Methioninase Cancer Gene Therapy with Selenomethionine as Suicide Prodrug Substrate[1]

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ABSTRACT

In this study, we report a novel approach to gene-directed enzyme prodrug therapy for cancer. This gene therapy strategy exploits the toxic pro-oxidant property of methylselenol, which is released from selenomethionine (SeMET) by cancer cells with the adenoviral-delivered *methionine α,γ-lyase* (*MET*) gene cloned from *Pseudomonas putida*. In MET-transduced tumor cells, the cytotoxicity of SeMET is increased up to 1000-fold compared with nontransduced cells. A strong bystander effect occurred because of methylselenol release from *MET* gene-transduced cells and uptake by surrounding tumor cells. Methylselenol damaged the mitochondria via oxidative stress and caused cytochrome *c* release into the cytosol, thereby activating the caspase cascade and apoptosis. Adenoviral *MET*-gene/SeMET treatment also inhibited tumor growth in rodents and significantly prolonged their survival. Recombinant adenovirus-encoding *MET* gene-SeMET treatment thereby offers a new paradigm for cancer gene therapy.

INTRODUCTION

A number of different strategies for cancer gene therapy have been developed; *e.g.,* the *p53* gene has been shown to inhibit tumor cell growth and induce apoptosis (1). The antitumor efficacy of the *p53* gene using retroviral (2), adenoviral (3), or lipid-based delivery (4) has been demonstrated. However, this strategy is limited only for tumors with defective p53. The herpes simplex virus *thymidine kinase* gene (HSV-*tk*) in combination with ganciclovir has also been shown to inhibit tumor growth (5). Tissue-specific promoters are now being used to target the expression of therapeutic genes to tumor cells (6–8). However, to date, gene therapy trials have shown very limited clinical efficacy (9) and have demonstrated recently (10) lethal toxicity. It appears, therefore, that other approaches to cancer gene therapy are necessary.

We have based our therapeutic strategy and target on methionine dependence, the elevated minimal methionine requirement for tumor cell proliferation, which is a key property of the majority of tumor cell types evaluated (11–19). There have been several therapeutic strategies to target the methionine dependence of cancer cells. Methionine starvation therapy such as with a methionine-free diet or methionine-depleted total parenteral nutrition prolonged the survival time of tumor-bearing rodents (20–22). Methionine-free total parenteral nutrition in combination with chemotherapeutic drugs showed preliminary evidence that methionine restriction enhanced the activity of chemotherapy of patients with high-stage gastric cancer (23).

rMETase[3] cloned from *Pseudomonas putida* (24, 25), which degrades methionine to α-ketobutyrate, ammonia, and methanethiol, has antitumor efficacy *in vitro* and *in vivo* (26, 27). rMETase has synergistic efficacy in combination with 5-fluorouracil (26) and cisplatin (27) on rodent and human tumors in mouse models.

Recently, we have introduced the *P. putida methioninase* (*MET*) gene (24, 25) into human lung cancer cells using a retroviral vector (28). We demonstrated prolonged survival of mice orthotopically implanted with the *MET* gene-transformed lung tumor cells in combination with rMETase treatment, compared with animals with the lung tumor cells without the *MET* gene. However, the relatively low transduction efficiency of retroviruses makes it difficult to fully explore the potential of the *MET* gene as a therapeutic strategy.

To increase transduction efficiency, we have constructed a recombinant adenoviral vector with the *MET* gene (rAdMET). rAdMET infection of the cancer cells resulted in methioninase expression of up to approximately 10% of the total cellular protein. The interaction between rAdMET and rMETase was evaluated for synergy on human ovarian carcinoma cells *in vitro*. The combination indices (29) of rMETase and rAdMET tested together were less than 0.7, indicating strong synergy. In contrast, the combination indices of all of the combinations of rMETase and control adenovirus had only an additive effect (30).

Selenols or diselenides catalyze oxidation of thiols (31) at ambient pO_2 to generate toxic ROS such as superoxide (32, 33). SeMET cannot be converted to selenol by mammalian cells and is relatively nontoxic (34, 35). However, rMETase catalyzes an α,γ-elimination reaction of SeMET to methylselenol, α-ketobutyrate, and ammonia (36). We have taken advantage of the production of methylselenol from SeMET by the *MET*-gene product to design a gene-directed enzyme prodrug therapy system for cancer.

MATERIALS AND METHODS

Recombinant Adenoviruses. The construction and propagation of recombinant adenovirus rAdMET, in which the *MET* gene is driven by the CMV-5 promoter and enhancer, has been described (30).

IC_{50} Determination of rAdMET-SeMET. Human cancer cell lines were tested for the *in vitro* efficacy of rAdMET-SeMET. Cytotoxicity was evaluated using the MTT assay as described (37). Briefly, cells (4×10^3) were seeded in 96-well plates and transduced with rAdMET or control-rAd. Twenty-four h after transduction, SeMET was added. After 3 days incubation, the medium was replaced with 0.5 mg/ml MTT for 2 h. The supernatants were removed, and 200 μl of isopropanol were added to each well, each of which was measured for absorbance at 540 nm in a microtiter plate reader (Bio-Rad Laboratories, Hercules, CA). IC_{50} values were determined by the median-effect equation (29).

[3] The abbreviations used are: rMETase, recombinant L-methionine α-γ-lyase; SeMET, selenomethionine, MET, *methionine α-γ-lyase* gene; rAd-MET, recombinant adenovirus encoding *MET* gene; control-rAd, recombinant E1 E3 depleted adenovirus; GSH, reduced glutathion; MOI, multiplicity of infection; ROS, reactive oxygen species; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SOD, superoxide dismutase; CL, chemiluminescence; pfu, plaque-forming unit(s).

Flow Cytometry. A549 human lung cancer cells were transduced (MOI, 50) with rAdMET and were incubated with SeMET (20 μM) 24 h later. At each time point in Fig. 2A, cells were harvested and fixed with 70% ethanol and stained with propidium iodide in staining buffer [PBS (pH 7.4) containing 0.1% Triton X-100, 0.1 mM EDTA, 0.05 mg/ml RNase A, and 50 μg/ml propidium iodide]. The DNA content of the stained cells was evaluated with FACSscan (Becton Dickinson, San Jose, CA).

Nucleosomal DNA Fragmentation. A549 cells were transduced with rAdMET (MOI, 50). Twenty-four h later, SeMET (20 μM) was added. At each time point in Fig. 2B, cells were harvested and genomic DNA was isolated and electrophoresed on 2% agarose gels and stained with ethidium bromide.

Lucigenin-dependent CL. A549 cells (1×10^6 cells) were transduced with rAdMET (MOI, 50). Twenty-four h later, cells (1×10^6) were incubated in 1 ml of RPMI with various concentrations of SeMET with or without 10 units of SOD for 30 min. Lucigenin (50 μg) was added to the cell suspension just before the measurement. Integrated CL was measured for 10 s three times with a TD21 Luminometer (Turner Designs, Sunnyvale, CA). Results are shown as the fold increase in CL relative to untreated control.

GSH Measurement. A549 cells (2×10^6) were transduced with rAdMET or control-rAd (MOI, 20). Twenty four h later, SeMET (20 μM) was added to the cells. At the indicated time points in Fig. 3B, cells were harvested, homogenized in phosphate-EDTA buffer, and centrifuged at $10,000 \times g$. After determination of protein levels of supernatants using the Lowry Reagent Kit (Sigma Chemical Co., St. Louis, MO), GSH contents in supernatants were measured fluorometrically using o-phthaldialdehyde (350 nm excitation and 420 nm emission) as described (38). Results are shown as μg GSH/mg protein.

Mitochondrial Membrane Potential. A549 cells were transduced with rAdMET (MOI, 20). Twenty-four h later, SeMET (20 μM) was added. After a 24-h incubation, mitochondrial membrane potential changes were monitored with the fluorescent dye, Mitosensor (Clontech, Palo Alto, CA), according to the manufacturer's instructions.

Cytochrome c Detection in Cytosolic Fraction. Cells (2×10^7) were harvested in 500 μl of isotonic buffer (210 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM HEPES, and 1 mM DTT with 1 μg/ml each of chymostatin, leupeptin, antipain, and pepstain) and homogenized with a Dounce homogenizer. Samples were centrifuged at $900 \times g$ to remove nuclei, which was followed by centrifugation at $10,000 \times g$ for 30 min at 4°C to remove the heavy membrane fraction. The supernatants containing the cytosolic fraction were used for Western blotting of cytochrome c. Cytosolic protein extracts (40 μg) were subjected to 10–20% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and incubated with the antibody against cytochrome c (Clontech), followed by enhanced CL detection (Amersham, Piscataway, NJ).

Caspase Measurements. Intracellular caspase-3, caspase-8, and caspase-9 were assayed by the ApoAlert Fluorescent assay kit (Clontech). A549 cells were treated with rAdMET (MOI, 20)/SeMET (20 μM). Cell lysates were incubated with the following substrates: DEVD-AFC (caspase-3), IETD-AFC (caspase-8), and LEDH-AMC (caspase-9) for 1 h. The specific activities were determined fluorometrically at 400-nm excitation and 505-nm emission for caspase-3 and caspase-8 and at 380-nm excitation and 460-nm emission for caspase-9. Results are shown as the fold increase in activity relative to untreated controls.

Electron Microscopy. A549 cells treated with rAdMET (MOI, 50) and SeMET (20 μM) were fixed with 3% glutalaldehyde in PBS (pH 7.4) followed by postfixation with aqueous 1% osmium tetroxide and 2% uranyl acetate. After dehydration, cells were embedded in resin. Ultrathin sections after counterstaining were visualized with an electron microscope (D7082 EM109/ EM109R; Zeiss, Jena, Germany).

Mouse Experiments. N1S1 rat hepatoma tumor cells (1×10^6 cells) were implanted into the peritoneal cavities of female nude mice. Two days after implantation, rAdMET (5×10^8 pfu), control-rAd (5×10^8 pfu), or mock controls were injected every other day for a total of five doses. Mice were also treated i.p. with either SeMET (1 μmol/mouse) or normal saline as a control, every day from day-3 to day-13. Ascites tumor growth was evaluated by body weight gain. All of the animal studies were conducted in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals under assurance number A3873-1.

RESULTS AND DISCUSSION

Cellular Toxicity of the Ad-MET and SeMET Combination. The addition of subtoxic levels of SeMET (20 μM) resulted in approximately a 100-fold enhancement of the toxicity of rAdMET to human tumor cells *in vitro* (Fig. 1A) attributable to the release of toxic
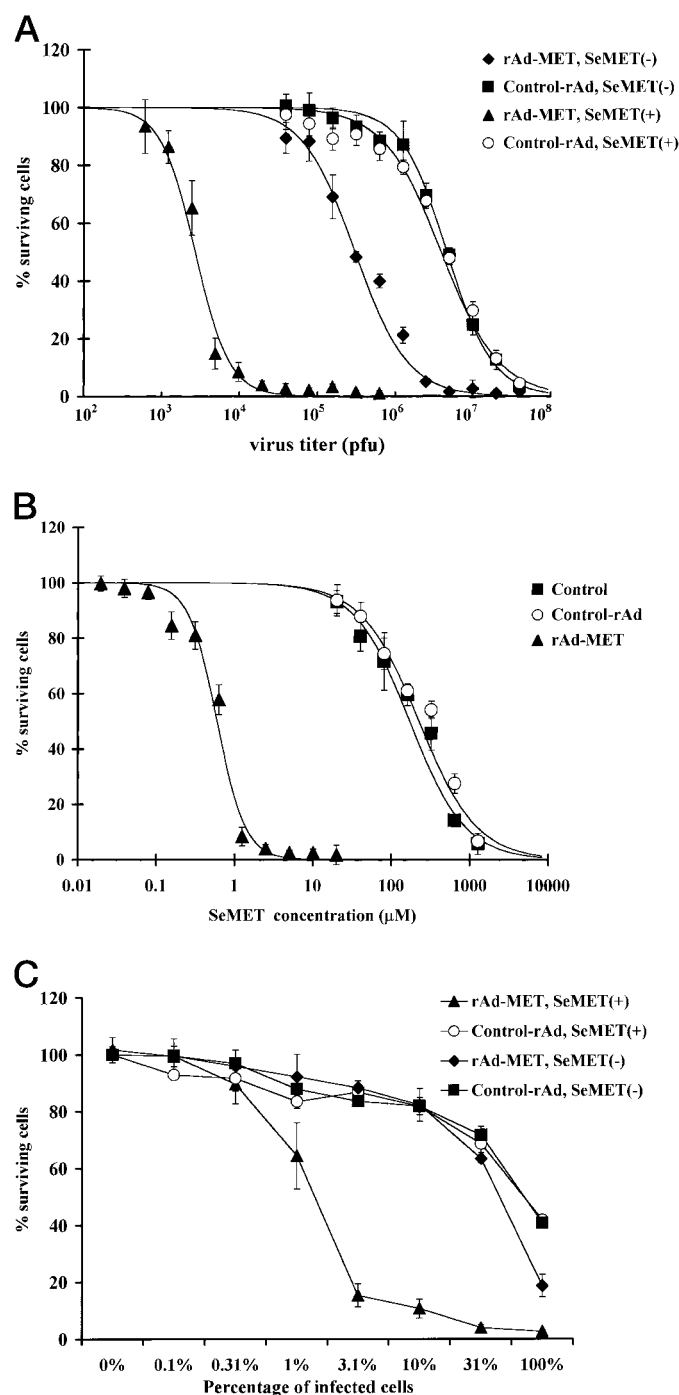

Fig. 1. A, efficacy of the combinations of rAdMET and SeMET. OVCAR-8 cells (4×10^3) were transduced with rAdMET or control-rAd at various titers with and without SeMET (20 μM). Cytotoxicity was measured by MTT reduction after 3 days of incubation. Approximately 100-fold enhancement of cytotoxicity of Ad-MET was seen. B, induction of SeMET cytotoxicity by rAdMET. OVCAR cells transduced with either rAdMET or control-rAd (10^5 pfu/well) and were treated with various concentrations of SeMET for 3 days. Cytotoxicity was measured by the MTT reaction. See "Materials and Methods" for details. C, bystander effect of rAdMET-SeMET. A549 cells, transduced with rAdMET or control-rAd (MOI, 50), were cocultured with nontransduced cells at different ratios with or without SeMET (20 μM). Approximately 80% of the cells in the culture, containing only 3% Ad-MET transduced cells, were killed as determined by the MTT assay.
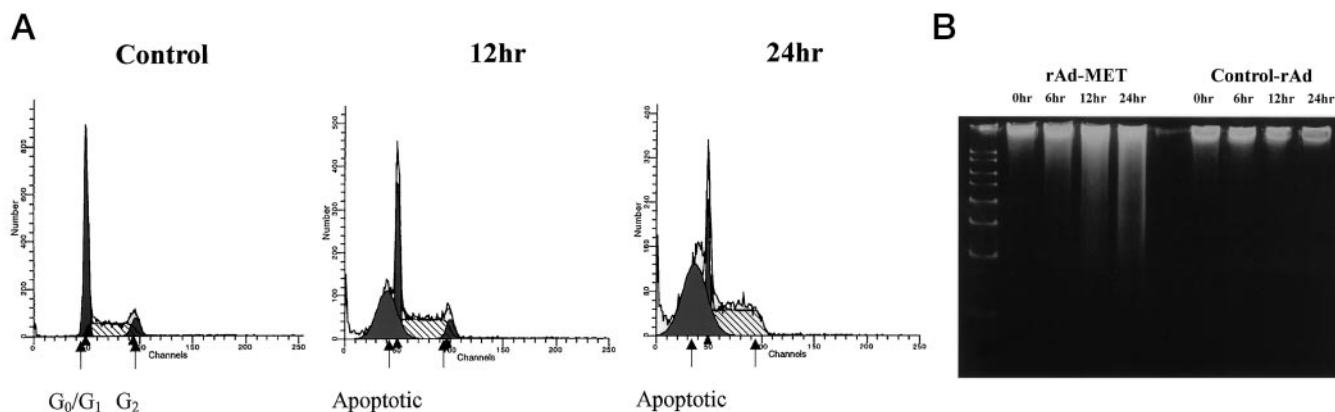
Fig. 2. Induction of apoptosis by rAdMET-SeMET. *A*, FACSscan. rAdMET-transduced A549 cells were incubated with SeMET (20 μM). Cells were harvested and fixed with 70% ethanol and stained with propidium iodide (50 μg/ml). The DNA content of the stained cells was evaluated with FACSscan. The sub-G_o population indicates apoptotic cells. *B*, nucleosomal DNA fragmentation. A549 cells were treated with rAdMET-SeMET as in *A*. Nucleosomal DNA was extracted and electrophoresed on 2% agarose gels and stained with ethidium bromide.

methylselenol by the rAdMET gene product, rMETase, as seen in reaction 1.

The IC_{50} values of SeMET for nontransduced or control-rAd-transduced human tumor cells (MOI, 50) were between 100 and 1000 μM. In contrast, the IC_{50} values of SeMET on rAdMET-transduced (MOI, 50) cells were greatly reduced to 0.3 to 5 μM. Thus, rAdMET increased the toxicity of SeMET up to 1000-fold in tumor cells (Fig. 1*B*; Table 1). The combination was highly toxic on all of the human tumor cell lines tested, including A549 lung cancer cells, OVCAR-8 ovarian cancer cells, Mia-PaCa and BxPC3 pancreatic cancer cells, Hep-2 head and neck cancer cells, and EKVX lung cancer cells.

Bystander Effect of rAdMET-SeMET. To evaluate the bystander effect of the *MET*-gene/SeMET combination, we cocultured nontransduced A549 cells with rAdMET-transduced A549 cells (MOI, 50) at various ratios. The administration of SeMET (20 μM) to cultures containing just 3% rAdMET-transduced cells resulted in the killing of more than 80% of the total cells in the culture (Fig. 1*C*). This strong bystander effect was thought to be caused by the diffusion of methylselenol from the *MET*-gene-transduced cells to the nontransduced cells.

Apoptosis Induction by rAdMET-SeMET. Twelve h after exposure to SeMET, sub-G_0 populations of A549 cells appeared, indicating apoptosis (Fig. 2*A*). Nucleosomal DNA fragmentation also occurred in the rAdMET-SeMET-treated A549 cells (Fig. 2*B*). To investigate the mechanism of rAdMET-SeMET induction of apoptosis, we first determined the extent of ROS generation as measured by lucigenin-dependent CL (39). The level of CL in rAdMET-treated A549 cells was dependent on the SeMET concentration (Fig. 3*A*). At 160 μM SeMET, CL was 100-fold higher than control and was completely inhibited by addition of SOD. In contrast, nontransduced cells did not produce ROS, even at the highest concentration of SeMET tested (Fig. 3*A*). It is thought that extracellular superoxide
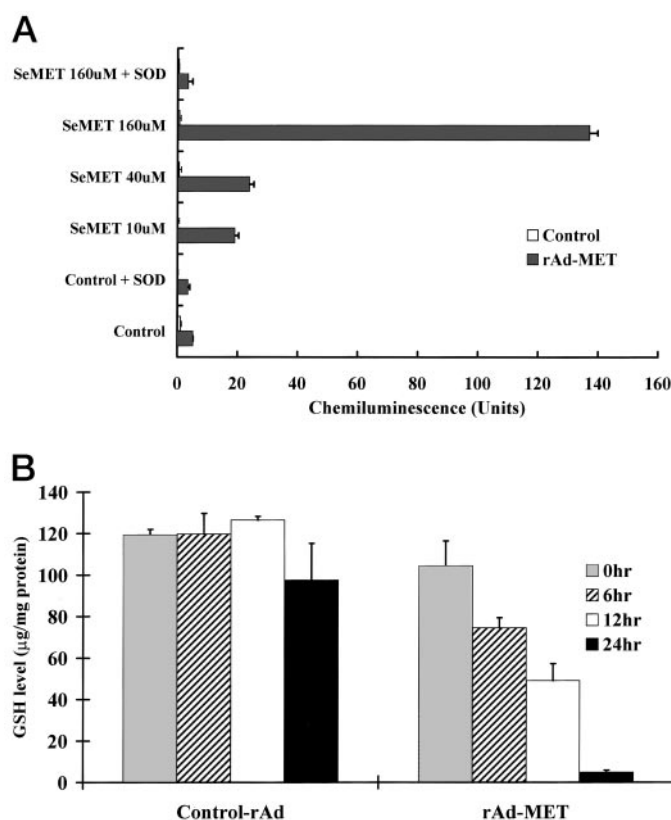

Fig. 3. *A*, induction of superoxide production by rAdMET-SeMET. Superoxide production in rAdMET-SeMET-treated cells was measured by lucigenin-dependent CL using a TD21 Luminometer (Turner Designs, Sunnyvale, CA). rAdMET-transduced A549 cells (1×10^6) were incubated in 1 ml of RPMI medium in various concentrations of SeMET with or without 10 units of SOD for 30 min. Lucigenin (50 μg) was added to the cell suspension just before the measurement. Integrated CL was measured for 10 s. Data shown are expressed as fold increase of untreated control. The level of CL (and hence superoxide production) in rAdMET-treated cells was dependent on the SeMET concentration and was inhibited by SOD. *B*, depletion of glutathione by rAdMET-SeMET. Cells (2×10^6) transduced with rAdMET or control-rAd (MOI, 20) were treated with SeMET (20 μM) 24 h later. Cell lysates, obtained at the time periods indicated, were incubated with 0.05% *o*-phthaldialdehyde for 15 min. GSH was measured fluorimetrically using 350 nm excitation and 420 nm emission. There was a marked reduction in GSH levels in treated cells after 24 h, suggesting its involvement in redox reactions with methylselenol. GSH content is shown as μg/mg protein (mean $\pm$ SD).

Table 1 *Comparison of SeMET IC_{50} in human tumor cell lines transduced with rAdMET*

The indicated cell lines were treated *in vitro* with rAdMET or control-rAd or were untreated (wild-type). The cells were then treated with SeMET and cytotoxicity determined with the MTT assay. See "Materials and Methods" for details.

Cell lines	SeMET IC_{50} (μM)		
	rAdMET	Control-rAd	Wild Type
OVCAR-8	0.59	170.3	223.6
A549	0.5	327.1	485.9
Hep-2	1.66	387	411
MIA PaCa-2	3.86	338	422
BxPC-3	3.38	222.6	311.8
EKVX	1.66	689.4	1360

released from the treated cells was detected by lucigenin. Detection of CL started immediately after adding lucigenin, suggesting that reaction between lucigenin and superoxide occurred in the cell-culture medium. Therefore, SOD probably depleted extracellular superoxide.
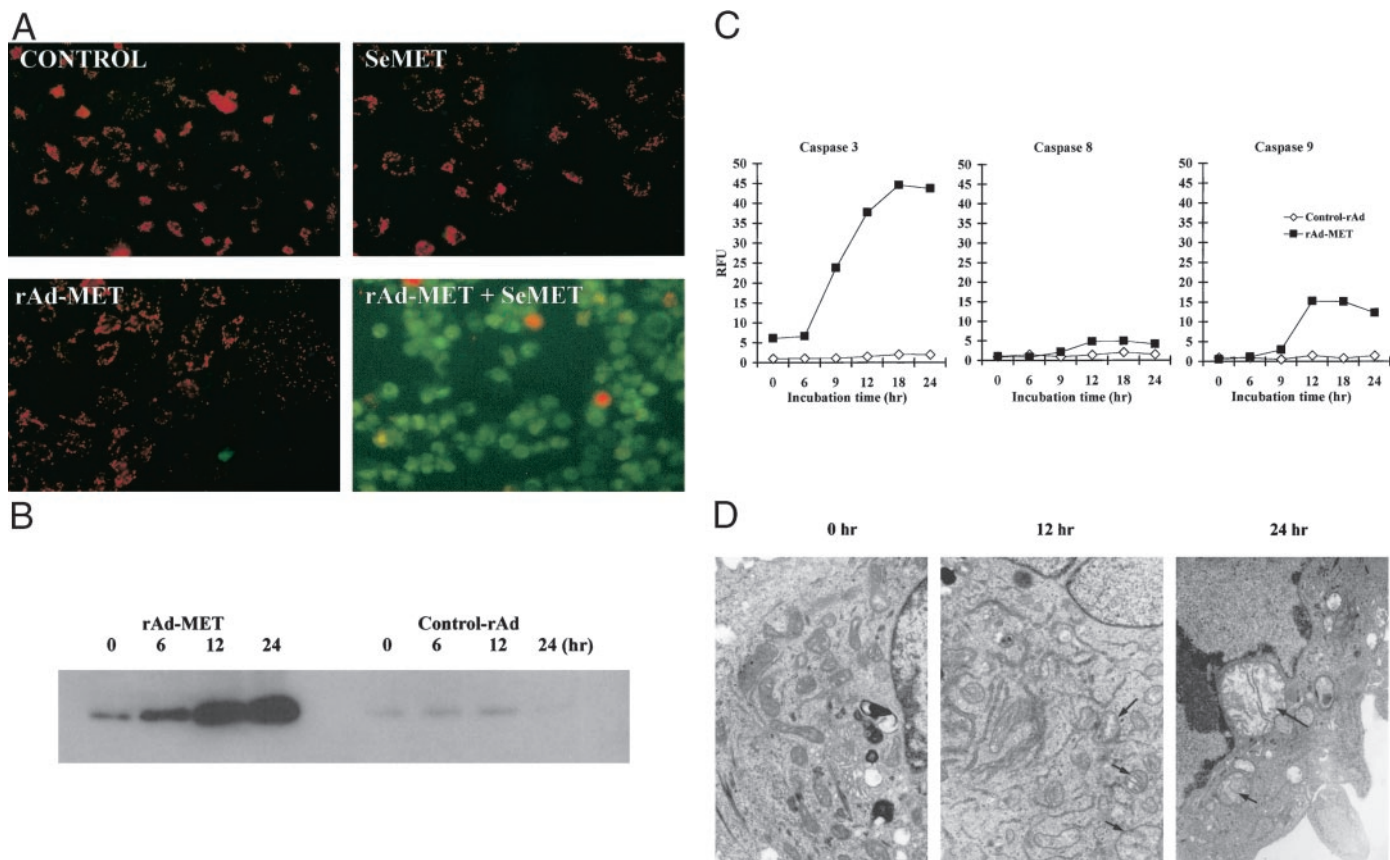
Fig. 4. *A*, mitochondrial membrane potential alteration in rAdMET-SeMET-treated cells. A549 cells were transduced with rAdMET (MOI, 20). Twenty-four h later, SeMET (20 μM) was added. After 24 h, mitochondrial membrane potential changes were monitored with the fluorescent dye Mitosensor (Clontech). Mitosensor is normally taken up in the mitochondria, where it forms aggregates that exhibit intense red fluorescence. In rAdMET-SeMET-treated cells, Mitosensor cannot accumulate in the mitochondria because of altered mitochondrial membrane permeability. As a result, the dye remains in monomeric form in the cytoplasm, where it fluoresces green. *B*, cytochrome *c* release in cytosol in rAdMET-SeMET-treated cells. Cytosols from cells treated with rAdMET-SeMET were measured by Western blotting using an anti-cytochrome *c* antibody (Clontech). Cells were homogenized in isotonic buffer with a Dounce homogenizer. The cytosolic fraction of the cell lysates was obtained by centrifugation at $10,000 \times g$ for 30 min at 4°C. The time-dependent release of cytochrome *c* was observed 6 h after addition of SeMET (20 μM) to rAdMET-transduced cells. Only a trace amount of cytochrome *c* was detected in control-rAd-transduced cells. *C*, activation of intracellular caspases in rAdMET-SeMET-treated cells. Caspase activity was assayed with the ApoAlert Fluorescent assay kit (Clontech). Cell lysates were incubated with DEVD-AFC, substrate for caspase-3, IETD-AFC, substrate for caspase-8, and LEDH-AMC, substrate for caspase-9, for 1 h. The specific activities were determined fluorometrically at 400-nm excitation and 505-nm emission for caspase-3 and caspase-8 and at 380-nm excitation and 460-nm emission for caspase-9. Results are shown as fold increase in activity relative to untreated control. *D*, mitochondrial swelling in rAdMET-SeMET-treated cells. rAdMET (MOI, 50)/SeMET (20 μM)-treated A549 cells were fixed with 3% glutalaldehyde in PBS (pH 7.4) followed by postfixation with aqueous 1% osmium tetroxide and 2% uranyl acetate. After dehydration, cells were embedded in resin. Ultrathin sections, after counterstaining, were viewed and photographed with an electron microscope (D7082 EM109/EM109R; Zeiss, Jena, Germany). Mitochondrial swelling was prominent after 12 h of treatment. After 24 h, most of the mitochondria were extremely swollen (*arrows*). All of the micrographs are shown at the same magnification.

ROS, especially the superoxide anion, may be formed during the oxidation of methylselenol by ambient dioxygen (33), followed by the oxidation of glutathione by the oxidized diselenide, which is retransformed to methylselenol (33). Indeed, the rAdMET-SeMET-treated cells had marked depletion of GSH in contrast to control-rAd/SeMET-treated cells (Fig. 3*B*).

rAdMET-SeMET-induced Mitochondrial Permeability and Release of Cytochrome *c*. The mitochondrial permeability transition is activated by oxidants, which causes mitochondrial swelling and loss of membrane potential (40, 41), resulting in mitochondrial cytochrome *c* release to the cytosol. The released cytochrome *c* binds to Apaf-1 and activates caspase-9 (42), which in turn activates downstream caspases and induces apoptosis (40–43). rAdMET-SeMET induces this apoptotic pathway in A549 human lung adenocarcinoma cells. Mitochondrial membrane potential is lowered by rAdMET-SeMET treatment but not by rAdMET or SeMET alone, as determined with the Mitosensor fluorescent dye (Clontech; Fig. 4*A*). A significant release of cytochrome *c* to the cytosolic fraction was observed beginning at 6 h after SeMET treatment, and it increased in a time-dependent manner (Fig. 4*B*). rAdMET-SeMET activated caspase-9 and caspase-3 in the treated cells up to 15-fold and 45-fold of

untreated control, respectively. Caspase-8 was activated only 5-fold (see Fig. 4*C* for representative results). Control-rAd/SeMET did not activate these caspases. Mitochondrial swelling was visualized by electron microscopy in rAdMET-SeMET-treated cells after 12 h (Fig. 4*D*).

These results suggested that induction of apoptosis by *MET*-gene/SeMET is caused by ROS generation from methylselenol, resulting in mitochondrial damage to release cytochrome *c* and activate the apoptotic cascade.

***In Vivo* Efficacy of rAdMET-SeMET.** Treatment of rat hepatoma N1S1 ascites in nude mice with rAdMET-SeMET inhibited ascites tumor growth as measured by body weight gain relative to similar nontumor-bearing animals (Fig. 5, *A* and *B*). rAdMET-SeMET significantly prolonged their survival with three of five rAdMET-SeMET animals alive at day-72. In contrast, all of the rAdMET- or SeMET-only treated mice were dead by day-24 (Fig. 5*C*).

The rAdMET-SeMET combination is a novel approach to cancer gene therapy exploiting the production of the apoptosis-inducing methylselenol from SeMET in rAdMET-transduced cancer cells. This approach is thus a new paradigm for cancer gene therapy. Selective
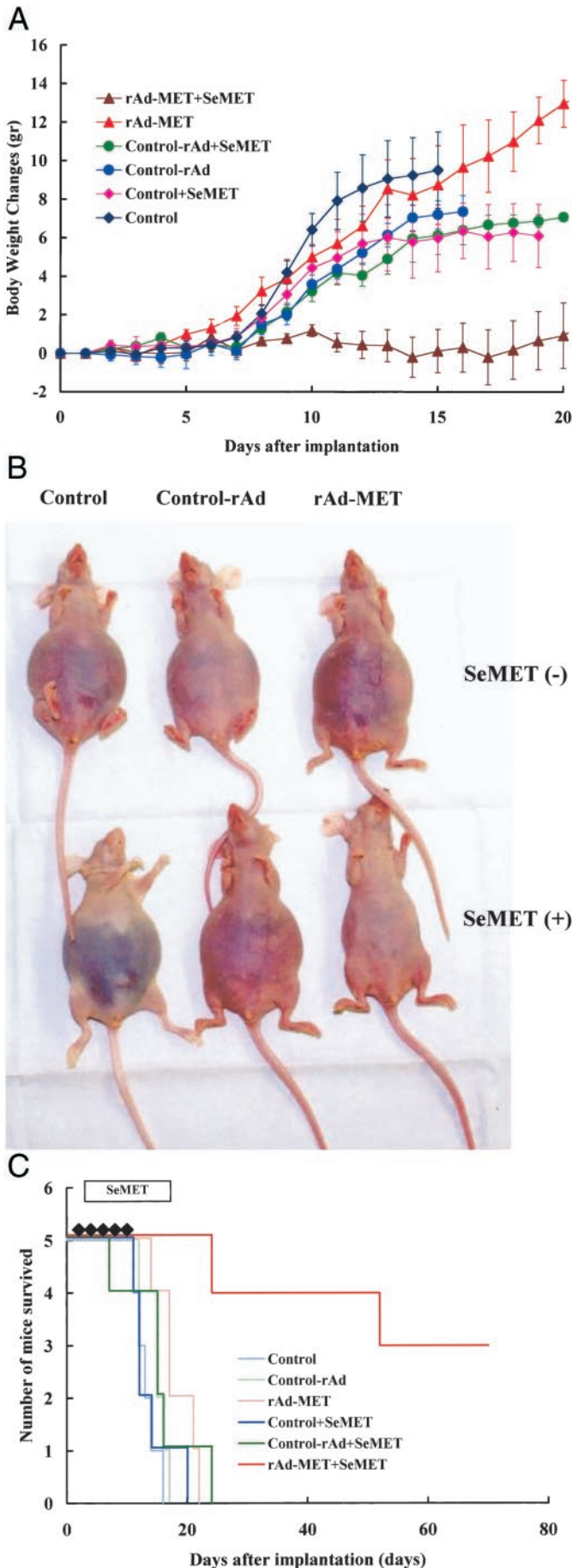
expression and delivery systems can now be developed with the goal of clinical application.

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Fig. 5. In vivo efficacy of rAdMET-SeMET. N1S1 rat hepatoma tumor cells (1×10^6 cells) were implanted into the peritoneal cavity of female nude mice. Two days after implantation, either rAdMET (5×10^8 pfu), control-rAd (5×10^8 pfu), or mock control were injected every other day for a total of five doses. Mice were treated i.p. with either SeMET (1 μmol/mouse) or normal saline as a control every day from day-3 to day-13. Ascites tumor growth was evaluated by body weight gain. A, determination of ascites tumor growth by body weight gain. Data are shown as the mean and SE. Only the combination of rAdMET-SeMET inhibited ascites growth. B, photographs of rAdMET-SeMET-treated mice compared with controls. Representative mice at day-10 after tumor implantation. rAdMET-SeMET-treated mice had no ascites. In contrast, other mice developed significant ascites. C, survival efficacy of rAdMET-SeMET-treated mice compared with controls.

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