion of surviving animals at the end of the experiment than the higher dose of docetaxel at 10 mg/kg (70 days) and was as efficient as the highest dose tested at 15 mg/kg (113 days). When the AdΔ Δ mutant was combined with a higher dose of docetaxel (10 mg/kg), >50% of animals were still alive when the study was terminated at day 170. Mutants and wild-type virus had similar efficacy in the DU145 xenograft model with no significant differences between treatment groups (Supplementary Fig. S4). Efficacy was also shown in the PC3 xenograft model (Fig. 4B). Neither wild-type nor the mutant viruses prolonged time to progression when administered alone at $1 \times 10^7$ vp and only slightly inhibited tumor growth at a higher dose (Fig. 4B; Supplementary Fig. S4; data not shown). The median time to progression was 18 to 29 days for all viruses at $1 \times 10^9$ vp and was not significantly different from mock-, Ad312-, or docetaxel-treated animals. Whereas docetaxel administered at 10 mg/kg had no significant antitumor efficacy, when combined with AdΔΔ, the median time to progression was increased from 18 to 39 days and was significantly ($P < 0.002$) different from single-agent treatments. These results suggest that tumor regression and prolonged survival can be obtained with the AdΔΔ virus even in relatively treatment-resistant cancer cells such as PC3. No significant differences in efficacy were observed between AdΔΔ and Ad5tg in these models.

Antitumor efficacy in DU145 xenografts was caused by viral gene expression and replication. In a separate study, DU145 tumors treated once with virus were analyzed for viral replication and genome amplification in tumors 3 to 10 days later (Fig. 4C). Higher levels of viral genomes were detected 3 days after treatment for all viruses compared with the nonreplicating Ad312 mutant. The amount of virus recovered after 10 days was slightly lower for all viruses. However, in animals treated with AdΔΔ in combination with docetaxel, slightly higher levels of viral genome copies were retained (Fig. 4C, right). In parallel, viral replication assays (TCID$_{50}$) were done on three tumors per group. Three days after infection, viral replication per micromgram tumor was $1.45 \times 10^5 \pm 3 \times 10^4$ pfu (Ad5tg), $6.7 \times 10^7 \pm 5 \times 10^6$ pfu (AdΔ19K), $5.23 \times 10^5 \pm 8 \times 10^4$ pfu (AdΔCR2), $1.05 \times 10^6 \pm 4 \times 10^5$ pfu (AdΔΔ), and $5.1 \times 10^5 \pm 2 \times 10^5$ pfu (AdΔΔ + docetaxel 5 mg/kg). After 10 days, a slight decrease in replication was detected for all viruses; Ad5tg was $5.23 \times 10^5 \pm 1 \times 10^4$ pfu and AdΔ19K and AdΔΔ were 90% and 80%, respectively, of the Ad5tg

### Table 1. Combination index for DU145 and PC3 cells treated with docetaxel and viral mutants at four constant dilution ratios

<table>
<thead>
<tr>
<th>Cells</th>
<th>Ratio Ad/docetaxel (ppc/nmol/L)</th>
<th>Ad5tg</th>
<th>AdΔ19K</th>
<th>AdΔCR2</th>
<th>AdΔΔ</th>
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<td></td>
<td></td>
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<td>1.19</td>
<td>1.04</td>
<td>0.81</td>
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<td>0.80</td>
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<td>0.81</td>
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<td>0.80</td>
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</tr>
<tr>
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<td>PC3</td>
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</tr>
<tr>
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<td>0.78</td>
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</table>

NOTE: Combination index values: <0.9 = synergy (S), >1.1 = antagonism (A), and 0.9 to 1.1 = additive effects (ad).
value. Other treatments including the AdΔΔ mutant in combination with docetaxel resulted in similar levels of replication to that of wild-type alone, in agreement with the genome amplification data. Histologic examination of the corresponding tumor tissue revealed increased levels of necrosis 10 days after treatment with Ad5tg or mutants compared with mock-infected tumors. Hexon expression was shown with all viruses from 3 to 10 days and was paralleled by E1A expression (Fig. 4D; data not shown).

Novel mutants with intact E3 region have higher efficacy in immunocompetent animals. To evaluate efficacy of the novel mutants in hosts with functional immune responses, the murine prostate cancer cells TRAMPC were implanted subcutaneously in intact mice. Antitumor efficacy was not significant for any mutant in this model likely due to the rapid growth progression and the lack of viral replication. However, when docetaxel (15 mg/kg) was combined with the AdΔΔ mutant, a trend towards reduced tumor growth was observed (Fig. 5A), although this
was not significantly different from animals treated with docetaxel and dl309 ($P > 0.3$; Fig. 5A). Despite lack of replication, E1A expression was present in a heterogeneous pattern throughout the tumors (4-15 days) treated with all tested mutants (Fig. 5B, day 15, right). Interestingly, both mock-infected and untreated TRAMPC tumors had high levels of CD68+ macrophages, making a quantitative determination of infiltrating macrophages in the presence of virus difficult (Fig. 5B, left). However, after infection with the E3B-deleted mutants dl309, dl922-947, and
Discussion

The aim of this study was to generate a novel oncolytic adenoviral deletion mutant with improved antitumor efficacy targeting solid cancers. We hypothesized that replication selectivity would be greatly improved through elimination of E1B19K antiapoptotic functions while limiting replication to cancer cells through the E1ACR2 deletion. Retention of the E1B55K gene would prevent attenuation of the viral life cycle observed for the dl1520 mutant (7). We also included the entire E3 region to further enhance viral efficacy in vivo in the presence of intact antiviral immune responses. The first generation of oncolytic adenoviruses tested in the clinic had the E3B genes deleted to facilitate clearance of virus (4, 6, 8). However, later findings showed that this deletion severely attenuated viral efficacy [10]. Furthermore, retention of the E3B genes did not appear to decrease safety. In this report, we showed that replication in normal cells and could efficiently replicate in all tested cell lines. Most normal tissues have few proliferating cells and replication of the AdΔΔ mutant in nonarrested normal cells was significantly attenuated compared with wild-type virus, with the greatest decrease in NHBE cells. In contrast, the single-deleted AdΔCR2 mutant replicated to higher levels under these conditions. Hence, the additional deletion of the E1B19K function attenuated viral replication, suggesting improved safety by preventing spread in normal tissue. These findings are in agreement with a previous report showing that the CR2-deleted dl922-947 mutant could still replicate in normal SAEC and MVEC cells (11). Most normal tissues have fewer proliferating cells and replication of the AdΔΔ mutant would likely be further attenuated in vivo due to its inability to bind to pRb in addition to impaired defense against the antiviral tumor necrosis factor response. Taken together, these results suggest that the AdΔΔ virus has potential as a new improved oncolytic candidate for future evaluation in clinical trials.

To this end, AdΔΔ was combined with cytotoxic drugs currently used in the clinic to treat prostate cancer. Enhanced cell killing was observed in DU145 and PC3 cells in combination with both docetaxel and mitoxantrone through synergistic interactions. Synergistic cell death also occurred with the single-deleted mutants and to a lesser degree with wild-type virus. Although it has been shown previously that enhancement of virus and cytotoxic drug-induced cell death is dependent on E1A expression, the specific E1A regions necessary for the sensitization have not yet been identified (29, 43–45). Here, we show that viruses deleted in the E1ACR2 region could potentially synergize with docetaxel in both the presence and the absence...

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of E1B19K expression, an interesting finding in light of the potential for future combination therapies. Docetaxel is currently the drug of choice in the United Kingdom for metastatic, hormone-refractory prostate cancers. Previous reports showed that antitumor efficacy could be enhanced by combining Δ24 mutants with DNA-damaging cytotoxins such as CPT-11 (46), radiotherapy (22), or gemcitabine (23). Recently, we showed that the E1B19K deletion alone could also greatly enhance tumor cell killing of pancreatic xenografts in combination with gemcitabine (39). However, to our knowledge, this is the first report showing that deletion of both E1ACR2 and E1B19K interacted synergistically with docetaxel, a drug whose main mechanism of action is the inhibition of cell division rather than DNA damage. A similar double-deleted virus was reported previously to efficiently kill melanoma cells (19). However, this mutant had viral gene expression and replication regulated by a tyrosinase promoter/enhancer element resulting in higher levels of expression than with the viral E1A promoter/enhancer in this study, and combinations with other cytotoxic factors were not reported. Even the PC3 cells, which are insensitive to both virus and docetaxel alone, could be greatly sensitized to docetaxel by the AdΔΔ mutant. Enhancement of cell death was less potent in the LNCaP and 22Rv cells, perhaps reflecting the greater sensitivity to mutants and docetaxel or alone, respectively. The greater responses to the AdΔΔ mutant but not the corresponding E3B-deleted mutants, in effect decreasing the drug concentrations required to kill LNCaP and 22Rv cells analogous to the results in PC3 and DU145 cells.

A contributing factor to the greater increase in cell death with the AdΔΔ mutant was the absence of a functional antiapoptotic E1B19K gene (30, 31, 34, 39). We showed that the mutants without E1B19K could activate apoptotic pathways in the 22Rv cells in contrast to Ad5tg and the AdΔΔ mutant. In addition, the AdΔΔ mutant but not the E1B19K-expressing viruses induced caspase-3 activation when combined with docetaxel, likely contributing to the greater enhancement of cell killing with AdΔΔ. Although it is known that adenovirus-induced cell death occurs through nonapoptotic pathways (47, 48), we showed that viruses deleted in the E1B19K gene released virus earlier than a virus with this gene intact, suggesting a more rapid induction of cell death mechanisms (30, 31). We also observed caspase-3 activation after infection with the E1B19K-deleted viruses at later time points even in the DU145 cells (>72 h; data not shown), indicating a more general activation of death programs due to viral replication and not through direct activation of apoptotic factors by viral proteins. Further studies are necessary to distinguish what viral mechanisms are responsible for the early activation of apoptosis-like events in the 22Rv cells. In addition, we recently determined that the AdΔ19K virus in combination with suboptimal doses of gemcitabine increased drug-induced apoptosis, whereas the virus alone had no effect on either caspase activation or mitochondrial depolarization in pancreatic cancers (39). This difference in responses to AdΔ19K mutants alone in prostate and pancreatic cells is probably the result of specific functional inactivating/activating mutations in each cell line, for example, intact p53 function in 22Rv but not in PT45 cells (41, 42). Our data also indicate that other mechanisms are equally important to enhance cell-killing activity because both efficient cell death and synergy were achieved with wild-type virus and the AdΔΔCR2 mutant.

Greatly improved antitumor efficacy was also seen when the novel AdΔΔ was combined with docetaxel in PC3 and DU145 tumor xenografts. In fact, at doses where no effects were detected when either the virus or drug was administered alone, significantly prolonged survival was observed when combined. At a higher dose of 1 × 10^10 vp, tumor growth inhibition was achieved with all mutants in the absence of drug with the greatest effects for the AdΔΔ in DU145 xenografts. Using the murine immunocompetent TRAMPC model, we also showed that mutants with intact E3 genes attenuated CD68+ macrophage infiltration. As expected, the corresponding E3B-deleted mutants dl309, dl922, and dl337 did not prevent this infiltration. Due to high basal levels of CD68+ cell infiltration in the TRAMPC xenografts, only qualitative assessments were possible. Although efficacy could not be optimized due to the rapid proliferation of TRAMPC cells, significantly prolonged survival was achieved in the slower-growing CMT-93 model with the AdΔΔ but not the corresponding E3B-deleted mutants. A similar trend was also seen in the CMT-64 model, the only murine syngeneic model that supports Ad5 replication, albeit at low levels (28). Nevertheless, a trend towards higher efficacy with AdΔΔ was observed in all three immunocompetent models with the lowest responses in the more aggressive and rapidly proliferating TRAMPC and CMT-64 models in combination with docetaxel or alone, respectively. The greater responses to the AdΔΔ mutant were likely caused by potent E1A expression in the absence of the E1B19K antiapoptotic function and presumably prolonged retention of virus compared with E3B-deleted mutants. These findings verified our previous results that quantitatively assessed viral gene expression and macrophage infiltration in animals with intact immune system showing high viral gene expression accompanied by low levels of macrophage infiltration for E3B-intact mutants and lower viral gene expression in the presence of higher levels of macrophage infiltration for E3B-deleted viruses (10). Additional contributions to the higher efficacy in the CMT-93 model are likely due to the more immunogenic nature of these tumors in the presence of virus as indicated previously (10).

From the data described here, the novel AdΔΔ virus has great potential for future clinical evaluation showing improved therapeutic index and oncolytic potency that was further enhanced in combination with chemotherapeutics.

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No potential conflicts of interest were disclosed.

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