BIOLOGY CONTRIBUTION

ADENOVIRAL E4ORF3 AND E4ORF6 PROTEINS, BUT NOT E1B55K, INCREASE KILLING OF CANCER CELLS BY RADIOTHERAPY IN VIVO

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Purpose: Radiotherapy is widely used for treatment of many tumor types, but it can damage normal tissues. It has been proposed that cancer cells can be selectively sensitized to radiation by adenovirus replication or by using radiosensitizing transgenes. Adenoviral proteins E1B55K, E4orf3, and E4orf6 play a role in radiosensitization, by targeting the Mre11, Rad50, and NBS1 complex (MRN) and inhibiting DNA double-strand break (DSB) repair. We hypothesize that combined with irradiation, these adenoviral proteins increase cell killing through the impairment of DSB repair.

Methods and Materials: We assessed the radiosensitizing/additive potential of replication-deficient adenoviruses expressing E1B55K, E4orf3, and E4orf6 proteins. Combination treatments with low-dose external photon beam radiotherapy were studied in prostate cancer (PC-3MM2 and DU-145), breast cancer (M4A4-LM3), and head and neck cancer (UT-SCC8) cell lines. We further demonstrated radiosensitizing or additive effects in mice with PC-3MM2 tumors.

Results: We show enhanced cell killing with adenovirus and radiation combination treatment. Co-infection with several of the viruses did not further increase cell killing, suggesting that both E4orf6 and E4orf3 are potent in MRN inhibition. Our results show that adenoviral proteins E4orf3 and E4orf6, but not E1B55K, are effective in vivo. Enhanced cell killing was due to inhibition of DSB repair resulting in persistent double-strand DNA damage, indicated by elevated phospho-H2AX levels at 24 h after irradiation.

Conclusions: This knowledge can be applied for improving the treatment of malignant tumors, such as prostate cancer, for development of more effective combination therapies and minimizing radiation doses and reducing side effects. © 2010 Elsevier Inc.

Cancer gene therapy, Adenovirus, Radiotherapy, Radiosensitivity, DNA repair inhibition.

INTRODUCTION

Despite improvements in current treatments for prostate cancer, there are no curative treatments for locally advanced, recurrent, or metastatic disease. Radiotherapy (RT) is widely used for treatment of prostate cancer and outcomes correlate with dose (1). However, higher doses carry greater risks for side effects (2). Combining RT and oncolytic adenovirus treatment shows promising results in prostate cancer treatment (3). Another

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approach for decreasing the required radiation dose, while maintaining curative potential, is combination with nonreplicating radiosensitizing adenoviruses.

The cell killing activity of radiation therapy can be increased with radiosensitizing transgenes (4, 5). Moreover, radiosensitization potential has been proposed for innate adenoviral gene products early region 1B 55-kDa (E1B55K), early region 4 11-kDa (E4orf3), and 34-kDa (E4orf6) (6–9). These proteins maintain effective replication of wild-type adenovirus in host cells by preventing cell cycle arrest in response to the presence of linear double-stranded adenovirus genomes, which the cell can interpret as DNA double-strand breaks (DSB) (10).

DSB repair inhibition has been suggested as a mechanism for adenovirus-mediated cell cycle modulation. The cellular MRN complex (Mre11, Rad50, and NBS1) is prerequisite in DSB recognition (11), and this is counteracted by adenoviral E4orf6 and E4orf3, which target the MRN complex for degradation (6–8, 12). The E1B55K protein has been suggested to enhance E4orf6 and E4orf3 activity (8, 13). Nevertheless, the role of these proteins and their combinations and their potential in radiosensitization in vivo remain unknown.

We hypothesized that when combined with irradiation, the impairment of DSB repair by adenoviral proteins would lead to accumulation of DNA damage and subsequent cancer cell death.

MATERIALS AND METHODS

Cell lines

Prostate cancer PC-3MM2 and DU-145 (ATCC, Manassas, VA) (9) and head-and-neck cancer explant UT-SCC8 (14) and cervical cancer HeLa cells (ATCC, Manassas, VA) were cultured in Dulbecco’s modified Eagle medium (Lonza, Basel, Switzerland), and breast cancer cell line M4A4-LM3 (ATCC, Manassas, VA) in RPMI-1640 (Lonza, Basel, Switzerland).

Recombinant adenoviruses

E1- and E3-deleted recombinant adenoviruses rAdE4orf3, rAdE1B55K, and rAdE4orf6 were a gift from Matthew D. Weitzman (12, 15, 16). The transgenes are located in the E1 region under the cytomegalovirus promoter. rAdE4orf3 also encodes green fluorescent protein (GFP). Replication-deficient control virus Ad5/GL is an E1- and E3-deleted serotype 5 adenovirus expressing GFP and luciferase under the cytomegalovirus promoter in the deleted E1 region (17). Replication-competent Ad300wt is a wild-type (wt) serotype 5 adenovirus (ATCC, Manassas, VA). Viral particle (VP) concentration was determined by spectrophotometry (λ = 260 nm).

Irradiation experiments

Cells were irradiated through a 1-cm thick plastic phantom bottom with 1 cm of water. Whole-body irradiation was used for the mice to avoid the need for anesthesia. For cell lines, in vitro radiation doses were chosen according to the relative sensitivity of each cell line, respectively (9, 18).

Western blot

PC-3MM2 cells were seeded on six-well plates and infected with 100 VP/cell. For γH2AX, immunoblotting cells were irradiated 24 h after infection with 0 or 8 Gy. Ultraviolet-B irradiated (150 J/m²) HeLa cells were used as a positive control (19). Thirty minutes or 24 h later, cells were harvested, sonicated, and acid-extracted overnight with 0.2M HCl (for γH2AX immunoblotting). Primary antibodies were 2A6 (anti-E1B55K) 1:20, 6A11 (anti-E4orf3) 1:25, Rsa#3 (anti-E4orf6) 1:20, and phospho-H2AX (#9718, Cell Signaling Technology, Danvers, MA; 1:1000) and GAPDH (#39-8600, Invitrogen, Carlsbad, CA; 1:2000).

Immunofluorescence assays

PC-3MM2 cells were infected with 100 VP/cell and 24 h later irradiated with 0 or 8 Gy. Twenty-four-hour posttreatment cells were fixed 20 min with 4% paraformaldehyde, permeabilized 5 min with ice-cold 70% EtOH, and blocked 1 h with 0.5% bovine serum albumin (BSA), 0.05% sodium azide in phosphate-buffered saline. Slides were incubated with primary phospho-H2AX mouse monoclonal antibody (Upstate, clone JBW301, Boston, MA; 1:1000) for 1 h at 37°C, washed twice, and incubated with secondary antibody Alexa Fluor 594 (Invitrogen, Carlsbad, CA; 1:500).

For adenovirus capsid detection, PC-3MM2 cells were treated similarly, permeabilized 10 min at room temperature with 0.1% Triton (×100), 0.5% BSA in phosphate-buffered saline, and blocked 1 h with immunomix (5% normal donkey serum, 0.2% BSA, and 0.05% sodium azide in phosphate-buffered saline). Primary adenovirus antibody was Virostat #1401 (Portland, ME; 1:200) and secondary antibody was Alexa Fluor 594 (Invitrogen, Carlsbad, CA; 1:500).

Cell viability assay

PC-3MM2 cells were infected for 2 h and 24 h later irradiated with 0 or 8 Gy. After cytotoxic effect was observed, cell viability was measured using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI).

Clonogenic assay

PC-3MM2 cells (5 × 10⁴) were seeded into 24-well plates and infected the next day in triplicate with the respective indicated viruses at 100 VP/cell. After 24 h, plates were irradiated with 0 or 8 Gy and the cells were transferred into six-well plates at densities of 1,000 cells/well. After 10 days, the cells were fixed with 10% paraformaldehyde and stained with 1% crystal violet in 70% ethanol. Colony formation for each was counted under a microscope.

Animal experiments

Animal experiments were approved by Experimental Animal Committee of the University of Helsinki and the Provincial Government of Southern Finland. PC-3MM2 xenografts were established by injecting 5 × 10⁶ cells in 100 μL of MEM into both flanks of 5-week-old male Nude/NMRI mice (Scanbur BK, Sollentuna, Sweden). Mice were randomized into 12 groups (n = 6 tumors): mock (injected with growth media only), rAdE1B55K, rAdE4orf3, rAdE4orf6, Ad5/GL, and Ad300wt, with and without RT. Growth media was used as virus diluent. Intratumoral virus injections of 1 × 10⁷ VP were administered every other day (total of 4 × 10⁸ VP), starting 7 days after tumor cell injection when tumors were 0.55 cm³ on average. Fractionated whole-body irradiation was given every other day (4 × 2 Gy) during 8 treatment days.

Statistical analysis

Statistical analyses were performed using two-tailed Student’s t-test and the nonparametric Mann–Whitney U test (SPSS 15.0, Chicago, IL). For all analyses, a p value of <0.05 was considered statistically significant.
RESULTS

Recombinant adenoviruses rAdE1B55K, rAdE4orf3, and rAdE4orf6 show efficient transduction of PC-3MM2 prostate cancer cells

Expression of adenoviral E1B55K, E4orf3, and E4orf6 proteins by recombinant viruses rAdE1B55K, rAdE4orf3, and rAdE4orf6, respectively, was confirmed by Western blot in PC-3MM2 cells (Fig. 1A). Replication-deficient E1- and E3-deleted control virus Ad5(GL) showed some expression of E4orf3 and E4orf6, but not E1B55K, which is due to some activation of the E4-region despite the lack of E1 activity (20). Wild-type adenovirus was used as a positive control.

Transduction efficiency was assayed in PC-3MM2 cells by detection of GFP expression for rAdE4orf3 (>90%) and non-replicative control virus Ad5(GL) (>90%) (Fig. 1B) or by immunofluorescence staining for adenovirus antigens for rAdE1B55K (>85%), rAdE4orf6 (>90%), and replicative control virus Ad300wt (>95%) (Fig. 1C).

Infection with recombinant adenoviruses expressing E4orf6, E4orf3, and E1B55K 24 h before irradiation significantly increases cell killing in vitro

Radiation doses were selected according to relative sensitivity to radiation of each cell line (9, 18). RT alone or combined with replication-deficient control virus Ad5(GL) did not affect cell viability at treatment with 8 Gy (Fig. 2A). rAdE4orf6 with an 8-Gy radiation dose presented a statistically significant cell killing effect compared to Ad5(GL) with RT in PC-3MM2, DU-145 (both p < 0.001), and M4A4-LM3 (p < 0.001) cell lines and, respectively, with a 2-Gy radiation dose in UT-SCC8 (p < 0.01) explant. A lower radiation dose was used for UT-SCC8 because these cells are low passage explants and therefore more radiosensitive than established cell lines (19). Similarly, rAdE4orf3 showed significant increase in cell killing in PC-3MM2 (p < 0.001), DU-145 (p < 0.05), and M4A4-LM3 (p < 0.05) when combined with 8 Gy, but not in UT-SCC8 combined with 2 Gy. In contrast, rAdE1B55K showed significant increase in cell killing only in DU-145 (p < 0.05).

The cell killing effect was then studied as a function of virus dose. rAdE4orf6 sensitized or enhanced cell killing significantly with 1 VP/cell (p < 0.05), whereas rAdE4orf3 and rAdE1B55K had additive or sensitizing effects with 100 VP/cell (both p < 0.01) (Fig. 2B). The radiosensitizing effect of Ad300wt has been published previously (9). Taken together, in vitro data suggest that E4orf6 and E4orf3 proteins cause radiosensitization or additive cell killing in various cancer cell lines, where E1B55K contributes little, if at all, to the effect.
Infection with multiple recombinant viruses does not further improve cell killing in vitro

Although there have been molecular level indications that E1B55K and E4orf6 as well as E1B55K and E4orf3 might work in complexes to inhibit the DSB repair (21–23), we did not observe further improved cell killing when using viral co-infections combined with RT (Fig. 3). Furthermore, there was no improved cell killing effect in other tested cell lines (M4A4-LM3, UT-SCC8) (data not shown).

Clonogenic assays

Radiosensitizing or additive effects were confirmed in clonogenic assays (Fig. 4). E4orf3- or E4orf6-expressing viruses without radiation had an effect on clonogenicity, as reported (20, 24, 25), and the effect of rAdE4orf6 was particularly pronounced, but combined with radiation the effect of both viruses was further enhanced.

Recombinant adenoviruses rAdE4orf6 and rAdE4orf3, but not rAdE1B55K, enhance cancer cell killing by radiotherapy in vivo

rAdE4orf3 was the most effective of transgene expressing viruses in vivo when used with radiation and resulted in significant reduction of tumor growth compared to Ad5(GL) with RT (p < 0.01, Fig. 5). Also, rAdE4orf6 with radiation resulted in a statistically significant reduction of tumor growth (p < 0.05). In contrast, rAdE1B55K with radiation did not inhibit tumor growth. Replicative control virus Ad300wt presented significant reduction of tumor growth combined with RT (p < 0.001), but not alone (data not shown). Interestingly, and in accord with in vitro results by us and others (20, 24, 25), recombinant viruses had antitumor efficacy even in the absence of radiation (Fig. 5).

Infection with rAdE4orf6 and rAdE4orf3 results in persistence of γH2AX foci at 24 h after irradiation

Inhibition of DSB repair has been suggested to be the main mechanism contributing to the radiosensitizing effect of replication-competent adenoviruses (7, 9). We used phospho-H2AX (γ-form) immunofluorescence staining to visualize DNA DSB at 24 h after 8 Gy irradiation (26, 27) (Fig. 6A). DSBs were present in 40–50% of cells infected with rAdE4orf3 or rAdE4orf6 or Ad300wt as indicated by γH2AX foci, whereas control virus Ad5(GL) or RT-only presented fewer γH2AX foci (10% and <5%, respectively). rAdE1B55K showed some persistence of γH2AX (25%). We further analyzed γH2AX levels by Western blot to quantify DSBs (27) (Fig. 6B). All 8 Gy irradiated cells presented elevated γH2AX levels at 30 min posttreatment.
whereas virus-only treated cells presented a weak signal. However, rAdE4orf6, rAdE4orf3, and Ad300wt showed persistent γH2AX levels at 24 h after combination treatment, suggesting inhibition of DSB repair. The radiosensitizing effect of wt adenovirus has been previously reported (9, 24, 25).

**DISCUSSION**

E1B55K, E4orf3, and E4orf6 have evolved to inhibit cell cycle arrest and DSB repair signaling to maintain effective virus replication (6–8, 28). However, their relative radiosensitizing/additive potentials and effects in vivo have not been investigated before. We show that either E4orf3 or E4orf6 are sufficient and potent enhancers of cancer cell killing in vitro and in vivo, whereas E1B55K does not significantly contribute to the effect.

The MRN complex (Mre11, Rad50, and NBS1) is important in DSB sensing, stabilization, signaling, and repair (11, 29). It upregulates ataxia-telangiectasia mutated and ataxia-telangiectasia mutated-Rad3–related signaling pathways (29, 30), which are involved in both homologous recombination and nonhomologous end-joining (10). The E4orf6 protein is involved in degradation of the MRN complex, leading to abrogation of ataxia-telangiectasia mutated/ataxia-telangiectasia mutated-Rad3–related signaling and DSB repair inhibition (7, 8). MRN degradation is an early event and does not require viral DNA replication to occur (31). Similarly, our results indicate major DSB repair impairment by rAdE4orf6 already at 30 min after irradiation.
Moreover, there is evidence that E4orf6 might initiate cell death programs alone by prolonging DNA-damage signaling in late stages after irradiation (32), explaining persistence of DSBs at 24 h after irradiation. E4orf3 and E4orf6 target the MRN complex to aggresomes, inhibiting activity in nuclear adenoviral replication sites, and concomitantly, at DSB repair sites (12, 33). Recent evidence suggests that this prevents ATR signaling, impedes recognition of viral genomes, and blocks checkpoint signaling (28). In our study, both E4orf6 and E4orf3 added to radiation mediated cell killing in vitro, and the activity of E4orf6 was higher. Further studies are needed to understand this, but one possible explanation is the higher effect of E4orf6 on DSB repair. In the sensitive clonogenic assay, E4orf6 was quite potent, whereas in vivo E4orf6 and E4orf3 had similar activity, suggesting a more complex scenario. E4orf6 and E4orf3 act in a different fashion: both target the MRN to the aggresomes; however, E4orf6 associates with E1B55K affecting nuclear export of host and viral mRNAs while E4orf3 directly affects the solubility of the cellular MRN complex (34, 35).

The E1B55K protein has been suggested to augment E4orf6 and E4orf3 in the MRN complex inhibition (21–23). However, it seems that it is not needed for enhancing the effects of radiation (8, 13), and infection with E4-deleted adenovirus elicits a cellular DNA damage response (7, 30)—both suggesting that E1B55K alone is not sufficient to inhibit DSB repair signaling. This is consistent with our results in vivo. Corroborating the lack of importance of E1B55K in this regard, E1B55K is deleted in oncolytic adenovirus ONXY-015 (dl1520), which has been clinically most effective

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Moreover, there is evidence that E4orf6 might initiate cell death programs alone by prolonging DNA-damage signaling in late stages after irradiation (32), explaining persistence of DSBs at 24 h after irradiation.
when used in combination with either chemotherapy or radiation therapy (36, 37).

Our findings show that combinations of E4orf3, E4orf6, and E1B55K proteins do not increase cell killing vs. E4orf3 or E4orf6 alone. Although E1B55K is not required for DSB repair inhibition, E4orf6 and E4orf3 seem to act independently of each other (21, 38). Therefore, it is logical that their combination is not needed as either can sufficiently disrupt the DSB repair signaling of an individual cell (either MRN degradation or mislocalization).

Although treatments with transgene-expressing virus or low-dose RT were rather ineffective alone, the combination was found highly effective, especially in vivo. PC-3MM2 prostate carcinoma xenografts model a treatment resistant...
cancer, and therefore inhibition of tumor growth by rAdE4orf3 with low-dose RT is intriguing. In previous studies on prostate cancer, murine models up to >50 Gy local external beam radiation doses have been used with oncolytic virotherapy (39). We show that tumor growth inhibiting effect can be achieved using only 8 Gy when combined with gene therapy.

Our data show that endogenous adenoviral proteins E4orf3 and E4orf6 can enhance prostate cancer cell killing by radiotherapy in vivo. Further, we show that multiple administrations of adenovirus together with fractionated radiotherapy can restrict aggressive prostate tumor growth. These data support combining adenoviral gene therapy and RT for the treatment of solid tumors. This might allow reduction of the radiation dose for reduction of harmful side effects. Further increases in efficacy could be realized by combining the approach with prodrug-converting enzymes, such as cytosine deaminase (40, 41), or by modifying the adenoviral vehicle to improve delivery (18, 42–46). As an alternative to viral delivery, E4orf3 or E4orf6 could be injected in plasmid form or produced in a recombinant manner and delivered within, for example, liposomal complexes to tumors.

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