Adenoviral endoplasmic reticulum–targeted mda-7/interleukin-24 vector enhances human cancer cell killing

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Abstract
We developed several adenoviral vectors designed to target MDA-7 expression to different subcellular compartments (endoplasmic reticulum (ER), mitochondria, nucleus, and cytosol) and evaluated their ability to enhance apoptosis. Adenoviral ER-targeted mda-7/interleukin-24 vector (Ad-ER-mda7) selectively and effectively inhibited the growth and proliferation of lung (A549 and H1299) and esophageal (Seg1 and Bic1) cancer cells by enhancing cell killing. Both Ad-mda7 and Ad-ER-mda7 activated a novel pathway of ER stress-induced apoptosis characterized by unregulated expression of phosphorylated JNK, phosphorylated c-Jun, and phosphorylated RNA-dependent protein kinase. Caspase-4 activation mediated Ad-mda7- and Ad-ER-mda7-induced cell death. In addition, Ad-mda7- and Ad-ER-mda7-mediated growth inhibition correlated with activation of ER molecular markers RNA-dependent protein kinase and JNK both in vitro (in Ad-mda7- or Ad-ER-mda7-treated lung cancer cells) and in vivo. These findings suggest that vectors targeting the ER (Ad-ER-mda7) may be more effective in cancer gene therapy possibly through more effective induction or ER stress pathways. [Mol Cancer Ther 2008;7(8):2528–35]

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Introduction
The melanoma differentiation-associated gene-7 (mda-7) is a tumor suppressor gene that induces apoptosis in a wide range of cancer cells, both in vivo and in vitro, when overexpressed through a replication-incompetent adenoviral vector (1–4). The cytotoxic activity of the mda-7 gene product is specific to tumor cells and independent of the status of other tumor suppressor gene products (e.g., p53, Rb, ras, or p16INK4; ref. 5). The cDNA of mda-7 encodes an evolutionarily conserved protein (MDA-7) that, despite having only 19% identity with the homodimeric cytokine interleukin (IL)-10, has been assigned to the IL-10 family and renamed IL-24 (6, 7). This assignment was based on the amino acid identity of MDA-7 with IL-10 and incorporation of an IL-10 family motif, its chromosomal localization within an IL-10 family cluster of genes, its translational regulation, and its predicted structural features (e.g., a four-helix bundle structure characteristic of the IL-10 family; refs. 6, 7). Ectopic expression of mda-7 in cancer cells by Ad-mda7 or plasmid DNA vectors results in the overproduction of intracellular MDA-7 protein and its secreted form, IL-24 (1, 5).

In laboratory studies using lung cancer cell lines, we have shown that MDA-7 overexpression leads to the up-regulated expression and phosphorylation of the RNA-dependent protein kinase (PKR) necessary for Ad-mda7-induced apoptosis (8, 9). In addition, we and others have shown that MDA-7/IL-24 intracellular-mediated apoptosis may involve the endoplasmic reticulum (ER) signaling pathway. For example, we have shown the consistent overexpression of several ER stress proteins (GRP78/BiP, GADD34, and P2PA) in Ad-mda7-treated lung cancer cells (10). Meanwhile, Fisher’s group has shown that the ER chaperone protein GRP78/BiP, by serving as an intracellular target of MDA-7/IL-24 and thereby mediating MDA-7/IL-24’s activation of its downstream targets p38 mitogen-activated protein kinase and GADD, selectively mediates apoptosis of prostate and breast cancer cells (11, 12). Molecular chaperones, such as GRP78/BiP and HSP70, play important roles in the unfolded protein response pathway by preventing the aggregation of misfolded proteins and shuttling them to the 20S proteasome for degradation (13). Because ER is a principal site for MDA-7/IL-24 protein synthesis and folding (10, 13), the ER stress-mediated cell death pathway can be triggered by disparate perturbations in normal ER function, such as the accumulation of unfolded, misfolded, or excessive MDA-7/IL-24 protein.

Assuming that Ad-mda7 treatment induces the accumulation of MDA-7 proteins and consequently ER and/or cytoplasmic stress, we hypothesized that the subcellular
Materials and Methods

Cell Lines and Reagents

Human lung (A549 and H1299) and esophageal (Seg1 and Bcl) cancer cell lines were obtained from the American Type Culture Collection. PKR+/+ and PKR−/− mouse embryo fibroblasts (MEF) were obtained from Dr. Glen Barber (University of Miami School of Medicine; ref. 14). MEF cells were maintained in DMEM containing 10% fetal bovine serum, 10 mmol/L glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (Life Technologies) in a 5% CO2 atmosphere at 37°C. Caspase-4 and JNK inhibitors were obtained from Calbiochem. Final working solutions were diluted in medium to contain <0.01% DMSO. All experiments using this compound were done under subdued lighting conditions.

Adenoviral Vector Construction

Constructions of the Ad-mda7, Ad-LacZ, and Ad-luc vectors have been reported previously (8). Constructions of plasmids targeted to the ER (pCMV/myc/ER/mda-7), mitochondria (pCMV/myc/Mito/mda-7), nucleus (pCMV/myc/Nuc/mda-7), and cytosol (pCMV/myc/Cyto/mda-7) have been reported previously (10). The ER-mda-7 fragment from pCMV/myc/ER/mda-7 was obtained by digestion at the BglII and BclI sites. The Mito-mda-7 fragment from pCMV/myc/Mito/mda-7 was obtained by digestion at the PmlI and BclI sites. The Nuc-mda-7 fragment from pCMV/myc/Nuc/mda-7 was obtained by digestion at the HincII and BclI sites. The Cyto-mda-7 fragment from pCMV/myc/Cyto/mda-7 was obtained by digestion at the HincII and BclI sites.

Individual shuttle vectors were obtained as follows. The PLJ37/pAD-RAP/Shuttle vector was subjected to EcoRV and BclI digestion and ligated with ER-mdma7, Mito-mdma7, Nuc-mdma7, or Cyto-mdma7 fragments to obtain their respective shuttle vectors (PLJ37/pAD-RAP/ER-mdma7/Shuttle vector, PLJ37/pAD-RAP/Mito-mdma7/Shuttle vector, PLJ37/pAD-RAP/Nuc-mdma7/Shuttle vector, or PLJ37/pAD-RAP/Cyto-mdma7/Shuttle vector). The resulting shuttle vectors were then subjected to further digestion with BstBI and ClaI to obtain large fragments. Each of the resulting large fragments was then inserted into a plJ34 vector between the BstBI and ClaI sites to create adenoviral vectors specifically targeted to the ER, mitochondria, nucleus, and cytosol, respectively (Ad-ER-mdma7, Ad-Mito-mdma7, Ad-Nuc-mdma7, and Ad-Cyto-mdma7). Adenoviral transduction efficiency in cancer cell lines was determined by infecting cells with Ad-LacZ vectors and quantifying the titers needed to transduce the LacZ gene into at least 70% of the cells.

Immunofluorescent Cellular Localization Studies

A549 lung cancer cells (5 × 10⁶ per well) were grown on chamber slides to 70% confluence and then transected with Ad-luc, Ad-mdma7, Ad-ER-mdma7, Ad-Mito-mdma7, Ad-Nuc-mdma7, or Ad-Cyto-mdma7 or treated with PBS as a negative control. Seventy-two hours later, cells were washed with PBS and fixed with 4% paraformaldehyde/PBS for confocal microscopic analysis described as previously (9). In brief, cells were blocked with 1% normal goat serum for 1 h and then incubated overnight at a dilution of 1:100 with the primary mouse monoclonal anti-MDA-7 antibody. Next, the slides were washed to remove primary antibody, rinsed with PBS, and placed in a prewarmed staining solution containing ER-Tracker red dyes or MitoTracker Deep Red 633 (M-22426; Molecular Probes) for ~15 to 20 min at 37°C. Then, the slides were washed and incubated with a FITC- or rhodamine-conjugated secondary antibody (Invitrogen) for 1 h. Next, the slides were mounted with ProLong Gold antifade reagent containing 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) and analyzed under an Olympus Fluoview FV500 laser confocal microscope (Olympus America) after adjustment for background staining.

Flow Cytometric Analysis

Apoptosis was assessed by propidium iodide staining and fluorescence-activated cell sorting analysis of cells. In brief, cells were harvested, pelleted by centrifugation, resuspended in PBS containing 50 μg/mL propidium iodide, 0.1% Triton X-100, and 0.1% sodium citrate, vortexed, and then subjected to fluorescence-activated cell sorting analysis (Becton Dickinson FACScan; FL-3 channel).

Immunoblot Analyses

Seventy-two hours after adenoviral transfection, A549 or H1299 cell extracts were prepared and immunoblot assays were done as described previously (8, 9). Antibodies to JNK, phosphorylated JNK (p-JNK), c-Jun, phosphorylated c-Jun, PKR (K-17), eIF-2α, and β-actin (control) were obtained from Santa Cruz Biotechnology. The caspase-4 was obtained from StressGen. Antibodies to phosphorylated PKR (pT451) and phosphorylated eIF-2α (Ser51) were obtained from BioSource International. Polyclonal and monoclonal antibodies to MDA-7 were obtained from Introgen Therapeutics.

Analysis of In vivo Tumor Growth after Ad-mda7 or Ad-ER-mdma7 Treatment

A549 cells (5 × 10⁶ per 0.2 mL) were injected s.c. into the flanks of female athymic nu/nu mice 4 to 6 weeks old. Once a tumor grew to ~5 × 5 mm, PBS, Ad-luc, Ad-mdma7, or Ad-ER-mdma7 was injected directly into it via a single pass of a 25-gauge hypodermic needle at dose of 3 × 10¹² viral particles (vp). A second injection was given 3 days later and a third injection 3 days after that. Thus, each mouse received a total of three injections (total 9 × 10¹² vp) over...
6 days. After the third injection, the maximal and minimal diameters of each tumor were measured by slide calipers placed on the skin, every 2 days for 30 days. Tumor volume was calculated by assuming a spherical shape and using the following formula: volume = \( \frac{a \times b^2}{2} \), where \( a \) and \( b \) are the maximal and minimal diameters, respectively. Results for each treatment group (6-9 mice per group) were averaged and expressed as the mean (SD).

Apoptosis was measured by histologic analysis. In brief, tumors were excised 24 h after the third injection in vivo, fixed in 10% formalin, embedded in paraffin blocks, and processed for histologic analysis and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assays. For terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay, sections were dehydrated in a graded series of ethanol and redistilled water. Tissue sections were then incubated with proteinase K, permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate, and labeled with the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling reaction mixture.

**Figure 1.** Immunofluorescent confocal microscopic analysis of intracellularly targeted mda-7 adenoviral vectors. A, analysis of antibody against MDA-7 stained green, ER stained red with ER-Tracker dyes, and nuclei stained blue with DAPI showed colocalization of both MDA-7 protein and the ER marker in Ad-ER-mda7-treated A549 cancer cells 72 h after transfection. Analysis of antibody against MDA-7 stained green, mitochondria (Mitochondria) stained red with MitoTracker Deep Red 633 (M-22426), and nuclei stained blue with DAPI showed colocalization of both MDA-7 protein and the Mitochondria marker in Ad-Mito-mda7-treated A549 cancer cells 72 h after transfection. Analysis of antibodies against MDA-7 stained green and nuclei stained blue or red with DAPI showed nuclear localization of MDA-7 protein in Ad-Nuc-mda7-treated A549 cancer cells 72 h after transfection. Analysis of antibodies against MDA-7 stained green and nuclei stained blue with DAPI showed cytosolic localization of MDA-7 protein in Ad-Cyto-mda7-treated A549 cancer cells 72 h after transfection. B, Western blot analysis of MDA-7 expression in A549 cell lysates and supernatant 72 h after treatment with PBS, Ad-Luc (2,500 vp or 24.5 moi), Ad-mda7 (2,500 vp or 24.5 moi), Ad-ER-mda7 (2,500 vp or 27.4 moi), Ad-Mito-mda7 (2,500 vp or 22.1 moi), Ad-Nuc-mda7 (2,500 vp or 26.59 moi), or Ad-Cyto-mda7 (2,500 vp or 24 moi). \( \beta \)-Actin expression was analyzed as a control.

**Statistical Analysis**

ANOVA and a two-tailed Student’s \( t \) test were used for statistical analysis when appropriate. Significance was set at \( P < 0.05 \).

**Results**

**Mda-7 Adenoviral Vectors Induce Targeted Intracellular Expression of MDA-7 Protein**

As confirmed by immunofluorescent confocal microscopic analysis of A549 lung cancer cells 72 h after transfection, the adenovirus-mediated intracellular targeting vectors Ad-ER-mda7, Ad-Mito-mda7, Ad-Nuc-mda7, and Ad-Cyto-mda7 successfully targeted MDA-7 protein expression to specific subcellular regions of the transduced cancer cells (Fig. 1A). The precise subcellular localization of the targeted proteins was confirmed by comparison of their expression patterns with those of molecular markers known to reside in these compartments. For example, nuclear targeted MDA-7 colocalized with DAPI-stained nuclei, and ER-targeted MDA-7 colocalized with ER-Tracker red dye. To determine whether these intracellularly
targeted mda-7 vectors caused MDA-7 secretion, post-transfection MDA-7 expression in lung cancer A549 cell lysates and supernatants was assessed by Western blot analysis. These analyses confirmed that each intracellularly targeted adenoviral vector successfully promoted the expression of correctly sized MDA-7 protein (Fig. 1B; ref. 10), thereby showing that Ad-mda7 transduction did cause the secretion of MDA-7 protein. Unexpectedly, however, the ER-targeted MDA-7 protein was shown to be secreted although it contained an ER retention signal.

Figure 2. Ability of adenoviral ER targeting mda-7 vector (Ad-ER-mda7) to induce signaling along the ER stress-mediated cell death pathway. A, flow cytometric analysis of apoptosis in A549, H1299, Seg1, Bic1, NHBE, and WI-38 cells 72 h after treatment with PBS, Ad-Luc (2,500 vp or 24.5 moi), Ad-mda7 (2,500 vp or 25.5 moi), Ad-ER-mda7 (2,500 vp or 24.27 moi), Ad-Mito-mda7 (2,500 vp or 22.1 moi), Ad-Nuc-mda7 (2,500 vp or 26.59 moi), or Ad-Cyto-mda7 (2,500 vp or 24 moi). Experiments involving each cell line were done in triplicate. B, Western blot analysis of p-JNK, JNK, phosphorylated c-Jun, c-Jun, caspase-4, phosphorylated PKR, and PKR protein expression in A549 and H1299 cell lysates 72 h after treatment with Ad-Luc (2,500 vp or 24.5 moi), Ad-mda7 (2,500 vp or 25.5 moi), Ad-ER-mda7 (2,500 vp or 24.27 moi), Ad-Mito-mda7 (2,500 vp or 22.1 moi), Ad-Nuc-mda7 (2,500 vp or 26.59 moi), or Ad-Cyto-mda7 (2,500 vp or 24 moi). β-Actin expression was analyzed as a control.
Adenoviral ER-Targeted Vector Induces Activation of the ER Stress-Mediated Cell Death Pathway

As shown by flow cytometric analysis of human lung (A549 and H1299) and esophageal (Seg1 and Bic-1) cancer cells after transfection, all of the intracellularly targeted mda-7 adenoviral vectors, except Ad-Nuc-mda7 vector, induced cell death within 72 h of transfection (Fig. 2A). Of those vectors that did induce apoptosis, the ER-targeted MDA-7 adenoviral vector (Ad-ER-mda7) was much more lethal than either mitochondria-targeted (Ad-Mito-mda7) or cytosol-targeted mda-7 (Ad-Cyto-mda7) vectors and also more lethal than the untargeted mda-7 vector (Ad-mda7). Moreover, as shown by flow cytometric analysis of NHBE normal human bronchial epithelial cells and WI-38 normal human fibroblasts 72 h after their adenoviral exposure, only one of the intracellularly targeted adenoviral mda7 vectors (Ad-Mito-mda7) was toxic to normal cells (Fig. 2A).

We hypothesize that the ER stress (accumulation of MDA-7 proteins in ER) is essential to Ad-ER-mda7-mediated cell death activity in cancer cells. To test our hypothesis, we determined the most important ER stress markers (JNK, p-JNK, c-Jun, and phosphorylated c-Jun) in A549 and H1299 lung cancer cells following PBS, Ad-mda7, Ad-ER-mda7, Ad-Mito-mda7, Ad-Nuc-mda7, and Ad-Cyto-mda7 treatment for 72 h by immunoblot analysis (15). As shown in Fig. 2B, expression of p-JNK and phosphorylated c-Jun was regulated after Ad-mda7 and Ad-ER-mda7 transduction but not after Ad-Mito-mda7, Ad-Nuc-mda7, or Ad-Cyto-mda7 transduction (Fig. 2B), suggesting that activation of JNK or c-Jun was the mechanism by which Ad-mda7- and Ad-ER-mda7-mediated cell killing. Caspase-4 is an ER-resident caspase that is processed in human cells in response to ER stress and is required for ER stress-induced cell death (15). We next investigated whether caspase-4 was involved in Ad-Mda7- and Ad-ER-mda7-induced cell death (15). Caspase-4 was cleaved to its active form in A549 and H1299 human lung cancer cells treated with Ad-mda7 or Ad-ER-mda7 but not in those treated with Ad-Mito-mda7, Ad-Nuc-mda7, or Ad-Cyto-mda7 (Fig. 2B). To more directly determine the importance of JNK and caspase-4 activation to Ad-ER-mda7 and Ad-ER-mda7-mediated cell death, A549 cells were exposed to Ad-mda7 and Ad-ER-mda7 in the absence or presence of a chemical JNK inhibitor (SP600125, 10 μmol/L) or a peptidic inhibitor of caspase-4 (Ac-LEVDC-CHO, 10 μmol/L) and subjected to fluorescence-activated cell sorting analysis to determine the extent of the resulting cell death. In both cases, inhibitory treatment reduced the amount of cell death induced by both Ad-mda7 (from 22.3% to 17% after JNK inhibition and from 22.3% to 13% after caspase-4 inhibition) and Ad-ER-mda7 (from 52% to 41% after JNK inhibition and from 52% to 27% after caspase-4 inhibition; Supplementary Data S1). Conversely, neither inhibitor reduced the amount of cell death induced by Ad-Mito-mda7 or Ad-Cyto-mda7 (Supplementary Data S1). Together, this suggested that both Ad-mda7- and Ad-ER-mda7-mediated cell killing involved the activation of JNK and caspase-4.

We have shown that MDA-7 overexpression in human lung cancer cells leads to the up-regulation and phosphorylation of PKR necessary for Ad-mda7-induced apoptosis (8, 9). We next determined if PKR activation might also play a role in Ad-ER-mda7-infected cancer cells. Human lung cancer cells revealed that both Ad-mda7 and Ad-ER-mda7 up-regulated and promoted the phosphorylation of PKR (Fig. 2B). In contrast, Ad-Mito-mda7, Ad-Nuc-mda7, and Ad-Cyto-mda7 did not. This contribution of PKR activation to the apoptotic activity of Ad-ER-mda7 was confirmed in experiments using MEF from PKR knockout mice. That Ad-mda7-induced cell killing was dependent on PKR was confirmed by the fact that only MEF from PKR+/+ (wild-type) mice underwent apoptosis induction after Ad-mda7 treatment (Supplementary Data S2; ref. 8). Ad-ER-mda7-mediated cell killing was also dependent on PKR with background level of apoptosis occurring in PKR-null and 20% apoptosis occurring in wild-type MEF (Supplementary Data S2). Conversely, neither Ad-Mito-mda7- nor Ad-Cyto-mda7-induced apoptosis appeared to be dependent on PKR genomic status, apoptosis occurring at similar rates in both PKR−/− and PKR+/− MEF (Supplementary Data S2).

In vivo Regression of Tumors Follows Intratumoral Injection of Ad-mda7 or Ad-ER-mda7 in nu/nu Mice

As shown by in vivo experiments in a s.c. nu/nu tumor mouse model and in confirmation of previous studies showing that intratumoral administration of Ad-mda7 inhibits the growth of lung tumor xenografts (16), intratumorally injected Ad-mda7 and Ad-ER-mda7 were both significantly more growth inhibitory than other PBS or Ad-luc [controls; P = 0.01 (Ad-mda7) and P = 0.005 (Ad-ER-mda7) versus controls; Fig. 3A]. In addition, intratumorally injected Ad-ER-mda7 was significantly more growth inhibitory than Ad-mda7 (P = 0.02; Fig. 3A). No significant tumor inhibition was observed in either Ad-luc- or PBS-treated mice. Analysis of A549 lung tumor tissue showed that (a) the growth inhibition observed in

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Figure 3. In vivo regression of tumors after intratumoral injection of Ad-mda7 or Ad-ER-mda7 in nu/nu mice. A, A549 tumor growth was significantly inhibited after injection with Ad-mda7 or Ad-ER-mda7 as opposed to PBS or Ad-luc. In each case, mice received a total of three injections over 6 d. Tumor volumes (Y axis) were measured in six to nine mice per group over 30 d. Mean (SD), B, histology of A549 tumors 24 h after last treatment with PBS, Ad-luc, Ad-mda7, or Ad-ER-mda7 (H&E staining; magnification, ×40). Analysis of A549 lung tumor tissues showed that the growth inhibition observed in Ad-mda7- or Ad-ER-mda7-treated mice was caused by MDA-7 protein expression and apoptosis. In addition, MDA-7 protein expression was associated with increased p-JNK and PKR expression in tumor tissues treated with Ad-mda7 or Ad-ER-mda7 as opposed to controls (PBS or Ad-luc).
Ad-mda7- or Ad-ER-mda7-treated mice was caused by MDA-7 protein expression and (b) this MDA-7 protein expression in both cases was associated with higher levels of p-JNK and PKR expression than those seen in control tumor tissues (Fig. 3B). Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assays showed increased terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling–positive staining cells in Ad-mda7- or Ad-ER-mda7-treated mice tissue (Fig. 3B). Thus, Ad-ER-mda7 effectively inhibited lung tumor growth in vivo, and intratumoral injection of Ad-mda7 or Ad-ER-mda7 caused no significant systemic toxicity. Together, these results showed the potential antitumor effect of Ad-mda7 or Ad-ER-mda7 when injected directly into tumors.

Discussion

In the present study, we have shown that Ad-mda7 or Ad-ER-mda7 treatment results in ER stress-induced apoptosis. We have also shown that Ad-mda7- or Ad-ER-mda7-mediated growth inhibition in vivo correlates with the activation of ER molecular markers such as PKR and JNK observed in vitro in Ad-mda7- or Ad-ER-mda7-treated cancer cells.

The replication-incompetent adenoviral vector Ad-mda7 is minimally toxic to normal cells but a potent inducer of apoptosis in a variety of cancer cell lines (1–5). As we and our collaborators have shown, mda-7 gene transfer via Ad-mda7 induces tumor-specific apoptotic, growth-inhibitory, and antiangiogenic effects independently of the status of other tumor suppressor genes (12, 17–19). In addition to its direct cytotoxic effects, Ad-mda7 transduction elicits the secretion of MDA-7, a protein that is also known as IL-24 and has unique apoptotic properties (21, 20, 21). In phase 1 trials in patients resistant to conventional therapy, intratumoral Ad-mda7 injection has produced minimal adverse effects and exhibited clinical activity in a subset of patients heavily pretreated with chemotherapy and radiation (22). Previously, we have shown that Ad-mda7 transduction of human lung cancer cells can increase expression of stress-related proteins, including GRP78/BiP, GADD34, PP2A, caspase-7, and XBP-1, consistent with activation of the unfolded protein response pathway, a key sensor of ER-mediated stress (23). The unfolded protein response pathway is an ER-to-nucleus signal transduction pathway that regulates the expression of a wide variety of target genes and is responsible for maintaining cellular homeostasis (13). Thus, activation of unfolded protein response signaling appears to be important in mediating apoptosis induced by Ad-mda7.

In the present study, we used a set of intracellularly targeted mda-7 adenoviral vectors that we designed to drive the expression of protein in specific subcellular compartments (ER, mitochondria, nucleus, and cytoplasm). Of all these vectors, the ER-targeted Ad-ER-mda7 vector was by far the most lethal to cells.

Interestingly, our demonstration that Ad-ER-mda7 transduction can still induce the secretion of MDA-7 protein warrants further studies to determine whether a secreted extracellular form from Ad-ER-mda7 could induce STAT3 activation and apoptosis in cancer cells. Indeed, after Ad-mda7 transduction of lung cancer cells, MDA-7 protein exists in both an intracellular form and a secreted extracellular form (1). The secreted extracellular form can bind to two different receptors: the type 1 IL-20 heterodimeric receptor complex comprising IL-20R1/IL-20R2 and the type 2 complex comprising IL-22R1/IL-20R2, which leads to activation of the STAT signaling pathways (12).

We next examined the expression of several downstream proteins including JNK, c-Jun, and caspase-4. The ER stress-mediated cell death pathway involves recruitment of the cytosolic adaptor TRAF2 to the ER membrane, where TRAF2 activates the apoptosis signaling kinase 1. Activation of apoptosis signaling kinase 1 leads in turn to activation of JNK and mitochondria-dependent caspase (24). Meanwhile, human caspase-4, which is 48% homologous to murine caspase-12, has been shown to be localized to the ER membrane and to be specifically activated by and required for ER stress-induced apoptosis (13). In this light, we showed that JNK and c-Jun activation is essential for Ad-mda7- and Ad-ER-mda7-mediated cell death, that Ad-mda7 and Ad-ER-mda7 treatment induces caspase-4 cleavage, and that p-JNK and caspase-4 are major drivers of apoptosis in Ad-mda7- and Ad-ER-mda7-transduced A549 lung cancer cells. Like Ad-mda7, which has been reported to use caspase-dependent and caspase-independent pathways of activation (8), our studies suggest that Ad-ER-mda7-induced apoptosis is partially dependent on caspase. Taken together, our data indicate that induction of ER stress-induced apoptosis by JNK and caspase-4 mediates Ad-mda7- and Ad-ER-mda7-induced apoptosis.

PKR is well characterized in the literature as an antiviral immune mediator that responds to viral dsRNA by activating eIF-2α, inhibiting protein synthesis inhibition, and blocking viral protein production (25). Apparently, its activation in HeLa, Cos1, U937, and NIH3T3 tumor cells leads to apoptosis induction (26, 27). Building on our previous demonstrations that Ad-mda7 transfection of lung cancer cells leads to the up-regulation and phosphorylation of PKR necessary for Ad-mda7-induced apoptosis (8, 9), we have now shown that Ad-ER-mda7 transduction does the same. We also found that up-regulation and phosphorylation of PKR is specific for Ad-mda7 and Ad-ER-mda7 because we saw no change in PKR expression after transduction with Ad-Mito-mda7, Ad-Nuc-mda7, or Ad-Cyto-mda7. In turn, we also conclude that the PKR up-regulation and phosphorylation induced by Ad-mda7 and Ad-ER-mda7 is not solely a response to adenoviral transfection because all of the other vectors we tested failed to induce PKR induction. Indeed, PKR induction appears to be critical for Ad-mda7 or Ad-ER-mda7 apoptosis because normal MEF lacking PKR were unable to undergo apoptosis as opposed to MEF-expressing wild-type PKR. This induction of apoptosis appeared to be specific to both Ad-mda7 and Ad-ER-mda7 because transduction of MEF lacking PKR with the Ad-Mito-mda7 vector did not impair...
apoptosis. In light of our previous identification of MDA-7 as a novel binding partner for PKR (9), further study is warranted to determine whether Ad-ER-mda7-induced MDA-7 might also interact with PKR.

Ad-ER-mda7 appears to be extremely growth inhibitory. This was shown by our experiments in an in vivo xenograft tumor model in which intratumoral administration of Ad-mda7 or Ad-ER-mda7 in mice bearing s.c. tumors significantly inhibited tumor growth and by tumor tissue studies showing an association between MDA-7 protein expression and the increased induction of PKR and p-JNK expression in Ad-mda7- or Ad-ER-mda7-treated tumor tissues as opposed to untreated (control) tissues.

In summary, by targeting the ER, Ad-ER-mda7 can induce cell death to a much greater extent through the ER stress-mediated cell death pathway involving caspase-4, JNK, and PKR activation. Ad-mda7 or Ad-ER-mda7 treatment results in ER stress-induced apoptosis and tumor growth inhibition in vivo. Going forward, our preliminary identification of the molecular mechanisms responsible for Ad-mda7-induced apoptosis of human cancer cells bodes well for the rational development of strategies for enhancing the antitumor effects of MDA-7, and our development of intracellularly targeted mda-7 adenoviral vectors will allow for the clinical optimization of this gene transfer therapy and the identification of patients unresponsive to Ad-mda7 therapies who might benefit from targeted therapy.

Disclosure of Potential Conflicts of Interest

S. Chada: Introgen Therapeutics employee. J.A. Roth: Introgen Therapeutics ownership interest. The other authors reported no potential conflicts of interest.

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