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Ionizing Radiation Enhances Adenoviral Vector Expressing *mda-7/IL-24*-mediated Apoptosis in Human Ovarian Cancer

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Abstract

Ovarian cancer is the fifth most common cause of cancer-related death in women. Current interventional approaches, including debulking surgery, chemotherapy, and/or radiation have proven minimally effective in preventing the recurrence and/or mortality associated with this malignancy. Subtraction hybridization applied to terminally differentiating human melanoma cells identified melanoma differentiation associated gene-7/interleukin-24 (*mda-7/IL-24*), whose unique properties include the ability to selectively induce growth suppression, apoptosis, and radiosensitization in diverse cancer cells, without causing any harmful effects in normal cells. Previously, it has been shown that adenovirus-mediated *mda-7/IL-24* therapy (Ad.*mda-7*) induces apoptosis in ovarian cancer cells, however, the apoptosis induction was relatively low. We now document that apoptosis can be enhanced by treating ovarian cancer cells with ionizing radiation (IR) in combination with Ad.*mda-7*. Additionally, we demonstrate that *mda-7/IL-24* gene delivery, under the control of a minimal promoter region of progression elevated gene-3 (*PEG-3*), which functions selectively in diverse cancer cells with minimal activity in normal cells, displays a selective radiosensitization effect in ovarian cancer cells. The present studies support the use of IR in combination with *mda-7/IL-24* as a means of augmenting the therapeutic benefit of this gene in ovarian cancer, particularly in the context of tumors displaying resistance to radiation therapy.

Ovarian cancer is the fifth leading cause of cancer deaths among US women and has the highest mortality rate of all gynecologic cancers (Jemal et al., 2004). The majority of patients initially present with stage III or IV disease and display poor prognosis. Unfortunately, despite advances

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in initial debulking surgery and chemotherapy, many patients will have recurrence in the abdomen or pelvis that frequently is not responsive to further chemotherapy and carries a negative prognosis (Christian and Thomas, 2001). Therefore, treatments that improve initial disease control in the abdomen and pelvis have the potential to extend the progression-free interval and possibly also survival. Additionally, mortality from epithelial ovarian carcinoma has remained high for the past few decades. These facts underscore the need for development of new effective therapies.

Since human ovarian carcinoma is considered to be the result of acquired genetic alterations, gene therapy offers a novel approach for treating this neoplastic disease (Gomez-Navarro et al., 1998; Wolf and Jenkins, 2002). However, to realize the promise of gene therapy, identifying a gene therapeutic that does not harm normal cells and can kill only tumor cells would represent an ideal weapon to combat this prevalent female disease and cancer in general. A number of diverse gene therapy approaches for ovarian cancer have been endeavored using adenoviral vectors (Ad), in both animal models (Barnes et al., 1997; Robertson et al., 1998; Alvarez et al., 2000; Casado et al., 2001) and human clinical trials (Alvarez and Curiel, 1997; Collinet et al., 2000). Some of these approaches have included Ad-mediated delivery of *p53*, a tumor suppressor gene, or *Bax*, a pro-apoptotic gene, or toxin encoding genes, such as *HSV-TK* and *cytosine deaminase*. Although many therapeutic approaches have been developed, most of them are limited in their application due to their low therapeutic potential and high toxicity to normal tissues. Of note, a recent Phase II/III trial of *p53* gene therapy failed due to low therapeutic benefit (Zeimet and Marth, 2003). Therefore, identification and development of novel genes with high therapeutic potential and minimal toxicity to normal tissues (high therapeutic index) are warranted. In principle, such genes would provide significant benefit in treating this prevalent cancer.

Melanoma differentiation associated gene-7 (*mda-7*) is a secreted cytokine belonging to the interleukin (IL)-10 family that has been designated IL-24. Multiple independent studies have confirmed that delivery of *mda-7*/IL-24 by a replication-incompetent adenovirus, Ad.*mda-7*, or as a GST-MDA-7 fusion protein selectively kills diverse cancer cells, without harming normal cells, and radiosensitizes various cancer cells, including non-small cell lung carcinoma, renal carcinoma, and malignant glioma (Kawabe et al., 2002; Su et al., 2003, 2005a; Yacoub et al., 2003a,b,c; Sauane et al., 2004; Lebedeva et al., 2005b). In addition, *mda-7*/IL-24 possesses potent anti-angiogenic, immunostimulatory, and bystander activities (Fisher, 2005). The sum of these attributes makes *mda-7*/IL-24 a significant candidate for cancer gene therapy (Fisher, 2005). Indeed, Ad.*mda-7* has been successfully used for Phase I/II clinical trials for solid tumors and has shown promising results in tumor inhibition (Fisher et al., 2003; Cunningham et al., 2005; Fisher, 2005; Lebedeva et al., 2005a; Tong et al., 2005). Although the ultimate end-result of Ad.*mda-7* infection is induction of apoptosis, it employs different signal transduction pathways, such as activation of p38 MAPK, PKR, TRAIL, in different tumor cell types to achieve this objective (Pataer et al., 2002; Saeki et al., 2002; Sarkar et al., 2002).

The therapeutic potential of *mda-7*/IL-24 gene therapy in the context of epithelial ovarian carcinoma has not been investigated extensively. We have previously demonstrated that Ad.*mda-7* gene therapy induces apoptosis in ovarian cancer cells but not in normal mesothelial cells (Leath et al., 2004). However, the apoptosis induction efficiency was low. The purpose of the present study was: (1) to develop combinatorial approaches to improve apoptosis induction in ovarian carcinoma cells; and (2) to evaluate ovarian cancer cell targeted gene therapy by tumor-specific transgene expression using the novel promoter derived from progression elevated gene-3 (*PEG-3*) (Su et al., 1997, 1999). We now document that this resistance to Ad.*mda-7* is reversible in ovarian cancer cells by employing ionizing radiation (IR). Additionally, we demonstrate that, *mda-7*/IL-24 gene delivery under the control of a

minimal promoter region of *PEG-3* (Su et al., 1997, 1999, 2000, 2001b) which functions selectively in diverse cancer cells with minimal activity in normal cells (Su et al., 2000, 2001b, 2005b; Sarkar et al., 2005a,b), displays a selective radiosensitization effect in ovarian cancer cells. The present study confirms that the therapeutic potential of *mda-7/IL-24* can be augmented by IR with the added advantage of being able to sensitize both radiation- and *mda-7/IL-24*-resistant cells to programmed cell death.

MATERIALS AND METHODS

Cell lines, culture conditions, and radiation protocol

The SKOV3 (p53 null), human ovarian adenocarcinoma cells, and human mesothelial cells were purchased from the American Type Culture Collection (Manassas, VA). Human ovarian adenocarcinoma cell lines HEY (p53 wild-type), SKOV3.ip1 (p53 null), and OV-4 (p53 mutant) were kindly provided by Dr. Judy Wolf and Dr. Janet Price (M.D. Anderson Cancer Center, Houston, TX) and Dr. Timothy J. Eberlein (Harvard Medical School, Boston, MA), respectively. SKOV3, SKOV3.ip1, and OV-4 cells were cultured and maintained in complete medium composed of DMEM:F12 (Cellgro; Media-tech, Washington, DC) supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT), 5 mM glutamine (Cellgro; Mediatech), and 1% penicillin and streptomycin (Cellgro; Mediatech). HEY cells were cultured and maintained in complete medium composed of RPMI (Cellgro; Mediatech) supplemented with 10% FBS, 5 mM glutamine, and 1% penicillin and streptomycin. Human mesothelial cells were cultured and maintained in complete medium composed of a 1:1 mixture of Media 199 (Cellgro; Mediatech) and MCDB 105 Media (Sigma-Aldrich, St. Louis, MO) supplemented with 20% FBS, 5 mM glutamine, 1% penicillin and streptomycin, and 10 ng/ml human epidermal growth factor (Life Technologies, Grand Island, NY). All cells were grown in a humidified atmosphere of 5% CO₂ at 37°C. Infections of all cells utilized respective infection medium which contained 2% FBS. Cells were irradiated with 2 Gy of IR using a ¹³⁷Cs γ-irradiation source. SB203580 was purchased from Sigma Chemical Co.

Construction of adenovirus (Ad) and infection protocol

The recombinant replication-incompetent Ads, *Ad.CMV-GFP*, *Ad.PEG-GFP*, *Ad.CMV-Luc*, *Ad.PEG-Luc*, *Ad.CMV-mda-7* (CMV promoter driving *mda-7/IL-24* expression), and *Ad.PEG-mda-7* (PEG-Prom driving *mda-7/IL-24* expression) were created in two steps as described previously and plaque purified by standard procedures (Su et al., 2005a). As a control, a replication-incompetent *Ad.vec* without any transgene was used. Cells were infected with a multiplicity of infection (moi) of 100 plaque-forming units (pfu) per cell with different Ads.

Fluorescence microscopy for GFP evaluation

Cells (2 × 10⁵/well) were plated in triplicate in 6-well plates. The next day, cells were infected with *Ad.CMV-GFP* and *Ad.PEG-GFP* at a moi of 100 pfu/cell for 2 h in 2% infection medium. Subsequently, 2 ml of complete media was added and incubated at 37°C. After 48 h, the cells were analyzed for green fluorescent protein (GFP) expression by observing under a fluorescent microscope.

Luciferase assay

Cells were plated in 12-well plates in 2 ml of complete medium. The next day, supernatant was aspirated and the cells were infected with *Ad.CMV.Luc* and *Ad.PEG.Luc* at a moi of 100 pfu/cell in 200 µl of 2% medium. Following incubation for 2 h, complete medium was added. Luciferase assays were performed using commercial kits (Promega, Madison, WI) 48 h after infection. Protein concentration was determined using BCA Protein Assay Kit from Pierce Biotechnology (Rockford, IL) as per manufacturer's recommendations. The luciferase activity

was then determined by calculating relative luciferase activity (RLU)/mg of protein as previously described (Leath et al., 2004).

Cell viability assay

Cells were seeded in 96-well tissue culture plates (1.5×10^3 cells per well) and treated the next day as described in the figure legends. At the indicated time points, the medium was removed, and fresh medium containing 0.5 mg/ml MTT was added to each well. The cells were incubated at 37°C for 4 h and then an equal volume of solubilization solution (0.01 N HCl in 10% SDS) was added to each well and mixed thoroughly. The optical density from the plates was read on a Dynatech Laboratories MRX microplate reader at 540 nm.

Apoptosis and necrosis (Annexin-V binding assay)

Cells were trypsinized and washed with complete media and then twice with PBS. The aliquots of the cells (2×10^5) were resuspended in $1 \times$ binding buffer (0.5 ml) and stained with APC (allophycocyanin)-labeled Annexin-V (BD Biosciences, Palo Alto, CA) according to the manufacturer's instructions. DAPI was added to the samples after staining with Annexin-V to exclude late apoptotic and necrotic cells. The flow cytometry was performed immediately after staining.

Clonogenic survival assay

Cells were treated as described in the Figure legends. The following day, after trypsinization and counting, cells were replated in 60-mm dishes (500 cells per dish) in triplicates. After 2 or 3 weeks, cells were fixed with 4% formaldehyde and stained with 5% (w/w) solution of Giemsa stain in water. Colonies >50 cells were scored and counted.

Western blot assay

Cell lines were grown on 10-cm plates and protein extracts were prepared with RIPA buffer containing a cocktail of protease inhibitors. A measure of 50 μ g of protein was applied to 12% SDS-PAGE and transferred to nitrocellulose/PVDF membranes. The membranes were probed with polyclonal or monoclonal antibodies to *mda-7/IL-24*, anti-PEA-3, anti-c-JUN, phospho-P38 MAPK, total P38 MAPK, and EF-1 α .

RNA isolation and Northern blot analysis

Total RNA was extracted from cells using the Qiagen RNeasy mini kit according to the manufacturer's protocol and was used for Northern blotting as previously described (Su et al., 2001b). Briefly, 10 μ g of RNA for each sample was denatured, electrophoresed in 1.2% agarose gels with 3% formaldehyde, and transferred onto nylon membranes. The blots were probed with an α - 32 P[dCTP]-labeled, full-length human *mda-7/IL-24* cDNA probe. Following hybridization, the filters were washed and exposed for autoradiography.

Statistical analysis

All of the experiments were performed at least three times. Results are expressed as mean $\pm$ SE. Statistical comparisons were made using an unpaired two-tailed student's *t*-test. A *P* < 0.05 was considered significant.

RESULTS

Ovarian cancer cell lines exhibits low levels of adenoviral-mediated gene expression

Previous studies document that ovarian cancer cell lines have low coxsackie adenovirus receptor (CAR) expression (Leath et al., 2004), which is a primary receptor for adenovirus

infection. Two replication-incompetent adenoviruses expressing GFP, one under the control of the CMV promoter (Ad.CMV-GFP) and the other under the control of the PEG-Prom (Ad.PEG-GFP) were constructed (Su et al., 2005b). To determine the status of adenoviral infectivity we examined GFP expression by fluorescence microscopy following infection with Ad.CMV-GFP and Ad.PEG-GFP at a moi of 100 pfu/cell 2 days post-infection. Infection with Ad.CMV-GFP resulted in high GFP expression in normal human mesothelial cells and in SKOV3 cells and low level GFP expression in SKOV3.ip1, OV-4, and HEY cells. With Ad.PEG-GFP infection, none to barely detectable GFP could be observed in normal human mesothelial cells and in ovarian cancer cell lines the GFP expression level was comparable to that observed after Ad.CMV-GFP infection (Fig. 1A). These findings indicate that ovarian cancer cells have variable levels of CAR that preclude efficient adenoviral transduction and subsequent transgene expression.

PEG-Prom functions selectively in cancer cells

To quantify PEG-Prom activity in ovarian cancer and normal human mesothelial cells a luciferase-based reporter gene assay was employed. We constructed Ad.PEG-Luc in which the expression of the luciferase reporter gene was under the control of the PEG-Prom in a replication-incompetent adenoviral vector and infected normal mesothelial cells or the different ovarian cancer cell lines. Luciferase activity was measured 48 h after infection and expressed as RLU per mg protein. Normal human mesothelial cells demonstrated no activity supporting the limited GFP expression detected immunohistochemically following infection with Ad.PEG-GFP (Fig. 1B, upper part). When compared to normal human mesothelial cells, all of the ovarian cancer cells showed significantly increased PEG-Prom activity, although the activity in HEY cells was much lower than that in the other three ovarian cancer cell lines. The findings from GFP expression and luciferase reporter studies further confirm that the PEG-Prom functions selectively in cancer cells and is a useful tool to ensure cancer cell-targeted transgene expression.

PEA-3 and AP-1 controls PEG-Prom activity in ovarian cancer cells

PEA-3 and AP-1 are two transcription factors conferring cancer cell-selective function of the PEG-Prom in multiple cancer cell types, such as prostate, breast, central nervous system (malignant glioma), and pancreatic cancers (Sarkar et al., 2005a,b; Su et al., 2005b). To determine the involvement of these two factors in regulating PEG-Prom activity in ovarian cancer cells, the relative abundance of PEA-3 and Jun, a component of AP-1, proteins in ovarian cancer, and normal human mesothelial cells was analyzed by Western blot analysis (Fig. 1B, lower part). The expression of PEA-3 and Jun was significantly higher in all of the ovarian cancer cells when compared to that in normal human mesothelial cells, except for HEY cells, which showed comparable level of Jun expression to normal human mesothelial cells. These findings confirm that like other cell types previously examined, PEA-3 and AP-1 also control PEG-Prom activity in ovarian cancer cells.

Infection with Ad.CMV-*mda-7* and Ad.PEG-*mda-7* generates MDA-7/IL-24 protein

To extend our studies further we used two additional replication-incompetent adenoviruses expressing *mda-7*/IL-24, one under the control of CMV promoter (Ad.CMV-*mda-7*) and the other under the control of PEG-Prom (Ad.PEG-*mda-7*). The authenticity of these Ad were confirmed by Western blot analysis for MDA-7/IL-24 following infection of ovarian cancer cells and normal human mesothelial cells at a moi of 100 pfu/cell. Normal human mesothelial cells showed abundant MDA-7/IL-24 protein following Ad.CMV-*mda-7* infection, but no MDA-7/IL-24 protein after Ad.PEG-*mda-7* infection (Fig. 1C). In ovarian cancer cells, infection with Ad.CMV-*mda-7* resulted in more intracellular MDA-7/IL-24 protein than infection with Ad.PEG-*mda-7*. The relative levels of MDA-7/IL-24 and GFP proteins

correlated well in different ovarian cancer cell lines, with SKOV3 cells showing the highest level of trans-genes and HEY cells showing the lowest. These findings further confirm that although the PEG-Prom is functionally less active than CMV promoter, it ensures cancer cell-specific expression of MDA-7/IL-24.

Ad.CMV-*mda-7* and Ad.PEG-*mda-7* induce apoptosis in ovarian cancer cells

Experiments were next performed to determine whether infection with Ad.CMV-*mda-7* or Ad.PEG-*mda-7* could induce growth suppression and apoptosis in ovarian cancer cells. Cell viability was assessed by standard MTT assay 3 and 6 days after Ad infection. As expected neither Ad.CMV-*mda-7* nor Ad.PEG-*mda-7* affected the viability of normal human mesothelial cells (Fig. 2A). Although both Ad.CMV-*mda-7* and Ad.PEG-*mda-7* decreased cell viability in all the ovarian cancer cells, the effect, although significant, was marginal, except for SKOV3 cells, which displayed a more pronounced effect (Fig. 2A). Analysis of apoptosis by Annexin-V staining produced similar corroborating results as the MTT assays (Fig. 2B). No significant increase in apoptotic cells was observed in normal human mesothelial cells upon infection with Ad.CMV-*mda-7* or Ad.PEG-*mda-7*. Both of these Ads induced marginal but significant increases in apoptotic cells in the ovarian cancer cells.

Ad.CMV-*mda-7* and Ad.PEG-*mda-7* induce apoptosis by activating p38 MAPK

Experiments were performed to determine whether Ad.CMV-*mda-7* and Ad.PEG-*mda-7* infection in ovarian cancer cells activates the p38 MAPK pathway that has been shown to mediate apoptosis induction by Ad.*mda-7*. In normal human mesothelial cells, infection with Ad.CMV-*mda-7* or Ad.PEG-*mda-7* did not increase phospho-p38 MAPK levels 2 days post-infection (Fig. 3A). In SKOV3, SKOV3.ip1, and OV-4 cells, which showed significant susceptibility to growth inhibition, Ad.CMV-*mda-7* or Ad.PEG-*mda-7* infection resulted in a significant increase in phospho-p38 MAPK levels. However, in HEY cells, which are relatively resistant to growth inhibition, the increase in phospho-p38 MAPK was marginal. Treatment with the specific p38 MAPK inhibitor SB203580 protected the cells from growth inhibition following infection with Ad.CMV-*mda-7* or Ad.PEG-*mda-7* (Fig. 3B). These findings indicate that activation of p38 MAPK also plays an important role in mediating growth inhibition by *mda-7*/IL-24 in ovarian cancer cells.

Combination of γ -irradiation and Ad.CMV-*mda-7* or Ad.PEG-*mda-7* infection overcomes resistance of ovarian cancer cells to both ionizing radiation and *mda-7*/IL-24

Since Ad.CMV-*mda-7* or Ad.PEG-*mda-7* alone showed relatively low apoptosis induction in ovarian cancer cells, we hypothesized that a combinatorial approach with other anti-cancer strategies might be useful in augmenting growth suppression and apoptosis. Previous studies confirm that *mda-7*/IL-24 is a potent radiosensitizer in multiple cancer subtypes, including non-small cell lung carcinoma, breast carcinoma, prostate carcinoma, and glioblastoma multiforme (Kawabe et al., 2002; Su et al., 2003; Yacoub et al., 2003a,b,c). These studies prompted us to test the effect of γ -radiation (IR) in combination with Ad.CMV-*mda-7* or Ad.PEG-*mda-7* in the context of ovarian cancer. In all four ovarian cancer cell lines, Ad.CMV-*mda-7*, Ad.PEG-*mda-7*, or 2 Gy of IR alone produced a marginal enhancement in the percentage of Annexin-V positive cells. However, a combination of 2 Gy of IR with Ad.CMV-*mda-7* or Ad.PEG-*mda-7* significantly enhanced Annexin-V staining in all four ovarian cancer cell lines (Fig. 4A). Similarly, in colony formation assays, infection with Ad.CMV-*mda-7* or Ad.PEG-*mda-7* alone significantly reduced colony formation only in the SKOV3 cell line; however, a combination treatment with 2 Gy IR further reduced the number of colonies not only in SKOV3 cell but also in the other three ovarian cancer cell lines (Fig. 4B). No significant induction of apoptosis or reduction in colony formation was apparent with this combinatorial treatment approach in normal human mesothelial cells. These results suggest that the

combination of *mda-7*/IL-24 and IR might be a useful and efficacious approach for treating ovarian cancer patients.

MDA-7/IL-24 protein production in ovarian cancer cells directly correlates with a decrease in cell survival by combination treatment with Ad.CMV-*mda-7* or Ad.PEG-*mda-7* and IR

We next endeavored to define the possible mechanism of augmentation of *mda-7*/IL-24-induced cell killing by IR. Infection with Ad.CMV-*mda-7* generated high levels of *mda-7*/IL-24 mRNA as early as 1 day after infection in SKOV3 and OV-4 cells (Fig. 5A). Although in SKOV3.ip1 and HEY cells *mda-7*/IL-24 mRNA level was not high 1 day after infection, which can be explained by low infectivity, it accumulated gradually and by 3 days post-infection all four ovarian cancer cells had high and comparable levels of *mda-7*/IL-24 mRNA. This finding contrasts with the observation that in HEY cells very little MDA-7/IL-24 protein levels were observed even at 2 days post-infection (Fig. 1C and 5B). An inherent block in *mda-7*/IL-24 mRNA translation into protein has been observed in pancreatic cancer cells that can be overcome by multiple combinatorial approaches such as inhibition of K-*ras* or induction of reactive oxygen species (Su et al., 2001a; Lebedeva et al., 2005b, 2006). When ovarian cancer cells were treated with γ -radiation elevated levels of MDA-7/IL-24 protein were detected upon infection with both Ad.CMV-*mda-7* and Ad.PEG-*mda-7* in all four ovarian cancer cells. These findings indicate that γ -radiation treatment helps overcome the translational block delimiting MDA-7/IL-24 protein production, thus facilitating augmentation of apoptosis induction.

DISCUSSION

We presently demonstrate a potentially clinically relevant phenomenon that a combination of *mda-7*/IL-24 with IR promotes apoptosis in ovarian cancer cells that are initially resistant to either agent used alone. In ovarian cancer cells, IR (at 2 Gy) or *mda-7*/IL-24 (either as Ad.CMV-*mda-7* or as Ad.PEG-*mda-7*) alone did not significantly affect cell growth, except for SKOV3 cells, whereas a combination of both agents significantly reduced cell viability in all four ovarian cancer cell lines examined in the present study. Furthermore, we demonstrate that simultaneous treatment of IR plus *mda-7*/IL-24 could override the translational block of *mda-7*/IL-24 mRNA into protein in ovarian cancer cells, which could explain the enhanced efficacy of this combinatorial approach.

Several tumor-specific or ovarian cell carcinoma-specific promoters have been investigated in the preclinical settings. Robertson et al. (1998) found that a plasmid containing the HSV/TK gene, under the SLP1 (secretory leukoproteinase inhibitor gene, which is highly expressed in a variety of epithelial tumors, including ovarian cancer) promoter, could kill ovarian cancer cell. Another study (Tanyi et al., 2002) reported increased Luc production in several types of epithelial cancer cell lines, including ovarian cancer cell lines, with minimal activity in non-epithelial and immortalized normal cell lines when delivered under the control of SLP1 promoter. However, the same group found some non-specific Luc activity in many cell types, using OSP1, a retroviral promoter reported to be transcriptionally active only in rat ovaries. In our present study, besides the CMV promoter, we used the PEG-Prom to control *mda-7*/IL-24 gene expression (Su et al., 2000, 2001b, 2005b) selectively in cancer cells. We demonstrate that Ad.PEG-*mda-7* induced apoptosis selectively in cancer cells without inducing harmful effects to normal human mesothelial cells. The advantage of using the PEG-Prom is that its cancer cell specificity is attributed to two transcription factors, PEA-3 and AP-1, which are overexpressed in >90% of cancers. Additionally, the PEG-Prom is functionally active in all cancer cells irrespective of their p53, Rb, or other tumor suppressor gene status. Thus, employing the PEG-Prom to drive transgene expression only in cancer cells has selective advantages over other tissue- or cancer-specific promoters.

Consistent with a previous report (Leath et al., 2004), this study demonstrates that *mda-7/IL-24* induces a low and variable degree of apoptosis in ovarian cancer cells when delivered via a replication-incompetent adeno-virus under the control of either the CMV or PEG promoters. One of the reasons for this finding is low expression of MDA-7/IL-24 protein due to low Ad-mediated *mda-7/IL-24* gene delivery. However, defects (or blocks) in signaling mechanisms contributing to this low apoptosis induction require investigation. Currently, the signaling mechanisms involved in *mda-7/IL-24*-mediated apoptosis induction in specific ovarian cancer cells remain incompletely understood. Previous studies showed that activation of p38 MAPK plays an important role in mediating Ad.*mda-7*-induced apoptosis in melanoma, prostate carcinoma, and malignant glioma cells (Sarkar et al., 2002; Lebedeva et al., 2005a; Gupta et al., 2006). Using only one ovarian cancer cell line, a recent report showed that induction of the FAS-FASL pathway mediates Ad.*mda-7*-mediated cell killing (Gopalan et al., 2005). However, in this study we demonstrate that both Ad.CMV-*mda-7* and Ad.PEG-*mda-7* activated the p38 MAPK pathway in ovarian cancer cells and the level of phosphorylated p38 MAPK correlated with the killing effect of Ad.CMV-*mda-7* and Ad.PEG-*mda-7* in these cells. Additionally, SB203580, a selective p38 MAPK inhibitor significantly abolished the *mda-7/IL-24*-induced killing effect. Thus, activation of p38 MAPK might be a key element in *mda-7/IL-24*-induced apoptosis induction in multiple cell types, including ovarian carcinoma.

To circumvent the limitation of low transduction efficiency, we were interested in defining strategies that could be utilized to improve delivery of *mda-7/IL-24*, especially to those cells that were most resistant (SKOV3.ip1, HEY). Treatment options for ovarian cancer patients frequently involve debulking surgery combined with various therapeutic approaches, including radiation, chemotherapy, immunotherapy, and recently anti-angiogenic therapy (Wolf and Jenkins, 2002; Jemal et al., 2004). In this context, methods enhancing the effectiveness of anti-cancer agents, without promoting toxicity, would be of immense value and could provide a means of significantly improving clinical responses. Studies in NSCLC (Kawabe et al., 2002) and malignant gliomas (Su et al., 2003; Yacoub et al., 2003b,c) formally showed that radiation enhances tumor cell sensitivity to *mda-7/IL-24*. In two recent studies (Su et al., 2005a, 2006), we have uncovered an important property of *mda-7/IL-24*: an ability to induce a potent radiation enhancement “bystander anti-tumor effect” not only in cancer cells inherently sensitive to this cytokine but also in tumor cells overexpressing the anti-apoptotic proteins BCL-2 or BCL-x_L and exhibiting resistance to the cytotoxic effects of both radiation and MDA-7/IL-24. These findings offer promise for dramatically expanding the use of *mda-7/IL-24* for cancer therapy, especially in the context of radiation therapy. Additionally, recent reports indicate that Ad.*mda-7* lethality in NSCLC cells can also be augmented by combination treatment with the non-steroidal anti-inflammatory drug sulindac (Oida et al., 2005) and that Ad.*mda-7* amplifies the anti-tumor effects of Herceptin (trastuzumab), an anti-p185ErbB2 murine monoclonal antibody (mAb) that binds to the extracellular domain of ErbB2, in breast carcinoma cells overexpressing HER-2/neu (McKenzie et al., 2004). Overall, these studies employing radiation, a chemotherapeutic agent, and a mAb reinforce the possibility of augmenting the anti-cancer activity of *mda-7/IL-24* and increasing its therapeutic index as a gene therapy for diverse cancers by using combinatorial therapy approaches (Fisher, 2005; Gupta et al., 2006). Our present studies reveal that radiation might be an effective component of the combinatorial treatment for ovarian cancer cells along with Ad.CMV-*mda-7* or Ad.PEG-*mda-7*.

In summary, a combination of IR plus *mda-7/IL-24* (administered as Ad.CMV-*mda-7* or Ad.PEG-*mda-7*) enhances apoptosis induction in ovarian cancer cells, which initially demonstrated relative resistance to either of these agents alone. In contrast, similar experimental protocols applied to normal human mesothelial cells do not induce significant harmful effects. The unique synergy in ovarian cancer cells correlates with the ability of IR to relieve the translational block preventing conversion of *mda-7/IL-24* mRNA into protein.

These in vitro data suggest that combination treatment of Ad.*mda-7* and IR has potential to increase tumor response to radiotherapy and/or gene therapy and warrants further investigation using in vivo tumor models.

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Fig. 1. GFP, Luc, and MDA-7/IL-24 expression following infection of normal mesothelial and ovarian carcinoma cells with various adenoviruses and de novo levels of Jun, PEA-3, and EF-1 α protein in mesothelial and ovarian carcinoma cells. **A:** Variable infectivity of ovarian cancer cells by adenovirus as determined by GFP expression. The indicated cells were infected with either Ad.CMV-GFP or Ad.PEG-GFP at a moi of 100 pfu/cell, and GFP expression was analyzed by fluorescence microscopy at 2d post-infection. **B:** Upper part: PEG-promoter drives high luciferase expression in ovarian cancer cells, but not in normal human mesothelial cells. The indicated cells were infected with Ad.PEG-luc at a moi of 100 pfu/cell, and luciferase activity was measured at 48 h post-infection and expressed as RLU/mg protein. The data represent the

mean $\pm$ SD of three independent experiments, each performed in triplicate. Lower part: The expressions of JUN, PEA-3, and EF-1 α protein were analyzed by Western blot analysis in ovarian cancer cells and normal human mesothelial cells. **C:** Infection of ovarian cancer cells and normal human mesothelial cells with Ad.CMV-*mda-7* or Ad.PEG-*mda-7* results in variable amounts of intracellular MDA-7/IL-24 protein. Cells were infected with the indicated viruses at a moi of 100 pfu/cell and total cell lysates were prepared at d 1, d 2, and d 3 post-infection. The cell lysate used for Ad.*vec* was the lysate prepared at d 3 post-infection. The expression of MDA-7/IL-24 and EF-1 α proteins were evaluated by Western blot analysis.

Fig. 2.

Effect of infection of normal mesothelial and ovarian carcinoma cells with Ad.CMV-*mda-7* or Ad.PEG-*mda-7* on cell viability and apoptosis induction. **A:** Infection with Ad.CMV-*mda-7* or Ad.PEG-*mda-7* results in a decrease in cell viability in ovarian cancer cells. Cells were infected with the indicated viruses and cell viability was assessed by MTT assay at 3 d and 6 d post-infection. A significant loss in cell viability was evident in the SKOV3 and OV-4 cell lines. Results are the average from at least three experiments $\pm$ SD. **B:** Infection with Ad.CMV-*mda-7* or Ad.PEG-*mda-7* variably induces apoptosis in different ovarian cancer cells. The indicated cell type was infected with the designated viruses for 72 h, and Annexin-V binding assay was performed as described in Materials and Methods. Results are the average from at least three experiments $\pm$ SD.

Fig. 3.

Infection with Ad.CMV-*mda-7* or Ad.PEG-*mda-7* induces p38 MAPK phosphorylation and treatment with SB203580 protects ovarian cancer cells from *mda-7*-mediated apoptosis. **A**: Cells were infected with the indicated viruses as described in Figure 1C. Total cellular extracts were prepared at 48 h post-infection and the levels of phospho-p38 and total p38 MAPK were analyzed by Western blot analysis. **B**: Cells were infected with the indicated viruses and treated with 1 μ M SB203580. Cell viability was assessed by MTT assay at 4 d post-infection. Results are the average from at least three experiments $\pm$ SD.

Fig. 4.

Combination of Ad.CMV-*mda-7* or Ad.PEG-*mda-7* plus IR induces enhanced killing and apoptosis in ovarian cancer cells. **A:** Effect of single and combination treatment with Ad. CMV-*mda-7* or Ad.PEG-*mda-7* plus IR on apoptosis induction in ovarian cancer cells. The indicated cell type was either uninfected or infected with Ad.*vec*, Ad.CMV-*mda-7*, or Ad.PEG-*mda-7* and the next day was exposed to 2 Gy of IR. An Annexin-V binding assay was performed at 48 h post-irradiation as described in Materials and Methods. Results are the average from at least three experiments $\pm$ SD. **B:** Effect of combination treatment with Ad.CMV-*mda-7* or Ad.PEG-*mda-7* plus IR on growth of ovarian cancer cells. Cells were either uninfected or infected and untreated or treated with IR as described in Figure 4A and colony forming assays were performed as described in the Materials and Methods section. Data are presented as a percentage of colony formation to that of the uninfected and untreated group. Results shown are averages $\pm$ SD of triplicate samples.

Fig. 5.

Combination treatment with *mda-7*/IL-24 and IR reverses the translational block in ovarian cancer cells. **A:** Infection of Ad.CMV-*mda-7* induces an abundant amount of MDA-7/IL-24 RNA. Total RNA was isolated at d 1, d 2, and d 3 after infection as described in Materials and Methods, and analyzed by Northern blotting. The blots were probed with a α - ^{32}P [dCTP]-labeled, full-length human *mda-7*/IL-24 cDNA probe and exposed for autoradiography. **B:** Combination treatment of IR plus MDA-7/IL-24 enhances *mda-7*/IL-24 protein. Cells were infected with Ad.*vec*, Ad.CMV-*mda-7*, or Ad.PEG-*mda-7* and untreated or treated with IR the next day. Protein lysates were prepared 24 h after IR and Western blotting was performed as described in Materials and Methods to determine the levels of MDA-7/IL-24 protein. Equal protein loading was confirmed by Western blotting with an EF-1 α antibody.