Melanoma Differentiation-associated 7 (Interleukin 24) Inhibits Growth and Enhances Radiosensitivity of Glioma Cells 

in Vitro and in Vivo


ABSTRACT

Purpose: Despite therapeutic interventions including surgery, chemotherapy, and radiotherapy, glioblastoma multiforme (GBM) has a very poor prognosis and novel therapies are required.

Experimental Design: Melanoma differentiation-associated 7 (mda-7) (interleukin 24), when expressed via a recombinant replication-defective adenovirus, adenovirus-associated 7 (mda-7), has profound antiproliferative and cytotoxic effects in a variety of tumor cells but not in nontransformed cells. The present studies examined the combined impact of mda-7 and radiation enhancing effects of radiation.

Results: mda-7 caused a dose-dependent reduction in the proliferation of glioma cells in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. The antiproliferative effects of mda-7 were enhanced by radiation in a greater than additive fashion. These effects were not observed in cultures of nontransformed primary astrocytes. Purified MDA-7 protein caused a similar dose-dependent reduction in GBM cell growth that was enhanced after radiation exposure. The enhanced reduction in growth correlated with increased necrosis and DNA degradation. These modifications in cell phenotype correlated with reduced expression of Bcl-X(L) and enhanced expression of BAX. Overexpression of Bcl-X(L) protected cells from the antiproliferative and cytotoxic effects of Ad.mda-7 + radiation. Incubation of cells with N-acetyl cysteine abolished the enhancing effects of radiation. In vitro, Ad.mda-7 and radiation reduced colony formation ability, which was significantly increased when the two treatments were combined. In vivo, Ad.mda-7 enhanced the survival of Fischer 344 rats implanted intracranially with glioma cells. Radiation did not alter survival in control infected animals, whereas it prolonged survival in those infected with Ad.mda-7.

Conclusions: These findings demonstrate that mda-7 reduces the proliferation and enhances the radiosensitivity of GBM cells in vitro and in vivo.

INTRODUCTION

In the United States, GBM is diagnosed in ~20,000 patients/annum. High-grade tumors such as anaplastic astrocytoma and GBM account for the majority of astrocytic tumors and constitute >40% of malignancies of the central nervous system (1, 2). Radiation therapy is typically used in the management of gliomas. Even under the best of circumstances in which essentially all of the tumor can be surgically removed and the patients treated with combinations of radiation and chemotherapy, the mean survival of this disease is only extended from ~3 months (1, 3) to 1 year with rare long-term survivors (4, 5). A number of novel gene therapy approaches have been recently developed with the aim of killing neoplastic cells via the transfer of lethal genes to the tumor cells, including glioma, using recombinant viruses, or more recently, to infect tumor cells with viruses that have been modified to become nonpathogenic to normal tissues but remain lytic to tumor cells (6, 7). The efficacies and potential side effects of these strategies are in the early investigational state.

The gene for mda-7 (IL-24) was isolated from human melanoma cells induced to undergo terminal differentiation by the treatment of melanoma cells induced to undergo terminal differentiation by...
treatment with IFN and mezerein (8). It is a member of the IL-10 family, which includes IL-10, IL-19, IL-20, IL-22, and AK155 (IL-26; Refs. 9–14). MDA-7 protein expression is decreased in advanced melanomas (8, 15, 16), with nearly undetectable levels in metastatic disease (15–21). 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, including those derived from the skin, prostate, breast, gastrointestinal tract and lung without exerting deleterious effects in normal human epithelial or fibroblast cells (9, 16–25).

The pathways by which Ad.mda-7 enhances apoptosis in tumor cells are not fully understood; however, evidence from several studies suggests the involvement of proteins important for the onset of growth inhibition and apoptosis, including BCL-\textsubscript{XL}, BCL2, BAX, and APO2/TRAIL (17–26). The ability of Ad.mda-7 to suppress growth in cancer cells appears to be independent of RB and p53 status (20, 27). In melanoma cell lines, but not in normal melanocytes, infected by Ad.mda-7, it was noted that a significant decrease in both BCL-2 and/or BCL-\textsubscript{XL} levels, with only a modest up-regulation of BAX and/or BAK expression (21). This data supports the hypothesis that Ad.mda-7 enhances the ratio of proapoptotic to antiapoptotic proteins in cancer cells, thereby facilitating induction of apoptosis (14, 17–21, 24). The ability of Ad.mda-7 to induce apoptosis in the prostate cancer cell line, DU145, which does not produce BAX, indicates that mda-7 can mediate apoptosis in tumor cells by a BAX-independent pathway (17, 18, 22, 24).

The ability of Ad.mda-7 to alter radiosensitivity has recently been investigated by ourselves and others. Kawabe et al. (37) demonstrated that lung cancer cells were radiosensitized by Ad.mda-7, and Su et al. (38) demonstrated that Ad.mda-7 radiosensitized human glioma cells in vitro. To extend our in vitro observations in glioma, we have investigated the ability of and mechanisms by which Ad.mda-7 and purified GST-MDA-7 protein radiosensitizes RT2 glioma cells in vitro and in vivo. Ad.mda-7 synergized with radiation-induced free radicals to reduce expression of BCL-\textsubscript{XL} and enhance expression of BAX, which lead to enhanced radiosensitivity in vitro. Animals implanted intracranially with glioma cells expressing MDA-7 survived longer and were more radiosensitive. These findings demonstrate that MDA-7 reduces the proliferation and enhances the radiosensitivity of GBM cells both in vitro and in vivo.

MATERIALS AND METHODS

Reagents. DMEM and penicillin-streptomycin were from Life Technologies, Inc. (New York, NY). MTT reagent was from Sigma (St. Louis, MO). Anti-caspase 3, Anti-Bcl-2, Anti-BCL\textsubscript{XL}, anti-FAS receptor, anti-FAS ligand, anti-BAX, and all of the secondary antibodies (antirabbit-HRP, antimouse-HRP, and antigoat-HRP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-PARP (1:2500, mouse monoclonal; Calbiochem) was raised in rabbits and used at a 1:3000 dilution for immunoblotting. A polyclonal anti-GST-MDA-7 antibody was generated using recombinant in HEK293 cells as described previously (30, 34). 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 (29). Expression of protein was performed by incubating an overnight culture at 1:100 dilution followed by incubation at 25°C until an A\textsubscript{\textsmaller{600nm}} of 0.4–0.6 was reached followed by induction with 0.1 μM isopropyl-1-thio-β-ν-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 PBS and 10 volumes PBS with 500 mM NaCl. Elution of bound protein was performed by passing 20 mM 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 50 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. Fischer 344 rat RT2 glioblastoma cells (University of Alabama, Birmingham, AL) and primary rodent astrocytes (kindly provided by Dr. Earl Ellis, Department Pharmacology and Toxicology, Virginia Commonwealth University) were cultured in DMEM supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and 1% penicillin/streptomycin. Cells were incubated in humidified atmosphere of 5% CO\textsubscript{2} at 37°C.

Recombinant Ad Infection. The Ad.mda-7 and control adenoviral vectors used were identical to those described previously (19–21). The viral titers for each Ad and infection efficiency for each cell type were determined by plaque formation assay. In vitro adenoviral infections were performed 24 h after plating (35). 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% CO\textsubscript{2}/95% air with gentle agitation. After 3 h, fresh growth medium with 10% fetal bovine serum was added.

Assessment of Apoptosis and Cell Viability. The extent of apoptosis was evaluated by assessing Wright-Giemsa stained cytospin 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 at least 15,000 cells (36). To confirm the results of morphological analysis, in some cases, cells were also evaluated by terminal deoxynucleotidyltransferase-mediated nick end labeling staining and oligonucleosomal DNA fragmentation assay as follows: staining, cytospin slides were fixed with 4% formaldehyde/PBS for 10 min, treated with acetic acid/ethanol (1:2) for 5 min, and incubated with terminal transferase reaction mixture containing 1X terminal transferase reaction buffer, 0.25 units/liter terminal transferase, 2.5 mM CoCl\textsubscript{2}, and 2 pmol fluorescein-12-dUTP (Boehringer
Mannheim, Indianapolis, IN) at 37°C for 1 h. The slides were mounted with Vectashield containing propidium iodide (Vector Laboratories, Burlingame, CA) and visualized using fluorescence microscopy (37).

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 (36). Floating and attached cells were isolated by trypsinization, recovered by centrifugation, resuspended in phenol red-free DMEM, and mixed 1:1 with trypan blue reagent. Cells (×400) were counted in all four fields of a hemocytometer.

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. Cells were plated (5–10,000 cells/well of a 12-well plate) and 24 h after plating infected with either Ad.mda-7 or control virus at the indicated MOI. In other experiments, 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. Twenty-four h after infection/protein treatment, cells were treated with kinase inhibitor drugs and then irradiated. 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 (38). The plates were read on a Dynatech MR600 Microplate Reader at 540 nm. All data were normalized relative to the control, nontreated unirradiated cells of the corresponding cell type.

Cell Survival Analyses. Cells were assayed for the effect(s) of Ad.mda-7 and radiation on cell survival. Cells were plated (10,000 cells/60-mm dish) and 24 h after plating infected with either Ad.mda-7 or control virus at the indicated MOI. Twenty-four h after infection, cells were irradiated. Ninety-six h after irradiation, cells were isolated by trypsinization and viable trypan blue negative cells replated in 60-mm dishes at 250-1000 cells/plate. Colonies were allowed to from surviving cells for 7–9 days before fixing and staining with crystal violet. Colonies that contain >50 cells were then counted. To generate the survival data, individual assays were performed at multiple dilutions with a total of 4 plates/data point.

Fig. 1 Ad.mda-7 suppresses glioma cell growth and enhances radiosensitivity. Glioma cells were cultured for 24 h after plating then infected with Ad.mda-7 or CMV control viruses at the following MOIs: A, RT2 cells, 5 MOI; B, RT2 cells, 25 MOI; C, RT2 cells, 50 MOI; D, primary rodent astrocytes, 50 MOI. The cells were irradiated, as indicated, 24 h after infection. MTT assays were performed 4 days after radiation as described in "Materials and Methods." The values were normalized to the control unirradiated cells, which is defined as 1.00. Data are the means of 12 data points ± SE from a representative experiment (n = 3, *, P < 0.05 less than corresponding control value when corrected for the growth suppressive effects of Ad.mda-7 alone).
Western Blot Analysis. Protein concentration was determined using a kit from Bio-Rad. Aliquots (40 μg) were solubilized in Laemmlli buffer, separated by SDS-PAGE, and transferred to nitrocellulose membranes as described. 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 × 15 min with TBST). The following antibodies were used: mouse anti-BCL-XXL, BAX, and BCL-2 monoclonal antibody; mouse anti-PARP and anti-β-actin (Santa Cruz Biotechnology); anti-p53 [polyclonal antibody (Oncogene Research Products, Cambridge, MA)]; and mouse anti-Fas antibody (Pharmigen, San Diego, CA). Membranes were incubated with HRP-conjugated antimouse or antirabbit IgG antibody, followed by washing with TBST (3 × 15 min). Proteins were visualized by enhanced chemiluminescence and quantified by densitometry.

DNA Fragmentation. Equal number of cells from each test sample (10⁶) were homogenized with 1 ml of lysis buffer (10 mM Tris at pH 7.4, 5 mM EDTA, and 1% Triton X-100). RNase A (100 μg/ml) was added to each sample and incubated at 50°C for 1 h. Proteinase K was then added (100 μg/ml), and the samples were incubated overnight for 50°C. The DNA was extracted using phenol and chloroform and centrifuged at 10,000 x g for 5 min at 4°C. The aqueous phase mixed with 2 volumes of ice-cold ethanol and then precipitated by centrifugation at 15,000 x g for 10 min, supernatants were removed, and DNA pellets were washed with 80% ethanol once (15,000 x g for 10 min), air-dried, and dissolved in Tris-EDTA buffer at pH 7.6. DNA concentration was determined, and 10 μg 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 of <0.05 were considered statistically significant. For animal studies, a log-rank test was applied to data. Experiments shown are the means of multiple individual points (±SE).

RESULTS

Ad.mda-7 Enhances the Radiosensitivity of RT2 as Measured in MTT Assays. RT2 cells were infected with increasing amounts of Ad.mda-7 or control virus and the expression of MDA-7 determined 48 h after infection. Increasing the viral particle MOI enhanced the amount of MDA-7 protein produced in each cell (data not shown).

Previous studies have shown that infection of tumor cells, but not nontransformed cells, with Ad.mda-7-inhibited tumor cell growth (16–25, 31, 29). To assess the effect of Ad.mda-7 on the growth and survival of RT2 glioma cells, we assayed cells for proliferation via MTT assay and viability via trypan blue staining after Ad.mda-7 infection and radiation exposure. Cells were plated, infected, and irradiated 24 h after infection with increasing radiation doses (Figs. 1, A–D). Increasing the viral MOI resulted in a dose-dependent reduction in glioma cell growth. Furthermore, although radiation reduced proliferation, it interacted with Ad.mda-7, but not control virus, to further reduce cell growth. These effects were not observed in either primary rodent astrocytes (Fig. 1D) or primary human astrocytes (data not shown; Ref. 29).

In general agreement with the findings in Fig. 1, Ad.mda-7 enhanced cell death, as judged by trypan blue staining, which was significantly increased after radiation exposure (Table 1). These effects were not observed in primary rodent astrocytes (Table 1). In contrast, cell survival, as measured by nuclear apoptotic morphology, was more weakly enhanced by the Ad.mda-7 + radiation combination. Expression of MDA-7 or irradiation enhanced the levels of apoptotic cells from 0.7 ± 0.2 to 1.8 ± 0.2 and 1.3 ± 0.1%, respectively. However, combined treatment of cells with Ad.mda-7 and radiation increased the percentage of apoptotic cells to 5.0 ± 0.4% (P < 0.05 greater than the combined effects of Ad.mda-7 and radiation individually).

Purified GST-MDA-7 Protein Has Similar Growth Inhibitory and Radiosensitizing Effects as Ad.mda-7. To confirm that our findings in Fig. 1 and Table 1 were not attributable to an effect related to viral infection, RT2 cells were treated with bacterially synthesized GST-MDA-7 or GST (Fig. 2) followed by exposure to ionizing radiation (29). GST-MDA-7, but not GST, caused a dose-dependent reduction in RT2 cell growth (Fig. 2A, data not shown); however, only at concentrations of GST-MDA-7 > 15 nm was any increase in cell killing by GST-MDA-7 observed. In agreement with data in Fig. 1, GST-MDA-7 suppressed the growth of RT2 cells that was enhanced in a greater than additive fashion by ionizing radiation (Fig. 2B). These findings also correlated with enhanced cell killing as judged by Giemsa staining (Fig. 2C, left set of bars) and by trypan blue staining (Fig. 2C, right set of bars).

Expression of MDA-7 Down-Regulates Expression of the Mitochondrial Antiapoptotic Molecule BCL-XXL. The BCL-2 gene family consists of both positive and negative regulators of apoptosis and interactions between these molecules can modulate the apoptotic threshold for a wide variety of noxious stimuli. Two family members in particular, BCL-2 and BCL-XXL, function as potent inhibitors of cell death and have

Table 1 Percentage of viable cells as determined by trypan blue exclusion staining

<table>
<thead>
<tr>
<th>Cell type/multiplicity of infection</th>
<th>Ad.CMV</th>
<th>Ad.mda-7</th>
<th>Ad.CMV + 6 Gy</th>
<th>Ad.mda-7 + 6 Gy</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT2 (MOI 5)</td>
<td>3.3 ± 1.7</td>
<td>7.0 ± 1.8</td>
<td>6.3 ± 1.7</td>
<td>18.1 ± 3.4,a,b</td>
</tr>
<tr>
<td>RT2 (MOI 25)</td>
<td>3.8 ± 1.6</td>
<td>11.9 ± 2.5a</td>
<td>8.0 ± 3.2</td>
<td>33.9 ± 3.6,a,b</td>
</tr>
<tr>
<td>RT2 (MOI 50)</td>
<td>3.7 ± 1.8</td>
<td>18.7 ± 1.2a</td>
<td>6.2 ± 2.3</td>
<td>47.4 ± 3.3,a,b</td>
</tr>
<tr>
<td>Primary astrocyte (MOI 50)</td>
<td>3.7 ± 0.9</td>
<td>3.6 ± 1.1</td>
<td>7.9 ± 2.1a</td>
<td>8.5 ± 2.1a</td>
</tr>
</tbody>
</table>

a P < 0.05 greater than control infected cells.

b P < 0.05 greater than irradiated cells, corrected for the toxic effects of Ad.mda-7.
been shown to enhance resistance to chemotherapeutic agents and ionizing radiation (39).

In parallel to studies in Fig. 1 and Table 1, the expression of pro- and antiapoptotic BCL-2 family members as well as the levels of FAS and tumor necrosis factor α receptors was determined by immunoblotting. Infection of RT2 cells with Ad. mda-7, but not control virus, reduced expression of BCL-XL and enhanced expression of BAX (Fig. 3A). Radiation did not alter BCL-XL expression but also enhanced BAX levels. The combination of Ad.mda-7 and radiation resulted in an additional modest increase in BAX expression and a large decrease in BCL-XL levels. Little alteration was observed in the expression of BCL-2, as well as the FAS and tumor necrosis factor α death receptors or their ligands (data not shown). The reduction in BCL-XL levels and enhancement in BAX expression also correlated with a reduction in p32 procaspase 3 levels and cleavage of PARP (data not shown).

As an additional separate measure of cell survival, we examined the integrity of nuclear DNA, 96 h after irradiation/120 h after viral infection. Irradiation and/or infection of cells with control virus did not significantly alter DNA integrity (Fig. 3B, two separate studies shown). However, infection of cells with Ad.mda-7 caused the appearance of a smear of lower molecular weight DNA as well as the appearance of low molecular weight DNA fragments that electrophoresed at or near the dye front, suggestive of necrosis. Irradiation of cells exhibiting Ad.mda-7-induced DNA degradation resulted in a large decrease in high molecular weight nucleosomal DNA and an increase in amount of the low molecular weight DNA fragments.

To test whether altered BCL-XL expression was causal in the modified proliferation and survival of glioma cells exposed to MDA-7 and radiation, RT2 cells were infected with Ad.BCL-XL, Ad.mda-7, or control virus and 24 h afterward irradiated. Cell growth and viability were determined 96 h after irradiation. Constitutive overexpression of Ad.BCL-XL abrogated the growth inhibitory effect of Ad.mda-7 and Ad.mda-7 + radiation (Fig. 3C). Overexpression of BCL-XL also reduced the antiproliferative effect of ionizing radiation. These findings correlated with a reduction in cell killing by Ad.mda-7 and Ad.mda-7 + radiation (Fig. 3D).

The Free Radical Scavenger NAC Blunted the Antiproliferative Interaction between Ad.mda-7 and Radiation. Ionizing radiation has been shown to enhance the production of mitochondria-derived free radicals that may be linked to altered cell signaling and survival processes (40). Expression of BCL-XL can, in part, reduce the generation of free radicals by mitochondria (40, 41). To determine whether radiation-induced free radicals interact with MDA-7 to alter cell viability a free radical-scavenging agent, NAC was added to infected RT2 cells prior radiation. NAC abolished the enhancement in cell survival of MDA-7-infected cells by up to 6 Gy of radiation (data not shown) and abrogated the antiproliferative effect of radiation (Fig. 3D).

Fig. 2 GST-MDA-7 reduces the proliferation of glioma cells and enhances radiation-induced cell killing. Cells were cultured for 24 h then treated with GST-MDA-7 or GST at the concentrations indicated, as described in “Materials and Methods.” As indicated, 24 h after GST-MDA-7 treatment, cells were irradiated (6 Gy). Cells were isolated 96 h after irradiation and cell numbers and viability determined by trypan blue exclusion staining and by Wright Giemsa staining of fixed cells. In parallel, cell numbers were also determined 96 h after irradiation by MTT assay. A, GST-MDA-7 inhibits the proliferation of RT2 cells in a dose-dependent fashion and enhances apoptotic cell death as judged by Giemsa staining for nuclear DNA fragmentation. B, GST-MDA-7 (0.5 nM) interacts with radiation in a greater than additive fashion to suppress RT2 cell growth in MTT assays. C, left set of bars, GST-MDA-7 (5.0 nM) interacts with radiation in a greater than additive fashion to enhance apoptotic cell killing as judged by Giemsa staining. Right set of bars, GST-MDA-7 (5.0 nM) interacts with radiation in a greater than additive fashion to enhance apoptotic cell killing as judged by trypan blue exclusion staining. Data are the means ± SE of three separate experiments; #, P < 0.05 greater than control infected cells; *, P < 0.05 less than control infected cells. © 2003 American Association for Cancer Research.
killing and reduction in proliferation of RT2 cells treated with Ad.mda-7 + radiation (Fig. 4A). This finding also correlated with a reduction in cell killing (Fig. 4B).

**Ad.mda-7 Enhances the Radiosensitivity of RT2 cells as Measured in Colony Formation Assays.** In Fig. 1, short-term MTT growth assays demonstrated that Ad.mda-7 interacted with radiation to cause a greater than additive reduction in proliferation that correlated with increased cell death. Additional studies were performed to determine whether Ad.mda-7 altered glioma cell colony formation after irradiation in vitro. Infection of cells with Ad.mda-7 (5 MOI) weakly reduced the colony formation of cells (0.89 ± 0.07), compared with control...
viral infection (1.00 ± 0.08), which was not significant. Radiation (6 Gy) caused a significant reduction in cell survival (0.29 ± 0.03). The combination of Ad.mda-7 and radiation reduced colony formation that was significantly greater than the additive effects of either treatment alone (0.02 ± 0.005, P < 0.05).

**DISCUSSION**

GBM is known to be relatively resistant to a variety of conventional anticancer therapies, including chemotherapy and radiotherapy. The studies in this manuscript were designed to examine the impact of the novel therapeutic cytokine MDA-7 (IL-24) on the growth and radiosensitivity of malignant glioma cells.

Expression of MDA-7, mediated by an adenoviral vector Ad.mda-7, reduced cell proliferation and enhanced cell death in RT2 glioma cells. This effect was not observed in primary rodent astrocytes. We have also discovered that expression of MDA-7 significantly inhibited the growth and enhanced the radiosensitivity of various human glioma cell lines, e.g., U251, U373, U87-MG, T98-G, without significant effect on primary human astrocytes (data not shown, in agreement with Ref. 29).

**Fig. 5** Ad.mda-7 prolongs animal survival and radiosensitizes RT2 cells in vivo. Cells were cultured for 24 h then infected with Ad.mda-7 or CMV control viruses (25 MOI) as described in “Materials and Methods.” Fischer 344 rats were implanted intracranially with infected RT2 cells, and 4 days after implantation, the head of each animal was irradiated. Animal survival was noted on a daily basis. Data are the total from four separate experiments of 4 animals/condition/experiment. Statistical analyses were performed using the log-rank test. ***, P < 0.05 greater than unirradiated animals; #, P < 0.01 greater than Ad.mda-7 alone animals.**

Ad.mda-7 Enhances the Survival of Rats Implanted Intracranially with RT2 Cells. The studies in this manuscript have used the RT2 rodent glioma cell line, in part, because it is syngeneic to the Fischer 344 rat (42). RT2 cells were infected with either control virus or Ad.mda-7 in vitro, and 24 h after infection, 10⁴ cells were implanted into the brains of Fischer 344 rats. Four days after implantation, the head of each rat was irradiated (6 Gy). The survival of the rats was noted on a daily basis. Rats implanted with control virus-infected cells, regardless of whether they were irradiated, died within ~15–20 days (Fig. 5). Rats implanted with Ad.mda-7-infected cells survived significantly longer than control virus alone or control virus + radiation animals (***, P < 0.05). Irradiation of rats implanted with Ad.mda-7-infected cells resulted in an additional significant increase in animal survival beyond that of Ad.mda-7 alone (#, P < 0.05).
Recent studies in lung carcinoma cells have noted that Ad.mda-7 can act as a radiosensitizer (28). In our studies using glioma cells, Ad.mda-7 and purified MDA-7 protein enhanced the growth inhibitory and cytotoxic effects of ionizing radiation. These effects correlated with reduced expression of the anti-apoptotic protein BCL-XL and enhanced expression of the pro-apoptotic protein BAX. Constitutive overexpression of BCL-XL abolished the toxic and growth inhibitory effects of Ad.mda-7, suggesting that MDA-7 inhibits the expression of anti-apoptotic BCL-2 family members, which, in turn, leads to mitochondrial dysfunction that initiates cell death processes. Incubation of cells with the free radical scavenger NAC also abolished the interaction between Ad.mda-7 and radiation, suggesting that radiation-induced free radicals interact with MDA-7 to enhance cell death. As radiation-induced free radicals are generated by the mitochondria (40), our findings argue that MDA-7- and radiation-induced alterations in mitochondrial function act in concert to promote cell death.

The modes of cell death induced by Ad.mda-7 and radiation were also investigated. On the basis of previous studies by our group (17, 18, 29) and our present findings with BCL-XL, we had expected that Ad.mda-7 and radiation would interact, primarily, to increase apoptosis. Although Ad.mda-7 and radiation caused procaspase 3 and PARP cleavage as well as significantly enhancing apoptosis in RT2 cells, the increase did not correspond to the large reduction in cell growth observed 96 h after irradiation. However, when cell viability was measured via trypan blue staining, the combination of Ad.mda-7 and radiation caused a large (>30% of total) 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 as a smear that subsequently resolved into small molecular weight fragments, rather than into a classical DNA ladder indicative of apoptosis. These findings suggest that the combination of Ad.mda-7 and ionizing radiation primarily promote cell death through a necrotic mechanism.

Enhanced cell numbers in G2-M phase of the cell cycle at the time of irradiation have been linked to increased radiosensitivity as has abolition of the radiation-induced G2-M cell cycle checkpoint (30, 43). Previously, MDA-7 has been linked to altered cell cycle progression (17, 20, 22, 28). Ad.mda-7 significantly enhanced cell numbers in G2-M phase in U251 human glioma cells that were additionally enhanced after radiation exposure (A. Yacoub, P. B. Fisher, and P. Dent, unpublished observations, see also Ref. 29). In contrast, Ad.mda-7 enhanced cell numbers in G1 phase in RT2 cells, which were additionally enhanced after radiation exposure (A. Yacoub, P. B. Fisher, and P. Dent, unpublished observations). In both cell types, the percentage of cells residing in S phase was almost abolished in the Ad.mda-7 + radiation treatment group. Both cell types were radiosensitized by Ad.mda-7. Thus in general agreement with the data of Kawabe et al. (28), these findings demonstrate that Ad.mda-7-induced alterations in cell cycle distribution are not a key mechanism by which radiosensitization occurs. In addition, as cells are depleted from the most radioresistant portion of the cell cycle by combined Ad.mda-7 + radiation treatment (43), our findings suggest that repeated radiation exposures may cause additional radiosensitization and cell killing via the synchronization of cells in G2-M and G1 phases.

The increase in BAX expression after infection of cells with Ad.mda-7 suggests that one mechanism of radiosensitization may be attributable to this proapoptotic protein. This concept is further supported by our findings using Ad.BCL-XL. To test whether BAX is an essential mediator of Ad.mda-7 radiosensitivity, we examined the impact of Ad.mda-7 on the radiosensitivity of the prostate carcinoma cell line DU145, which is BAX null. Ad.mda-7 as a single agent was more toxic to DU145 cells than to either RT2 or U251 cells; furthermore, Ad.mda-7 also radiosensitized DU145 cells (A. Yacoub, P. B. Fisher, and P. Dent, unpublished observations). These findings suggest that BAX is not required for Ad.mda-7-induced radiosensitization.

The studies in this manuscript used the RT2 rodent glioma cell line, in part, because it is syngeneic to the Fischer 344 rat (42). Animals implanted with RT2 cells infected in vitro with Ad.mda-7 survived longer than either control virus alone or control virus + radiation animals. Irradiation of rats implanted with Ad.mda-7-infected cells resulted in an additional increase in animal survival beyond that of Ad.mda-7 alone. Collectively, these findings demonstrate that Ad.mda-7 can increase the radiosensitivity of glioma cells in vivo and argue that Ad.mda-7 has the potential for clinical translation as a cytotoxic and radiosensitizing agent in patients. Additional studies will be required to understand the precise molecular mechanisms by which Ad.mda-7 and MDA-7 protein cause radiosensitization of glioma cells.

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Melanoma Differentiation-associated 7 (Interleukin 24) Inhibits Growth and Enhances Radiosensitivity of Glioma Cells \textit{in Vitro} and \textit{in Vivo}

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