MDA-7 (Interleukin-24) Inhibits the Proliferation of Renal Carcinoma Cells and Interacts with Free Radicals to Promote Cell Death and Loss of Reproductive Capacity


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Abstract
The median survival of metastatic renal cell carcinoma (RCC) is 12 months, and the majority of treatment options are palliative. MDA-7 (interleukin-24), when expressed via a recombinant replication defective adenovirus, Ad.mda-7, has profound antiproliferative and cytotoxic effects in a wide variety of tumor cells but not in nontransformed cells. The studies in this study examined the impact of MDA-7 on RCC proliferation and survival. RCC lines (A498 and UOK121N), but not primary renal epithelial cells, were resistant to adenoviral infection that correlated with a lack of coxsackievirus and adenovirus receptor expression. Additional studies were performed using purified preparations of bacterially synthesized glutathione S-transferase (GST)-MDA-7 protein. GST-MDA-7, but not GST, caused a dose-dependent inhibition of RCC proliferation but not of primary renal epithelial cells. Clinically achievable concentrations of the novel therapeutic agent arsenic trioxide (0.5–1 μM) were found to have little effect on RCC growth. However, the combination of GST-MDA-7 and arsenic trioxide resulted in a greater than additive reduction in cell growth that correlated with a large increase in tumor cell death. The free radical scavenger N-acetyl cysteine abolished the potentiating effect of arsenic trioxide. Although pro-caspase 3, poly(ADP-ribose) polymerase, and Bcl-xL levels, as well as nucleosomal DNA integrity, were reduced by combined treatment, cell killing was predominantly nonapoptotic. Combined treatment of RCC lines with GST-MDA-7 and arsenic trioxide also resulted in a substantial reduction in clonogenic survival compared with either treatment individually. Collectively, these findings demonstrate that MDA-7 protein, in combination with agents that generate free radicals, may have potential in the treatment of RCC.

Introduction
There are ~30,000 new cases of RCC diagnosed each year in the United States of America and >10,000 die/annum of metastatic disease. Metastatic RCC is highly resistant to the majority of conventional chemotherapies, with low response rates, and a 5-year survival of <10% (1–3). More recent attempts at treating this disease, using growth inhibitory and proinflammatory cytokines, such as IL-2 and IFN-α, have also proved of marginal benefit (4, 5). Thus, the development of new therapies that more potently control RCC growth and survival is warranted.

Gene therapy is a novel approach to treat cancer: in this therapy, recombinant viruses that contain tumor suppressor genes or which contain genes that encode toxic cytokines are used to reduce tumor cell growth and survival (6). Other approaches have used modified adenoviruses that are selectively replicative and lytic only in tumor, but not in nontransformed, cells (7). In the majority of gene therapeutic approaches currently under investigation, the potency of cancer cell killing depends on the rate of infection of the target tumor cells (8). In this regard, the laboratory of Dr. Curiel recently demonstrated that RCC cells were resistant to infection by type 5 adenoviruses that recognize the CAR, because RCC lines appeared to express low levels of CAR (9). The lack of CAR expression not only results in a loss of adenoviral infection but has also been associated with enhanced tumor cell growth in vivo and invasiveness (10, 11).

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3 The abbreviations used are: RCC, renal cell carcinoma; CAR, coxsackievirus and adenovirus receptor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HRP, horseradish peroxidase; GST, glutathione S-transferase; JNK, c-Jun NH2-terminal kinase; PARP, poly(ADP-ribose) polymerase; ERK, extracellular signal-regulated kinase; EGF, epithelial growth factor; CMV, cytomegalovirus; IL, interleukin.
The gene encoding mda-7 (IL-24) was isolated from human melanoma cells induced to undergo terminal differentiation by IFN and mezerein (12–14). It is a member of the IL-10 family, which includes IL-10, IL-19, IL-20, IL-22, and AK155 (IL-26) family (12, 13, 15, 16). MDA-7 expression is decreased in advanced melanomas, with nearly undetectable levels in metastatic disease (12–14, 17). Enforced expression of MDA-7, by use of a recombinant adenovirus Ad.mda-7, has been shown to inhibit the growth of a broad spectrum of cancer cells in vitro, including those derived from skin, prostate, breast, central nervous system, cervical, sarcoma, colorectal, and lung, without exerting deleterious effects in normal human epithelial or fibroblast cells (12, 13, 18–24). Of particular note, MDA-7 was shown to be secreted from infected cells in vitro and in vivo, which may in part explain its profound antitumor bystander effects in vivo (21, 22).

The pathways by which MDA-7 enhances apoptosis in tumor cells are not fully understood, although published evidence suggests the involvement of multiple genes important for the onset of growth inhibition and apoptosis, such as p21, p53, GADD genes, BCL-xL, BCL-2, BAX, and APO2/tumor necrosis factor-related apoptosis-inducing ligand (12, 13, 18–23). Of note, the ability of MDA-7 to suppress growth in cancer cells appears to be independent of retinoblastoma protein and p53 status (19–24). In melanoma cell lines infected by Ad.mda-7, it was noted that both BCL-2 and BCL-xL levels significantly decreased but that BAX and BAK levels were only slightly increased (22). Furthermore, the ability of Ad.mda-7 to induce apoptosis in the prostate cancer cell line, DU145, which is BAX null, indicates that Ad.mda-7 can mediate apoptosis via a BAX-independent pathway (12, 13, 25).

The impact of Ad.mda-7 on the survival of RCC lines and primary renal epithelial cells is unknown, and the present studies examined whether Ad.mda-7 altered the growth and survival of RCCs. In agreement with a previous report (9), we discovered that RCC lines, but not primary renal epithelial cells, were resistant to type 5 adenoviral infection. In addition, purified GST-MDA-7 protein, but not GST, caused a dose-dependent reduction in RCC line proliferation but not that of primary renal epithelial cells. Free radicals species, generated by clinically relevant concentrations of arsenic trioxide, synergized with subnanomolar concentrations of GST-MDA-7 to inhibit the proliferation, viability, and long-term survival of RCCs. These findings argue that MDA-7, in combination with agents that generate free radicals, may have potential in the treatment of RCC.

Materials and Methods
Reagents. DMEM and penicillin-streptomycin were from Invitrogen Life Technologies, Inc. (Carlsbad, CA). Nonessential amino acids and sodium pyruvate were from Cellgro (Hendon, VA). MTT reagent and Giemsa stain were from Sigma (St. Louis, MO). Anti-caspase 3, phospho-/-total-ERK1/2, phospho-/total-P38a/b, phospho-/total-JNK1/2, anti-Bcl-2, anti-Bcl-xL, anti-FAS receptor, anti-FAS ligand, anti-Bax, and all of the secondary antibodies (anti-rabbit-HRP, antimouse-HRP, and antigoat-HRP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-PARP (1:2500, mouse monoclonal) was purchased from Calbiochem. Enhanced chemiluminescence kit was purchased from NEN Life Science Products (Boston, MA). Other constructs and reagents were as described in Refs. 18–20, 23, and 24.

Generation of Ad.mda-7 and Synthesis of GST-MDA-7. Recombinant type 5 adenovirus to express MDA-7 (Ad.mda-7), control (CMV vector), or control (p-galactosidase) was generated using recombination in HEK293 cells. Standard cloning procedures were used to generate a bacterial expression vector comprising in-frame fusion of the mda-7 open reading frame 3’ to the GST open reading frame in GST-4T2 vector (Amersham Pharmacia), using BamHI and NotI sites introduced into mda-7 by PCR (20). Expression of protein was performed by inoculating an overnight culture at 1:100 dilution followed by incubation at 25°C until an A560 of 0.4–0.6 was reached, followed by induction with 0.1 μM isopropyl-1-thio-β-D-galactopyranoside for 2 h. Cells were harvested by centrifugation and sonicated in PBS followed by centrifugation to obtain soluble protein. The lysate was bound to a glutathione-agarose column (Amersham Pharmacia) at 4°C for 2 h followed by washing with 50 volumes of PBS and 10 volumes of PBS with 500 mM NaCl. Elution of bound protein was performed by passing 20 ml reduced glutathione through the column and collecting 1-ml fractions. Fractions were analyzed by gel electrophoresis, and positive samples were dialyzed against 1000 volumes of PBS for 4 h with one change, followed by 500 volumes of DMEM for 4 h. Protein concentration was estimated by Bradford assays, as well as gel electrophoresis, in conjunction with Coomassie blue staining. Samples were tested for activity using GST protein as control. Using gel-purified GST-MDA-7, a polyclonal anti-GST-MDA-7 antibody was raised in rabbits and used at a 1:3000 dilution for immunoblotting.

Cell Culture. Human U373 glioma cells (American Type Culture Collection) and human RCC cells: A498 (American Type Culture Collection UOK121N (Dr. Lineham, National Cancer Institute) and primary human renal epithelial cells (Clonetics, Cambrex Corp., East Rutherford, NJ) were cultured in DMEM supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and 1% penicillin/streptomycin according to the suppliers’ instructions. Cells were incubated in humidified atmosphere of 5% CO2 at 37°C.

Primary Culture of Rodent Hepatocytes. Hepatocytes were isolated from adult male Sprague Dawley rats by the two-step collagenase perfusion technique (26, 27). The freshly isolated hepatocytes were plated on rat-tail collagen (Vitrogen) coated at a density of 2.5 × 105 cells/well and cultured in DMEM supplemented with 250 μM insulin, 0.1 nM dexamethasone, 1 nM thyrone, and 100 μg/ml penicillin/streptomycin, at 37°C in a humidified atmosphere containing 5% CO2. An initial medium change was performed 2 h after cell seeding, at the time of viral infection, to remove dead or mechanically damaged cells.

Recombinant Adenovirus Infection. The Ad.mda-7 and control adenoviral vectors used were identical to those described previously (18, 20–25). The viral titers for each virus control adenoviral vectors used were identical to those described previously (18, 20–25). The viral titers for each virus were determined by plaque formation assay. In vitro adenoviral infections were performed 24 (RCC) and 4 h (hepatocytes) after plating.

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Monolayer cultures were washed in PBS and incubated with purified virus in 1 ml of growth medium without serum for 1 h at 37°C in a humidified atmosphere of 5% CO₂/95% air with gentle agitation. For RCC lines, after 3 h, fresh growth medium with 10% fetal bovine serum was added.

Assessment of Apoptosis and Cell Death. The extent of apoptosis and necrosis (cell death) was evaluated by examining Wright-Giemsa-stained cytosin slides under light microscopy and scoring the number of cells exhibiting the classic morphological features of apoptosis. For each condition, 10 randomly selected fields/slide were evaluated, encompassing $\approx 15,000$ cells (27, 28). To confirm the results of morphological analysis, in some cases, cells were also evaluated by terminal deoxynucleotidyl transferase-mediated nick end labeling staining and oligonucleosomal DNA fragmentation assay as follows: staining, cytosin slides were fixed with 4% formaldehyde/PBS for 10 min, treated with acetic acid/ethanol (1:2) for 5 min, and incubated with terminal deoxynucleotidyl transferase reaction mixture containing 1 $\times$ terminal deoxynucleotidyl transferase reaction buffer, 0.25 units/terminal deoxynucleotidyl transferase, 2.5 mM CoCl₂, and 2 pmol fluorescein-12-dUTP (Boehringer Mannheim, Indianapolis, IN) at 37°C for 1 h. The slides were mounted with Vectashield containing propidio iodide (Vector Laboratories, Burlingame, CA) and visualized using fluorescence microscopy.

Assessment of Cell Viability. Cell viability was also evaluated by assessing trypan blue inclusion/exclusion of isolated cells under light microscopy and scoring the percentage of cells exhibiting blue staining (29). Floating and attached cells were isolated by trypsinization, recovered by centrifugation, resuspended in phenol red-free DMEM, and visualized under light microscopy.

MTT Assay for Determination of Cellular Viability. The MTT test is based on the enzymatic reduction of the tetrazolium salt MTT in living, metabolically active cells but not in dead cells. Cells were plated (5–10,000 cells/well of a 12-well plate) and 24 h after plating treated with either GST or GST-MDA-7 at the indicated concentrations. Thirty min after protein treatment, cells were treated with arsenic trioxide at the indicated concentrations. The cytotoxicity of the various treatments was assessed 4 days after irradiation by measurement of cell viability by use of the MTT assay, as described previously (30). The plates were read on a Dynatech MR600 Microplate Reader at 540 nm. All data were normalized relative to the GST control, nontreated cells of the corresponding cell type. 

Cell Survival Analyses. Cells were assayed for the effect(s) of GST-MDA-7 and arsenic trioxide on cell survival. Cells were plated (10,000 cells/60-mm dish) and 24 h after plating treated with GST-MDA-7 or GST. Thirty min later, cells were treated with arsenic trioxide. Fifty-six h later, cells were isolated by trypsinization, and viable trypan blue-negative cells were replated in 60-mm dishes at 250–1,000 cells/plate. Colonies were allowed to form surviving cells for 10–14 days, before fixing and staining with crystal violet (29, 30). Colonies that contain $>50$ cells were then counted. To generate the survival data, individual assays were performed at multiple dilutions with a total of six plates per data point repeated for a total of three experiments.

Western Blot Analysis. Protein concentration was determined using a kit from Bio-Rad. Aliquots (40 $\mu$g) were solubilized in Laemmli buffer, separated by SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were blocked 2 h at 4°C in TBST [5% nonfat milk in 10 mM Tris-HCl, 100 mM NaCl, and 0.1% Tween 20 (pH 7.6)]. Membranes were exposed to the primary antibodies, followed by washing (3 $\times$ 15 min with TBST). Membranes were incubated with HRP-conjugated antimouse or antirabbit IgG antibody, followed by washing with TBST (3 $\times$ 15 min). Proteins were visualized by enhanced chemiluminescence.

DNA Fragmentation. An equal number of cells from each test (10⁵) was homogenized with 1 ml of lysis buffer [10 mM Tris (pH 7.4), 5 mM EDTA, and 1% Triton X-100]. RNase A (100 $\mu$g/ml) was added to each sample and incubated at 50°C for 1 h. Proteinase K was then added (100 $\mu$g/ml), and the samples were incubated overnight at 50°C. The DNA was extracted using phenol and chloroform and centrifuged at 10,000 $\times$ g for 5 min at 4°C. The aqueous phase mixed with two volumes of ice-cold ethanol and then precipitated by centrifugation at 15,000 $\times$ g for 10 min, supernatants were removed, and DNA pellets were washed with 80% ethanol once (15,000 $\times$ g for 10 min), air dried, and dissolved in TE buffer (pH 7.6). DNA concentration was determined, and 10 $\mu$g of each sample were then electrophoresed on a 1.5% agarose gel and analyzed for the presence of a laddering pattern.

Statistical Analyses. Comparison of the effects of various treatments was performed using one-way ANOVA and a two-tailed t test. Differences with a $P < 0.05$ were considered statistically significant. Experiments shown are the means of multiple individual points (±SE).

Results

Previous studies by our group have shown that tumor cells, but not nontransformed cells, infected with the type 5 recombinant adenovirus, Ad.mda-7, undergo growth arrest and apoptosis (12, 13, 20–23, 25). Initial studies in RCC lines attempted to recapitulate our previous findings using Ad.mda-7 in other tumor cell types. However, we were unable to observe any effect of Ad.mda-7 on A498 cell growth and observed only a weak effect on the growth of UOK121N cells (data not shown). In contrast, transfection of RCC lines with a plasmid to express MDA-7 reduced colony formation and cell growth (Fig. 1; Refs. 19 and 21).

Because transfection, but not infection, of RCC lines to express MDA-7 resulted in reduced cell growth, we determined whether RCC lines expressed the CARs, which are necessary for adenovirus entry into cells. In the two RCC lines tested, CAR levels were very low, in contrast to either U373 malignant glioma cells or primary human renal epithelial cells (Table 1). This is similar to the findings of Haviv et al. (9). Furthermore, when cells were exposed to increasing doses of an adenovirus to express β-galactosidase, it was noted that the virus more easily infected U373 and primary renal epithelial cells than A498 or UOK121N RCC lines (Fig. 2).
A representative experiment is shown (Fig. 1). Higher numbers reflect enhanced binding and increased CAR availability. Colonies were transferred into the culture media of RCC lines (Fig. 3). To confirm that the effect of GST-MDA-7 was caused by MDA-7 and not a contaminating bacterial protein, we made use of MDA-7 protein synthesized in primary rodent hepatocytes. Cultures of primary rat hepatocytes were infected with Ad.mda-7 or control virus, and 96 h after infection, the culture media from the hepatocytes were transferred into the culture media of RCC lines (Fig. 3D). RCC cells incubated with either fresh media or media from control virus-infected hepatocytes had similar growth rates. However, kidney cancer cells incubated with media from GST-MDA-7, arsenic trioxide, nor GST-MDA-7 altered the growth potential in RCC lines was examined. Arsenic trioxide enhanced the growth suppressive effects of GST-MDA-7, but not GST, in RCC lines (Fig. 5, A–C). Neither low concentrations of arsenic trioxide nor GST-MDA-7 altered the growth potential of primary renal epithelial cells (Fig. 5C).

Because RCC lines were resistant to adenoviral infection, we synthesized MDA-7 as a GST fusion protein in Escherichia coli and determined whether purified MDA-7 protein could alter cell growth and survival. GST-MDA-7, but not GST, caused a dose-dependent reduction in the proliferation of A498, UOK121N, and primary renal epithelial cells at concentrations \( \geq 1 \mu M \) (Fig. 4), which correlated with enhanced cell killing at higher concentrations (data not shown). Previously, several groups have shown that ionizing radiation, which also generates free radicals, can interact with MDA-7 to enhance tumor cell killing (20, 33–35). Thus, the ability of arsenic trioxide to enhance the antiproliferative and cell killing effects of MDA-7 in RCC lines was examined. Arsenic trioxide enhanced the growth suppressive effects of GST-MDA-7, but not GST, in RCC lines (Fig. 5, A and B). Neither low concentrations of arsenic trioxide nor GST-MDA-7 altered the growth potential of primary renal epithelial cells (Fig. 5C).

In parallel to the MTT proliferation assays in Fig. 5, the viability of cells was determined 96 h after GST-MDA-7/arsenic trioxide treatment. As judged by Wright Giemsa staining of fixed cells and microscopic examination of nuclear morphology, neither GST-MDA-7, arsenic trioxide, nor their combination enhanced “classical” nuclear apoptosis. Cell nuclei appeared to be degraded in a nonapoptotic fashion.
ion, more indicative of necrosis. In general agreement with this finding, significantly more trypan blue-positive cells were present in cells treated with GST-MDA-7 and arsenic trioxide than under any other condition (Fig. 6). Arsenic trioxide is believed to enhance cell killing by the generation of free radicals, and incubation of cells with the free radical scavenger N-acetyl-cysteine significantly reduced cell killing (Fig. 6, A and B). The protective effect of N-acetyl-cysteine was also reflected in a blockade of enhanced growth suppression after arsenic trioxide and GST-MDA-7 treatment (data not shown).

The expression of pro-caspase 3, PARP, and Bcl-XL, as well as the integrity of nuclear DNA, was also examined after GST-MDA-7 and arsenic trioxide treatment. Despite only observing a weak enhancement in "classical" nuclear apoptotic morphology, p32 pro-caspase 3 and PARP were both cleaved, and the expression of Bcl-XL was reduced (Fig. 7 A). Near complete nucleosomal DNA degradation was observed 96 h after combined treatment with both agents, which was also suggestive of a necrotic form of cell death (Fig. 7 B).

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**Fig. 3.** GST-MDA-7 causes a dose-dependent reduction in the proliferation of renal carcinoma cells but not primary renal epithelial cells. Cells were plated in 12-well plates (1 × 10⁴ cells/well) and 24 h later treated with increasing concentrations of GST and GST-MDA-7 as indicated. Cell growth was determined via MTT assay 96 h after GST-MDA-7 treatment. Data are the means of 12 wells (one plate per condition, ±SE) from a representative experiment using different preparations of GST-MDA-7 (n = 3). A, UOK121N cells; B, A498 cells; C, primary renal epithelial cells. In D, primary rat hepatocytes were isolated and cultured as described in “Materials and Methods.” Four h after plating, hepatocytes were infected with either control CMV virus or Ad.mda-7 (at a multiplicity of infection of 50). Five days after infection, the media from the hepatocytes were removed and mixed with an equal volume of RCC line culture media. These media were added to cultures of A498 and UOK121N cells, plated in 12-well plates (1 × 10⁴ cells/well), 24 h previously. Cell growth was determined via MTT assay 96 h after MDA-7 treatment. Data are the means of 12 wells (one plate per condition, ±SE; *P < 0.05 less than control CMV media or fresh media) from a representative experiment using different hepatocyte infections (n = 2).

**Fig. 4.** Arsenic trioxide causes a concentration-dependent reduction in primary and renal carcinoma cell growth. Cells were plated in 12-well plates (1 × 10⁴ cells/well) and 24 h later treated with increasing concentrations of As₂O₃ as indicated. Cell growth was determined via MTT assay 96 h after GST-MDA-7 treatment. Data are the means of 12 wells (one plate per condition, ±SE) from a representative experiment using separate As₂O₃ formulations (n = 3). Arsenic trioxide caused a dose-dependent enhancement in cell killing at higher concentrations (>10 μM) (data not shown).
Fig. 5. GST-MDA-7 and arsenic trioxide interact in a greater than additive fashion to reduce renal carcinoma cell growth. Cells were plated in 12-well plates (~1 x 10^4 cells/well) and 24 h later treated with increasing concentrations of GST, GST-MDA-7, and As_2O_3 (0.5 μM) as indicated. Cell growth was determined via MTT assay 96 h after GST-MDA-7 treatment. Data are the means of 12 wells (one plate per condition, ±SE) from a representative experiment using different preparations of GST and GST-MDA-7 (n = 3). *P < 0.05 less than GST value cells; **P < 0.05 greater than corresponding GST-MDA-7 value without As_2O_3 cotreatment.

Fig. 6. GST-MDA-7 and arsenic trioxide interact in a greater than additive fashion to enhance renal carcinoma killing that is blocked by the free radical scavenger N-acetyl cysteine. Cells were plated in 6-well plates (~5 x 10^4 cells/well) and 24 h later treated with GST, GST-MDA-7 (both 0.5 nM), and As_2O_3 (0.5 μM) as indicated. Where indicated, cells were pretreated with 10 mM N-acetyl cysteine (NAC) 1 h before the addition of As_2O_3. Cells were isolated 96 h after GST-MDA-7 treatment. Cell death was determined on isolated fixed cells by Giemsa staining for apoptosis and necrosis (presented as a single value) as described (30). Data are the means of six wells (one plate per condition, ±SE) from a representative experiment using different preparations of GST and GST-MDA-7 (n = 3). *P < 0.05 greater than GST value cells; **P < 0.05 greater than corresponding GST-MDA-7 value without As_2O_3 cotreatment; ***P < 0.05 less than value in cells not incubated with NAC.
interaction of GST-MDA-7 and arsenic trioxide (data not shown).

GST-MDA-7 and arsenic trioxide significantly enhanced tumor cell death as judged by enhanced trypan blue inclusion in treated cells, and to confirm that this combination also reduced the long-term proliferative capacity of tumor cells, we performed clonogenic survival assays. Treatment of cells with low concentrations of GST-MDA-7 did not significantly alter long-term proliferation/cell survival, whereas arsenic trioxide significantly reduced clonogenicity (Fig. 8, A and B). The combination of GST-MDA-7 with arsenic trioxide caused a large, greater than additive reduction in the survival of renal carcinoma cells.

Discussion

RCC is known to be relatively resistant to a variety of “conventional” anticancer therapies, including combination chemotherapy and radiotherapy. The studies in this study were designed to examine the impact of the novel therapeutic cytokine MDA-7 (IL-24; Refs. 12 and 13) on the growth and survival of RCC lines.

The adenoviral vector Ad.mda-7 did not alter RCC proliferation, which correlated with a lack of CAR expression and weak infectivity in RCC lines. These findings were noted previously by Haviv et al. (9). In contrast to our data in kidney cancer cells, primary renal epithelial cells were much more easily infected by type 5 adenovirus. Collectively, this demonstrates that kidney cancer cells have reduced expression of CAR protein compared with nontransformed kidney epithelial cells, which is in general agreement with the concept that several cell types, including bladder carcinomas and prostate carcinomas, lose expression of CAR proteins as they become transformed (10, 11).

Recent studies in brain, lung, and mammary carcinoma cells have noted that Ad.mda-7 can act as a radiosensitizer (20, 33–35). In our studies using glioma cells, Ad.mda-7 and purified MDA-7 protein enhanced the growth inhibitory and cytotoxic effects of ionizing radiation that were blocked by the free radical scavenger N-acetyl cysteine. These effects correlated with reduced expression of the antiapoptotic protein BCL-XL; constitutive overexpression of BCL-XL abolished the toxic and growth inhibitory effects of Ad.mda-7. In RCC lines, GST-MDA-7 but not GST caused a dose-dependent reduction in proliferation without causing cell killing; as a single agent, high concentrations of GST-MDA-7 caused a further reduction in growth and modestly enhanced cell death. That GST did not alter cell biology argues that the effect is specific to MDA-7 and not because of bacterial endotoxins in our purified protein preparations, which was supported by the use of MDA-7 synthesized in primary hepa-

Fig. 7. GST-MDA-7 and arsenic trioxide interact to enhance cleavage of pro-caspase 3 and PARP in renal carcinoma cells that correlates with reduced expression of BCL-XL and enhanced activity of p38 and JNK1/2. Cells were plated in 100-mm plates (2 × 10⁶ cells/well) and 24 h later treated with GST, GST-MDA-7 (both 0.5 nM), and As₂O₃ (0.5 μM) as indicated. Cells were isolated 96 h after GST-MDA-7 treatment. Protein expression levels were determined using Bradford assay for total protein followed by SDS PAGE and immunoblotting, as described in “Materials and Methods.” In parallel plates, nucleosomal DNA integrity was determined using agarose gel electrophoresis as described in “Materials and Methods.” A, expression of PARP, p32 pro-caspase 3, and BCL-XL in RCC lines. B, nucleosomal DNA integrity in RCC lines. C, ERK1/2, JNK1/2, and p38 activity in RCC lines. Data are from a representative experiment (n = 3).
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reduction in Bcl-XL expression. The free radical scavenger greater than additive reduction in cell growth, and a supra-arrest without a large increase in cell death, whereas one to that correlates with enhanced cell killing.

tact with MDA-7 to promote additional growth suppression concentrations of MDA-7 and that free radical species can in-
strate that tumor cell growth can be modulated by low con-
lethal effects of MDA-7. Collectively, these findings demon-
suggestive that the generation of free radicals enhanced the
-Acetyl cysteine blocked the effects of arsenic trioxide,
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of three separate experiments (±SE). *P < 0.05 less than control cells;

Fig. 8. GST-MDA-7 and arsenic trioxide interact in a greater than additive fashion to reduce renal carcinoma cell colony formation ability. Cells were plated in 6-well plates (~5 × 10⁴ cells/well) and 24 h later treated with GST, GST-MDA-7 (both 0.5 nM), and As₂O₃ (0.5 μM) as indicated. Cells were isolated 96 h after GST-MDA-7 treatment, and cell viability was determined using trypan blue exclusion assay (see Fig. 6). On the basis of trypan blue-negative viable cell values, 250, 500, and 2000 viable cells were replated in Linbro plates. The plating density for colony formation assays depended on the previous treatment of the cells and data obtained in Figs. 5 and 6. Days (10–14) after plating, cells were fixed and stained with crystal violet. Colony formation was determined using visual counting, and a colony was defined as a group of >50 cells. Data are the means of three separate experiments (±SE). *P < 0.05 less than control cells; *P < 0.05 less than As₂O₃ or GST-MDA-7-treated cells.

tocytes. These effects also correlated with reduced expres-
sion of the antiapoptotic protein Bcl-XL.

Arsenic trioxide interacted with GST-MDA-7 to cause a greater than additive reduction in cell growth, and a supra-
additive increase in cell death that correlated with a further reduction in Bcl-XL expression. The free radical scavenger N-acetyl cysteine blocked the effects of arsenic trioxide, suggestive that the generation of free radicals enhanced the lethal effects of MDA-7. Collectively, these findings demonstrate that tumor cell growth can be modulated by low concentrations of MDA-7 and that free radical species can interact with MDA-7 to promote additional growth suppression that correlates with enhanced cell killing.

Low concentrations of GST-MDA-7 caused ~50% growth arrest without a large increase in cell death, whereas one to two orders of magnitude higher concentrations of the cytokine caused arrest and cell death. These findings have similarity to those in A431 squamous carcinoma cells treated with EGF (37). In A431 cells, low picomolar concentrations of EGF promote cell growth; however, above ~100 pmol EGF, growth arrest is promoted. At EGF concentrations above >1 ng/ml, A431 cells undergo growth arrest followed by apoptosis. It has also recently been suggested by another group that the ability of MDA-7 to promote growth arrest and cell death can, to a certain extent, be dissociated (15).

MDA-7 has been proposed to bind to the IL-20 receptor complex and thus regulate cell behavior via Janus-activated kinase/signal transducers and activators of transcription signaling (38). Studies by our group et al. (20, 33, 34, 36) have implicated signaling by mitogen-activated protein kinase pathways in the cellular response to MDA-7, with signaling by p38 and JNK1/2 being implicated in MDA-7-induced cell death. Arsenic trioxide-induced cell death, using high concentrations of As₂O₃, has been linked to reduced ERK1/2 activity and enhanced p38 activity, with JNK1/2 surprisingly also acting as a protective signal (39). Our findings suggest that enhanced JNK1/2 signaling, but not p38 signaling, plays a central role in the toxic interaction between MDA-7 and As₂O₃.

The novel therapeutic arsenic trioxide is currently being examined as a drug that can enhance the toxicity of established chemotherapeutic agents (40). Arsenic trioxide is thought to generate free radical species (primarily oxygen derived; Ref. 41), thereby enhancing oxidative stress and DNA damage, leading to the promotion of cell death (e.g., in renal carcinoma cells; Ref. 42). It has also been linked in a cell type and dose-dependent fashion to enhanced proliferation and to the promotion of cellular differentiation (43, 44). The cytotoxic effects of arsenic trioxide were reported to be less in nontransformed cells (43). Differentiation therapy for the treatment of cancer, particularly hematologic malignancies, is also under intense investigation (40). In this regard, MDA-7 has also been linked to cell differentiation and the induction of growth arrest in undifferentiated tumor cells but not differentiated nontransformed cells (12, 13, 45). Thus, it is possible that MDA-7 and arsenic trioxide interact to cause growth arrest and inappropriate differentiation in renal carcinoma cells leading to cell death. That a mixed population of differentiated nontransformed human kidney epithelial cells were resistant to the combination of MDA-7 and arsenic trioxide suggests that normal tissue toxicity may not be dose limiting.

The modes of cell death induced by GST-MDA-7 and arsenic trioxide were also investigated. On the basis of previous studies by our group (12, 13), and our present findings with BCL-XL, we had expected that GST-MDA-7 and arsenic trioxide would interact, primarily, to increase apoptosis. Although GST-MDA-7 and arsenic trioxide caused procaspase 3 and PARP cleavage, the increase did not correspond to a large induction of “classical” apoptotic nuclear morphology. However, when cell viability was measured via trypan blue staining, the combination of GST-MDA-7 and arsenic trioxide caused a large (~30%) increase in cell morbidity. In agreement with a nonapoptotic mode of cell
death, agarose gel DNA fragmentation analyses indicated that nuclear DNA was being degraded into small molecular weight fragments, rather than into a "classical DNA ladder" indicative of apoptosis. These findings suggest that GST-MDA-7 and As$_2$O$_3$ promote cell death through a necrotic mechanism.

Colony formation assays are arguably the best in vitro test to determine whether a particular therapeutic agent may have any impact on tumor cell growth in vivo (46). Treatment of RCC lines with a low concentration of GST-MDA-7 reduced cell growth by -50% as judged in MTT assays and by manual cell counting, yet when treated cells were grown in colony formation assays in the absence of the cytokine, little alteration in colony-forming ability was observed. This argues that low concentrations of GST-MDA-7 cause a reversible growth arrest in RCC lines. Arsenic trioxide had little effect on short-term cell growth as measured in MTT assays but significantly reduced colony formation ability, suggestive that the effects of free radical generation are manifested many cell doublings after the initial exposure, which is similar to the findings of many laboratories using ionizing radiation (46). The combination of GST-MDA-7 and arsenic trioxide caused a -10-fold increase in cell killing compared with either agent individually. Of note, these colony formation data do not include the [mteq]50% cell killing achieved before replating by GST-MDA-7 and arsenic trioxide exposure. These provocative findings suggest that MDA-7 and agents that generate free radicals, e.g., arsenic trioxide, have potential for the treatment of RCC.

References

MDA-7 (Interleukin-24) Inhibits the Proliferation of Renal Carcinoma Cells and Interacts with Free Radicals to Promote Cell Death and Loss of Reproductive Capacity

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