Methioninase Cancer Gene Therapy with Selenomethionine as Suicide Prodrug Substrate

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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 methioninase \(_{\gamma}\)-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 \(\alpha\)-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 \(P_2\)O 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 \(\alpha,\gamma\)-elimination reaction of SeMET to methylselenol, \(\alpha\)-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 \(\times\) 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 \(\mu\)l 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).

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\(^3\) The abbreviations used are: rMETase, recombinant L-methionine \(\alpha,\gamma\)-lyase; SeMET, selenomethionine; MET, methionine \(\alpha,\gamma\)-lyase gene; rAd-MET, recombinant adenovirus encoding MET gene; control-rAd, recombinant E1 E3 depleted adenovirus; GSH, reduced glutathione; 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 × 10⁶ cells) were transduced with 24-h incubation, mitochondrial membrane potential changes were monitored with the fluorescent dye, Mitosensor (Clontech, Palo Alto, CA), according to the manufacturer’s instructions.

GSH Measurement. A549 cells (2 × 10⁶) were transduced with SeMET 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 × 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-phthalaldialdehyde (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 × 10⁶) 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 pepstatin) and homogenized with a Dounce homogenizer. Samples were centrifuged at 900 × g to remove nuclei, which was followed by centrifugation at 10,000 × 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 measured fluorometrically using o-phthaldialdehyde (350 nm excitation and 460 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% glutaraldehyde 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. NISI rat hepatoma tumor cells (1 × 10⁶ cells) were implanted into the peritoneal cavities of female nude mice. Two days after implantation, rAdMET (5 × 10⁶ pfu), control-rAd (5 × 10⁶ 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 rAd-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
methylselenol by the rAdMET gene product, rMETase, as seen in reaction 1.

The IC\textsubscript{50} values of SeMET for nontransduced or control-rAd-transduced human tumor cells (MOI, 50) were between 100 and 1000 \(\mu\)M. In contrast, the IC\textsubscript{50} values of SeMET on rAdMET-transduced (MOI, 50) cells were greatly reduced to 0.3 to 5 \(\mu\)M. Thus, rAdMET increased the toxicity of SeMET up to 1000-fold in tumor cells (Fig. 1B; 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 \(\textit{MET}\)-gene/SeMET combination, we cocultured nontransduced A549 cells with rAdMET-transduced A549 cells (MOI, 50) at various ratios. The administration of SeMET (20 \(\mu\)M) to cultures containing just 3% rAdMET-transduced cells resulted in the killing of more than 80% of the total cells in the culture (Fig. 1C). This strong bystander effect was thought to be caused by the diffusion of methylselenol from the \(\textit{MET}\)-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. 2A). Nucleosomal DNA fragmentation also occurred in the rAdMET-SeMET-treated A549 cells (Fig. 2B). 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 and was inhibited by SOD. In contrast, nontransduced cells did not produce ROS, even at the highest concentration of SeMET tested (Fig. 3A). It is thought that extracellular superoxide 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.

### Table 1 Comparison of SeMET IC\textsubscript{50} in human tumor cell lines transduced with rAdMET

The indicated cell lines were treated \textit{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.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>rAdMET</th>
<th>Control-rAd</th>
<th>Wild Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVCAR-8</td>
<td>0.59</td>
<td>170.3</td>
<td>223.6</td>
</tr>
<tr>
<td>A549</td>
<td>0.5</td>
<td>327.1</td>
<td>485.9</td>
</tr>
<tr>
<td>Hep-2</td>
<td>1.66</td>
<td>387</td>
<td>411</td>
</tr>
<tr>
<td>Mia PaCa-2</td>
<td>3.38</td>
<td>338</td>
<td>422</td>
</tr>
<tr>
<td>BxPC3-3</td>
<td>3.38</td>
<td>222.6</td>
<td>311.8</td>
</tr>
<tr>
<td>EKVX</td>
<td>1.66</td>
<td>689.4</td>
<td>1360</td>
</tr>
</tbody>
</table>

Fig. 2. Induction of apoptosis by rAdMET-SeMET. A, FACScan. rAdMET-transduced A549 cells were incubated with SeMET (20 \(\mu\)M). Cells were harvested and fixed with 70% ethanol and stained with propidium iodide (50 \(\mu\)g/ml). The DNA content of the stained cells was evaluated with FACScan. The sub-G\(_0\) 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 3% agarose gels and stained with ethidium bromide.

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 \(\times\) 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 \(\mu\)g) 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-SeMET-treated cells was dependent on the SeMET concentration and was inhibited by SOD. B, depletion of glutathione by rAdMET-SeMET. Cells (2 \(\times\) 10\(^5\)) transduced with rAdMET or control-rAd (MOI, 20) were treated with SeMET (20 \(\mu\)M) 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 \(\mu\)g/mg protein (mean ± SD).
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. 3B).

**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. 4A). 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. 4B). 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. 4C 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. 4D).

These results suggested that induction of apoptosis by MEGene/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. 5C).

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.

REFERENCES


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.
METHIONINASE/SELENOMETHIONINASE GENE THERAPY


