Activation of the Fas-FasL Signaling Pathway by MDA-7/IL-24 Kills Human Ovarian Cancer Cells

Began Gopalan,† Anya Litvak,† Sikha Sharma,† Abner M. Mhashilkar,§ Sunil Chada,∥ and Rajagopal Ramesh†

Departments of Thoracic and Cardiovascular Surgery and Experimental Therapeutics, The University of Texas M.D. Anderson Cancer Center; and Introgen Therapeutics, Inc., Houston, Texas

Abstract

The tumor-suppressive activity of melanoma differentiation–associated gene-7 (mda-7), also known as interleukin 24 (IL-24), has been shown in a spectrum of human cancer cells in vitro and in vivo. However, mechanisms responsible for antitumor activity of mda-7 in human ovarian cancer cells have not been identified. We investigated the therapeutic activity and underlying mechanisms of adenovirus-mediated mda-7 gene (Ad-mda7) transfer in human ovarian cancer cells. Ad-mda7 treatment resulted in overexpression of MDA-7/IL-24 protein in both ovarian cancer and normal ovarian epithelial cells. However, Ad-mda7 significantly (P = 0.001) inhibited cell proliferation and induced apoptosis only in tumor cells and not in normal cells. Studies addressing the mechanism of action of Ad-mda7–induced tumor cell apoptosis revealed early activation of the transcription factors c-Jun and activating transcription factor 2, which in turn stimulated the transcription of an immediate down-stream target, the death-inducer Fas ligand (Fasl), and its cognate receptor Fas. Associated with the activation of Fas-Fasl was the activation of nuclear factor κB and induction of Fas-associated factor 1, Fas-associated death domain, and caspase-8. Promoter-based reporter gene analyses showed that Ad-mda7 specifically activated the Fas promoter. Inhibition of Fas using small interfering RNA resulted in a significant decrease in Ad-mda7–mediated tumor cell death. Additionally, blocking of Fas with NOK-1 antibody abrogated Ad-mda7–mediated apoptosis. Collectively, these results show that Ad-mda7–mediated killing of human ovarian cancer cells involves activation of the Fas-Fasl signaling pathway, a heretofore unrecognized mediator of MDA-7 apoptosis induction. (Cancer Res 2005; 65(8): 3017-24)

Introduction

Ovarian cancer is the second most common gynecologic malignancy in the United States and is the fifth leading cause of death among women (1). An estimated 25,000 women will be diagnosed with ovarian cancer and 50% of these patients will die of the disease. Despite advances made in the treatment of ovarian cancer, the overall long-term survival of patients has not significantly improved (1). Hence, developing and testing of new therapeutic agents and strategies are warranted.

The human melanoma differentiation–associated gene-7 (mda-7), also known as interleukin 24 (IL-24), encodes a protein of 206 amino acids with a predicted molecular mass of 23.8 kDa (2). Gene transfer studies have shown that mda-7 exerts its antitumor activity in a spectrum of cancer cells via multiple cell type–dependent signaling pathways resulting in apoptosis (reviewed in ref. 2, 3). Information related to the antitumor activity of mda-7 on human ovarian cancer cells is limited. In this study, we show the potent and selective antitumor effects of adenovirus-mediated mda-7 gene (Ad-mda7) in human ovarian cancer cells and show that activation of Fas-FasL is critical for Ad-mda7–mediated apoptosis.

Materials and Methods

Cell lines and cell culture. Human ovarian cancer cell line MDAH 2774 (p53 mutant) was grown as previously described (4). Human normal ovarian epithelial (NOE) cells were generously provided by Dr. Robert Bast.

Cell viability and cell cycle assay. Tumor cells and normal cells (10^5) treated with PBS, Ad-luc, or Ad-mda7 (3,000 viral particles [vp]/cell) were subjected to cell viability and cell cycle analysis as described previously (5–7).

Western blot analysis. Cells treated with PBS, Ad-mda7, or Ad-luc were harvested at 24 and 48 hours after treatment and subjected to Western blotting. The antibodies used are listed in Supplementary Methods. The blots were subjected to semiquantitative analysis using a densitometer with values for PBS-treated samples set to 1.

Detection of cytochrome c release from the mitochondria into the cytosol was determined as previously described (6).

Electrophoretic mobility shift assay. Cytoplasmic and nuclear extracts from Ad-luc or Ad-mda7–treated cells were prepared (9) and subjected to electrophoretic mobility shift assay (EMSA) for nuclear factor κB (NF-κB) and activator protein 1 (AP-1) according to the protocol of the manufacturer (Promega, Madison, WI). The radioactive bands were visualized by a PhosphoImager (Molecular Dynamics, Sunnyvale, CA) and quantitated using Image Quant software (Amersham-Pharmacia, Piscatway, NY).

RNase protection assay. Total RNA from PBS-, Ad-luc–, or Ad-mda7–treated tumor cells was isolated at 24 and 48 hours after treatment using Trizol reagent (Invitrogen, Carlsbad, CA) and RNase protection assay was done using RiboQuant Kit with hApo-3 Multi-Probe template set (BD PharMingen, San Diego, CA) according to the protocol of the manufacturer.

Fas expression analysis. For analysis of total and cell-surface Fas expression, tumor cells (1×10^5 to 1×10^6) treated with PBS, Ad-luc, or Ad-mda7 were analyzed by immunofluorescence using anti-Fas antibody (1 μg/mL; Santa Cruz Biotechnology, Santa Cruz, CA) and by flow cytometry using FITC-labeled anti-human Fas monoclonal antibody (1 μg: DX-2, DX-5; Biosource International, Camarillo, CA). Detailed protocol is available in Supplementary Methods.

Fas promoter analysis. MDAH 2774 cells (5×10^5) were transfected with FPR-Luc plasmid containing the luciferase gene under the control of the human wild-type Fas (CD95) promoter or with ΔΔ-Luc plasmid that contained a mutation in the Fas promoter. Construction and use of the
plasmids have previously been described (10). Cells were transfected with 1,2-dioleoyl-3-trimethylammonium-propanoic acid (DOPE) and 1,2-dioleoyl-3-trimethylammonium-propane-3-yl-parinarate (DOPE-Par) nanoparticle (11). Three hours after transfection, cells were treated with Ad-β-gal or Ad-md7. Cells were harvested at 48 hours after treatment and luciferase expression was determined (11).

Experiments using dominant negative IκB expression vector. Tumor cells were transfected with 2.5 μg of dominant negative IκB (dn-IκB) plasmid expression vector provided by Dr. Paul Chiao (12) that overexpresses IκB-β. Plasmid vector carrying neomycin (neo) gene was used as vector control. Twenty-four hours after transfection, cells were treated with Ad-md7 and analyzed for IκB expression by Western blot analysis and for Fas expression by flow cytometry.

Experiments using dominant negative Fas-associated death domain expression vector. MDAH 2774 cells were transfected with enhanced yellow fluorescent protein (EYFP)-dominant negative Fas-associated death domain (FADD) plasmid expression vector or with EYFP plasmid vector (12). The EYFP-dnFADD plasmid expresses the EYFP and dnFADD protein as a fusion protein, enabling both visualization and function of the FADD protein. Pilot studies showed more than 80% of the cells were transduced when transfected with 2.5 μg of plasmid encapsulated in nanoparticles as described above (data not shown). Based on these results, cells were transfected with 2.5 μg of plasmid DNA. At 24 hours after transfection, cells were either not treated or treated with Ad-md7. Untransfected cells treated only with Ad-md7 were included in these experiments. At 48 hours after Ad-md7 treatment, cells were harvested, washed, and analyzed for apoptotic cells by flow cytometry (5). The number of cells in the sub-G0 phase, an indicator of cells undergoing apoptosis, was determined and expressed as percent apoptotic cells. Expression of FADD, caspase-9, and caspase-8 was determined by Western blotting.

Small interfering RNA analysis. MDAH 2774 cells were transfected with 200 nmol/L of Fas-specific or control small interfering RNA (siRNA) using lipofectamine (Invitrogen, Carlsbad, CA). At 48 hours after transfection, cells were treated with Ad-md7. Cells were harvested 48 hours after Ad-md7 treatment, fixed, and analyzed for apoptotic cells by flow cytometry (5).

The sequences used to synthesize Fas and control siRNA are given in Supplementary Methods.

Experiments using anti-Fasl antibody. Tumor cells (5 × 10^5) were either not treated or pretreated with antihuman FasL blocking antibody (BD Pharmingen) for 2 hours. Cells were subsequently treated with Ad-md7. Cells were harvested at 48 hours after Ad-md7 treatment, washed with PBS twice, and analyzed for apoptotic cells by flow cytometry as described above and for caspase-8 activity by Western blotting.

Statistical analysis. All experiments were done twice and experimental results were analyzed for statistical significance using t test and ANOVA. The significance level was set at P < 0.05.

Results and Discussion

MDA-7 inhibits ovarian cancer cell proliferation by inducing cell cycle arrest and apoptosis. Before the start of the experiment, the transduction efficiencies of tumor cells and normal ovarian epithelial cells were determined. More than 80% of tumor and normal cells were transduced at 3,000 vp/cell (data not shown). Based on this result, we used Ad-luc or Ad-md7 at 3,000 vp/cell in all subsequent experiments. Exogenous MDA-7 protein expression that increased over time was observed in both tumor and normal cells treated with Ad-md7 but not in cells treated with PBS or Ad-luc (Fig. 1A). Significant (P = 0.001) inhibition of cell proliferation was observed in tumor cells but not in normal cells treated with Ad-md7 (Fig. 1B). No significant inhibitory effect on proliferation was observed in PBS- and Ad-luc–treated tumor and normal cells. These results show that Ad-md7 selectively inhibits ovarian tumor cell growth with minimal effects to normal cells. Our results concur with the findings of Leath et al. (13).

The mechanism by which Ad-md7 inhibited proliferation was next investigated. Ad-md7–treated tumor cells showed a more elevated G2-M cell cycle arrest (27%) than PBS-treated (8.3%) and Ad-luc–treated cells (18.9%; Fig. 1C). In normal cells, no significant difference in the G2-M phase was observed among Ad-md7 or control cells (Fig. 1C). Associated with the cell cycle arrest was the induction of apoptosis in Ad-md7–treated tumor cells, as evidenced by the activation of caspase-3 and caspase-9 and cleavage of poly(ADP-ribose) polymerase (PARP; Fig. 1D). Cleavage of caspases and PARP was not observed in normal cells treated with PBS, Ad-luc, or Ad-md7 (Fig. 1D). These results show that Ad-md7 effectively and selectively inhibits ovarian cancer cell proliferation by inducing G2-M cell cycle arrest and apoptosis, while sparing normal cells. The ability of Ad-md7 to induce G2-M cell cycle arrest and apoptosis is similar to that observed in other tumor types (2, 3, 14).

MDA-7 regulates various apoptosis-associated signaling proteins. A role for RNA-dependent protein kinase (PKR), phosphorylated c-Jun-NH2-kinase (pJNK), and p38 mitogen-activated protein kinase (p38MAPK) in Ad-md7–mediated apoptosis has previously been reported (2, 3). Reports also exist showing p38MAPK and pJNK convert signals of various extracellular stimuli for the expression of specific target genes through phosphorylation and activation of transcription factors such as c-Jun and activating transcription factor 2 (ATF2; refs. 15, 16). Based on these reports, we examined the expression of pPKR, pJNK, and p38MAPK and their downstream targets, c-Jun and ATF2, in ovarian cancer and normal cells after treatment. Increased expression of phosphorylated PKR (pPKR), eukaryotic initiation factor 2 (p-eIF2), p38MAPK (pp38MAPK), and JNK (pJNK) proteins was observed in Ad-md7–treated tumor cells after 48 hours but not at 24 hours (Fig. 2A). No significant change in expression of these proteins was observed in PBS- and Ad-luc–treated cells at both 24 and 48 hours. Only two proteins showed greatly increased expression at 24 hours in Ad-md7–treated tumor cells compared with control cells: phosphorylated c-Jun (pc-Jun) and ATF2 (pATF2). Increased pc-Jun and pATF2 expression in Ad-md7–treated cells compared with PBS- and Ad-luc–treated cells continued at 48 hours. In normal cells, no significant difference in the expression of these proteins was observed at 24 or 48 hours among Ad-md7–, Ad-luc–, and PBS-treated cells (Fig. 2A). These results show that activation of pc-Jun and pATF2 by Ad-md7 in ovarian cancer cells at 24 hours does not involve p38MAPK and pJNK. The possibility that pc-Jun and pATF2 are activated by extracellular signal-regulated kinase 1/2 was also examined and was ruled out (data not shown).

Because pc-Jun and pATF2 were activated in Ad-md7–treated cells, we speculated that MDA-7–mediated apoptotic signaling involves NFκB or AP-1. NFκB and AP-1 are major transcription factors that are activated through binding of the Jun family of transcriptional proteins (c-Jun, JunD, and JunB) in association with members of the Fos family or with other transcription factors such as ATF2, cAMP-responsive element binding protein, and nuclear factor of activated T cells (17). Analysis of nuclear lysates from Ad-md7–treated cells revealed higher NFκB binding activity than those from Ad-luc– and PBS-treated cells at both 24 and 48 hours after treatment (Fig. 2B). Maximal NFκB activation was observed at 24 hours. AP-1, unlike NFκB, was activated only at 48 hours and not at 24 hours after Ad-md7 treatment (Fig. 2B),
Figure 1. MDA-7/IL-24 inhibits ovarian cancer cell proliferation and induces G2 arrest and apoptosis. Ovarian tumor (MDAH 2774) and normal ovarian epithelial (NOE) cells were treated with PBS, Ad-luc, or Ad-mda7. Cells were harvested at various time points and analyzed for exogenous MDA-7 protein expression, cell proliferation, cell cycle arrest, and apoptosis. A, MDA-7 expression was observed in Ad-mda7–treated cells but not in PBS- or Ad-luc–treated cells. β–Actin was used as an internal loading control. B, proliferation of tumor cells, but not normal cells, treated with Ad-mda7 was significantly lower (P = 0.001) than in cells treated with PBS or Ad-luc. Bars, SE. C, cell cycle analysis showed induction of G2-M arrest was higher in Ad-mda7–treated tumor cells than in PBS- and Ad-luc–treated cells. G2-M arrest was not observed in Ad-mda7–treated normal cells. D, activation of caspase-9 and caspase-3 and cleavage of PARP were observed in Ad-mda7–treated tumor cells and not in normal cells. β–Actin was used as a loading control.
suggesting that NFκB, and not AP-1, is playing a role in initiating early molecular signaling events. AP-1 activation was also observed in Ad-luc–treated cells at 48 hours albeit less than that observed in Ad-mda7–treated cells. In normal cells, no increases in apoptosis-related proteins were observed in Ad-mda7–treated cells compared with PBS- or Ad-luc–treated cells. β-Actin was used as a loading control. B, NFκB and AP-1 activity in MDAH 2774 tumor cells showed increased activation of NFκB, but not AP-1, in Ad-mda7–treated cells at 24 hours compared with PBS- and Ad-luc–treated cells. AP-1 activity, however, was increased at 48 hours after Ad-mda7 treatment. Activity is expressed as percent increase over PBS.

**Figure 2.** MDA-7 regulates expression of apoptosis-related proteins. Tumor (MDAH 2774) and normal ovarian epithelial (NOE) cells were treated with PBS, Ad-luc, or Ad-mda7. At 24 and 48 hours after treatment, cells were harvested and analyzed for expression of apoptosis-related proteins by Western blot analysis, and for NFκB and AP-1 activation by EMSA, as described in Materials and Methods. A, increased expression of several apoptosis-related proteins was observed in Ad-mda7–treated tumor cells but not in PBS- or Ad-luc–treated cells. In normal cells, no increases in apoptosis-related proteins were observed in Ad-mda7–treated cells compared with PBS- or Ad-luc–treated cells. β-Actin was used as a loading control. B, NFκB and AP-1 analysis in MDAH 2774 tumor cells showed increased activation of NFκB, but not AP-1, in Ad-mda7–treated cells at 24 hours compared with PBS- and Ad-luc–treated cells. AP-1 activity, however, was increased at 48 hours after Ad-mda7 treatment. Activity is expressed as percent increase over PBS.

MDA-7 activates Fas and Fas-related proteins in ovarian cancer cells. The presence of NFκB and AP-1 binding sites in the promoter region of Fas and Fasl has been previously reported (18). Additionally, recent reports showed NFκB and AP-1 are involved in DNA damage–induced CD95L expression and apoptosis in T-cell
Figure 3. MDA-7–mediated activation of Fas and Fas-related proteins in ovarian cancer cells. Tumor (MDAH 2774) cells were treated with PBS, Ad-luc, or Ad-mda7, harvested, and analyzed for expression of Fas and Fas-related proteins, as described in Materials and Methods. A, mRNA analyses by RNase protection assay showed increased caspase-8, Fasl, Fas, FADD, FAP, and FAF1 in Ad-mda7–treated cells compared with PBS- and Ad-luc–treated cells at 24 hours. No change in TRAIL, TNF, TRADD, and RIP was observed between the treatment groups. L32 and GAPDH are shown as loading controls. B, Western blotting showed increased expression of Fas and Fas-related protein in Ad-mda7–treated cells compared with PBS- and Ad-luc–treated cells at 24 hours. β-Actin was used as a loading control. C, Western blotting showing activation of caspase-8 after Ad-mda7 treatment at 24 and 48 hours. D, inhibition of FLIP<sub>S/L</sub> in Ad-mda7–treated cells compared with that in PBS- and Ad-luc–treated cells at 24 hours. E, cleavage of BID in Ad-mda7–treated cells compared with that in PBS- and Ad-luc–treated cells at 24 hours. F, cytochrome c release from mitochondria into cytoplasm in Ad-mda7–treated cells occurs at both 24 and 48 hours after treatment. G, immunofluorescence analysis showed increased cell-surface expression of Fas in Ad-mda7–treated cells compared with PBS- and Ad-luc–treated cells. Magnification, ×100. H, flow cytometry showed Ad-mda7 increased cell-surface Fas expression compared with PBS- or Ad-luc–treated cells. Fas expression was determined by measuring the mean fluorescent intensity. I, inhibition of NFκB activation by overexpressing dnIFκB aborts Ad-mda7–induced cell-surface Fas expression.
and ovarian cancer cell lines (19). Because of these reports and our observation of NF-κB activation, we tested whether Ad-mda7 treatment results in the activation of the Fas-FasL family of proteins. As shown in Fig. 3A, mRNA analysis showed higher expression of caspase-8, Fasl, FasL, FADD, FAP, and Fas-associated factor 1 (FAF1) in Ad-mda7–treated cells than in PBS- and Ad-luc–treated cells at 24 hours. A moderate increase in caspase-8 and Fasl expression was also observed in Ad-luc–treated cells compared with PBS-treated cells. No difference in tumor necrosis factor (TNF) receptor 1–associated death domain protein (TRADD), TNF, and receptor-interacting protein (RIP) expression was observed among the various treatment groups, suggesting that activation of Fas-related, but not TNF-related, family members may be responsible for initiating the apoptotic cascade in Ad-mda7–treated cells.

Because changes in mRNA levels do not always correlate with protein expression, Western blot analysis was done. Elevated expression of Fas, Fasl, and FADD proteins was observed in Ad-mda7–treated cells compared with control cells at 24 hours (Fig. 3B). Both the magnitude and the kinetics of Fas, Fasl, and FADD protein expression were in agreement with our mRNA results. No change in TRADD expression was observed among the various treatment groups. Activation of caspase-8 was also observed after Ad-mda7 treatment compared with PBS or Ad-luc treatment (Fig. 3C).

Additional evidence for Ad-mda7–mediated regulation of Fas-Fasl signaling is provided by demonstrating the down-regulation of FLICE-like inhibitory protein (FLIP), cleavage of BH3 interacting domain (BID), and release of cytochrome c from the mitochondria into the cytoplasm, all of which are associated with the Fas-Fasl pathway (20). Treatment with Ad-mda7 resulted in the inhibition of short and long forms of FLIPS/L (Fig. 3D), cleavage of BID (Fig. 3E), and release of cytochrome c from the mitochondria into the cytoplasm (Fig. 3F). Regulation of these molecules was observed as early as 24 hours after Ad-mda7 treatment; however, cytochrome c release from the mitochondria was significantly higher at 48 hours than at 24 hours. Our results showing increased Fas, Fasl, FADD, and caspase-8 expression in Ad-mda7–treated cells suggest that MDA-7–induced apoptosis of ovarian cancer cells occurs via the Fas-Fasl pathway. The possibility that Ad-mda7 enhances the sensitivity of ovarian cancer cells to Fas-induced apoptosis, in part by inhibiting FLIP, exists. However, we have not further investigated the role of FLIP as it is beyond the scope of the present study.

To further determine whether the increased Fas protein expression resulted in increased cell-surface expression, tumor cells were stained with anti-Fas antibody and analyzed by immunohistochemistry and flow cytometry. Immunohistochemical analysis showed increased Fas expression in Ad-mda7–treated cells than in control cells at 24 hours after treatment (Fig. 3G). Flow cytometry analysis showed more than 90% of tumor cells exhibited cell-surface Fas positivity after staining with anti-Fas antibody. After Ad-mda7 treatment, however, there was an increase from 7.11 (PBS) and 7.17 (Ad-luc) to 9.74 (Ad-mda7) in mean fluorescent intensity of cell-surface Fas expression (Fig. 3H), indicating that Ad-mda7 caused a 73% increase over controls in cell-surface Fas expression.

To test whether the increased Fas expression in Ad-mda7–treated cells was mediated by NF-κB, cotransfection experiments were carried out using dnIκB or control plasmid vector. As shown in Fig. 3J, increased Fas expression was observed in Ad-mda7–treated cells transfected with a control plasmid vector. However, Ad-mda7–induced Fas expression was significantly abrogated in cells transfected with dnIκB plasmid. Flow cytometry results correlated with Western blotting data (Fig. 3I). These results show that NF-κB activates Fas expression in Ad-mda7–treated cells. Furthermore, our results show that activation and expression of Fas occurred in a p53-independent fashion because MDAH 2774 tumor cells are mutant for p53 (4). Fas-mediated apoptosis independent of p53 status has been reported previously (21).

**MDA-7 activates Fas promoter.** We next investigated the effect of Ad-mda7 treatment on Fas promoter activation. Tumor cells transfected with FPR-Luc plasmid were significantly activated following Ad-mda7 treatment compared with cells treated with Ad-βgal (P = 0.001; Fig. 4A). A slight increase in luc expression was also observed in Ad-βgal–treated cells, suggesting some nonspecific activation (P = 0.04). In contrast, no significant increase in luc expression was observed in Ad-βgal– or Ad-mda7–treated cells transfected with Δ6-Luc plasmid, indicating that Ad-mda7 treatment results in specific activation of wild-type Fas promoter. The increased Fas promoter activity observed in the present study is likely mediated by the binding of NF-κB induced by Ad-mda7.

**Inhibition of MDA-7–mediated Fas activation abrogates apoptosis.** Studies have shown that the adapter protein FADD and caspase-8 are part of the death-inducing signaling complex, which is formed following FAS-induced signaling and is essential for Fas-mediated apoptosis (22). Therefore, we examined the effect of overexpression of dnFADD on Ad-mda7–mediated apoptosis. Cells were transfected with EYFP or EYFP-dnFADD and treated with PBS or Ad-mda7. Cells were analyzed for apoptosis using flow cytometry and for caspase-8 and caspase-9 using Western blot analysis. A significantly higher number of cells treated with Ad-mda7 alone were apoptotic (22.7%; P = 0.001) compared with cells treated with EYFP plasmid (3.8%) alone or with EYFP-dnFADD plasmid (4.2%; Fig. 4B). However, Ad-mda7–mediated apoptosis was significantly inhibited in EYFP-dnFADD–transfected cells overexpressing dnFADD (8.1%; Fig. 4B). In contrast, Ad-mda7 treatment in EYFP-transfected cells showed increased apoptosis (33.2%). Furthermore, Ad-mda7 treatment of parental cells or cells transfected with EYFP plasmid resulted in the activation of caspase-8 and caspase-9 (Fig. 4C). No caspase activation was observed in cells treated with EYFP or EYFP-dnFADD. These results show that Ad-mda7 activates the Fas-Fasl pathway, resulting in the recruitment of death-inducing signaling complex, which leads to the activation of caspase-8 and caspase-9.

To further test whether Fas plays a role in Ad-mda7–mediated apoptosis, siRNA experiments were conducted. Significantly fewer MDA-7–induced apoptotic cells were observed in Fas siRNA-transfected cells (9.2%; P = 0.001) than in cells transfected with control siRNA (18.8%; Fig. 4D). Thus, inhibition of Fas expression abrogated Ad-mda7–mediated apoptosis.

**Additional evidence for Fas-Fasl.** In Ad-mda7–mediated apoptosis is provided using Fasl blocking antibody (NOK-1). Previous studies show NOK-1 can effectively block FasL-induced apoptosis (23, 24). Treatment of cells with Ad-mda7 resulted in activation of caspase-8 and increased apoptosis (22.3%, P < 0.05; Fig. 4E) compared with PBS- or NOK-1–treated cells. However, Ad-mda7–mediated caspase-8 activation and apoptosis were abrogated in the presence of NOK-1 blocking antibody (8.8%). These results show the involvement of Fas-Fasl in Ad-mda7–mediated apoptosis.

The inability to completely abrogate Ad-mda7–mediated apoptosis using dnFADD, siRNA, or NOK-1 antibody remains unclear. One possibility could be the experimental conditions employed.
Figure 4. MDA-7 activates Fas promoter and inhibition of MDA-7–mediated Fas activation abrogates apoptosis. A, MDAH 2774 cells seeded in six-well plates were transfected with FPR-Luc plasmid or Δ6-Luc plasmid. Cells were subsequently treated with Ad-Δgal or Ad-mda7 and analyzed for luciferase expression as described in Materials and Methods. Ad-mda7 significantly (P = 0.001) increased luciferase expression in FPR-Luc–transfected cells compared with Ad-Δgal–treated cells. No change in the luciferase expression was observed in Δ6-Luc–transfected cells treated with Ad-mda7 or Ad-Δgal. B, MDAH 2774 cells transfected with EYFP or EYFP-dnFADD plasmid were either not treated or treated with Ad-mda7 and analyzed for apoptosis by fluorescence-activated cell sorting. An increased number of apoptotic cells were observed in Ad-mda7–treated cells compared with untreated cells. However, Ad-mda7–mediated apoptosis was significantly (P = 0.001) abrogated in cells overexpressing EYFP-dnFADD. No inhibition of Ad-mda7–mediated apoptosis was observed in cells overexpressing only EYFP. Bars, SE. C, Western blotting analysis showed activation of caspase-8 and caspase-9 in Ad-mda7–treated cells compared with untreated cells. However, Ad-mda7–mediated activation of caspase-8 and caspase-9 was inhibited in cells overexpressing EYFP-dnFADD. No inhibition of caspase-8 and caspase-9 by Ad-mda7 was observed in cells overexpressing EYFP. β-Actin was used as a loading control. D, cells transfected with siRNA targeted to Fas or with scrambled siRNA were treated with Ad-mda7 and analyzed for Fas expression by Western blotting (top) and for apoptosis by fluorescence-activated cell sorting analysis (bottom). Ad-mda7 induced apoptosis in cells transfected with scrambled siRNA targeted to Fas. Bars, SE. E, cells were pretreated with PBS or with FasL blocking antibody (NOK-1) for 2 hours. Subsequently, cells were either not treated or treated with Ad-mda7 and analyzed for caspase-8 expression by Western blotting (top) and for apoptosis by fluorescence-activated cell sorting analysis (bottom). Ad-mda7 induced apoptosis in PBS-treated cells that correlated with activation of caspase-8. However, Ad-mda7–mediated apoptosis was significantly (P < 0.05) abrogated in cells pretreated with NOK-1 antibody. Activation of caspase-8 or marked induction of apoptosis was not observed in NOK-1–treated cells compared with PBS-treated cells. Bars, SE.
Another possibility is the involvement of additional molecular mechanisms that play a role in apoptosis. Whatever these possibilities may be, it is clear that in ovarian cancer cells activation of the Fas-FasL signaling pathway at early time points is important in Ad-mda7–mediated apoptosis. In conclusion, we have shown that Ad-mda7–mediated apoptosis in ovarian cancer cells occurs via the Fas-FasL pathway.

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5. Ramesh R, Mhashilkar A, Chada S, et al. Ad-mda7–mediated apoptosis in ovarian cancer cells occurs via the Fas-FasL pathway. In conclusion, we have shown that Ad-mda7–mediated apoptosis in ovarian cancer cells activation of the Fas-FasL signaling pathway at early time points is important in Ad-mda7–mediated apoptosis. In conclusion, we have shown that Ad-mda7–mediated apoptosis in ovarian cancer cells occurs via the Fas-FasL pathway.
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