Gene therapy with E2F-1 up-regulates the protein kinase PKR and inhibits growth of leiomyosarcoma in vivo

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Abstract
Overexpression of the transcription factor E2F-1 induces apoptosis in a variety of carcinoma cells and inactivates murine double minute protein 2, a factor associated with poor prognosis in soft tissue sarcomas. We have shown previously that the double-stranded RNA-activated protein kinase PKR plays an important role in mediating this apoptotic response in carcinoma cells to E2F-1. We sought to evaluate the potential of E2F-1 gene therapy in soft tissue sarcomas and to study the involvement of PKR in the response to E2F-1 overexpression in mesenchymal cells. A replication-deficient adenovirus carrying the E2F-1 gene (Ad5E2F) was used to induce E2F-1 overexpression in the p53 mutated leiomyosarcoma cell line, SKLMS-1. Western blot analysis confirmed E2F-1 overexpression and up-regulation of the antiapoptotic factor Bcl-2 48 hours following infection with Ad5E2F. Apoptosis in Ad5E2F-treated cells was confirmed by fluorescence-activated cell sorting analysis and by poly(ADP-ribose) polymerase cleavage and DNA fragmentation assays. Vector-dependent up-regulation of PKR correlated with the amount of Ad5E2F-induced apoptosis. In vivo treatment of SKLMS-1 tumor-bearing BALB/c mice with intratumoral injections of Ad5E2F at a dose of 2 × 1010 viral particles resulted in significant inhibition in tumor growth compared with control-treated animals (P < 0.016). Complete disappearance of all tumors was seen in two of seven mice in the Ad5E2F-treated animals. Immunohistochemical analysis of tumor specimens showed overexpression of E2F-1 and up-regulation of PKR in Ad5E2F-treated tumors. These findings show that adenovirus-mediated overexpression of E2F-1 results in up-regulation of PKR and significant growth suppression of leiomyosarcomas in vivo. Taken together, these data suggest that E2F-1 gene therapy and PKR modulation might be a promising treatment strategy for these tumors that are highly resistant to conventional therapies. [Mol Cancer Ther 2005;4(11):1710–6]

Introduction
Transcription factors of the E2F family are essential for cellular transition from the G1 phase to the S phase of the cell cycle. Although all the E2F transcription factors can induce proliferation and differentiation, only E2F-1 is known to effectively induce a broad variety of cancer cells to undergo apoptosis (reviewed in ref. 1). Among >1,240 genes found to be significantly altered after E2F activation, only p53, p73, and apoptosis protease-activating factor 1 have been identified to be E2F-1-inducible genes capable of mediating an apoptotic response (1–4). Because some cancer cells undergo apoptosis despite mutations or deletions of p53 and p73, we have investigated alternative pathways through which E2F-1 induces apoptosis. We found recently that the absence or inhibition of the double-stranded RNA-activated protein kinase PKR leads to significantly decreased sensitivity to E2F-1-induced apoptosis in carcinoma cells, suggesting that PKR plays an important role in the apoptotic response to E2F-1 (5). PKR is known to mediate the antiproliferative and antiviral actions of IFN (reviewed in ref. 6). Up-regulation and activation of PKR leads to homodimerization and phosphorylation of PKR and its downstream targets. The best studied of these targets is the α-subunit of the eukaryotic translation initiation factor-2α. Phosphorylation of eukaryotic translation initiation factor-2α by PKR inhibits the initiation of RNA translation by the 40S ribosomal subunit. This leads to inhibition of protein synthesis, suppression of growth, and apoptotic cell death. However, PKR also regulates several other transcription factors that have been shown to play a crucial role in cell growth, differentiation, proliferation, and induction of apoptosis in different tumor cell lines, including nuclear factor-κB, p53, and signal transducers and activators of transcription 1. Hence, PKR activation can mediate apoptosis through several pathways.

Soft tissue sarcomas are a group of histologically heterogeneous, putatively mesenchymal malignancies. Despite intensive research, therapeutic advances in the
treatment of these tumors have been limited over the past several decades, and many patients will die of metastatic disease (7). New and innovative treatment strategies are therefore desperately needed.

Clinical studies have shown a direct correlation between worsening prognosis and increase of murine double minute protein 2 (mdm2) gene expression in a variety of soft tissue sarcomas (58, 9). Mdm2 is normally bound to p53, inducing its degradation. E2F-1 antagonizes this action by inducing the transcription of p14ARF, which binds to mdm2 and stabilizes p53 (10). Interestingly, it has been reported that the 3-hydroxy-3-methylglutaryl CoA reductase inhibitor lovastatin rapidly induced apoptosis in Ewing’s sarcoma through the activation of E2F-1 (11). These reports suggest that E2F-1 overexpression may be a promising strategy to induce apoptosis in soft tissue sarcomas. We therefore investigated the efficacy of E2F-1 gene therapy in leiomyosarcoma in vitro and in vivo. Additionally, our interest was focused on the role of PKR and other downstream targets of E2F-1 known to be important in cell cycle control and apoptosis.

Materials and Methods

Cell Lines

We used the human leiomyosarcoma cell line SKLMS-1 obtained from the American Type Culture Collection (Rockville, MD), which has a codon 245 p53 point mutation. SKLMS-1 cells were grown in DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FCS, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were grown in monolayers to a confluency of ~80% before treatment.

Recombinant Adenovirus Vectors

Cells were treated with replication-defective recombinant adenovirus vectors at a multiplicity of infection (MOI) of 2,000 viral particles per cell. Transduction efficiency for the replication-deficient adenovirus type 5 (Ad5) was determined previously using an adenoviral vector containing the β-galactosidase reporter gene under control of the cytomegalovirus promoter (Ad5βGal). The adenovirus vector containing the E2F-1 transgene under the control of the cytomegalovirus promoter (Ad5E2F) has a deletion in the E1 subunit, which renders it replication defective. A recombinant adenovirus containing the luciferase reporter (Ad5Luc) under control of the cytomegalovirus promoter was used as a control. Cells were treated with PBS, vector control (Ad5Luc), or Ad5E2F and allowed to incubate under standard tissue culture conditions (37°C and 5% CO2).

Western Blot Analysis

Protein extracts were prepared from cells at days 2, 3, 4, and 6 following infection with recombinant adenovirus vectors. Western blot analysis was done as described previously (12). Protein concentrations were determined using the Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA). Lysates were analyzed by Western blot analysis using 10% SDS gels. Lanes were loaded with 50 μg protein and electrophoresed for 2 hours at 90 V. Gels were transferred to nitrocellulose membranes that were blocked with 5% nonfat dry milk and incubated with primary antibodies overnight at 4°C. Primary antibodies included mouse monoclonal antibody IgG to β-actin (Amersham Life Science, Inc., Arlington Heights, IL), monoclonal antibody to E2F-1 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal antibody IgG to PKR, Bax, Bb, cyclin E, and poly(ADP-ribose) polymerase (Santa Cruz Biotechnology), hamster purified IgG antibody to human Bcl-2, mouse purified IgG antibody to human p21 (PharMingen, San Diego, CA), and monoclonal antibody to p53 (Onco-gene Research Products, Cambridge, MA). Conjugated secondary antibody (PharMingen) was added for 1 hour, and protein signals were detected using enhanced chemiluminescence Western blotting detection reagents (Amersham Biosciences, Buckinghamshire, United Kingdom).

Fluorescence-Activated Cell Sorting Analysis

Cells were harvested for fluorescence-activated cell sorting (FACS) analysis and fixed with ice-cold 80% ethanol, stained with propidium iodide (PI; Sigma, St. Louis, MO), and analyzed using a flow cytometer (EPICS XL-MCL, Coulter, Miami, FL) as described previously (13). Floating cells and adherent cells were harvested and pelleted. Cells were washed and resuspended in PI/RNase solution. Following incubation in the dark for 30 minutes at room temperature, flow cytometry was done to obtain the percentage of apoptotic cells (subdiploid cell population). Cell cycle profiles were analyzed with a program combined with the flow cytometer (Multicycle, Phoenix Flow System, San Diego, CA). DNA fragmentation assays were done to verify apoptotic cell death as described previously (13).

Animal Studies

BALB/c nu/nu mice ages 4 to 5 weeks were obtained from Taconic (Germantown, NY). All experimental studies were approved by The University of Texas M.D. Anderson Cancer Center Animal Care and Use Committee. Each animal was given a single s.c. injection of 5 × 10⁶ SKLMS-1 cells into the right flank. Once tumors reached 5 mm (single greatest dimension), serial intratumoral injections were done every other day for a total of 13 injections. Injections consisted of PBS (mock treatment; 6 mice), Ad5Luc vector control at 2 × 10¹⁰ viral particles (6 mice), or Ad5E2F at a dose of 2 × 10¹⁰ viral particles (7 mice). Tumor growth was measured in three dimensions every other day, and tumor volume was calculated. Mice were euthanized when tumors reached a predetermined size that was felt to interfere with quality of life (greatest diameter, >20 mm). At the time of euthanasia, tumors were excised and fixed in 10% formalin, embedded in paraffin, and then cut into 4-mm sections. Tumors were assessed for apoptosis using in situ terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assays. Immunohistochemical analysis was done on tissue sections with antibodies to E2F-1 and PKR and processed with avidin-biotin-peroxidase reagent Vectastain ABC kit according to the manufacturer’s recommendations (Vector Laboratories, Burlingame, CA).

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Results and Discussion
Ad5E2F Induces E2F-1 Overexpression and Apoptosis in Leiomyosarcoma Cells

Deregulation of the Rb-E2F-p14ARF-mdm2-p53 axis is a common feature of many tumor cells (reviewed in ref. 14). Several investigators have shown that E2F-1 overexpression can drive tumor cells into cell cycle progression and induce apoptosis (reviewed in ref. 1). Whereas the role of Rb and p53 mutations in tumor development are well established, it has only been reported recently that the functional silencing of p53 by up-regulation of mdm2 correlates with poor prognosis in soft tissue sarcomas (8, 15). Interestingly, mdm2 can directly inhibit the growth regulatory function of Rb in a p53-independent manner, and induction of apoptosis induced by deregulation of E2F-1 in mdm2-overexpressing bone tumor cells has been reported (16, 17). Because E2F-1 inactivates mdm2 through the transcriptional up-regulation of p14ARF, we were interested in determining whether overexpression of E2F-1 could induce apoptosis in leiomyosarcoma cells, which are often resistant to apoptosis by conventional chemotherapeutic agents (10). We used a recombinant adenovirus vector encoding the β-galactosidase reporter gene under the control of a cytomegalovirus promoter to determine the transfection efficiency of adenoviral vectors in SKLMS-1 cells. At a MOI ranging between 2,000 and 8,000 viral particles per cell, 60% to 95% of the sarcoma cells showed nuclear staining for β-galactosidase, indicative of transgene expression (data not shown). All of the subsequent experiments were done using adenovirus vectors at a MOI of 2,000 viral particles per cell. Western blot analysis of Ad5E2F-treated SKLMS-1 cells showed marked overexpression of E2F-1 and cleavage of poly(ADP-ribose) polymerase (PARP). Membranes were probed for β-actin to assess equal protein loading. Ad5E2F infection induced overexpression of E2F-1 and cleavage of poly(ADP-ribose) polymerase.

Figure 1. E2F-1 is overexpressed in leiomyosarcoma cells following treatment with Ad5E2F. A, FACS analysis of PI-stained cells revealed a 300% increase in apoptosis 48 h following Ad5E2F treatment compared with control treatment. B, Western blot analysis of whole-cell lysates from SKLMS-1 cells 48 h after treatment with either PBS, Ad5Luc, or Ad5E2F at a MOI of 2,000 viral particles per cell. Membranes were incubated with a monoclonal antibody against E2F-1 or poly(ADP-ribose) polymerase (PARP). Membranes were probed for β-actin to assess equal protein loading. Ad5E2F infection induced overexpression of E2F-1 and cleavage of poly(ADP-ribose) polymerase.

Figure 2. Induction of cell cycle progression by E2F-1. FACS analysis of PI-stained cells was done 48 h after treatment with PBS, Ad5Luc, or Ad5E2F. Ad5E2F-treated cells showed a reduction of the cell population in G1 phase and an accumulation of cells in the G2 phase compared with controls. The population of cells with a sub-G1 DNA content was significantly increased after Ad5E2F infection (white arrow).
cells. To confirm that these morphologic changes were due to apoptosis, we did FACS analysis and DNA fragmentation assays. The quantification of cells with a sub-diploid DNA content by FACS analysis after PI staining was done to assess the apoptotic cell population. There was a significant difference between Ad5E2F-treated and control-treated cells (PBS or Ad5Luc) within 48 hours (Fig. 1A). Treatment with Ad5E2F induced apoptosis in ~40% of the leiomyosarcoma cells compared with a 10% to 12% subdiploid cell population in control-treated cells. Analysis for poly(ADP-ribose) polymerase cleavage confirmed apoptosis in Ad5E2F-treated cells but not in control-treated cells (Fig. 1B).

**Cell Cycle Analysis and Expression of Cell Cycle–Related Proteins after Treatment with Ad5E2F**

E2F-1 plays a major role in cell cycle progression from G1 to S phase. Because resistance to conventional antitumor therapies is often associated with G1 arrest of tumor cells, we were interested to assess the potential of adenovirus-mediated E2F-1 overexpression to force leiomyosarcoma cells through cell cycle progression, potentially increasing their susceptibility to apoptotic cell death. As shown in Fig. 2, Ad5E2F treatment was associated with a decrease of the G1 population and a concomitant increase of the proportion of cells in G2-M phase within 48 hours of treatment (Fig. 2). As noted at 24 to 72 hours, the cell population with a sub-G1 DNA content increased rapidly in Ad5E2F-treated cells (white arrow), whereas PBS-treated or Ad5Luc-treated cells showed little change in cell cycle profiles over time. These data are consistent with previous findings in carcinoma cells, suggesting that E2F-1 overexpression can induce S-phase entry leading to a G2-M block ultimately resulting in apoptotic cell death.

Cell cycle progression is regulated by several proteins that interact with E2F-1, including pRb, p53, p21, and cyclin E. We therefore evaluated the expression of these proteins using Western blot analysis 48 hours following treatment with Ad5E2F or controls. No significant changes in expression levels of pRb, cyclin A, cyclin E, and p53 were observed. However, p21 was markedly up-regulated within 48 hours after E2F-1 transfection (Fig. 3A). Following DNA damage, p53 activates p21, suppressing Rb phosphorylation and consequently inhibits the activation of E2F-1 by enhancing E2F-Rb complex binding (10, 18–20). This leads to cell cycle arrest in G1, a mechanism that has been shown to protect tumor cells from apoptosis induced by chemotherapeutic agents (reviewed in ref. 21). Interestingly, despite up-regulation of p21, overexpression of...
E2F-1 led to apoptosis in sarcoma cells with mutated p53. Hence, increased p21 did not rescue E2F-1-overexpressing cells from apoptotic cell death.

**Study of Apoptosis-Related Factors Bcl-2 and Bax**

Previous studies on E2F-1-induced apoptosis reported that the activation of p53 was important in the induction of cell death. This p53-dependent pathway has been reported to depend on mitochondrial membrane destabilization, subsequent release of cytochrome c, and activation of caspase-9 and caspase-3 (22–25). We used Western blot analysis to evaluate potential apoptotic mediators in the mutant p53 SKLMS-1 cell line following treatment with Ad5E2F. The expression levels of two principal factors in the early activation of the mitochondrial caspase cascade, the antiapoptotic factor Bcl-2 and the proapoptotic factor Bax, were studied. Forty-eight hours following infection with Ad5E2F, Bcl-2 expression was increased compared with levels in the control-treated cells, whereas Bax levels remained unchanged following E2F-1 overexpression (Fig. 3B).

Paradoxically, overexpression of Bcl-2 is also known to block E2F-1-induced apoptosis or retard the entry of cells into S-phase (26, 27). Hence, our findings seem to contradict the relationship of E2F-1 and Bcl-2 described previously. However, recent reports have shown transcriptional up-regulation of Bcl-2 by E2F-1 in glioma, melanoma, and osteosarcoma cells (28, 29). We are currently investigating whether phosphorylated Bcl-2, which has the ability to inactivate unphosphorylated Bcl-2 on the mitochondria, is up-regulated following overexpression of E2F-1, which would provide an explanation for these paradoxical findings (30). On the other hand, up-regulation of Bcl-2 may indicate that the mechanism of E2F-1-induced apoptosis is partially independent of the classic mitochondrial pathway,

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**Figure 5.** **A,** Ad5E2F-1 inhibits tumor growth in vivo. Tumor growth of SKLMS-1 cells in the flanks of nude mice. Treatment consisted of 13 consecutive intratumoral injections with either PBS or 2 × 10^10 viral particles per injection of Ad5Luc or Ad5E2F. Treatment with either PBS, Ad5Luc, or Ad5E2F was initiated when the tumor diameter reached 5 mm. Significantly smaller tumor volumes were observed in animals treated with Ad5E2F compared with controls (P < 0.016, ANOVA). **B,** Ad5E2F-1 treatment induces apoptotic cell death in vivo. Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining (dark gray–stained nuclei) of paraffin-embedded slides showed significant amount of apoptotic cell death in the Ad5E2F-treated tumors but not in Ad5Luc-treated tumors.
which would corroborate our reports about the release of apoptosis-inducing factor on E2F-1 overexpression (12).

Ad5E2F Induces Up-Regulation of PKR
We reported recently that an important pathway of E2F-1-induced apoptosis is mediated through the activation of PKR (5). Because these findings were seen in carcinoma cell lines, we were interested to determine if PKR plays a role in E2F-1-induced apoptosis in sarcoma cells. SKLMS-1 cells were treated with increasing MOIs of the Ad5E2F vector. Apoptosis was quantified by assessing subdiploid cell populations with FACS analysis 48 hours after treatment. With increasing MOI, we noted increases in the amount of apoptosis measured (Fig. 4). Western blot analysis for PKR expression showed dose-dependent up-regulation of PKR 48 hours following E2F-1 gene transduction (Fig. 4). Hence, PKR expression and apoptotic response were strongly correlated, indicating that PKR is involved in the response to E2F-1 deregulation in sarcoma cells.

Antitumor Effect of Ad5E2F in Sarcoma In vivo
To determine if our in vitro findings could be confirmed in vivo, we used a sarcoma xenograft model in nude mice and treated established tumors with adenoviral vectors. SKLMS-1 cells (5 × 10⁶) were injected in the flank of immunodeficient BALB/c nu/nu mice, resulting in established tumors in >95% of the animals. Once the tumors reached 5 mm, tumors were treated with intratumoral injection of PBS (mock infection), Ad5Luc, or Ad5E2F. The viral vectors were delivered at a dose of 2 × 10¹⁰ viral particles per injection.

After 30 days, tumors in the control group reached 20 mm and the mice were sacrificed. Mean tumor volumes in the Ad5E2F-treated group were considerably lower than the mean tumor volumes recorded in the Ad5Luc-treated or PBS-treated animals (Fig. 5A). ANOVA showed a highly significant difference between the groups (P < 0.016). To confirm that growth inhibition of sarcoma xenografts was due to apoptosis, we did H&E staining and in situ terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling analysis. H&E staining revealed significant cell death in the tumors treated with Ad5E2F. We observed 20% to 30% terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling–positive (apoptotic) cells per high-power field examined in Ad5E2F-treated tumors but only 3% to 5% in Ad5Luc (and <3% in PBS-treated tumors; Fig. 5B). Two of seven tumors treated with Ad5E2F showed a complete regression of all visible tumor.

Figure 6. Immunohistochemistry analysis of SKLMS-1 tumors after direct intratumoral treatment with PBS (top), Ad5Luc (middle), or Ad5E2F (bottom). Paraffin-embedded tumors were sectioned and then stained red for E2F-1 expression (left) and dark red for PKR expression (right) and counterstained (blue). E2F-1 was significantly overexpressed in sarcoma cells near the needle tract. Although PKR is expressed at low levels in most SKLMS-1 cells, treatment with E2F-1 clearly up-regulated PKR protein levels.
E2F-1 and PKR Are Overexpressed in Ad5E2F-Treated Tumors

We used immunohistochemistry to verify overexpression of E2F-1 in Ad5E2F-treated tumors. Significant expression of E2F-1 was seen throughout the tumors injected with Ad5E2F but not in PBS- or Ad5Luc-treated sarcomas (Fig. 6). Staining for PKR expression showed increased PKR levels in all Ad5E2F-treated tumors. PKR activation plays a fundamental role in defense against viruses by limiting replication and pathogenesis of viruses. Therefore, one would expect to see increased expression of PKR following gene transfer treatments that depend on viral vectors. Accordingly, treatment of cell lines and tumors with adenoviral vectors carrying the luciferase reporter gene did result in a slight increase of PKR expression compared with levels in PBS-treated cells and xenografts. This slight increase in expression, however, was well below PKR levels seen following Ad5E2F therapy (Fig. 6).

This is the first time that PKR up-regulation after Ad5E2F treatment has been shown in vivo, adding to the evidence that PKR plays an important role in mediating the apoptotic response to E2F-1 gene therapy. Deregression of E2F-1 has the potential to not only drive cells into apoptosis but also, depending on their state of differentiation and development, induce cellular proliferation. Interestingly, there is evidence in the literature that the PKR status may be an essential regulator of tumorigenesis and that increased levels of PKR in lung and colon cancers are associated with improved survival (31).

Conclusion

Here, we report that adenovirus-mediated E2F-1 deregulation induces cell cycle progression and apoptosis in leiomyosarcomas in vitro and in vivo. Additionally, we showed the association of PKR with E2F-1 overexpression in vivo for the first time. Because forced cell cycle progression can overcome chemo-resistance and because PKR is a potential tumor suppressor, we advocate the further exploration of antitumor strategies aiming to induce deregulation of E2F-1.

References


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