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

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Abstract
We have previously shown 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 cells, LNCaP-Res cells [androgen insensitive with functional p53 and androgen receptor (AR)], and PC3 cells (androgen insensitive, p53null, and ARnull). A supra-additive radiosensitizing effect was observed in terms of clonogenic inhibition and induction of apoptosis (caspase-3 + caspase-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-Ser15 p53 with significant induction of p21waft-1cip1, phospho-γH2AX, PUMA, and Bax levels and reduction of AR and bcl-2 expression. Similarly, ARnull and p53null PC-3 cells showed elevated levels of Bax and phospho-γH2AX expression. These findings show 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.

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Introduction
Radiation therapy (RT) is an established and common treatment for prostate cancer. Yet, for men with high-risk disease, failure rates are ~40% over 5 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) caused pronounced radiosensitization in p53wild-type (LNCaP) and p53null (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% to 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 p21waft-1cip1 independently of p53 (13-17). MDM2 also mediates ubiquitination and proteolysis of the androgen receptor (AR; refs. 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 ADP ribosylation factor-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.

Results
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 were confirmed by immunofluorescence staining of E2F1 and
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 coverslips incubated with Ad-E2F1 (10 MOI) or AS-MDM2 (200 nmol/L) as described in Materials and Methods. The cells were fixed 24 h posttransfection, probed with anti-MDM2 or anti-E2F1 antibodies, counterstained with 4',6-diamidino-2-phenylindole (DAPI), and then visualized by immunofluorescence microscopy. Images shown are representatives of three experiments. Magnification, ×40 (MDM2); ×20 (E2F1). 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 (Lipofectinal alone) for 24 h. An equal concentration of protein from each reaction was then probed by immunoblot with antibodies against MDM2 or E2F1 and normalized with β-actin. Bottom, densitometry data for C and D.

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 with 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 multiplicities 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: (a) adenoviral luciferase (Ad-Luc) alone; (b) Ad-Luc + mismatch oligonucleotide (MM); (c) Ad-Luc + AS-MDM2; (d) Ad-E2F1 alone; (e) Ad-E2F1 + MM; (f) Ad-E2F1 + AS-MDM2; and (g) AS-MDM2 alone.

LNCaP cells were significantly radiosensitized by Ad-E2F1 ($D_0 = 76.9$, $SF_2 = 0.2946$, and $n = 4.5$ with $P < 0.045$) or Ad-E2F1 + AS-MDM2 ($D_0 = 64.6$, $SF_2 = 0.0536$, and $n = 1.2$).
with $P < 0.0035$) when compared with Ad-Luc ($D_0 = 93, SF_2 = 0.24$, and $n = 2.3$; Table 1; 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 $P < 0.001$) or Ad-E2F1 + AS-MDM2 ($D_0 = 45.9, SF_2 = 0.0216$, and $n = 11.7$ with $P < 0.00021$) when compared with Ad-Luc ($D_0 = 90.7, SF_2 = 0.4091$, and $n = 4$; Table 1; 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 with 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 in response to Ad-E2F1 + AS-MDM2 ($D_0 = 75, SF_2 = 0.0853$, and $n = 1.25$; $P < 0.00014$) when compared with Ad-Luc ($D_0 = 141.4, SF_2 = 0.4182$, and $n = 1.85$; Table 1; Fig. 2C).

In response to Ad-E2F1 + AS-MDM2, at SF2, LNCaP-Res cells had the highest radiation enhancement ratio (18.95), followed by PC3 (4.9) and LNCaP cells (4.5). The radiation enhancement ratio for SF2 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 radiation enhancement ratio at SF2 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 show that AS-MDM2 synergizes the radiosensitizing effect of Ad-E2F1, particularly in androgen-resistant LNCaP-Res cells. The $D_0$ radiation enhancement ratio was modestly present in all the cell lines except in PC-3 cells treated with Ad-E2F1.

Ad-E2F1 and AS-MDM2 in Combination with Radiation Increases Apoptosis

Apoptosis by Caspase-3/7 Assay. Caspase-3/7 activation precedes the onset of cell death (25, 26). Caspase-3/7 activity was highest in the irradiated Ad-E2F1 + AS-MDM2 group in all three cell lines (Fig. 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; Table 3). These findings show 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 terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling (TUNEL).

Apoptosis by TUNEL Assay. TUNEL assays were also done 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 with controls in LNCaP and LNCaP-Res.

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**Table 1. Radiation Inactivation Estimates of Prostate Cancer Cell Lines Treated with Ad-E2F1 or Ad-E2F1 + AS Obtained Using a Single-Hit Multitarget Model**

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>RT + Ad-Luc</th>
<th>RT + Ad-E2F</th>
<th>RT + Ad-Luc + MM</th>
<th>RT + Ad-Luc + AS</th>
<th>RT + Ad-E2F + MM</th>
<th>RT + Ad-E2F + AS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$D_0$ (cGy)</td>
<td>$SF_2$</td>
<td>$n$</td>
<td>$D_0$ (cGy)</td>
<td>$SF_2$</td>
<td>$n$</td>
</tr>
<tr>
<td>LNCaP</td>
<td>93</td>
<td>0.24</td>
<td>2.3</td>
<td>76.9</td>
<td>0.2946</td>
<td>4.5</td>
</tr>
<tr>
<td>LNCaP-Res</td>
<td>90.7</td>
<td>0.4091</td>
<td>4</td>
<td>65.6</td>
<td>0.1768</td>
<td>4</td>
</tr>
<tr>
<td>PC-3</td>
<td>141.4</td>
<td>0.4182</td>
<td>1.85</td>
<td>156</td>
<td>0.496</td>
<td>1.95</td>
</tr>
</tbody>
</table>

**FIGURE 2.** Clonogenic assays of LNCaP (A), LNCaP-Res (B), and PC3 (C) cells cultured in respective media and transfected with Ad-E2F1 + AS-MDM2 for 24 h before radiation (RT) at 2, 4, and 6 Gy as described in Materials and Methods. Points, mean percent surviving cells from three independent experiments for all three cell lines; bars, SE.
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 with 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 Up-Regulation 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 as shown in Fig. 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 (Fig. 4A-C). This up-regulation was mitigated when LNCaP, LNCaP-Res, and PC-3 cells were treated with AS-MDM2. These findings show 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, 27), a significant increase in p53 protein was observed in unirradiated LNCaP cells, 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 (Fig. 5A and B). Inhibition of MDM2 led to increased p53 stability.

Because p53 protein was elevated in response to Ad-E2F1 + AS-MDM2 + irradiation, we reasoned that this treatment would enhance p53 transactivation function. Figure 5A and B (24 hours after treatment) and Fig. 5D (6 hours 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 (28). 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 show that Ad-E2F1 + AS-MDM2 in the presence or absence of radiation induces 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 (29) and is a regulator of apoptosis (30), and AR activation is regulated in part by p53 and MDM2 (19, 31), 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 (Fig. 5A and B). Ad-E2F1 + AS-MDM2 treatment was effective at down-regulating radiation-induced AR protein.

Because p53 transactivation function was robustly observed with Ad-E2F1 plus AS in both LNCaP and LNCaP-Res cells (Fig. 5C), we further tested the effect of Ad-E2F1 + AS-MDM2 induced p53 transactivation function on p53 targets in LNCaP cells. In the LNCaP cells, we found that phospho-p53 increased as quickly as 6 hours with Ad-E2F1 or Ad-E2F1 + AS-MDM2 alone or in combination with radiation (Fig. 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 proapoptotic 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 γH2AX phosphorylation (32). Thus, we analyzed phosphorylated forms of γH2AX in LNCaP cells. A significant increase in phospho-γH2AX was observed with Ad-E2F1 or Ad-E2F1 + AS-MDM2 treatment with and without radiation (Fig. 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.

Table 2. Radiation Enhancement Ratios for Prostate Cancer Cell Lines Treated with Ad-E2F1 or Ad-E2F1 + AS

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Radiation Enhancement Ratios</th>
<th>Radiation Enhancement Ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RT + Ad-E2F</td>
<td>RT + Ad-Luc + MM</td>
</tr>
<tr>
<td></td>
<td>D0 RER SF2 RER</td>
<td>D0 RER SF2 RER</td>
</tr>
<tr>
<td>LNCaP</td>
<td>1.21</td>
<td>0.8</td>
</tr>
<tr>
<td>LNCaP-Res</td>
<td>1.4</td>
<td>2.3</td>
</tr>
<tr>
<td>PC-3</td>
<td>0.9</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Abbreviation: RER, radiation enhancement ratio.
Ad-E2F1 + AS-MDM2 Induces Phospho-γH2AX, Bax, and p21 Proteins in p53null PC-3 Cells

Androgen-independent and p53null 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-γH2AX to ascertain whether increased E2F1 is associated with phospho-γH2AX 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-MDM2 caused induction of phospho-γH2AX in PC3 cells; further induction was evident with irradiation (Fig. 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. Up-regulation of p21 after

**FIGURE 3.** Caspase-3/7 levels in LNCaP, LNCaP-Res, and PC3 cells after Ad-E2F1, AS-MDM2, and RT (5 Gy) treatment. LNCaP (A), LNCaP-Res (B), and PC3 (C) cells were cultured in their respective media for 3 d, and then incubated with Ad-E2F1 for 1 h (LNCaP, 10 MOI; LNCaP-Res, 20 MOI; PC3, 50 MOI) followed by 200 nmol/L of AS-MDM2 or MM for 36 h in the presence of Lipofectin. Caspase-3/7 activity (relative fluorescent units) was measured by fluorometric assay. Columns, average from three independent experiments; bars, SE. *, P < 0.0001, compared with respective MM control; †, P < 0.01, compared with Ad-Luc control (one-way ANOVA least significance difference test).

*p<0.001 – compared to MM
+t<0.01 – compared to Ad-Luc
Ad-E2F1 ± AS-MDM2 was seen in both p53wild-type and p53null 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-E2F1 or Ad-E2F1 + AS-MDM2. No changes in the induction of caspase-3 activity in response to Ad-E2F or Ad-E2F + AS-MDM2 was observed when endogenous p21 protein was inhibited using p21 small interfering RNA (data not shown). These findings suggest that p21 may not be directly required in the apoptotic response to Ad-E2F or Ad-E2F + AS-MDM2.

Because p21 was not a factor in eliciting apoptosis in response to Ad-E2F1 or Ad-E2F + AS-MDM2, 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-MDM2 with or without radiation; a concurrent reduction in Bcl-2 was observed in cells treated with the Ad-E2F1 + AS-MDM2 group (Fig. 6C). These findings suggest that Ad-E2F– or Ad-E2F1 + AS-MDM2–mediated death may be due to induction of Bax, and thus we observe an increase in caspase-3 activity (Fig. 3).

Normal Human Fibroblast Cells Are Resistant to Ad-E2F1 or Ad-E2F1 + AS-MDM2 Treatments

The above findings show that Ad-E2F1 or Ad-E2F1 + AS-MDM2 treatments were effective in killing prostate tumor cells. Next, we tested the effect of these treatments on normal human fibroblasts. Table 3 summarizes the results of these experiments.

Table 3. Other Significant Comparisons for All Three Cell Lines

<table>
<thead>
<tr>
<th>Caspase-3 + Caspase-7</th>
<th>LNCaP</th>
<th>LNCaP-Res</th>
<th>PC3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Groups</td>
<td>−RT</td>
<td>+RT</td>
<td>−RT</td>
</tr>
<tr>
<td>Ad-E2F1 vs Ad-E2F1 + AS</td>
<td>P &lt; 0.003</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>Ad-E2F1 + AS vs Ad-Luc + AS</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.0001</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>Ad-E2F1 + AS vs AS</td>
<td>P &lt; 0.002</td>
<td>P &lt; 0.0001</td>
<td>P &lt; 0.0001</td>
</tr>
</tbody>
</table>

FIGURE 4. E2F1 or MDM2 levels in prostate cells after Ad-E2F1, AS-MDM2, and RT treatments. Cells were cultured for 3 d and then transduced with Ad-E2F1 or Ad-Luc with AS-MDM2 as described in Materials and Methods. For groups with RT, cells were irradiated (5 Gy) after 24 h of gene transduction. Total protein was extracted after 24 h for groups without RT and after 48 h for the 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. Right, densitometry data (A–C).

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fibroblast cells. Radiation alone or radiation with or without Ad-Luc, Ad-Luc + AS-MDM2, Ad-E2F1, Ad-E2F1 + MM failed to cause an increase in caspase-3/7 activity (Fig. 7), as was observed significantly in LNCaP, LNCaP-Res, and PC-3 cells (Fig. 3). Further, with treatment of Ad-E2F1 + AS-MDM2, there was a weak increase in caspase-3/7 activity both in the unirradiated and irradiated groups (Fig. 7). These findings show that Ad-E2F1 or Ad-E2F1 + AS-MDM2 therapy is 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 (33). One such set of conflicting signals is the concomitant stimulation of E2F1 and p53 activity (23, 34). The p53 gene plays a key role in prevention of tumor formation through regulation of downstream targets, leading to growth arrest and apoptosis (35). 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 (36, 37). We investigated the phosphorylation status of p53 to understand the influence of E2F1 + AS-MDM2 on p53 activation. Using Ser15 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 (38) reported that p53 phosphorylation at Ser15 is critical for p53-mediated apoptosis. Other studies have shown that extracellular signal–regulated kinases and p38 kinase activation of p53 and apoptosis are mediated through phosphorylation of p53 at Ser15 (39). We found that transcriptional activation, measured by cotransfection 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, 33, 40). Our results using the PC3 (p53null) model show 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 because many advanced prostate cancers harbor mutant or no p53 expression (41-47).

Up-regulation 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 up-regulation of phospho-p53 in response to DNA-damaging agents (48). 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 and in the absence of functional p53 (49-51). p21 also blocks DNA replication by inhibiting proliferation cell nuclear antigen activity (52-54) and is required for or associated with apoptosis in some cell types (28).

Elevation of p21 expression by etoposide, a topoisomerase inhibitor, has been observed in human breast carcinoma cells to trigger apoptosis (55). Similarly, okadaic acid–induced apoptosis is coupled with the cell cycle–independent up-regulation of endogenous p21 (56). Furthermore, vascular smooth muscle

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**FIGURE 5 Continued.** 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-γH2AX or actin expression in LNCaP cells after Ad-E2F1 and AS-MDM2 followed by RT (5 Gy) treatment. Right, densitometry data.
cells transfected with p21 DNA exhibit the characteristic features of apoptotic cell death (57). In our study, p21 up-regulation by Ad-E2F1 + AS-MDM and radiation was not associated with greater levels of apoptotic cell death and clonogenic inhibition in p53<sup>null</sup> PC-3 cells because knockdown of p21 failed to reduce apoptosis in response to these treatments. On the other hand, up-regulation 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 AR were reduced after exposure to Ad-E2F1 + AS-MDM2, compared with Ad-E2F1 alone or Ad-Luc alone. Both MDM2 and E2F1 have a role in AR-mediated transcription (58). MDM2 indirectly affects AR-mediated signaling through inactivation of p53. For example, when p53 is overexpressed in hormone-refractory tumors (59-62), there is also increased expression of AR (31, 63) and vice versa (64, 65). Recently, Davis and colleagues (66) studied the expression patterns of AR and E2F1; increased levels of E2F1 were associated with decreased expression of AR in metastatic prostate tissues. 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 shown that down-regulation of AR is partly

**FIGURE 6.** γH2AX, p21, Bcl2, and BAX protein levels in PC3 cells. Equal amounts of PC3 cell lysates were loaded by SDS-PAGE and immunoblotted with phospho-γH2AX or actin antibodies after Ad-E2F1 and AS-MDM2, followed by RT (5 Gy) treatment. A. Phospho-γH2AX 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, densitometry data (A and B). 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.
responsible for the induction of apoptosis in prostate cancer cells (30). Thus, the down-regulation of AR protein in response to 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-γH2AX was significantly elevated in response to Ad-E2F1 or Ad-E2F1 + AS-MDM2 treatments with or without radiation. This observation is in concordance with Powers et al. (32). It is not clear whether the increase in phospho-γH2AX is an indicator of increased double-strand breaks caused by Ad-E2F1 because the role of phospho-γH2AX in DNA repair and apoptosis induction is controversial.

The mode of killing elicited in response to Ad-E2F1 or Ad-E2F1 + AS-MDM2 was primarily through apoptosis and reproductive death because we failed to observe any kind of mitotic catastrophe such as micronuclei or large nondividing cells. This is confirmed in another study, which found Ad-E2F1 to be involved directly in eliciting apoptosis rather than causing other mitotic changes (67).

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 sensitivities to androgen. Radiosensitization by E2F1 expression and inhibition of MDM2 was associated with up-regulation of Bax, phospho-γH2AX, and p21 in all prostate cancer cell lines tested, with down-regulation 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.

Materials and Methods

Cell Culture

LNCaP and PC3 cells were cultured in DMEM-F12 containing 10% fetal bovine serum, penicillin-streptomycin, and l-glutamine, as described previously (68). Androgen-insensitive LNCaP-Res cells were grown in androgen-deprived DMEM/F-12 medium. Androgen deprivation was achieved by culturing the cells in medium containing 10% charcoal-stripped serum for 3 d. LNCaP-Res cells were established by serial passage of LNCaP cells in androgen-deprived medium for 1 y. Normal human embryonic IMR-90 fibroblast cell line (ATCC CCL-186) was obtained from American Type Culture Collection and grown in DMEM/F-12 medium.

Oligonucleotides and Antibodies

Idera, Inc., provided the oligonucleotides. The antisense MDM2 oligonucleotide (AS-MDM2) and its mismatch control oligonucleotide (MM) are 20-mer mixed-backbone oligonucleotides with the following sequences; AS-MDM2, 5-UGACACCTGTTCTCACUCAC-3; MM, 5-UGTCACCTTTTTTTCATUCAC-3. They were stored as frozen aliquots at −20°C (13). E2F1, p53, p21, MDM2, phospho-p65, PUMA, and β-actin antibodies were obtained from Oncogene; phospho-γH2AX (Ser139) antibody from Abcam; and antimouse horseradish peroxidase–conjugated secondary antibody from Amersham.

Viral infection and Transient Transfections

LNCaP, LNCaP-Res, PC3, or IMR-90 cells (5 × 10^5) were grown for 3 d, and then infected with adenovirus-5 construct that harbors full-length E2F1 under the control of cytomegalovirus promoter (Ad-E2F1) or control adenoviral construct that harbors full-length luciferase cDNA under the control of cytomegalovirus promoter (Ad-Luc; control vector). The cells were incubated with the virus for 1 h followed by transfection with 200 nmol/L AS-MDM2 or mismatch (MM; control) oligonucleotide in 2 mL culture medium for 36 to 48 h in the presence of 7 μg/mL Lipofectin (Invitrogen). Groups with radiation (5 Gy) treatment were irradiated 24 h after AS-MDM2 or MM and reincubated for 24 h.

Western Blot Analysis

Western blot analyses were done to confirm transduction efficiency. Cells were harvested after 24 h posttransfection with Ad-E2F1 or AS-MDM2, lysed in buffer [50 mmol/L Tris-HCl (pH 6.8), 2% SDS with protease inhibitor cocktail set I (Calbiochem)], and were sonicated. Thirty micrograms of protein from each cell lysate were electrophoresed on a 4% to 20% SDS polyacrylamide gel. After transfer onto a polyvinylidene difluoride membrane (Millipore) in a transblot apparatus and blocking with 5% low-fat dried milk, the blots were incubated overnight at 4°C with specific

FIGURE 7. Normal human embryonic IMR-90 fibroblast cells were cultured in DMEM-F12 for (10 MOI) followed by 200 nmol/L of AS-MDM2 or MM for 36 h in the presence of Lipofectin. Caspase-3/7 activity (relative fluorescent units) was measured by fluorometric assay. Columns, average from three independent experiments; bars, SE.
primary antibodies. The membranes were washed and labeled with an antimouse horseradish peroxidase–conjugated secondary antibody (Amersham Pharmacia Biotech) at room temperature for ~1 h. Detection by chemiluminescence was done according to the manufacturer’s (Amershan) standard protocol.

Immunofluorescence/Confocal Microscopy

LNCaP cells grown on coverslips were fixed in ice-cold acetone/methanol (1:1) for 15 min at −20°C. Cells were then air-dried, rehydrated in PBS, blocked, and permeabilized in 0.2% Triton X-100/PBS. The cells were then washed in PBS and subsequently blocked in 5% bovine serum albumin. Primary antibodies were applied to the coverslips for 1 h and then overlaid with Alexa Fluor 568–conjugated goat antimouse secondary antibody (Molecular Probes) for 30 min. After three washes in PBS, the coverslips were further incubated in 4′,6-diamidino-2-phenylindole (1 μg/mL) to stain the nuclei. The coverslips were then mounted using an aqueous mounting medium (anti-fading agents; Biomeda 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 media as described above for 2 to 3 d. Appropriate dilutions of Ad-E2F1 or Ad-Luc in 1 mL 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) 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 d, colonies were stained with methylene blue and counted. Cell survival was adjusted for plating efficiency.

Detection of Apoptosis by TUNEL Assays

TUNEL was done 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 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 h. Following washing with TBS, the cells were analyzed by flow cytometric analysis on a FACSCalibur 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) 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). Cells were cultured in the respective media and treated with Ad-E2F1 or Ad-Luc and AS-MDM2 or MM as described above. A total of 2 × 10⁴ cells in 50 μL culture medium were mixed with 50 μL of Homogeneous Caspase-3/7 reagent in 96-well plates and incubated at room temperature for 18 h. Substrate cleavage was quantified fluorometrically at 485-nm excitation and 538-nm emission. Fluorescence was measured on a fluorescent plate reader (LabSystems, Inc.).

p53-Luciferase Reporter Assays

LNCaP, LNCaP-Res, and PC3 cells were incubated with Ad-E2F1 or Ad-Luc for 1 h, followed by cotransfection of p53 luciferase reporter constructs (1 μg/mL) and AS-MDM2 or MM treatments as described above. Cells were incubated for 24 h at 37°C; after 24 h, fresh medium was added to the cells. The cells were harvested 48 h posttransfection in 100 μL reporter lysis buffer (Promega). Luciferase and β-galactosidase activities were measured using kits from Promega and Roche, respectively. Luciferase values were normalized by transfection efficiency as measured by β-galactosidase. All data represent mean values of three independent experiments ± SD.

Statistical Analysis

Statistics were done 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.

Disclosure of Potential Conflicts of Interest

The Dr. A. Pollack received a commercial grant from Varian Medical Systems.

References


Correction: Article on AS-MDM2 and Radiation Enhances E2F1-Induced Apoptosis

In the article by Udayakumar and colleagues on AS-MDM2, beginning on page 1742 of the November 2008 issue of Molecular Cancer Research, there was an error in the title. The correct title appears here.

Antisense MDM2 Enhances E2F1-Induced Apoptosis and the Combination Sensitizes Androgen-Sensitive and Androgen-Insensitive Prostate Cancer Cells to Radiation.
