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Antisense MDM2 Enhances E2F1-Induced Apoptosis and The Combination Sensitizes Androgen Dependent and Independent Prostate Cancer Cells To Radiation

Thirupandiyur S Udayakumar¹, Paul Hachem¹, Mansoor M. Ahmed^{1,2}, Sudhir Agrawal³, and Alan Pollack^{1,*}

¹ Fox Chase Cancer Center, Philadelphia, PA

² Weis Center for Research, Geisinger Clinic, Danville, PA

³ Idera Pharmaceuticals, Cambridge, MA

Abstract

We have previously demonstrated in separate studies that MDM2 knockdown via antisense-MDM2 (AS-MDM2) and E2F1 overexpression via adenoviral-mediated E2F1 (Ad-E2F1) sensitized prostate cancer cells to radiation. Because E2F1 and MDM2 affect apoptosis through both common and independent pathways, we hypothesized that coupling these two treatments would result in increased killing of prostate cancer cells. In this study, the effect of Ad-E2F1 and AS-MDM2 in combination with radiation was investigated in three prostate cancer cell lines: LNCaP; LNCaP-Res (androgen insensitive with functional p53 and androgen receptor [AR]); and PC3 cells (androgen insensitive, p53^{null} and AR^{null}). A supra-additive radiosensitizing effect was observed in terms of clonogenic inhibition and induction of apoptosis (caspase 3+7 activity) in response to Ad-E2F1 plus AS-MDM2 treatments in all three cell lines. In LNCaP and LNCaP-Res, these combination treatments elevated the levels of phospho-Ser 15-p53 with significant induction of p21^{waf1/cip1}, phospho-γH2AX, PUMA and Bax levels and reduction of AR and bcl-2 expression. Similarly, AR^{null} and p53^{null} PC-3 cells showed elevated levels of Bax and phospho-γH2AX expression. These findings demonstrate that the combination of Ad-E2F1 and AS-MDM2 significantly increases cell death in prostate cancer cells exposed to radiation and that this effect occurs in the presence or absence of AR and p53.

Keywords

Apoptosis; E2F1; AS-MDM2; Prostate; Radiation

INTRODUCTION

Radiation therapy (RT) is an established and common treatment for prostate cancer. Yet, for men with high-risk disease, failure rates are about 40% over five years (1). Our previous studies indicate that manipulation of the apoptotic pathway will increase cell death in response to RT and that E2F1 and MDM2 are key regulators of this response (2,3). E2F1 is a transcription factor with multiple functions, including the regulation of apoptosis. E2F1 overexpression has been shown to enhance cell death via apoptosis in certain cell-types in response to chemotherapy and radiotherapy (4–6). We recently reported that adenoviral-E2F1 (Ad-E2F1)

Correspondence: Alan Pollack, M.D., Ph.D., University of Miami Miller School of Medicine, 1475 NW 12 Avenue Suite 1500 (D31), Miami, FL 33136, Phone: 305 243 4916, apollack@med.miami.edu.

caused pronounced radiosensitization in p53^{wild-type} (LNCaP) and p53^{null} (PC3) prostate cancer cell lines (2).

MDM2 is an oncogene and overexpression of this protein is linked to increased cell proliferation and predisposition to tumorigenesis (7). Our results show that MDM2 is overexpressed in 30–40% of diagnostic prostate tumor tissue from men referred for RT treatment (8). MDM2 regulates p53 function through a negative feedback loop involving p53 ubiquitination (9–12) and is a negative regulator of p21^{waf1/cip1} independently of p53 (13–17). MDM2 also mediates ubiquitination and proteolysis of the androgen receptor (AR) (18–20). Abrogating MDM2 expression by antisense-MDM2 (AS-MDM2) is an effective strategy for inducing apoptosis in vitro and in vivo (3,13,21,22).

E2F1 and MDM2 are two key proteins that promote apoptosis through common and independent apoptotic pathways. In p53 wild-type cells, E2F1 also increases endogenous MDM2 levels through ARF mediated p53 induction which, in turn, stabilizes p53 protein by reducing proteosomal degradation through MDM2 (23,24). The combination of Ad-E2F1 + AS-MDM2 should, therefore, enhance the killing of prostate cancer cells treated with RT.

MATERIALS AND METHODS

Cell Culture

LNCaP and PC3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM)-F12 medium, containing 10% FBS, penicillin–streptomycin, and L-glutamine, as described previously (25). Androgen insensitive LNCaP-Res cells were grown in androgen deprived (AD) DMEM/F-12 medium. Androgen deprivation was achieved by culturing the cells in medium containing 10% charcoal-stripped serum for 3 days. LNCaP-Res cells were established by serial passage of LNCaP cells in AD medium for one year. Normal human embryonic IMR-90 fibroblast cell line (ATCC CCL-186) was obtained from American Type Culture Collection (Rockville, MD) and grown in DMEM/F-12 medium.

Oligonucleotides and Antibodies

Idera, Inc. (Cambridge, MA) provided the oligonucleotides. The antisense MDM2 oligonucleotide (AS-MDM2) and its mismatch control oligonucleotide (MM) are 20-mer mixed-backbone oligonucleotides with the following sequence; AS-MDM2; 5'-UGACACCTGTTC-TCACUCAC-3 and MM; 5'-UGTCACCCTTTTTCATUCAC-3. They were stored as frozen aliquots at –20°C (13). Antibodies to E2F1, p53, p21, MDM2, phospho-p65, PUMA and β -actin were obtained from Oncogene (La Jolla, CA); for phospho- γ H2AX (serine 139) from Abcam (Cambridge, MA), and for anti-mouse horseradish peroxidase conjugated secondary antibody from Amersham, (Buckinghamshire, UK).

Viral infection and Transient Transfections

LNCaP, LNCaP-Res, PC3 or IMR-90 (5×10^5) cells were grown for 3 days, infected with adenovirus-5 construct that harbors full-length E2F1 under the control of CMV promoter (Ad-E2F1) or control adenoviral construct that harbors full-length luciferase cDNA under the control of CMV promoter (Ad-Luc; control vector). The cells were incubated with the virus for 1 hr followed by transfection with 200 nM AS-MDM2 or mismatch (MM; control) oligonucleotide in 2 ml culture medium for 36–48 hrs in the presence of 7 μ g/ml lipofectin (Invitrogen). Groups with radiation (5 Gy) treatment were irradiated 24 hours after AS-MDM2 or MM and re-incubated for 24h.

Western Blot analysis

Western blot analyses were performed to confirm transduction efficiency. Cells were harvested after 24 h post transfection with Ad-E2F1 or AS-MDM2, lysed using buffer (50 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS) with protease inhibitor cocktail set I (Calbiochem, San Diego, CA) and were sonicated. Thirty micrograms of protein from each cell lysate was electrophoresed on a 4–20% SDS polyacrylamide gel. After transfer onto a PVDF membrane (Millipore, Bedford, MA) in a transblot apparatus and blocking with 5% low-fat dried milk, the blots were incubated overnight at 4°C with specific primary antibodies. The membranes were washed and labeled with an antimouse horseradish peroxidase conjugated secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ) at room temperature for approximately 1 h. Detection by chemiluminescence was performed according to the manufacturer's (Amersham, Aylesbury, UK) standard protocol

Immunofluorescence/Confocal microscopy

LNCaP cells grown on cover slips were fixed in ice cold acetone/methanol (1:1) for 15 min at –20°C. Cells were then air dried, re-hydrated in PBS, blocked, and permeabilized in 0.2 % Triton X-100/PBS. The cells were then washed in PBS and subsequently blocked in 5% BSA. Primary antibodies were applied to the cover slips for 1 h and then overlaid with Alexa-Fluor 568-conjugated goat anti-mouse secondary antibody (Molecular Probes) for 30 min. Following 3 washes in PBS, the cover slips were further incubated in 4'6'-diamidino-2-phenyl hydrochloride (DAPI) (1 µg/ml) to stain the nuclei. The cover slips were then mounted using an aqueous mounting medium (Anti-fading Agents; Biomedica Corp.). The images were analyzed and captured using a Bio-Rad Radiance 2000 LSCM confocal microscope.

Clonogenic Assays

The techniques for clonogenic survival assays have been described previously (2). For clonogenic survival assays, LNCaP, LNCaP-Res, and PC3 cells were cultured in respective mediums as described above for 2–3 days. Appropriate dilutions of Ad-E2F1 or Ad-Luc in 1 mL of solution to achieve appropriate MOIs were gently placed onto the monolayer of cells in each dish and incubated for 1 h. Control dishes with medium alone or with Ad-Luc were exposed to identical conditions. After incubation, 4 mL of control medium with serum were added to each dish and incubated overnight. At 24 h after viral exposure, three sets of dishes at each RT dose level were irradiated with a high dose rate cesium unit (¹³⁷Cs irradiator, Model 81-14R, JL, Shepherd & Associates, San Fernando, CA) for a total of 2, 4 and 6 Gy. Immediately after irradiation, the cells were trypsinized, serially diluted, replated into 100-mm dishes, and incubated. After 14 days, colonies were stained with methylene blue and counted. Cell survival was adjusted for plating efficiency. The data represent the average from three independent experiments. D₀ and n were calculated using a single-hit multi-target model (26). Radiation Enhancement Ratios (RER) were calculated using SF₂ or D₀ values of radiation alone versus combined treatments as described earlier (27).

Detection of apoptosis by TUNEL Assays

Terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick end labeling (TUNEL) was performed with the fluorescein-FragEL DNA fragmentation detection kit according to the manufacturer's instructions (Oncogene Research Products, Biosciences Inc.). Briefly, cells were fixed with 4% paraformaldehyde at room temperature for 10 min. After being washed with phosphate-buffered saline (PBS), cells were treated with proteinase K (2 mg/ml) at room temperature for 5 min followed by addition of TdT equilibration buffer for 30 min. The cells were then stained with the fluorescein-FragEL-TdT labeling reaction mix and TdT enzyme at 37°C for 1 hr. Following washing with Tris-buffered saline (TBS), the cells were analyzed by flow cytometric analysis on a FACS calibur flow cytometer (BD

Biosciences). A sample population of 10,000 cells was used for analysis by cell count software. The data were analyzed with FlowJo (Tree Star, San Carlos, CA) software.

Determination of Caspase 3/7 activity

Caspase 3/7 activity was measured using a fluorometric substrate, Z-DEVD-Rhodamine (The Apo-ONE™ Homogeneous Caspase-3/7 Assay kit; Promega, Madison, WI). Cells were cultured in the respective medium and treated with Ad-E2F1 or Ad-Luc and AS-MDM2 or MM as described above. A total of 2×10^4 cells in 50 μ l culture medium were mixed with 50 μ l of Homogeneous Caspase-3/7 reagent in 96-well plates and incubated at room temperature for 18 hr. Substrate cleavage was quantified fluorometrically at 485 excitation and 538 nm emissions. Fluorescence was measured on a fluorescent plate reader (LabSystems Inc., Franklin, MA).

p53-luciferase reporter assays

LNCaP, LNCaP-Res, and PC3 cells were incubated with Ad-E2F1 or Ad-Luc for 1 hr followed by co-transfection of p53 luciferase reporter constructs (1 μ g/ml) and AS-MDM2 or MM treatments were as described above. Cells were incubated for 24 hr at 37 C; after 24 hr fresh medium was added to the cells. The cells were harvested 48 hr post-transfection in 100 μ l reporter lysis buffer (Promega). Luciferase and β -galactosidase activities were measured using kits from Promega and Roche (Mannheim, Germany), respectively. Luciferase values were normalized by transfection efficiency as measured by β -galactosidase. All data represent mean values $\pm$ s.d. of three independent experiments.

Statistical analysis

Statistics were performed using the Statistical Package for the Social Sciences (SPSS). Statistical significance among experimental groups was determined using the one-way ANOVA, least significance difference test. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Antisense-MDM2 (AS-MDM2) inhibits MDM2 protein induced in response to Adenoviral E2F1 therapy

In our previous studies, we successfully overexpressed E2F1 using an adenoviral vector and knocked down MDM2 using AS-MDM2 as single agents in prostate cancer cell lines (2,22). In this study, we used a combination approach. Overexpression of E2F1 by Ad-E2F1 and MDM2 suppression by AS-MDM2 was confirmed by immunofluorescence staining of E2F1 and MDM2 (Figs. 1A and B show the data for LNCaP). Similar results were observed in Western blot analysis (Figs. 1C and D) in all three cell lines. As a single agent Ad-E2F1 caused a modest increase in MDM2 protein in all LNCaP-Res cell line (but was weakly induced in LNCaP and PC3) that was manifestly reduced when AS-MDM2 was added. Previously, it was reported that E2F1 overexpression causes an increase in ARF activity leading to increased expression of p53 protein that in turn will upregulate MDM2 (23,24). The crosstalk between E2F1 and MDM2 supports a combined approach.

Effect of Ad-E2F1 + AS-MDM2 + Radiation on Overall Cell Death by Clonogenic Cell Survival Assay

To understand the potential cooperative benefit of Ad-E2F1 and AS-MDM2 on radiation response, we measured clonogenic cell survival in the three cell lines. Compared to previous studies (2), a low concentration of Ad-E2F1 was used to determine the relative gain from adding AS-MDM2. Based in part on previous dose response studies (2), we found that a multiplicity

of infection (MOI) of 10 for LNCaP cells, 20 for LNCaP-Res, and 50 for PC3 cells resulted in significant ectopic overexpression of E2F1 with minimal cytotoxicity. These MOI doses were used in combination with AS-MDM2 treatments. There were seven treatment groups: (1) Adenoviral-luciferase (Ad-Luc) alone; (2) Ad-Luc + mismatch oligonucleotide (MM); (3) Ad-Luc + AS-MDM2; (4) Ad-E2F1 alone; (5) Ad-E2F1 + MM; (6) Ad-E2F1 + AS-MDM2; and (7) AS-MDM2 alone.

LNCaP cells were significantly radiosensitized by Ad-E2F1 ($D_0=76.9$, $SF_2=0.2946$ and $n=4.5$ with a p value of <0.045) or Ad-E2F1 + AS-MDM2 ($D_0=64.6$, $SF_2=0.0536$ and $n=1.2$ with a p value of <0.0035) when compared to Ad-Luc ($D_0=93$, $SF_2=0.24$ and $n=2.3$) (Table 1 and Fig. 2A). LNCaP-Res cells displayed a slightly greater degree of radiosensitization from Ad-E2F1 ($D_0=65.6$, $SF_2=0.1768$ and $n=4$ with a p value of <0.001) or Ad-E2F1 + AS-MDM2 ($D_0=45.9$, $SF_2=0.0216$ and $n=11.7$ with a p value of <0.00021) when compared to Ad-Luc ($D_0=90.7$, $SF_2=0.4091$ and $n=4$) (Table 1 and Fig. 2B). In PC-3 cells, a slight increase in clonogenic cell survival was observed in response to Ad-E2F1 treatment ($D_0=156$, $SF_2=0.496$ and $n=1.95$) when compared to Ad-Luc ($D_0=141.4$, $SF_2=0.4182$ and $n=1.85$), apparently related to the low MOI used. However, significant radiosensitization of PC-3 cells was observed with Ad-E2F1 + AS-MDM2 ($D_0=75$, $SF_2=0.0853$ and $n=1.25$; p value of <0.00014) when compared to Ad-Luc ($D_0=141.4$, $SF_2=0.4182$ and $n=1.85$) (Table 1 and Fig. 2C).

In response to Ad-E2F1 + AS-MDM2, at SF_2 , LNCaP-Res cells had the highest radiation enhancement ratio (RER) (18.95), followed by PC3 (4.9) and, lastly, LNCaP cells (4.5). RER for SF_2 was modestly increased in response to Ad-E2F1 in LNCaP-Res (2.3) and was absent in both LNCaP (0.8) and PC-3 cells (0.85). A similar modest RER at SF_2 was observed in response to AS-MDM2 in all three cell lines (LNCaP: 1.4; LNCaP-Res: 1.3; and PC-3: 1.6) (Table 2). These results demonstrate that AS-MDM2 synergizes the radiosensitizing effect of Ad-E2F1, particularly, in androgen resistant LNCaP-Res cells. The D_0 RER was modestly present in all the cell lines except in PC-3 cells treated with Ad-E2F1.

Increased apoptosis when Ad-E2F1 and AS-MDM2 are added to radiation

Apoptosis by caspase 3/7 assay—Caspase 3/7 activation precedes the onset of cell death (28,29). Caspase 3/7 activity was highest in the irradiated Ad-E2F1 + AS-MDM2 group in all three cell lines (Figure 3). The degree of activation was greatest in LNCaP cells (Fig. 3A) followed by LNCaP-Res (Fig. 3B) and then PC-3 cells (Fig. 3C). These findings demonstrate that irrespective of androgen sensitivity, p53 function and AR status, the combination of Ad-E2F1 and AS-MDM2 results in high levels of radiosensitization via apoptosis. Activation of caspase 3/7 was found to parallel apoptosis as assessed by TUNEL.

Apoptosis by TUNEL Assay—TUNEL assays were also performed to assess the incidence of apoptosis in the seven treatment groups. The control virus or mismatch oligonucleotide transfection caused a basal increase in apoptosis as assessed by TUNEL in all three cell lines (data not shown). AS-MDM2 alone, Ad-E2F1 alone or Ad-E2F1 + AS-MDM2 increased cell death when compared to controls in LNCaP and LNCaP-Res, but not in PC-3 cells (data not shown). When radiation was added to these treatment groups, significant induction of apoptosis was observed in all three cell lines compared to the unirradiated groups. Interestingly, Ad-E2F1 + AS-MDM2 + 5 Gy resulted in cell death that was significantly greater ($p<0.0001$) than all other treatment groups studied in all cell lines, including in PC-3 cells.

Ad-E2F1 in combination with AS-MDM2 enhances phospho-p53 levels, increases p53 transactivation leading to upregulation of p53 targets, increases levels of phospho-γH2AX and down-regulates Bcl-2 and AR in LNCaP and LNCaP-Res cells

To further understand the mechanism of radiosensitization by Ad-E2F1 and MDM2, we first ascertained the modulation of MDM2 levels in response to these treatments and radiation. As stated earlier and shown in Figure 1C, Ad-E2F1 caused a weak to modest elevation of endogenous MDM2. Radiation also caused significant up-regulation of MDM2 in LNCaP, LNCaP-Res, and PC-3 cells treated with Ad-Luc or Ad-Luc + MM (Figs. 4A, 4B, 4C). This up-regulation was mitigated when LNCaP, LNCaP-RES, and PC-3 cells were treated with AS-MDM2. These findings demonstrate that AS-MDM2 counteracts the action of E2F1 overexpression and/or irradiation on MDM2 levels.

Next, we analyzed the effects of Ad-E2F1 and AS-MDM2 on p53 function in LNCaP and LNCaP-Res cells, which both have wild-type p53. As expected and described previously (2, 3,30), a significant increase in p53 protein was observed in unirradiated LNCaP, but not LNCaP-Res, cells treated with AS-MDM2. However, Ad-E2F1 significantly increased p53 in both cell lines. The highest relative increases in p53 over the Ad-Luc controls, were with the treatments of Ad-E2F1 + AS-MDM2 + irradiation (Figs. 5A and 5B). Inhibition of MDM2 led to increased p53 stability.

Since p53 protein was elevated in response to Ad-E2F1 + AS-MDM2 + irradiation, we reasoned that this treatment would enhance p53 transactivation function. Figures 5A and B (24hrs after treatment) and 5D (6hrs after treatment) show that phospho-p53 levels are increased by Ad-E2F1 + AS-MDM2 in Western blot analysis in LNCaP and LNCaP-Res cells. A significant increase in serine phosphorylation of p53 protein was observed in both unirradiated and irradiated cells. Moreover, p53 transactivation function was most pronounced in cells exposed to Ad-E2F1 + AS-MDM2 (Fig. 5C).

p21 is downstream of p53 and has been reported to play an important role in cell cycle arrest; however, the role of p21 in apoptosis is still controversial (31). The levels of p21 were significantly elevated in unirradiated LNCaP cells treated with Ad-E2F1, Ad-E2F1 + MM or Ad-E2F1 + AS-MDM2. In irradiated LNCaP cells, the greatest induction of p21 was seen with Ad-E2F1 + AS-MDM2 (Fig. 5A). In unirradiated and irradiated LNCaP-Res cells, p21 was found to be elevated in response to Ad-Luc + AS-MDM2, Ad-E2F1 alone, Ad-E2F1 + MM and Ad-E2F1 + AS-MDM2, and these levels were further increased in response to irradiation (Fig. 5B). These results demonstrate that Ad-E2F1 + AS-MDM2 in the presence or absence of radiation induce p53 protein that is functionally activated to enhance the levels of effector genes, such as p21.

Because AR plays a critical role in the development and progression of prostate cancer (32), is a regulator of apoptosis (33), and AR activation is regulated in part by p53 and MDM2 (19,34), we analyzed AR expression in response to Ad-E2F1 + AS-MDM2. Radiation caused a significant increase in AR protein in LNCaP and LNCaP-Res cells that were treated with Ad-Luc, Ad-Luc + MM and Ad-Luc + AS-MDM2. Lower levels of AR protein expression were observed in both unirradiated and irradiated LNCaP and LNCaP-Res cells treated with Ad-E2F1 ± AS-MDM2, with the greatest reduction seen after exposure to Ad-E2F1 and AS-MDM2 (Figs. 5A and B). Ad-E2F1 + AS-MDM2 treatment was effective at downregulating radiation-induced AR protein.

Since p53 transactivation function was robustly observed with Ad-E2F1 plus AS in both LNCaP and LNCaP-Res cells (Figure 5C), we further tested the effect of Ad-E2F1 + AS induced p53 transactivation function on p53 targets in LNCaP cells. In the LNCaP cells, we found that phospho-p53 increased as quickly as 6 hr by Ad-E2F1 or Ad-E2F1 + AS alone or

in combination with radiation (Figure 5D). This was associated with strong increases in PUMA and Bax with no significant changes in the Bcl-2 protein, suggesting that there was activation of pro-apoptotic events in response to Ad-E2F1 or Ad-E2F1 + AS with or without ionizing radiation.

E2F1 has been found to stimulate ATM through a unique mechanism that is distinct from agents that cause DNA double-strand breaks and cause delayed γ H2AX phosphorylation (35). Thus, we analyzed phosphorylated forms of γ H2AX in LNCaP cells. A significant increase in phospho- γ H2AX was observed with Ad-E2F1 or Ad-E2F1 + AS treatment with and without radiation (Figure 5E). These findings ascertain the functional effect of E2F1 leading to phosphorylation of p53 that may involve ATM kinase in both untreated and irradiated LNCaP cells.

Ad-E2F1 + AS-MDM2 induces phospho- γ H2AX, Bax and p21 protein in p53^{null} PC-3 cells

Androgen independent and p53^{null} PC-3 cells responded effectively to radiation when they were treated with Ad-E2F1 + AS-MDM2. To understand the mechanism of sensitization, we assessed the level of phospho- γ H2AX to ascertain whether increased E2F1 is associated with phospho- γ H2AX in the absence of wild-type p53 protein. In addition, we assessed the induction of p21 protein, bcl-2 and Bax to determine whether these p53 targets are induced in the absence of p53. Ad-E2F1 and Ad-E2F1 + AS caused induction of phospho- γ H2AX in untreated PC3 cells, further induction was evident with irradiation (Figure 6A).

Irradiated PC-3 cells were found to have increased elevation of p21 protein when treated with Ad-E2F1 or Ad-E2F1 + AS-MDM2 (Fig. 6B), in the presence or absence of radiation. Slightly higher levels of p21 were seen when Ad-E2F1 and AS-MDM2 were combined. The upregulation of p21 after Ad-E2F1 $\pm$ AS-MDM2 was seen in both p53^{wild-type} and p53^{null} prostate cancer cells, suggesting a role in radiation-induced apoptosis. To further understand the role of p21, we determined the effect of p21 inhibition on the induction of caspase-3 activity mediated by Ad-E2F or Ad-E2F + AS. No changes in the induction of caspase-3 activity in response to Ad-E2F or Ad-E2F + AS was observed when endogenous p21 protein was inhibited using p21 siRNA (data not shown). These findings suggest that p21 may not be directly required in the apoptotic response to Ad-E2F or Ad-E2F + AS.

Since p21 was not a factor in eliciting apoptosis in response to Ad-E2F1 or Ad-E2F + AS, we assessed the induction of bcl-2 and Bax in response to these treatments. Bax was found to be elevated in response to Ad-E2F1 or Ad-E2F1 + AS with or without radiation; a concurrent reduction in bcl-2 was observed in cells treated with Ad-E2F1 + AS group (Figure 6C). These findings implicate that Ad-E2F or Ad-E2F1 + AS mediated death can be due to induction of Bax and thus we observe an increase in caspase 3 activity (Figure 3).

Normal human fibroblast cells are resistant to Ad-E2F1 or Ad-E2F1 + AS treatments

The above findings demonstrate that Ad-E2F1 or Ad-E2F1 + AS treatments were effective in killing prostate tumor cells. Next, we tested the effect of these treatments on normal human fibroblast cells. Radiation alone or radiation with or without Ad-Luc/Ad-Luc + AS/Ad-E2F1/Ad-E2F1 + MM failed to cause an increase in caspase activity 3/7 activity (Figure 7) as was observed significantly in LNCaP, LNCaP-Res and PC-3 cells (Figure 3). Further, with treatment of Ad-E2F1 + AS, there was a weak increase in caspase-3/7 activity both in unirradiated or irradiated group (Figure 7). These findings demonstrate that Ad-E2F1 or Ad-E2F1 + AS therapy are weakly toxic to normal fibroblast cells but highly toxic to prostate cancer cells.

DISCUSSION

Both E2F-1 and MDM2 are key determinants of apoptosis and, as we have shown previously, each sensitizes LNCaP cells to radiation (2,3). We hypothesized that Ad-E2F1 and AS-MDM2 would work in concert to sensitize prostate cancer cells to radiation. The data presented here show that Ad-E2F1 + AS-MDM2 significantly enhanced clonogenic and apoptotic cell death when administered with radiation, over either agent applied individually. This is the first report describing the radiosensitization of prostate tumor cells with Ad-E2F1 + AS-MDM2.

We also investigated the molecular mechanisms involved in the enhancement of cell death from Ad-E2F1 + AS-MDM2 combined with radiation by measuring key proteins in apoptotic pathways. Although the mechanisms by which E2F1 induces apoptosis are not completely understood, it has been suggested that apoptosis results from incompatible signals for proliferation and cell cycle arrest (36). One such set of conflicting signals is the concomitant stimulation of E2F1 and p53 activity (23,37). The p53 gene plays a key role in prevention of tumor formation through regulation of downstream targets, leading to growth arrest and apoptosis (38). High levels of p53 protein were observed when either Ad-E2F1 or AS-MDM2 was given alone. The levels of p53 were highest when Ad-E2F1 + AS-MDM2 were combined with irradiation. Therefore, the conflicting signals from E2F1 and p53 were amplified by adding AS-MDM2.

Several studies have shown that E2F1 and p53 cooperate to mediate apoptosis. In fibroblasts, E2F1-induced apoptosis is potentiated by high levels of endogenous wild-type p53 (39,40). We investigated the phosphorylation status of p53 to understand the influence of E2F1 + AS-MDM2 on p53 activation. Using serine-15 phospho-specific antibodies, treatment with Ad-E2F1 + AS-MDM2 was confirmed to significantly activate p53 above that of the single treatment controls. Shono and colleagues reported that p53 phosphorylation at serine-15 is critical for p53-mediated apoptosis (41). Other studies have shown that ERKs and p38 kinase activation of p53 and apoptosis are mediated through phosphorylation of p53 at serine-15 (42). We found that transcriptional activation, measured by co-transfection with a p53 response element-luciferase reporter construct in LNCaP and LNCaP-Res cells, was maximal in response to Ad-E2F1 + AS-MDM2. These targeted treatments induced a high-level of p53 function (as observed by increased levels of phospho-p53) in p53^{wild-type} LNCaP and LNCaP-Res cells.

Although it was originally believed that p53 was essential for E2F1-mediated apoptosis, it is now clear that p53 is not always required (6,36,43). Our results using the PC3 (p53^{null}) model demonstrate that treatment with Ad-E2F1 plus AS-MDM2 is effective at inducing apoptosis and increasing cell death overall, through p53-independent mechanisms. The therapeutic applications are, therefore, broad, since many advanced prostate cancers harbor mutant or no p53 expression (44–50).

Upregulation and activation of p53 by Ad-E2F1 + AS-MDM2 + irradiation in LNCaP and LNCaP-Res cells heralded increased p21 protein expression. It is well known that expression of p21 is linked to the upregulation of phospho-p53 in response to DNA-damaging agents (51). In PC-3 cells, E2F1 + AS-MDM2 and radiation also resulted in an increase in p21, despite the absence of functional p53. p21 is a potent inhibitor of cyclin-dependent kinases and induces cell cycle arrest in response to DNA damage both in the presence or absence of functional p53 (52–54). p21 also blocks DNA replication by inhibiting PCNA (proliferation cell nuclear antigen) activity (55–57) and is required or associated with apoptosis in some cell types (31).

Elevation of p21 expression by etoposide, a topoisomerase inhibitor, has been observed in human breast carcinoma cells, to trigger apoptosis (58). Similarly, okadaic acid induced apoptosis is coupled with the cell-cycle-independent upregulation of endogenous p21 (59).

Furthermore, vascular smooth muscle cells transfected with p21 DNA exhibit the characteristic features of apoptotic cell death (60). In our study, p21 upregulation from Ad-E2F1 + AS-MDM2 and radiation was not associated with greater levels of apoptotic cell death and clonogenic inhibition in p53 null PC-3 cells since knockdown of p21 failed to reduce apoptosis in response to these treatments. On the other hand, upregulation of Bax related to increased caspase-3/7 activity may play a critical role in regulating the response to Ad-E2F1 + AS-MDM2.

We also observed that the levels of androgen receptor were reduced after exposure to Ad-E2F1 + AS-MDM2, compared to Ad-E2F1 alone or Ad-Luc alone. Both MDM2 and E2F1 have a role in androgen receptor (AR)-mediated transcription (61). MDM2 indirectly affects AR-mediated signaling through inactivation of p53. For example, when p53 is overexpressed in hormone refractory tumors (62–65) there is also increased expression of AR (34,66) and vice versa (67,68). Recently, Davis and colleagues studied the expression patterns of AR and E2F1; increased levels of E2F1 were associated with decreased expression of AR in metastatic prostate tissues (69). They also found that overexpression of E2F1 caused a decrease in the mRNA, protein and promoter activity of the AR, indicating that E2F1 and AR interactions have an opposing critical role in prostate cancer progression. Other reports have demonstrated that downregulation of AR is partly responsible for the induction of apoptosis in prostate cancer cells (33). Thus, the down regulation of AR protein in response Ad-E2F1 + AS MDM2 in LNCaP and LNCaP-Res cells may have contributed to the apoptotic response observed; however, AR is not required because apoptosis was still observed in PC-3 cells.

The other hallmark of this study is that in both LNCaP and PC-3 cells, phospho- γ H2AX was significantly elevated in response to Ad-E2F1 or Ad-E2F1 + AS treatments with or without radiation. This observation is in concordance with Powers et al (35). It is not clear whether increase in phospho- γ H2AX is an indicator of increased double-strand breaks caused by Ad-E2F1 since the role of phospho- γ H2AX in DNA repair and apoptosis induction is controversial.

The mode of killing elicited in response to Ad-E2F1 or Ad-E2F1 + AS was primarily through apoptosis and reproductive death, since we failed to observe any kind of mitotic catastrophe such as micro-nuclei or large non-dividing cells. This is confirmed in another study where Ad-E2F1 was found to be involved directly in eliciting apoptosis rather causing other mitotic changes (70).

We provide evidence for the first time that adenoviral mediated overexpression of E2F1 combined with AS-MDM2 mediated suppression of MDM2 significantly induces apoptosis and clonogenic death when combined with radiation. The enhancement in radiation-induced cell death was observed in three prostate cancer cell lines with varying molecular responses and sensitivity to androgen. Radiosensitization by E2F1 expression and inhibition of MDM2 was associated with up-regulation of Bax, phospho- γ H2AX, and p21 in all prostate cancer cell lines tested, with downregulation of functional AR and bcl-2. Thus, adenoviral-E2F1 and AS-MDM2 are promising adjuncts to radiation in the treatment of prostate cancer, with less or no toxicity to normal cells.

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Figure 1. MDM2 and E2F1 expression in LNCaP cells after Ad-E2F1 and AS-MDM2 treatment. MDM2 (A) and E2F1 (B) were detected in LNCaP cells grown on cover slips incubated with Ad-E2F1 (10MOI) or AS-MDM2 (200nM) as described in ‘materials and methods’. The cells were fixed 24 hr post-transfection, probed with anti-MDM2 or anti-E2F1 antibodies, counterstained with 4', 6'-diamidino-2-phenylindole (DAPI) and then visualized by immunofluorescent microscopy. Images shown here are representatives of three experiments MDM2 (40X) and E2F1 (20X) magnification. Same cells are shown in each row and images on the bottom row are formed by superimpositions of the other two rows. C and D. Total cell extract was prepared from LNCaP, LNCaP-Res and PC3 cells transfected with Ad-E2F1 or MM alone and combined treatments with Ad-E2F1+MM or AS and LNCaP control (lipofectin alone) for 24 hr. An equal concentration of protein from each reaction was then probed by immunoblot with antibodies against MDM2 or E2F1 and normalized with β -actin. Lower panels show the densitometry data from for figure C and D.

Figure 2. Clonogenic assays of LNCaP (A) and LNCaP-Res (B) and PC3 (C) cells cultured in respective mediums and transfected with Ad-E2F1+AS-MDM2 for 24 hr before radiation (RT) at 2, 4 and 6 Gy as described in materials and methods. The data shown in the figure represent the average from 3 independent experiments. Values were expressed as percent surviving (mean $\pm$ SEM) from three separate experiments for all three cell lines.

Figure 3.

Caspase 3/7 levels in LNCaP, LNCaP-Res and PC3 cells after Ad-E2F1, AS-MDM2 and RT (5Gy) treatment. LNCaP (A), LNCaP-Res (B) and PC3 (C) cells were cultured in their respective medium for 3 days, and then incubated with Ad-E2F1 for 1h LNCaP (10MOI), LNCaP-Res (20MOI) and PC3 cells (50MOI) followed by 200 nM of AS-MDM2 or MM for 36h in the presence of Lipofectin (LC). Caspase 3/7 activity (RFLU, relative fluorescent units) was measured by fluorometric assay. The data shown represent the average values ($\pm$ SEM) from three independent experiments. *, $p < 0.0001$, compared with respective MM control, §, $p < 0.01$ compared with Ad-Luc control (one way Anova, LSD test). The table represents other significant comparisons for all three cell lines.

Figure 4.

E2F1 or MDM2 levels in prostate cells after Ad-E2F1, AS-MDM2 and RT treatments. Cells were cultured for 3 days and then transduced with Ad-E2F1 or Ad-Luc with AS-MDM2 as described in the materials and methods. For groups with RT cells were irradiated with (5 Gy) after 24 hr of gene transduction. Total protein was extracted after 24 hr for groups without RT, and 48 hr for RT group. Immunoblot assays for E2F1, MDM2 and β -actin were measured after Ad-E2F1+AS-MDM2 and RT treatments in LNCaP, LNCaP+RT (A), LNCaP-Res, LNCaP-Res+RT (B) and PC3, PC3+RT (C) cells. Respective densitometry data from for figure A, B and C are shown in panels.

Figure 5.

Key apoptotic protein levels in LNCaP and LNCaP-Res cells after Ad-E2F1, AS-MDM2 and RT treatments. Cells were cultured for 3 days and then transduced with Ad-E2F1 or Ad-Luc with AS-MDM2. For groups with RT cells were irradiated with (5 Gy) after 24 hr of gene transduction. Total protein was extracted after 24 hr for groups without RT, and 48 hr for RT group. Immunoblot assays of key related proteins (p53, phos-p53 (ser15), p21, AR and β -actin) were measured after Ad-E2F1+AS-MDM2 and RT treatments in LNCaP (A), LNCaP-Res (B) cells. (C) For p53 transactivation studies LNCaP and LNCaP-Res cells were incubated with Ad-E2F1 or Ad-Luc for 1hr followed by co-transfection of p53 luciferase reporter constructs (1ng/ml) and AS-MDM2 or MM (200 nM) treatment as described. The cells were harvested 48 hr post-transfection in 100 μ l reporter lysis buffer (Promega). Luciferase and β -galactosidase activities were measured using kits from Promega and Roche (Mannheim, Germany), respectively. Luciferase values were normalized by transfection efficiency as measured by β -galactosidase. All data represent mean values $\pm$ SEM of three independent experiments. (D) Equal amounts of protein from the cell lysates were loaded by SDS-PAGE and immunoblotted with phospho-p53, PUMA, Bcl2, BAX or actin antibodies. (E) phospho- γ H2AX or actin expression in LNCaP cells after Ad-E2F1 and AS-MDM2 followed by RT (5 Gy) treatment. Right panel shows the densitometry data.

Figure 6.

γ H2AX, p21, Bcl2 and BAX protein levels in PC3 cells. Equal amounts of PC3 cell lysates were loaded by SDS-PAGE and immunoblotted with phospho- γ H2AX or actin antibodies after Ad-E2F1 and AS-MDM2 followed by RT (5 Gy) treatment. (A) phospho- γ H2AX or actin expression in PC3 cells after Ad-E2F1 combined with AS-MDM2 and RT (5 Gy) treatment. (B) p21 expression in PC3 cells after Ad-E2F1 and AS-MDM2 treatment. Right panels show the densitometry data for figure 6A and 6B respectively. (C) Bcl2 and BAX expression levels in PC3 cells after Ad-E2F1 combined with AS-MDM2 and RT (5 Gy) treatment. Actin was measured as a loading control.

Figure 7. Normal human embryonic IMR-90 fibroblasts cells were cultured in DMEM/F12 medium for 24 hr, and then incubated with Ad-E2F1 for 1h (10MOI) followed by 200 nM of AS-MDM2 or MM for 36h in the presence of Lipofectin (LC). Caspase 3/7 activity (RFLU, relative fluorescent units) was measured by fluorometric assay. The data shown represent the average values ($\pm$ SEM) from three independent experiments.

Radiation inactivation estimates of prostate cancer cell lines treated with Ad-E2F1 or Ad-E2F1 + AS obtained using single-hit multi-target model.

Cell lines	Inactivation Estimates																								
	RT + Ad-Luc				RT + Ad-E2F				RT + Ad-Luc + MM				RT + Ad-Luc + AS				RT + Ad-E2F + MM				RT + Ad-E2F + AS				
	D ₀	cGy	SF ₂	n	D ₀	cGy	SF ₂	n	D ₀	cGy	SF ₂	n	D ₀	cGy	SF ₂	n	D ₀	cGy	SF ₂	n	D ₀	cGy	SF ₂	n	
LNCaP	93	0.24	2.3	76.9	0.29448	4.5	95.1	0.2386	2.1	75.8	0.1753	2.6	86.8	0.2707	3	64.6	0.0536	1.2							
LNCaP-Res	90.7	0.4091	4	65.6	0.1768	4	95.5	0.4095	4	82.3	0.3218	4.2	66.2	0.0863	1.8	45.9	0.0216	1.7							
PC-3	141.4	0.4182	1.85	156	0.496	1.95	135.4	0.37	1.7	84	0.2522	3	146.9	0.2597	1.01	75	0.0853	1.25							

Table 2
Radiation enhancement ratios for prostate cancer cell lines treated with Ad-E2F1 or Ad-E2F1 + AS.

Cell lines	Radiation Enhancement Ratios (RER)											
	RT + Ad-E2F			RT + Ad-Luc + MM			RT + Ad-Luc + AS			RT + Ad-E2F + MM		
	D ₀	RER	SF ₂	D ₀	RER	SF ₂	D ₀	RER	SF ₂	D ₀	RER	SF ₂
LNCaP	1.21	0.8	0.97	1.03	1.23	1.4	1.1	0.9	1.44	1.44	1.44	4.5
LNCaP-Res	1.4	2.3	0.95	0.99	1.1	1.3	1.4	4.7	2	18.95	2	18.95
PC-3	0.9	0.8	1	1.13	1.7	1.6	0.96	1.6	1.8	1.8	1.8	4.9