Chapter 6

Infectivity enhanced adenoviral-mediated mda-7/IL-24 gene therapy for ovarian carcinoma.


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Abstract

Objective. Melanoma differentiation associated gene-7 [mda-7/Interleukin (IL)-24] has been identified as a novel anti-cancer agent, which specifically induces apoptosis in cancer cells but not in normal epithelial, endothelial and fibroblast cells. The objective of this study was to evaluate the anti-tumor effect of adenovirus-mediated mda-7/IL-24 (Ad.mda-7) gene therapy in ovarian carcinoma and further improve anti-tumor effect by enhancing infectivity of Ad.mda-7.

Methods. A panel of human ovarian carcinoma cells, OV-4, HEY, SKOV3, SKOV3.ip1 and control normal human mesothelial cells, were infected by a replication deficient recombinant adenovirus encoding mda-7/IL-24 and control virus Ad.CMV.Luc. After 72 h, apoptosis was evaluated by TUNEL and Hoechst staining and further quantified by fluorescent activated cell sorter (FACS) analysis. Infectivity of Ad.mda-7 was enhanced by retargeting it to CD40 or EGFR receptors overexpressed on ovarian cancer cells. Subsequently, enhancement in apoptosis of CD40- or epidermal growth factor receptor (EGFR)-retargeted Ad.mda-7 was evaluated.

Results. Adenoviral-mediated delivery of mda-7 induces apoptosis ranging from 10–23% in human ovarian cancer cells tested with the highest percentage of apoptosis noted in SKOV3 cells. Minimal apoptosis was noted in normal mesothelial cells. CD40- or EGFR-retargeted Ad.mda-7 increased apoptosis by 10–32% when compared to that achieved with untargeted Ad.mda-7.

Conclusion. Ad.mda-7 exhibits ovarian cancer-specific apoptosis, but does not affect normal human mesothelial cells. Infectivity enhanced CD40- and EGFR-retargeted Ad.mda-7 augments apoptosis induction, thus increasing the therapeutic index and translational potential of Ad.mda-7 gene therapy.

Keywords: mda-7/IL-24; Adenoviral vector; Retargeting; Ovarian carcinoma

Introduction

The American Cancer Society predicts that 25,400 of epithelial ovarian carcinoma cases will be diagnosed in 2003 with most patients having advanced tumor stage at the time of presentation. Although response to primary chemotherapeutic agents following initial debulking surgery is high, most patients will ultimately recur and will be treated with non-curative therapies. The mortality from
epithelial ovarian carcinoma has remained high for the past few decades. These facts underscore the need for development of new therapies.

Since human ovarian carcinoma is considered to be the result of acquired genetic alterations, gene therapy offers a novel approach for ovarian cancer therapy [1–3]. For effective gene therapy of cancer, most essential requirements are: (1) a therapeutic gene with high therapeutic potential and low or minimal toxicity to normal tissues and (2) a suitable vector to deliver genes with high in vivo gene delivery efficiency to tumors. In addition, features of the cancer should be ideal for application of gene therapy approaches. Specific ovarian cancer features such as confinement of ovarian cancer within the peritoneal cavity makes it a practical target for employment of gene therapy approaches [2,4–9]. A number of distinct gene therapy approaches for ovarian cancer have been endeavored using adenoviral vectors (Ad), in both animal models [2,4–9] and human clinical trials [10–12]. Some of these approaches have included adenovirus-mediated delivery of the p53, a tumor suppressor gene; or Bax, a pro-apoptotic gene; or toxin encoding genes such as HSV-TK and cytotoxic deaminase [10–13] and many other genes. Although many therapeutic approaches have been developed, most of them are limited in their application due to their low therapeutic potential [14] and high toxicity to normal tissues [8,15,16]. Of note, a recent phase II/III trial of p53 gene-therapy trial failed due to low therapeutic benefit [14]. Therefore, identification and development of novel genes with high therapeutic potential and minimal toxicity to normal tissues (high therapeutic index) are warranted. In this regard, we have identified a novel melanoma differentiation associated gene (mda-7/IL-24) by subtraction hybridization strategy, which specifically induces growth suppression and apoptosis in a wide range of cancer cells but does not harm normal cells such as normal melanocytes, endothelial cells, astrocytes, mammary and prostate epithelial cells, and skin fibroblasts [17]. Previous studies reported that mda-7/IL-24 induces apoptosis in cancer cells by acting at multiple levels and up-regulating p53, BAX (in a p53-independent manner), TRAIL, activating Caspas and GADD (growth arresting and DNA damaging) family genes [23,25,26]. In addition, mda-7/IL-24 was also shown to have potent antiangiogenic activity, which is essential for tumor growth inhibition [27]. Furthermore, recent studies reported that mda-7 is a secreted protein [20,22,24,26], exhibiting immunostimulatory activity, and is designated as IL-24 [25,26,28,29]. Thus, mda-7/IL-24 functions as a multi-modality anti-cancer agent, possessing pro-apoptotic, anti-angiogenic and immunostimulatory properties [30]. All these attributes make mda-7/IL-24 a most ideal candidate for cancer therapy [30]. In this regard it was shown that overexpression of mda-7/IL-24 via adeno viral vector (Ad.mda-7) infection of melanoma, breast carcinoma, colon carcinoma, prostate carcinoma, small cell lung carcinoma, malignant glioma and pancreatic carcinoma culminates in apoptosis [17–19,21,22,24,31]. However, normal melanocytes, endothelial cells, mammary and prostate epithelial cells, and skin fibroblasts are refractory to Ad.mda-7-induced killing [18,19,21,22,24]. Thus far, the therapeutic utility of mda-7/IL-24 gene therapy in the context of epithelial ovarian carcinoma has not been investigated. Studies evaluating mda-7/IL-24 in the context of ovarian carcinoma may prove to be very valuable for developing an effective gene therapy strategy for ovarian carcinoma.

For gene therapy of ovarian carcinoma, recombinant adenoviral vectors have been widely employed [2,3,5–9,32–39]. However, Ad-based human gene therapy clinical trials failed to show significant clinical responses due to low infectivity of tumor cells, which is attributed to deficiency of Coxsackie adenovirus receptor (CAR) [40–43]. We have previously shown that Ad transduction of ovarian tumor cells can be enhanced by epidermal growth factor receptor (EGFR) and CD40 retargeting strategies to achieve augmented gene transfer efficacy.

The purpose of this study is to evaluate the anti-tumor effects of Ad.mda-7 in the context of ovarian carcinoma and further evaluate the improvement of therapeutic potential of Ad.mda-7 gene therapy by employing CD40 and EGFR retargeting strategies.

Materials and methods

Cells and media

The 293 human kidney cells, SKOV3 (p53 null), human ovarian adenocarcinoma cells and human mesothelial cells were purchased from 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; Mediatech, Washington, D.C.) 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.

**Viruses**

Replication deficient adenovirus 5 encoding *mda-7/IL-24* (Ad.mda-7) was constructed previously [18]. Replication deficient, recombinant adenovirus 5 encoding enhanced green fluorescence protein, Ad.EGFP, was constructed as described previously [44]. Briefly, the fragment containing cDNA encoding the EGFP was obtained from pEGFP plasmid vector (Clontech, Palo Alto, CA) and was subcloned into pShuttle CMV followed by homologous recombination with viral vector DNA plasmid pAdEasy-1 (AdEasy system, Quantum Biotechnologies, Carlsbad, CA) and rescuing the virus [44]. Replication deficient adenovirus encoding luciferase reporter gene, Ad.CMV.Luc, was constructed by similar method. All adenoviruses were propagated in 293 cells and purified by ultra-centrifugation using a cesium chloride gradient as per standardized protocol [44]. Adenoviral viral particle number was determined spectrophotometrically as described previously by Maizel et al. [45] and viral titre (plaque forming units) was determined by the plaque assay method described previously [46].

**Determination of apoptosis by Hoechst staining**

SKOV3, HEY and OV-4 human ovarian cancer cells (2 × 10³) were plated in six-well plates in triplicate. Cells were infected next day with either Ad.CMV.Luc or Ad.mda-7 at an moi of 100 pfu/cell in 2% infection medium. Phosphate-buffered saline (PBS) was used in control wells. Complete medium was added after overnight infection. Seventy-two hours after infection, supernatant medium was removed and cells were washed with PBS. Cells were then trypsinized, recovered and (2 × 10⁴ cells) transferred to microscope slides by centrifugation at 750 g/ml (Sigma-Aldrich) was added for 5 min using a Shandon Cytospin 3 machine (Cheshire, England), and fixed with 10% buffered formalin for 10 min at room temperature (RT). After washing with PBS, 20 µl of Hoechst stain 33258 20 µg/ml (Sigma-Aldrich) was added and incubated at RT for 5 min. After washing with PBS, the coverslip was mounted using Gel Mount (Sigma, St. Louis, MO) aqueous mounting medium. Apoptosis induction was determined by visualization of condensed and fragmented nuclei of apoptotic cells under a fluorescent microscope.

**Determination of apoptosis by TUNEL staining**

Cells were treated with viruses as described above and after 72 h, 2 × 10⁴ cells were transferred to microscope slides as described above and fixed in 10% buffered formalin for 10 min at RT. After fixing the cells, apoptosis assay (TUNEL staining) was performed using Apop Tag kit reagents (Intergen, Purchase, NY) as per manufacturer’s recommendation. Cells were treated with proteinase K (20 µg/ml) for 15 min and washed with PBS. Subsequently, endogenous hydrogen peroxidase was quenched using 3.0% hydrogen peroxide for 5 min. Cells were then washed and incubated in a humidified chamber with TdT enzyme for 1 h at 37°C (for the negative control, water was used instead of TdT). After incubation, cells were soaked in a stop-wash buffer (Intergen) for 30 min, rinsed in PBS (three times) and finally incubated with antidigoxigenin antibody coupled to horseradish peroxidase at room temperature in a humidified chamber for 30 min. After washing again with PBS, the brown color development was achieved by incubating for 6 min at room temperature with a substrate solution containing 0.008% 3,3′-diaminobenzidine tetra hydrochloride and 0.02% hydrogen peroxide. The cells were counterstained by a weak hematoxylin stain for 30 s. After mounting the coverslips, slides were visualized under a light microscope to identify apoptotic cells by dark-brown color in the nuclei.

**Determination of adenovirus infectivity in ovarian cancer cells by luciferase assay**

The infectivity of adenovirus (Ad) in various human ovarian cancer cells was determined by luciferase assay. SKOV3, SKOV3.ip1, HEY and OV-4 (5 × 10⁴) human ovarian cancer cells and human mesothelial cells were plated in 24-well plates in 1 ml of complete medium. Next day, supernatant was aspirated and the cells were infected with Ad.CMV.Luc, at a multiplicity of infection (moi) of 100 pfu/cell in 200 µl of 2% medium. Following incubation for 2 h, 500 µl of complete medium was added. Forty-eight hours after infection, the medium was aspirated and cells were gently washed with PBS. Subsequently, 150 µl of 1× lysis buffer (Promega, Madison, WI) was added to completely cover the cells. The cells were then incubated at room temperature for 15 min and cell extracts were harvested from wells. The cell extracts obtained were transferred to 1.5 ml tubes and centrifuged down for 2 min at 14,000 rpm in a microcentrifuge at 4°C and the supernatant was obtained. Twenty microliters of this supernatant was then added to 100 µl of assay reagent buffer in luminometer tubes and vortexed. Relative light units (RLU) were determined using Berthold luminometer (Gaithersburg, MD) and 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 RLU/mg of protein.

**Determination of adenoviral infectivity by GFP evaluation**

Human ovarian cancer cells SKOV3, SKOV3.ip1, HEY and OV-4 (5 × 10⁴/well) were plated in triplicate in 24-well plates. Next day, cells were infected with Ad.EGFP at an moi of 100 pfu/cell for 2 h in 2% infection medium. Subsequently, 500 µl of complete media was added and
incubated. After 72 h, the cells were analyzed for the GFP expression by observing under a fluorescent microscope.

**Quantification of apoptosis by fluorescent activated cell sorter (FACS) analysis**

Human ovarian cancer cells SKOV3, SKOV3.ip1, HEY and OV-4 (2 × 10^5/well) were plated in six-well plates in triplicate for each experiment. Cells were infected the next day with either Ad.CMV.Luc or Ad.mda-7 at an moi of 100 pfu/cell in 2% infection medium. Following infection, plates were rocked at 15-min intervals for 1 h to ensure even infection. The wells were then washed with PBS and complete medium was added. Seventy-two hours after infection with Ad.mda-7 or Ad.CMV.Luc as described above, supernatant was removed and saved (to obtain apoptotic cells which were floating in supernatant), and adherent cells were washed with PBS and trypsinized. Following trypsinization, cells were resuspended in the supernatant obtained earlier. Cells were counted and divided into 5 × 10^5 aliquots and were then pelleted at 4°C in a tabletop centrifuge at 1000 × g for 5 min. Following centrifugation, cells were resuspended in 0.5 ml of complete medium, and stained with FITC-labeled Annexin-V (Oncogene Research Products Apoptosis Detection Kit, Boston, MA) according to the manufacturer’s recommended rapid protocol. After incubating the cell in the dark at RT for 20 min, propidium iodide (PI) was subsequently added and FACS analysis was performed immediately (FACScan, Becton Dickinson, San Jose, CA). The cells undergoing apoptosis (early apoptosis) and cells in late apoptotic stage were quantified based on their staining pattern. Cells stained positive for Annexin-V only were in early apoptotic stage, whereas cells stained positive for both Annexin-Vand PI were considered to be in late apoptotic stage.

**Determination of CD40 expression levels in SKOV3 and human mesothelial cells**

CD40 expression levels in SKOV3 and human mesothelial cells were determined by FACS analysis as described in our previous study [47]. Briefly, cells 2 × 10^5/well were plated in six-well plates in complete medium. Next day, after trypsinization, 2 × 10^5 cells were resuspended in fluorescence-activated cell sorting buffer (PBS containing 2% fetal bovine serum) and stained with either FITC-conjugated mouse anti-human CD40 monoclonal antibody or FITC-conjugated IgG1 isotype control antibody (BD Biosciences, San Diego, CA). Cells were then incubated at 4°C for 20 min and washed with fluorescence-activated cell sorting buffer and fixed with 1% paraformaldehyde/PBS solution before FACS analysis (FACScan; Becton Dickinson).

**CD40 targeting of Ad.mda-7**

For CD40 targeting of adenovirus, CAR/G28 fusion protein containing an anti-CD40 single chain Fv and the CAR ectodomain was made as described previously [48]. Briefly, anti-CD40 single chain Fv cDNA generated from the G28-5 hybridoma cell line was linked to cDNA of the CAR ectodomain, resulting in CAR/G28 fusion protein. The CAR/G28 was produced using recombinant baculoviruses, purified and characterized [48]. To enhance infectivity of Ad.mda-7, SKOV3, SKOV3.ip1, HEY and OV-4 (2 × 10^5) human ovarian cancer cells were plated in six-well plates in triplicate for each experiment. Cells were infected with either CD40-targeted or untargeted viruses next day utilizing 2% infection medium. To generate CD40-targeted Ad.mda-7 or Ad.CMV.Luc, 400 ng of CAR/G28 was added to 2 × 10^7 pfu/cell of Ad.mda-7 or Ad.CMV.Luc and incubated at RT for 1 h. Cells were infected with either CD40-targeted or untargeted Ad.mda-7 or Ad.CMV.Luc at an moi of 100 pfu/cell. Following infection, plates were rocked at 15-min intervals for 1 h to ensure even spreading of the medium containing the virus. The wells were then washed and complete medium was added. Seventy-two hours after infection, medium was removed, cells were trypsinized, counted and centrifuged to obtain cell pellet. The cell pellet was then resuspended in 0.5 ml of complete medium, and stained with FITC-labeled Annexin-V and PI and performed FACS analysis as described above.

**EGFR targeting of Ad.mda-7**

To enhance adenovirus infectivity by EGFR targeting, we used a bi-specific antibody Fab-425 that binds to the knob domain of adenovirus as well as EGFR. This antibody was generated as described in our previous study [49]. Human ovarian cancer cells, SKOV3.ip1 (2 × 10^5/well) cells, were plated in six-well plates in triplicate for each experiment. Either Ad.mda-7 or Ad.CMV.Luc (2 × 10^5 pfu) were incubated with either 100 ng of Fab-425 (in PBS) or PBS for 45 min at room temperature. The virus was then diluted with 2% infection medium, and the cells were infected at an moi of 100 pfu per cell for 1 h at 37°C. Cells were then washed with PBS, incubated in complete medium. After 72 h, cells were stained with Annexin-V and PI as described above and subjected to FACS analysis.

**Statistical analysis**

The percentage of apoptosis was quantified by FACS analysis. Luciferase activity was measured and expressed as RLU/mg protein. Descriptive statistics (mean and SD) were calculated from measurements taken from the experiments performed in triplicates. T tests were used to compare the apoptosis means of PBS-treated vs. Ad.CMV.-Luc-treated groups. The comparison of the means of Ad.mda-7-mediated apoptosis to PBS or Ad.CMV.Luc cells was performed using t tests, as well. Pairwise comparisons were done to test for a difference in apoptosis induction between cell lines SKOV3, HEY, SKOV3.ip1, OV-4 and
mesothelial cells. Correlations were calculated using Spearman Correlation. Comparisons of the mean percentage of apoptosis of Ad.mda7 vs. Ad.mda-7 + CD40 and Ad.Luc + CD40 vs. Ad.mda7 + CD40 were performed using \( t \) tests. In addition, the \( t \) test was used to determine if there was a significant difference between the total mean percentage of apoptosis utilizing EGFR-targeted Ad.mda-7 compared to untargeted Ad.mda-7. \( T \) tests were used to determine if there was a difference between Ad.mda-7-treated mesothelial cells and control virus-treated mesothelial cells and also CD40-targeted Ad.mda-7 and non-targeted Ad.mda-7-treated mesothelial cells. All analyses were done using SAS software (version 9.0; SAS Institute, Inc. Cary, NC). \( P < 0.05 \) was considered statistically significant in all of the analyses.

Results

Adenoviral-mediated mda-7 induces apoptosis in ovarian cancer cells

To determine the apoptosis-inducing effect of adenoviral-mediated \( mda-7/IL-24 \) gene therapy in ovarian cancer, a panel of human ovarian cancer cells, SKOV3, OV-4, HEY and SKOV3.ip1, was selected. In addition, normal human mesothelial cells were selected to determine the ovarian cancer specific apoptosis-inducing properties of Ad.mda-7. The results of Hoechst and TUNEL assays indicated that Ad.mda-7 but not Ad.CMV.Luc induced apoptosis in all of the ovarian cancer cells tested. Fig. 1 shows apoptosis induction in SKOV3 cells as demonstrated by Hoechst and TUNEL staining (other cell line data not demonstrated). FACS data analysis showed that apoptosis in control wells receiving PBS was 4.2% in HEY cells, 3.7% in SKOV3.ip1 cells, 5.6% in OV-4 cells and 7.8% in SKOV3 cells, suggesting background level apoptosis (Fig. 2A). Apoptosis induction in Ad.CMV.Luc control virus-treated cells was also background level, 6.7% in HEY cells, 2.8% in SKOV3.ip1 cells, 5.1% in OV-4 cells and 9.8% in SKOV3 cells. Comparison of apoptosis means of PBS-treated vs. Ad.CMV.Luc-treated groups indicated that there was no significant difference (\( P \) value of 0.377, 0.566, 0.16 and 0.19 for SKOV3, OV-4, HEY and SKOV3.ip1, respectively). In contrast, Ad.mda-7-mediated apoptosis was significantly higher compared to PBS-treated or Ad.CMV.Luc-treated cells: 20% in OV-4 cells (\( P = 0.0086, P = 0.0072 \) compared to PBS and Ad.CMV.Luc, respectively), highest 23% in SKOV3 cells (\( P = 0.0005, P = 0.0029 \)), 10% in HEY cells (\( P = 0.0055 \) and \( P = 0.163 \) compared to PBS and Ad.CMV.Luc, respectively) and 12% in SKOV3.ip1 cells (\( P = 0.0051, P = 0.0031 \)) (Fig. 2B). Although apoptosis was induced in all of the ovarian cancer cells tested, it was not to the same extent. Pairwise comparison of means of apoptosis % indicated that there was a significant difference in apoptosis induction between cancer cell lines [SKOV3 vs. HEY \( (P = 0.0004) \), SKOV3 vs. SKOV3.ip1 \( (P = 0.0049) \) and SKOV3 vs. mesothelial cells \( (P = 0.0004) \), except SKOV3 vs. OV-4 \( (P = 0.45) \)].

Fig. 1. Ad.mda-7 induces apoptosis in SKOV3 human ovarian cancer cells as demonstrated by Hoechst and TUNEL staining. (A) Apoptosis induction by Ad.mda-7 in human ovarian cancer cells (SKOV3 cells) as demonstrated by Hoechst staining. SKOV3 cells were infected by Ad.mda-7 (100 pfu/cell) and after 72 h, stained with Hoechst 33258. Apoptotic cells with fragmented nuclei are indicated by arrows. (1) Ad.mda-7-infected SKOV3 cells showing apoptosis. (2) SKOV3 cells treated with PBS did not show apoptosis. (3) SKOV3 cells infected with Ad.CMV.Luc did not exhibit apoptosis except for few cells, which undergo apoptosis normally. Apoptotic cells are indicated by arrows. (B) Apoptosis induction in human ovarian cancer cells (SKOV3 cells) as demonstrated by TUNEL staining. (1) TUNEL-stained SKOV3 cells infected by Ad.mda-7 (100 pfu/cell) showing apoptosis. Apoptotic cells are stained brown and are indicated by arrows. (2) SKOV3 cells treated with PBS did not show apoptosis. (3) SKOV3 cells infected with Ad.CMV.Luc also failed to show apoptosis.
These data suggest that Ad.mda-7 induces apoptosis in all ovarian carcinoma cells tested but to a varying degree.

**Apoptosis induction in ovarian cancer cells correlates with infectivity of Ad.mda-7**

Adenovirus infectivity assays showed that SKOV3 cells exhibited the highest infectivity as determined by luciferase expression $1.2 \times 10^8$ RLU/mg protein, followed by OV-4 $4.25 \times 10^8$ RLU/mg protein. HEY and SKOV3.ip1 expressed lower luciferase $2.9 \times 10^8$ RLU/mg protein and $1.43 \times 10^8$ RLU/mg protein, respectively (Fig. 3A). These
infectivity data correlated well with the degree of apoptosis induction in these cell lines (rho = 0.8). In contrast, although the infectivity was high in mesothelial cells, apoptosis induction was low, suggesting a no or negative correlation (rho = -1). We further validated the infectivity of these cell lines by infecting them with Ad.EGFP and determining the GFP expression by fluorescent microscopy. Once again, the data correlated with percentage apoptosis induction with SKOV3 exhibiting highest GFP expression followed by OV-4. HEY and SKOV3.ip1 expressed lower EGFP (Fig. 3B). These data suggest that the variation in apoptosis induction in ovarian cancer cell lines may be attributed to infectivity of Ad.mda-7.

**Infectivity enhancement of Ad.mda-7 by targeting to CD40 results in enhanced apoptosis**

Our initial results suggested that percentage of apoptosis induction correlated with level of Ad.mda-7 infectivity. On this basis, we decided to test whether increasing Ad.mda-7 infectivity would result in increased apoptosis induction. FACS analysis data showed that CD40 targeting of Ad.mda-7 resulted in an increase in apoptosis with significant increase in two cell lines, 12% in OV-4 ($P = 0.03$), 28% in HEY ($P = 0.015$), 10% in SKOV3 ($P = 0.24$), 1% in SKOV3.ip1 ($P = 0.83$) compared to untargeted Ad.mda-7 (Fig. 4). In CD40-targeted Ad.CMV.Luc (control virus) treated SKOV3 and HEY cells, there was no increase in apoptosis between targeted or untargeted Ad.CMV.Luc. However, in SKOV3.ip1, a minimal 3% increase and in OV-4, 10% increase was seen. Apoptosis induction by CD40-targeted Ad.mda-7 was significantly higher when compared to CD40-targeted Ad.CMV.Luc 18.8% higher apoptosis in OV-4 ($P = 0.0039$), 31.29% in HEY ($P = 0.007$), 24.17% in SKOV3 ($P = 0.04$) and 7.43% in SKOV3.ip1 ($P = 0.0047$) (Fig. 4). Overall, these results suggest that increased apoptosis induction by Ad.mda-7 can be achieved by increasing its infectivity via retargeting to CD40.

Although increase in apoptosis was observed in SKOV3, OV-4 and HEY, only 1% increase in apoptosis was observed in SKOV3.ip1 cells. This finding in SKOV3.ip1 cells may be attributed to low levels (13%) of CD40 expression in this cell line. These results suggest that increasing Ad.mda-7 infectivity by targeting to cellular receptors highly expressed in ovarian cancer cells increases apoptosis induction.

**EGFR-targeted Ad.mda-7 enhances apoptosis induction in SKOV3.ip1 cells**

Since epidermal growth factor receptor (EGFR) was shown to be highly expressed (higher than that of CAR or CD40) in SKOV3.ip1 cells, we tested whether augmenting...
infectivity of Ad.mda-7 by EGFR targeting will increase apoptosis of SKOV3.ip1 cell by using Fab-425 to retarget Ad.mda-7 to the SKOV3.ip1 cells. Apoptosis was readily apparent by 48 h utilizing this strategy. When FACS analysis was performed at 72 h, 13% of cells were in the early apoptotic stage (stained positive for Annexin-V only) and 32% of the cells were in late apoptosis stage (stained positive for both PI and Annexin-V) as compared to 7.47% in untargeted Ad.mda-7, thus resulting in a marked decrease in viable cells (Fig. 5). Total percentage (%) of apoptosis utilizing EGFR targeting was significantly greater than untargeted or CD40-targeted Ad.mda-7 (P = 0.0014). These results indicate that augmenting infectivity by retargeting Ad.mda-7 to EGFR receptors highly expressed in SKOV3.ip1 cells results in a significant increase in apoptosis.

Ad.mda-7 induces minimal apoptosis in normal human mesothelial cells

To determine if mda-7/IL-24 induces apoptosis in normal cells, human mesothelial cells (2 × 10⁵) were plated in six-well plates and infected with either Ad.mda-7 or Ad.CMV.Luc at an moi of 100 pfu/cell or with PBS. After 72 h, cells were subjected to FACS as described above. FACS data analysis revealed that apoptosis in control wells receiving PBS, Ad.CMV.Luc and Ad.mda-7 was 9.4%, 6.8% and 7.4%, respectively (Figs. 2 and 6), thus indicating no significant difference between Ad.mda-7-treated and control virus-treated cells (P = 0.5957). To determine if the low/background level apoptosis is not due to low infectivity, we determined Ad infectivity of mesothelial cells by luciferase and EGFP expression as described above. The luciferase expression was very high (8.96 × 10⁸ RLU/mg protein; Fig. 3A), almost comparable to SKOV3 cells, indicating that infectivity was very high. Furthermore, GFP expression was also high, similar to that seen in the SKOV3 ovarian cancer cell line (Fig. 3B), confirming the high infectivity. These results suggest that although infectivity is high in mesothelial cells, Ad.mda-7 induces very little apoptosis, suggesting its specificity to cancer cells. These results strengthen previously published results that mda-7/IL-24 does not affect normal endothelial, fibroblasts and other normal cells.

To determine if CD40 retargeting has any effect on apoptosis induction in mesothelial cells, we infected mesothelial cells with CD40-retargeted or non-targeted Ad.mda-7 or retargeted or non-targeted control virus and evaluated the apoptosis by FACS. There was no significant
difference in apoptosis between CD40-targeted or non-targeted Ad.mda-7 \((P = 0.2339)\), thus indicating that CD40-retargeted Ad.mda-7 does not cause any harmful effects in mesothelial cells. Of note, CD40 expression in mesothelial cell was only 3% (data not shown). This decreases the infectivity of CD40-retargeted Ad resulting in further decrease of vector-associated toxicity.

**Discussion**

Melanoma differentiation associated gene-7 (mda-7) is a novel anti-tumor agent because it selectively kills tumor cells without affecting normal cells. This property of mda-7 is very important to obtain high therapeutic index. It has been shown that mda-7 inhibits tumor growth by multiple mechanisms. mda-7 induces cell cycle arrest at G2/M phase, induces apoptosis in cancer cells, inhibits new blood vessel formation essential for tumor growth and stimulates the immune system. In addition, mda-7 is a secreted protein, which allows it to exhibit bystander effect resulting in amplified tumor cell killing. These multifunctional anti-cancer properties make it an ideal candidate for ovarian cancer gene therapy.

In this study, we have demonstrated that Ad.mda-7 gene therapy induces apoptosis in a panel of ovarian cancer cells but does not harm normal human mesothelial cells. We have also shown that apoptosis induction was variable in ovarian cancer cells and that it is directly related to infectivity of Ad.mda-7. We have further shown that infectivity of Ad.mda-7 can be enhanced by CD40 and EGFR retargeting strategies to achieve significant augmentation of apoptosis.

To determine the therapeutic utility of mda-7/IL-24 for ovarian carcinoma, we have performed in vitro apoptosis assays in a panel of ovarian cancer cells. In our initial evaluations, we noted that the amount of apoptosis in each ovarian cancer cell line was low and variable. The reason for these results was not clear. Previous studies have reported that the expression of CAR, the primary receptor for Ad transduction, was low in ovarian tumor cells and human primary tumor cells and that its expression varies between cell lines. In addition, it was also reported that transduction of Ad and its gene transfer efficiency directly correlates with expression of CAR. On this basis, we hypothesized that differential induction of apoptosis by Ad.mda-7 may be due to variable transduction efficiency of mda-7/IL-24 and disparate mda-7/IL-24 gene transfer in these cell lines. To test our hypothesis, we determined the transduction efficiency of Ad in each cell line by infecting ovarian cancer cell lines by Ad.CMV.Luc and Ad.EGFP which express marker genes, luciferase (Luc) and enhanced green fluorescent protein (EGFP), respectively, and subsequently evaluating the transfer of these genes to the cells by luciferase assays and fluorescent microscopy. Luciferase activity served as a quantitative index of Ad infectivity and EGFP expression serves as a visual index of Ad infectivity.

Luciferase activity was low in SKOV3.ip1 and HEY cells \(1.43 \times 10^5\) RLU/mg protein and \(2.15 \times 10^5\), respectively. In OV-4 cells, luciferase activity was higher \(4.25 \times 10^5\) RLU/mg protein and highest in SKOV3 cells \(1.2 \times 10^6\) RLU/mg protein. Although not quantitative, EGFP expression directly correlated with the results from Ad5.Luc assays. Both approaches were consistent in their findings and illustrated that SKOV3 cells were the most sensitive to infection, followed by OV-4, whereas HEY and SKOV3.ip1 were the most resistant. These results directly correlated with the apoptosis induction by Ad.mda-7 with highest being in SKOV3 followed by OV-4, HEY and SKOV3.ip1, suggesting that tumor cell transduction of Ad.mda-7 is the major limitation to achieve high anti-tumor effect in ovarian carcinoma cells.

To circumvent the limitation of low transduction efficiency, we were interested in defining strategies that could be utilized to improve infectivity of Ad.mda-7, specifically in those cells that were most resistant to infection. In this regard, we have shown that CD40, a type I transmembrane protein that belongs to the tumor necrosis factor receptor superfamily \([50]\), is highly expressed in ovarian carcinoma cell lines as well as in ovarian primary tumor cells \([47]\). We have previously shown the feasibility of exploiting this novel target for retargeting Ad to augment gene delivery \([47]\) using bispecific fusion protein CAR/G28. On this basis, we have employed CD40 retargeting strategy to augment Ad.mda-7 infectivity and gene transfer of mda-7/IL-24 to ovarian cancer cells. This retargeting strategy significantly increased the apoptosis induction compared to non-targeted Ad.mda-7, thus improving therapeutic potential. On this basis, employing the CD40 retargeting strategies for in vivo gene delivery may prove valuable to increase the clinical translatable potential of mda-7/IL-24 in ovarian cancer. The hallmark of cancer cells is that each cell line or primary tumor is highly variable in their expression of different surface receptors. Therefore, targeting a single receptor for Ad transduction may not achieve a significantly higher level of gene transfer. In this context, receptors that are known to be highly expressed in a particular cell line should be used for Ad retargeting. When we tested CD40 retargeting of Ad.mda-7, it significantly improved the apoptosis induction in all the cell lines tested except SKOV3.ip1. The reason being that CD40 expression was also very low only 13% compared to other cell lines (HEY, 64%; OV-4, 58%; SKOV3, 25%) \([47]\). Once again, the apoptosis induction directly correlated with the expression levels of CD40 with highest being in HEY and lowest in SKOV3.ip1. Therefore, utilizing an alternate receptor, which is highly expressed in SKOV3.ip1 for retargeting Ad.mda-7, may enhance apoptosis.

Previous research has shown that EGFR expression is markedly greater than that of CAR, which mediates Ad infectivity in SKOV3.ip cells \([51]\). On this basis, retargeting Ad.mda-7 to EGFR by means of a bispecific antibody conjugate Fab-425 produced significantly increased late
apoptosis to 32%, whereas in control virus group, it was only 6%. This result could be due to high mda-7/IL-24 gene transfer. Thus, EGFR targeting of Ad.mda-7 was effective for SKOV3.ip1. Evaluation of late apoptosis % via CD40-targeted Ad.mda-7 in HEY, OV-4, SKOV3 and SKOV3.ip1 ranged between 5% and 7%.

One of the essential requirements of a therapeutic gene to utilize in gene therapy is that its expression should not induce any deleterious effects in normal cells. In this regard, mda-7/IL-24 fits the requirements of a therapeutic gene. Previous research with mda-7/IL-24 has clearly illustrated the absence of deleterious effects on normal human cells such as fibroblasts, endothelial, melanocytes, astrocytes and epithelial cells [18,19,21,22,24]. Because ovarian carcinoma is an intraperitoneal disease, mesothelial cells were selected as our control cell line to evaluate possible deleterious effects of mda-7/IL-24. As predicted, FACS apoptosis assays on mesothelial cells infected with Ad.mda-7 failed to demonstrate any increase in apoptosis as compared to mock-infected cells or control vector-infected cells. This is of critical importance, because both Ad.CMV.Luc and Ad.EGFP infectivity assays demonstrated that mesothelial cells were extremely sensitive to Ad infection, trailing only SKOV3 cells in their ability to be infected. These results demonstrate that mda-7/IL-24 is an ideal candidate for ovarian cancer gene therapy and may prove to be a powerful therapeutic agent for the management of epithelial ovarian carcinoma. We are now analyzing multiple pathways that may be altered by mda-7/IL-24 to elicit apoptosis in ovarian cancer cells.

To determine the therapeutic utility of, in the clinical setting, evaluation of its anti-tumor effect in human ovarian cancer primary tumor material and in vivo tumor models is warranted. We are currently endeavoring the evaluation of apoptosis induction in primary human ovarian cancer cells, and in in vivo human ovarian cancer xenograft murine model systems.

In conclusion, we have shown that mda-7/IL-24 is an ideal gene for ovarian cancer gene therapy based on its cancer-specific apoptosis induction and minimal toxicity to normal mesothelial cells. In addition, employing CD40 and EGFR retargeting strategies can significantly increase therapeutic potential of Ad.mda-7. Retargeting Ad.mda-7 addresses limitations of tissue specificity and gene transfer efficiency that will be important in developing new vectors that are efficacious in the clinical setting. Development of new vectors with improved gene transfer efficiency in vivo may prove valuable in increasing clinical translational potential of Ad.mda-7 gene therapy.

References

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