Mre11 inhibition by oncolytic adenovirus associates with autophagy and underlies synergy with ionizing radiation

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New treatment approaches are needed for hormone refractory prostate cancer. Oncolytic adenoviruses are promising anti-cancer agents, and their efficacy can be improved by combining with conventional therapies such as ionizing radiation. The aim of this study was to determine the timing of oncolytic adenovirus treatment with regard to radiation and study the mechanisms of synergy in combination treatment. Prostate cancer cells were infected with oncolytic adenoviruses, irradiated and synergy mechanisms were assessed. In vivo models of combination treatment were tested. Radiation and oncolytic viruses were synergistic when viral infection was scheduled 24 hr after irradiation. Combination of oncolytic adenovirus with radiotherapy significantly increased antitumor efficacy in vivo compared to either agent alone. Microarray analysis showed dysregulated pathways including cell cycle, mTOR and antigen processing pathways. Functional analysis showed that adenoviral infection was accompanied with degradation of proteins involved in DNA break repair. Mre11 was degraded by frequent inactivation of Chk2-Thr68 in combination treated cells, while γH2AX-Ser139 was elevated implicating the persistence of DNA double strand breaks. Increased autophagy was seen in combination treated cells. Combination treatment did not increase apoptosis or virus replication. The results provide evidence of the antitumor efficacy of combining oncolytic adenoviruses with irradiation as a therapeutic strategy for the treatment of prostate cancer. Further, these findings propose a molecular mechanism that may be important in radiation induced cell death, autophagy and viral cytopathic effect.

Key words: oncolytic adenovirus; ionizing radiation; DNA repair inhibition; autophagy; synergism

Current treatments for prostate cancer include hormonal therapies, radiotherapy, chemotherapeutics and surgery. However, more than 30% of patients will relapse following therapy with curative intent, local recurrence being very common with positive biopsy evidence reported in up to 90% of patients previously treated with radiation therapy.1 Although androgen ablation therapies are often initially effective, the emergence of hormone refractory disease is often fatal. Current treatment options for recurrent and advanced disease are limited. Means to sensitize tumor cells to conventional therapies would be useful with regard to side effects and patients’ quality of life.

Prostate cancer metastasizes preferably to bone, lymph nodes, lungs and liver.7 When metastases are treated with radiation therapy, relatively high doses are needed to obtain efficacy. In disseminated disease radiation should be applied to extensive areas, which increases the risk for side effects. Moreover, tumor cells can become radiation resistant despite initial radiation responsiveness.3 Although radiation therapy is relatively safe and efficient for local tumors and early stage disease, complete response rates are low and long-term survival remains poor in metastatic disease.

Oncolytic adenoviruses (Ads) have shown promising efficacy in the treatment of prostate cancer in several preclinical3,26 and clinical studies.5,7,8 The utility of oncolytic viruses lies in the natural life cycle of the virus: viral replication kills infected tumor cells, and each dying cell releases thousands of virions into surrounding tumor tissue for effective local amplification of the tumor cell killing effect. Viruses can also disseminate through the vasculature to distant metastases. Replication is targeted to cancer cells by engineered alterations in the virus genome reducing the damage to normal cells.7,10 The combination of Ads and radiotherapy has been evaluated and useful combination effects have been reported in vitro and in vivo.11,12 However, the underlying mechanisms have been poorly characterized that has hindered the rational optimization of therapeutic regimens.

The mechanism by which adenoviral replication causes cell death (“oncolysis”) is poorly understood. It is known that infection results in a rounded shape and condensation of the cytoplasm,
that is, cytopathic effect (CPE). Apoptotic and necrotic mechanisms have been proposed and recent studies\(^4,15\) suggest that Ad infected cancer cells feature autophagy, a form of programmed cell death distinct from apoptosis, characterized by the degradation of intracellular components via the lysosome.\(^6\) Radiation alone has also been shown to induce autophagy.\(^7\)

Ionizing radiation causes DNA double strand breaks that are recognized by the cellular DNA repair machinery. The MRN complex (Mre11, Rad50 and NBS1) is essential in recognizing these breaks and acts as a part of the DNA repair machinery,\(^16\) which also recognizes viral genetic materials and initiates a response that leads to the concatemerization and inactivation of nonself DNA. Oncolytic Ads inhibit this response by binding of E4orf3, E4orf6, which leads to the concatemerization and inactivation of nonself DNA.\(^17\) The interaction of E1b55K/E4orf6 with Mre11 is a key step leading to MRN degradation and to inactivation of its downstream target proteins such as ataxia telangiectasia mutated (ATM), which is involved in DNA-damage signaling.\(^19,21\) MRN complex inhibition prevents correction of radiation induced double strand breaks and their constant accumulation leads to cell death. Here, we report that an oncolytic Ad targets Mre11 for degradation. This results in downregulation of Chk2-Thr68 and inhibition of double strand DNA break repair, which subsequently causes autophagy in prostate cancer cell lines in vitro and results in increased cell killing in vivo.

### Material and methods

#### Culture of human cells

Hormone refractory prostate cancer DU-145, PC-3 and lung adenocarcinoma A549 were purchased from ATCC (Manassas, VA). PC-3MM2 cells are a metastatic hormone refractory subline of PC-3 (Courtesy of Isaiah J. Fidler, MD Anderson Cancer Center, Houston, TX). 293 cells are from Microbix (Toronto, Ontario, Canada). All cell lines were cultured as reported previously.\(^6\)

#### Viruses

Replication deficient adenovirus Ad5/3Luc1 and replication competent Ads Ad5/3-A24 and Ad5/3A24hCCG are Ad5 based constructs retargeted to the Ad serotype 3 receptor. The tumor selectivity of Ad5/3-A24 and Ad5/3A24hCCG is based on a 24-bp deletion in the retinoblastoma (Rb) binding site of E1A.\(^24,25\) Ad5/3A24hCCG has a marker gene, hCGβ, inserted in the deleted gp19K/6.7K region of E3. Ad300WT is wild type Ad5 from ATCC.

#### Irradiation experiments

Irradiation was performed with a linear accelerator (Clinac 600C/D, Varian Medical Systems, Palo Alto, CA) using a 6-MV photon beam and dose rate 400 MU/min (~ 4 Gy/min). Cells were irradiated through a 1-cm thick plastic phantom bottom with 1 cm of water. In vivo, mice remained in standard plastic cages that were placed in the middle of radiation field.

#### Determination of optimal schedule for combination therapy

Cells were seeded on 96-well plates, incubated overnight and either irradiated (4 or 15 Gy) or infected (0.1–100 vp/cell) in 50 μl of growth media (GM) supplemented with 2% FCS for 1 hr at 37°C. Infection was stopped by washing with GM containing 5% FCS and then 100 μl of 5% GM was added. Twenty-four hours after the first treatment, the irradiated cells were infected and infected cells irradiated. Cell viability was measured 4–7 days after the last treatment using the MTS assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega, Madison, WI).

#### Caspase-3 apoptosis assay

Cells were lysed and the amount of caspase-3 activity was estimated by measuring proteolytic activity toward a synthetic caspase-3 substrate Ac-DEVD-pNa peptide (Calbiochem, Darmstadt, Germany). A 40-μg aliquot of each lysate was prepared as previously described.\(^26\)

#### Western blotting

**Dynamin 2 and Mre11.** Cell lysates containing 50 or 70 μg of soluble protein were fractioned on 7.5% sodium dodecyl sulfate/polyacrylamide electrophoresis (SDS-PAGE) gel (Bio-Rad, Hercules, CA) and transferred to a polyvinylidene difluoride membrane (Amersham Biosciences Corp., Piscataway, NJ). Primary mouse anti-Dynamin 2 antibody (BD Biosciences Pharmingen, Franklin Lakes, NJ) and rabbit anti-Mre11 antibody (Cell Signaling Technology, Danvers, MA) were diluted in blocking solution 1:250 and 1:1,000, respectively. Membranes were incubated with primary antibody and washed 3 times with 1× PBS/TBS-0.1% Tween followed by incubation for 1 hr with a horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (Amersham Biosciences), dilution 1:50,000, or anti-rabbit IgG secondary antibody (Amersham Biosciences), dilution 1:200,000. Chemiluminescence detection was performed with an enhanced chemiluminescence (ECL) kit (Amersham Biosciences) according to the manufacturer’s instructions.

**Chk2-Thr68 and γH2AX-Ser139.** Total cell lysates were prepared by resuspending cell pellets in urea-Tris buffer (9 M urea, 75 mM Tris-HCl, pH 7.5, 0.15M 2-mercaptoethanol) and sonicating briefly. Proteins (25 or 50 μg) were separated by SDS-PAGE, transferred to polyvinylidene fluoride membranes (Immobilon P, Millipore Corporation, Billerica, MA), and blocked in PBS with 5% nonfat milk. Chk2-Thr68 was detected by a rabbit antibody (Cell Signaling Technology, catalogue number 2661) using a dilution 1:800. γH2AX-Ser139 was detected by a mouse antibody (Upstate/Millipore, catalogue number 05-636) using a dilution 0.5 μg/ml. Membranes were then washed 3 times with 1× PBS/TBS-0.1% Tween for 10 min and incubated for 1 hr with either a horseradish peroxidase-conjugated anti-mouse secondary antibody (DakoCytomation, Glostrup, Denmark), 1:4,000 or with a biotinylated anti-rabbit antibody (DakoCytomation) 1:2,000 followed by incubation with a horseradish peroxidase-conjugated streptavidin (DakoCytomation) 1:4,000. Protein complexes were detected using ECL (Amersham Life Sciences).

#### Microarray analysis

Cells were irradiated with 4 Gy, collected 24 hr after the treatment, washed with PBS, snap frozen and stored into −80°C. Total RNAs were collected from 3 individual experiments that were pooled together for the RNA extraction and microarray analysis. Total RNA was extracted with TRIzol\(^16\) (Invitrogen, Carlsbad, CA) followed by purification with RNeasy columns (Qiagen, Valencia, CA). The quality of the total RNA was analyzed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Gene expression microarray analyses were carried out from 4 μg of total RNA using one-cycle target labeling and Affymetrix Human Genome U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA). After hybridization, washing and staining, the arrays were scanned by GeneChip 7G Scanner and analyzed using Affymetrix GeneChip Operating (GCOS) 1.4 Software. Analysis was carried out with default parameters and scaling was performed with a target intensity of 100. All genes denoted as absent by GCOS were discarded from further analysis. The microarray data were annotated using custom CDF files (HGU133Plus2_Hs_ENTREZG.cdf, ver 11.0.1)\(^27\) and normalized using the RMA method.\(^28\) Genes with fold-change at least 3 were considered as differentially expressed and analyzed for pathways using Signaling Pathway Impact Analysis (formerly known as Pathway-Express)\(^29,30\) method. The data are deposited at http://www.ebi.ac.uk/microarray-as/aer/entry, accession number E-MEXP-2172.

#### Fluorescence activated cell sorting (FACS) analysis

6 × 10⁶ cells were seeded in 16-mm plates in 5% FCS GM and incubated overnight. Cells were irradiated with 10 Gy and 24 hr
later infected with 100 vp/cell of oncolytic Ad5/3Δ24hCG. When CPE was observed by microscopy, cells were trypsinized and stained with 1 μg/ml acridine orange (AO, Sigma, St. Louis, MI) for 15 min. Cells were pelleted 10,000 rpm 1 min and resuspended in 0.5 ml of 1× PBS. FL3-H emission from 2 × 105 cells was measured with a LSR flow cytometer (BD Biosciences).

**LC3 immunofluorescence**

Cells were seeded at 1 × 106 cells/well on 6-well plates with coverslips in 5% GM and incubated overnight and transfected twice with pLC3-GFP using Superfect (Qiagen, Hilden, Germany). Twenty-four hours later cells were irradiated with 10 Gy and another 24 hr later, cells were infected with 100 vp/cell of Ad5/3Δ24hCG. Forty-eight hours later when CPE was observed, cells were fixed with 4% paraformaldehyde 10 min RT. Samples were mounted with Vectashield (Vector Laboratories, Burlingame, CA) and imaged using an LSM Meta 510 confocal microscope (Carl Zeiss, Jena, Germany).

LC3 distribution was quantitated in transfected cells with a Zeiss AxioVert 200 fluorescence microscope (Carl Zeiss). Percentage of punctate distribution of GFP-LC3 was counted in 5 nonoverlapping fields. Percentage of punctate distribution of GFP-LC3 was counted from a minimum of 200 GFP-positive cells in 5 nonoverlapping fields. Cells containing more than 3 dot-formation were determined positive for autophagy and the number of these cells relative to all GFP-positive cells was counted, normalized to untreated cells and presented as ratio of positive cells to all cells. Each treatment condition was carried out in duplicate in at least 3 independent experiments. Statistical analysis was done with Student’s t-test.

**Electron microscopy**

Cells were seeded at 1 × 106 cells/well on 6-well plates in 5% GM and incubated overnight. Twenty-four hours later cells were irradiated with 10 Gy and 48 hr later infected with 100 vp/cell of Ad5/3Δ24hCG. Forty-eight hours after infection cells were collected and pelleted down, fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 60 min and postfixed with 2% osmium tetroxide for 1 hr, dehydrated in series of ethanol and embedded in LX-112 resin. Ultra-thin section was cut at 50–60 nm, stained with uranyl acetate and lead citrate in Leica EMstain automatic stainer (Leica microsystem, Wetzelar, Germany) according to manufacturer’s recommendations.

Jeol JEM-1400 electron microscope (Jeol, Tokyo, Japan) was used for imaging using 80 or 100 kW accelerator voltage. Digital microphotographs were captured by Olympus-Sis Morada digital camera (Olympus, Münster, Germany).

**Animal experiments**

**Subcutaneous model.** 5 × 106 PC-3MM2 cells were inoculated in 200 μl of MEM (n = 16 tumors/group) into both flanks of 6-week-old male Nude/NMRI mice (Taconic, Ejby, Denmark). Mice were randomized into treatment groups and when tumors were 5 mm in one diameter, half of the mice received 2 Gy whole body irradiation while half were sham irradiated. Twenty-four and 48 hr later, tumors were injected with 2.35 × 109 vp of Ad5/3lacI, Ad5/3Δ24hCG or MEM. After 4 days, mice were again treated with or without 2 Gy irradiation and 24 and 48 hr later 3.0 × 106 cells was injected as above. Medetomidine-ketamine-0.9% saline (1:2:7) was used for anesthesia.

**Orthotopic lung metastasis model.** On Day 0, 1.5 × 106 PC-3MM2 cells were inoculated in 200 μl of MEM into left lungs of 6-week-old male Nude/NMRI mice. Medetomidine-ketamine-0.9% saline (1:2:7) anesthetized mice were put on their right side and the injection site was marked to be between mid-axillary and mid-sternal line. The tip of a 27 G needle was wiped with 70% ethanol to prevent the formation of s.c. tumors; the needle was set to 5-mm depth and the cell solution was injected into the lung. Mice were randomized into 4 groups (n = 11 mice/group). On Day 6, 2 groups received 2 Gy whole body irradiation and 2 groups were sham-irradiated. On Days 7 and 8, mice were injected intravenously with 2.0 × 1010 vp of Ad5/3Δ24hCG or MEM. Mice received another round of radiation on Day 10 and virus injections on Day 11. Animal experiments were performed in accordance to the Experimental Animal Committee of the University of Helsinki and the Provincial Government of Southern Finland regulations.

**hCGβ measurement**

Blood (40 μl) was collected from the great saphenous vein of mice and diluted into 120 μl of 1× PBS. Blood clots were allowed to form, samples were centrifuged, serum was collected and analyzed for hCGβ.

**Statistical analysis**

Chou and Talalay’s median-effect method32 was used to calculate combination index (CI) values under assumption of mutually nonexclusive drug interactions. One sample t-test was then performed to test statistically significant difference of mean CI values. A mean CI of less than 1 indicates synergy while greater than 1 suggests antagonism. A mean CI not significantly different from 1.0 is considered additive. Analysis of tumor volume was performed using a repeated measures model with PROC MIXED (SAS® Ver. 9.1, Cary, NC). The tumor volume measurements were log transformed for normality. The effects of treatment group, time in days and the interaction of treatment group and time were evaluated by F tests. The a priori planned comparisons of specific differences in predicted treatment means averaged over time and at each time point by t-statistics. Cox regression models were used to assess differences in survival between the 4 groups with the Ad5/3Δ24hCG + 2 Gy as reference group. For all analyses, a 2-sided p value of < 0.05 was deemed statistically significant.

**Results**

**Infection with oncolytic adenovirus 24 hr after irradiation results in synergistic oncoslysis of prostate cancer cells in vitro**

To explore whether irradiation enhances the oncolytic action of Ads, we assayed different time points to irradiate and infect prostate cancer cells with Ad5/3Δ24hCG. Synergy, as calculated by mean CI, was observed when PC-3MM2 cells were infected 24 hr after irradiation (Fig. 1b, mean CI = 0.525). A synergistic effect was observed also with DU-145 cells when infection was performed 24 hr after irradiation (Fig. 1a). When infection was performed 24 hr prior to 4 Gy irradiation, no synergy was observed (CI-mean > 1) in PC-3MM2 cells (Supporting Information Fig. 1a), but in DU-145 the CI-mean was 0.371 (Supporting Information Fig. 2). Other time points with regard to infection and irradiation were also tested (Supporting Information Figs. 1 and 2).

PC-3MM2 and DU-145 cells were irradiated and infected with other oncolytic viruses Ad5/3-Δ24, Ad300WT and Ad5/3Δ24hCG 24 hr after irradiation to confirm that the effect was not virus specific (Figs. 1c–i). The cells that were only irradiated were set as a reference (100%). At all irradiation and viral doses, oncolytic Ads showed significantly (p < 0.005) enhanced cell killing when compared to radiation with nononcolytic control virus Ad5/3lacI.

**Viru s 24 hr after 2 Gy irradiation inhibits subcutaneous tumor growth in mice**

To assess synergy in vivo, we established a subcutaneous model of aggressive, hormone refractory disease. Mice with PC-3MM2 tumors (n = 16/group) received 2 Gy irradiation followed by intratumoral virus 24 and 48 hr later in 2 cycles. Treatment with Ad5/3Δ24hCG suppressed tumor growth (Fig. 2a). By Day 11, tumor size was significantly (p < 0.05) smaller if mice had been treated with irradiation prior to Ad5/3Δ24hCG versus virus alone.
Combination therapy increases survival of mice with orthotopic lung metastatic prostate cancer

To test if the combination treatment would benefit the survival of mice, we used an orthotopic model of aggressive prostate cancer metastatic to lung. The mice were randomized into 4 different treatment groups: control (Mock), virus only (Ad5/3Δ24hCG), irradiation only (Mock 12 Gy) or irradiation and virus (Ad5/3Δ24hCG 12 Gy). On Day 6, mice received whole body irradiation and 24 and 48 hr later oncolytic adenovirus or only MEM intravenously. The mice were treated a second time with radiation followed by single injection of virus or MEM on Days 10 and 11, respectively. The irradiation dose was adjusted to 2 Gy at tumor depth. Mice treated with the combination of radiation and virus survived significantly longer than mice that received only virus ($p < 0.02$, Fig. 2b). Mean survival in the combination group was 30.2 days versus 22.1, 25.9 and 20.2 days in the mock, radiation only and virus only groups, respectively.

Ad5/3Δ24hCG contains human chorionic gonadotropin β-chain (hCGβ) inserted in the E3 region. This gene product functions as a marker for viral replication. We have previously shown that Ad5/3Δ24hCG replication can be monitored by measuring plasma hCGβ levels in mice. All mice that received Ad5/3Δ24hCG featured high serum hCGβ levels suggesting effective virus replication in human tumor cells (Fig. 2c).

Apoptotic activity, Dynamin 2 expression or virus replication are not increased in combination treated cells

Next, we studied the putative molecular mechanisms behind the observed synergy.

Apoptosis was assessed by measuring caspase-3 activity. No increase in activity was seen in combination treated cells (Fig. 3a). Hormone refractory prostate cancer cells are known to be frequently resistant to apoptosis and this was confirmed in our analyses as radiation alone did not increase caspase-3 activity levels.

Based on previous results, we studied the expression of Dynamin 2 as a marker for increased viral uptake. Western blot analysis showed no increase in Dynamin 2 levels after radiation implicating no increase in virus uptake (Fig. 3b). Our results did not indicate an increase in virus production in irradiated cells, as no difference in the amount of new virions produced was detected by a TCID50 analysis (Fig. 2d).
Gene expression profiles of radiated cells

We studied prostate cancer cells 24 hr after irradiation using microarray analysis to characterize the gene expression profiles at the time on virus infection. The individual upregulated and downregulated genes are listed (Supporting Information Table 1). Interestingly, E2F transcription factor 8 and cyclin E2, which play a key role in G1/S transition, were downregulated by 7.98- and 12.84-fold, respectively. Also other genes involved in DNA synthesis, replication and metabolism such as GINS2, PCNA and MCMs were downregulated.

To further examine what pathways are affected by highly upregulated or downregulated genes, we analyzed pathways using PathwayExpress29,30 (Supporting Information Table 2). Our results suggest that among the 3 most affected pathways that were inhibited were cell cycle, mTOR signaling pathway and MAPK signaling pathway. These pathways are implicated in various cellular functions, including cell proliferation, differentiation and migration. Moreover, radiation and mTOR signaling inhibition leads to increased autophagocytosis.33 This has also been seen in prostate cancer cells.34 Interestingly, in PC-3MM2 cells the Antigen processing and presentation-pathway and in DU-145 cells the Toll-like receptor signaling-pathway were downregulated after irradiation. Both of these pathways are important in the processing and initiating a defensive response in cells that have been disturbed by pathogens.

Based on microarray findings we proceeded to further analyze specific protein level interactions.

Oncolytic Ad decreases Mre11 in radiated hormone refractory prostate cancer cells

Ionizing radiation causes DNA double strand breaks that are recognized by the cellular DNA repair machinery. The MRN complex is critical in recognizing these breaks and acts as a part of the machinery.18 ATM is a downstream target protein for MRN and further regulates the phosphorylation of proteins in the cascade leading to cell cycle arrest and DNA repair.

We assessed the levels of Mre11 in PC-3MM2 and DU-145 cells, and found decreased Mre11 levels in combination treated cells (Fig. 3c). To confirm abrogation of the ATM activity, we analyzed the phosphorylated form of Checkpoint kinase 2 (Chk2-Thr68), a downstream target of ATM. Chk2-Thr68 levels were reduced in combination treated versus radiated cells (Fig. 3d).

We also analyzed another component of the MRN/ATM pathway, histone H2AX that is phosphorylated at Ser139 by ATM in response to DNA damage. We found that γH2AX-Ser139 levels were increased following Ad infection and the effect was further enhanced by combination treatment with radiation (Fig. 3e).

**FIGURE 2** – Radiation increases the antitumor effect of oncolytic viruses in vivo. (a) A subcutaneous model of hormone insensitive aggressive prostate cancer was established by injecting $5 \times 10^6$ PC-3MM2 cells into mice (Day 0). Mice were randomized into 3 treatment groups receiving no virus, replication deficient Ad5/3lac1 or oncolytic Ad5/3Δ24hCG intratumorally (Days 7 and 8). Half of the mice received 2 Gy whole body irradiation 24 hr before virus injection (Day 6). Two days later mice received a second treatment cycle (Days 10 and 11). On Day 11, tumors in mice treated with irradiation and Ad5/3Δ24hCG were significantly smaller than in groups treated with either modality alone ($^*p < 0.05$). (b) The effect of combination therapy was studied in a model of aggressive lung metastatic hormone refractory prostate cancer. On Day 0, PC-3MM2 cells were inoculated directly into the left lung of 6-week-old male nude/NMRI mice. Mice ($n = 11$) were randomized into 4 treatment groups, half of which received 2 Gy whole body irradiation on Day 6. Twenty-four and 48 hr later, $2.0 \times 10^{10}$ vp of Ad5/3Δ24hCG or no virus was injected intravenously. Four days later (Day 10), mice received a second identical cycle. Mice treated with 2 Gy irradiation and oncolytic Ad5/3Δ24hCG survived significantly longer than mice that received oncolytic virus only, $p < 0.02$. (c) High hCGβ expression in blood from mice in both groups on Day 9 suggests effective virus replication at early time points. (d) A TCID50 assay shows difference in the viral yield from irradiated versus unirradiated cells.
Induction of autophagy by oncolytic adenovirus

We studied the localization of transiently transfected eGFP-LC3 as marker for autophagy. LC3 proteins localize in the membranes of acidic vesicles accumulating in the cytosol when autophagy occurs and the conversion of soluble LC3-I to lipid bound LC3-II is associated with the formation of autophagosomes. A punctate pattern of LC3 localization, typical of autophagy, was seen in combination treated cells (Fig. 4a). Ad infection or radiation alone resulted in a lower number of autophagy positive cells (Fig. 4b). In all cell lines, oncolytic virus and irradiation alone also induced autophagy. For PC-3MM2, the number of cells positive for autophagy was greater in combination treated cells when compared to other treatments; however, the differences did not reach statistical significance.

Autophagy is associated with the accumulation of acidic vesicular organelles in the cytoplasm that can be detected with an AO staining. In FACS analysis of the stained cells, we found 0.43% and 0.03% of mock and virus infected PC-3MM2 cells positive for AO, respectively. Irradiated cells showed 1.83% positivity, while the combination treatment infection increased the positive population to 6.3% of total, which is a 15-fold increase compared to mock treatment (Fig. 4c).

The presence of autophagic vacuolar structures was confirmed with electron microscopy. Irradiation or treatment with Ad5/3A24hCG alone was sufficient to induce autophagic vacuoles. Moreover, the number of autophagocytic vacuoles was greater in combination treated cells (Fig. 5).

Discussion

The present study shows that infection with Ad5/3A24hCG 24 hr after irradiation results in synergistic cell killing. The same synergy was also seen with other oncolytic or wild type Ad and the combination resulted in a statistically significant oncolytic effect over virus only treatment. Importantly, the effect was observed also in subcutaneous and orthotopic lung metastatic in vivo models.

Though similar observations have been published recently with nonreplicating adenoviruses, the synergy mechanisms between oncolytic Ads and irradiation have not been clarified heretofore. Increased viral replication, apoptosis or viral entry have been suggested, however we did not detect any of these. Expression of Ad receptors coxsackie and adenovirus receptor (CAR), CD46 and αβ integrins is not increased by radiation, which supports our findings of unchanged Dynamin 2 levels. Lack of increased apoptosis induction might be explained by Ad E1B19k, which is a Bcl-2 related protein and one of the most potent known inhibitors of apoptosis. However, even cells infected with Ad5/3 Luc1 lacking the E1B were not more susceptible to apoptosis, which might reflect the apoptosis resistant nature of hormone refractory prostate cancer cells.

Radiation induced dsDNA breaks and subsequent signaling through the mitogen activated protein kinase (MAPK/ERK) pathway has recently been shown to enhance the transgene expression in the context of replication deficient adenoviruses. However, in our study we found this pathway to be downregulated in prostate cancer cell lines. Our microarray analysis revealed dysregulation of cell cycle and mTOR pathways. The mTOR pathway is a key regulator of autophagy, which has an important role in inducing cell death in cancer cells that frequently are resistant to apoptosis. mTOR suppresses autophagy and apoptosis under normal circumstances and is upregulated in response to cellular starvation. There is evidence that mTOR inhibitors positively affect the ability of radiation to induce autophagy. mTOR inhibitors also have been shown to sensitize tumor cells to DNA damaging agents. The mechanisms underlying these effects remain unclear, but enhanced apoptosis via interconnections through autophagic pathways and enhanced cell killing by dysregulation of DNA double-strand break repair have been implicated. We show here that
adenovirus infection is accompanied by downregulation of proteins involved in DNA damage repair.

Upon cellular infection, the Ad capsid initiates a rapid and strong response in host transcriptome that initiates the transcription of defensive molecules. Many Ad gene transcripts repress this response. As irradiation downregulates the antigen processing and presentation-pathway in PC-3MM2 cells, it may suppress the initiation of cellular response to Ad infection and thus facilitate and accelerate the Ad induced cytotoxicity. In addition, the TLR signaling-pathway was significantly downregulated in radiated DU-145 cells. TLRs recognize motifs from pathogens and the pathway activation leads to type I interferon and proinflammatory cytokine production. These cytokine responses are important in controlling pathogen replication and they also provide an initiation signal for the adaptive immune response. This observed pre-existing vulnerability of irradiated prostate cancer cells to Ad infection might help Ads to escape the defensive cellular mechanisms against pathogens and be particularly important in vivo, where also the innate and adaptive immune systems participate in defensive processes induced by the Ad infection.

Ad proteins have been shown to inhibit the cellular double strand break repair machinery. We observed a reduction in Mre11 levels and in ATM down-stream component, Chk2, in combination treated cells, a phenomenon well described in previous studies. Ad proteins account for the reduction by binding to proteins of the MRN complex leading to their degradation. MRN inhibition therefore prevents correction of radiation induced double strand breaks and the constant accumulation of mutations eventually makes it impossible for even cancer cells to survive.

Another downstream component of ATM, γH2AX-Ser139, was increased in combination treated cells. This may be explained by ATM-Rad3 related (ATR) and DNA-PK mediated phosphorylation of H2AX in ATM compromised cells, as suggested in a recent study where wild-type infection was sufficient to induce γH2AX foci. Interestingly, other recent publication showed that adenoviral proteins can also inhibit ATR signaling and in the light of other studies the stronger contribution of DNA-PK seems more probable. γH2AX is a marker of double strand breaks and DNA configurations misinterpreted as such. Our data are in accord with previous reports suggesting that the short dsDNA genomes resulting from Ad replication are recognized by the DNA signaling pathway, which results in induction of the double strand break repair response. However, Ad proteins E3orf3, E4orf6 and E1b55K counteract this mechanism to maximize cellular takeover, S-phase like state, and productive Ad replication. In cells that have sustained radiation injury, these Ad proteins inhibit double strand break repair and thus DNA damage accumulates. Our findings suggest that this eventually leads to autophagy, when cells are resistant to apoptosis. Autophagy is an evolutionarily conserved intracellular lysosomal pathway that is responsible for degradation of long-lived proteins and cell organelles. It is known not only to be induced by cellular starvation, but also viral infection or radiation can result in autophagy in certain conditions. Our
and this pathway may be central in controlling autophagy. In Thr68/Rus infected cells. Our data suggest that the Mre11/ATM/Chk2-of cellular mechanisms underlying the CPE observed in adenovi-

ous data suggest that the Mre11/ATM/Chk2-pathway may be important in this regard,

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summary, this study sheds light on the mechanism responsible for synergy between Ads and radiation, providing a strong rationale for combining these modalities in patient treatment.

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