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Methioninase gene therapy with selenomethionine induces apoptosis in bcl-2-overproducing lung cancer cells

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We have previously shown that the toxic pro-oxidant methylselenol is released from selenomethionine (SeMET) by cancer cells transformed with the adenoviral methionine α,γ-lyase (methioninase, MET) gene cloned from *Pseudomonas putida*. Methylselenol damaged the mitochondria via oxidative stress, and caused cytochrome *c* release into the cytosol thereby activating caspase enzymes and thereby apoptosis. However, gene therapy strategies are less effective if tumor cells overexpress the antiapoptotic mitochondrial protein bcl-2. In this study, we investigated whether rAdMET/SeMET was effective against bcl-2-overproducing A549 lung cancer cells. We established two clones of the human lung cancer A549 cell line that show moderate and high expression levels of bcl-2, respectively, compared to the parent cell line, which has very low bcl-2 expression. Staurosporine-induced apoptosis was inhibited in the bcl-2-overproducing clones as well as in the parental cell line. In contrast to staurosporine, apoptosis was induced in the bcl-2-overproducing clones as well as the parental cell line by AdMET/SeMET. Apoptosis in the rAdMET–SeMET-treated cells was determined by fragmentation of nuclei, and release of cytochrome *c* from mitochondria to the cytosol. A strong bystander effect of AdMET/SeMET was observed on A549 cells as well as the bcl-2-overproducing clones. rAdMET/SeMET prodrug gene therapy is therefore a promising novel strategy effective against bcl-2 overexpression, which has blocked other gene therapy strategies.

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We have previously reported[1] a novel approach to gene-directed enzyme prodrug therapy for cancer that exploits the toxic pro-oxidant property of methylselenol. When selenomethionine (SeMET) is given to cancer cells infected with the adenoviral-delivered methionine α,γ-lyase (methioninase, MET) gene cloned from *Pseudomonas putida*, methylselenol is released. This vector was termed rAdMET. The mitochondria of the cancer cells are damaged via oxidative stress because of the treatment causing release of cytochrome *c* into the cytosol thereby activating the caspase cascade and subsequently apoptosis.

However, gene therapy strategies and other antitumor approaches are less effective if tumor cells overexpress the mitochondrial protein bcl-2. Bcl-2 is a 28 kDa protein and mainly resides at the outer mitochondrial membrane.[2] Bcl-2 is an anti-apoptotic protein that inhibits permeability changes in the mitochondrial membrane,[3,4] thereby preventing cytochrome *c* from being released from the mitochondria into the cytosol and subsequent apoptosis.[5–7] Bcl-2 overproduction inhibits many therapeutic approaches to cancer, including gene therapy.[8–10] For example, the HSV-tk gene and gancyclovir prodrug is not effective against bcl-2-overproducing tumor cell lines.[10,11] The purpose of this study is to determine the potential of rAdMET/SeMET therapy against bcl-2-overproducing cancer cells.

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Materials and methods

Cell culture

The A549 cell line, derived from human lung cancer, and its clones were cultured in RPMI 1640 (Mediatech, Inc., Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum at 37°C and 5% CO_2. Exponentially growing cells were used for all experiments. The cells were subcultured every 3 or 4 days.

Bcl-2 transfection

Bcl-2 cDNA (0.8 Kb[13]) (kindly provided by Dr Y Tsujimoto; Osaka University Medical School) was ligated

into the pLXIN retroviral vector at the *Eco*R-I site using T4 ligase. pLXIN-bcl-2 and pVSV-G vectors (Clontech, Palo Alto, CA) were cotransfected into GP-293 retroviral packaging cells (Clontech, Palo Alto, CA) using Lipofectamine (Gibco BRL Life Technologies, Rockville, MD). Supernatants, of transfected GP-293 cells, containing bcl-2 retroviruses, were used to infect A549 cells to establish bcl-2-overexpressing clones. Bcl-2-transformed cells were selected using G-418 up to 2.2 mg/ml. Two clones, named BB for bcl-2 moderate expression and B1S for bcl-2 high expression, were selected.

Western blotting for detection of bcl-2 and cytochrome c

To detect bcl-2 expression, in each cell line (A549, BB, B1S), 5×10^7 cells were harvested and collected by centrifugation. After washing twice with ice-cold PBS, the cell pellet was treated according to the manufacturer's protocol with the Cell Fractionation Kit (Clontech, Palo Alto, CA). The cells were disrupted by douncing 100 times using a tissue grinder with a type-B pestle (Fisher, Pittsburgh, PA) on ice. Supernatants were obtained by centrifugation at 700 *g* for 10 minutes in a Beckman (Microfuge® 18) centrifuge. Protein concentrations were determined at OD-280 with a Hitachi U-2000 spectrophotometer.

Protein (25 μg) was loaded per lane on a 10–20% SDS-polyacrylamide gel (Invitrogen, Carlsbad, CA) at 125 V. After electrophoresis, the proteins were electrotransferred to nitrocellulose membranes (Amersham Life Science, Piscataway, NJ) at 200 mA for 1 hour. The membranes were blocked for 3 hours in a solution of 7% powdered nonfat milk in TTBS (0.1% Tween 20, 150 mM NaCl, and 10 mM Tris-HCl pH 7.6). After gently rinsing with TTBS, the membranes were probed with a rabbit polyclonal primary antibody against bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA) at a concentration of 3 μl/ml at 4°C overnight. After washing three times, the membrane was treated with donkey anti-rabbit secondary antibody (Amersham) at a concentration of 1 μl/5 ml for 1 hour. For signal detection, chemiluminescence reagent ECL + Plus (Amersham) was used according to the manufacturer's protocol.

For cytochrome *c* extraction, 5×10^7 cells were harvested for each cell line and treated with the Cell Fractionation Kit. The cells were disrupted by douncing 60 times using a tissue grinder with a type A pestle on ice. After centrifugation at 700 *g* for 10 minutes, this supernatant was centrifuged at 10,000 *g* for 25 minutes to obtain the cytosol fraction. Supernatant protein (30 μg) was loaded in each lane on 10–20% Tris-Glycine SDS gels (Invitrogen) at 120 V. After membrane transfer and blocking, the membrane was probed with rabbit monoclonal primary antibody against cytochrome *c* (BD Biosciences Clontech, Palo Alto, CA) at a concentration of 4 μl/ml. For detection, a chemiluminescence reagent ECL + Plus (Amersham) was used with donkey anti-rabbit secondary antibody (Amersham).

ELISA for detection of bcl-2 and cytochrome c

Bcl-2 and cytochrome *c* were also quantitated by ELISA. A bcl-2 ELISA kit (R&D Systems Inc., Minneapolis, MN) was used for bcl-2. The Quantikine human cytochrome *c* kit (Alexis, San Diego, CA) was used for cytochrome *c*. Each procedure was carried out according to the manufacturer's protocols. Absorbance at 450 nm was read on a Benchmark microplate reader (Bio-Rad Laboratories, Hercules, CA) and the data were analyzed by Microplate Manager-4.0 PC Data Analysis software (Bio-Rad Laboratories) on an IBM PC.

Hoechst 33258 staining for apoptosis

Hoechst 33258 (Sigma),[12] a DNA stain, was used as a nuclear marker of apoptosis. Staurosporin[13,14] (Alexis, San Diego, CA) was used to introduce apoptosis. Cells (5×10^4) were seeded on chamber slides (Nalge Nunc International, Naperville, IL) in wells with culture medium (RPMI 1640 with 10% FBS). Staurosporine was added the next day to each well at a concentration of 3 μM and incubated for 3 hours. The medium was changed to a medium containing Hoechst 33258 at a concentration of 2 μM with incubation for 20 minutes. After gently washing with PBS, fluorescent nuclei were observed with an Olympus model BH-2 fluorescence microscope with a 360 nm filter for excitation and 500 nm filter for emission. Morphology of the stained nuclei was used to determine apoptosis. The parental A549 clone and derived clones, BB and B1S, were seeded (5×10^3 cells) in culture medium in each well of four chamber plates. After 24 hours, rAdMET (MOI 20) was added to the cells. At 24 hours after transfection, SeMET was added to each well at the final concentration of 20 μM. After 12 hours, cells were stained with Hoechst 33258 to observe nuclear condensation.

MTT cell viability assay

Cells (5×10^3) were seeded in each well of 96-well plates. After 24 hours, cells were infected with rAdMET at an MOI of 20. SeMET was added to each well at a final concentration of 20 μM the next day. After 3 days incubation, the medium was replaced with methylthiazolyldiphenyl-tetrazolium bromide (MTT) reagent (Sigma) in culture medium (0.5 mg/ml) for 2 hours. After removing the fluid in each well, 200 μl isopropanol was added to each well and plates were vibrated for 1 hour. Absorbance at 540 nm was measured with the Benchmark microplate reader and data were analyzed by Microplate Manager-4.0 PC Data Analysis software on an IBM PC. The surviving cells were calculated as a percentage of absorbance of untreated controls. IC_{50} values were determined by the median-effect equation.

Bystander effect

To evaluate the bystander effect of rAdMET/SeMET gene therapy for bcl-2-overexpressing clones, we cocultured rAdMET-transduced cells and nontransduced cells at various ratios for each clone. The cells were mixed at

various ratios and seeded onto 96-well plates adjusted to 5×10^3 cells in each well. After 24 hours, SeMET was added to each well at a final concentration of 20 μM. After 3 days of incubation, the surviving cell ratio was evaluated with the MTT assay as described above.

Results and discussion

Bcl-2 overproducing cell lines

The A549 human lung cancer cell line has very low endogenous bcl-2 levels (Figs 1, 2). The parental cells were transduced with a bcl-2-containing retrovirus vector.[29] Two new clones, which showed different expression levels of bcl-2, were selected. Bcl-2 expression was confirmed by Western blotting (Fig 1) and ELISA (Fig 2). The highest bcl-2-expressing clone (B1S) had almost twice the bcl-2 expression of clone BB (Figs 1, 2).

Staurosporine induces apoptosis via a mitochondrial pathway.[15] The A549 parental line entered apoptosis within 3 hours after incubation with 3 μM staurosporine. Hoechst 33258 staining showed that 20% of the cells had extensive nuclear condensation with the other cells showing moderate condensation. In contrast, when the BB and B1S clones were similarly treated with staurosporine, none of the cells showed nuclear condensation (Fig 3a–c). Therefore, the clones that express elevated levels of bcl-2 protein were resistant to a standard apoptosis-inducing agent.

Figure 1 Bcl-2 Western blot. A549, BB, and B1S cells (5×10^7) were harvested as described in the Materials and methods. Protein (25 μg) was applied on each lane of 10–20% SDS-polyacrylamide gels. After electrophoresis, the proteins were electrotransferred to nitrocellulose membranes and the signal was detected with chemiluminescence reagent.[28]

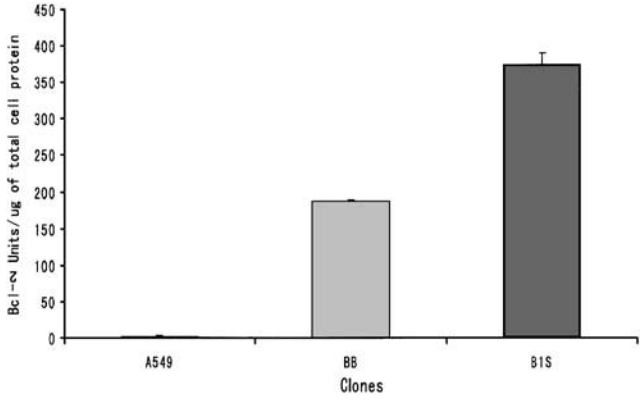

Figure 2 Bcl-2 ELISA. A549, BB, and B1S cells (1×10^6) were resuspended in 1 ml of PBS and lysed, and assayed according to the manufacturer's protocol at a dilution of 1:20 of the cell lysates. A standard curve was prepared with the standards supplied with the kit. Bcl-2 was quantitated with the standard curve as units per μg of total protein content of the sample. One unit of bcl-2 is defined as the amount of bcl-2 in 1000 lysed cells using the manufacturer's standards.

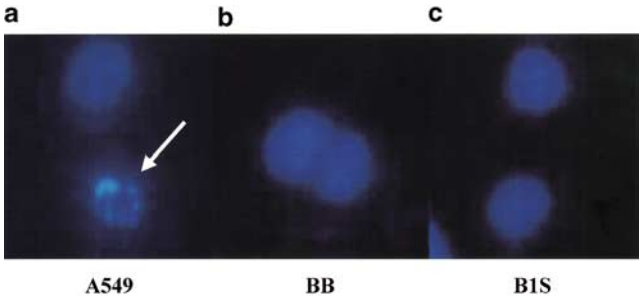

Figure 3 Staurosporine-induced apoptosis. A549 parental cells and bcl-2-overexpressing clones BB and B1S (5×10^4 cells) were incubated with 3 μM staurosporine for 3 hours. Subsequently, cells were stained with Hoechst 33258 at a concentration of 2 μM for 20 minutes. Images were observed with a fluorescence microscope using 360 nm excitation and 500 nm emission. (**a**) A549, (**b**) BB, (**c**) B1S; magnified $\times$ 400.

Comparison of efficacy of rAdMET/SeMET gene therapy on A549, BB, and B1S

A549, BB, and B1S equally showed nuclear condensation 24 hours after rAdMET/SeMET gene therapy as seen by Hoechst 33258 staining (Fig 4a–c). In all, 20% of each clone showed extensive nuclear condensation and the rest showed moderate nuclear condensation. This result showed that rAdMET/SeMET gene therapy caused apoptosis in the bcl-2-overexpressing BB and B1S clones as well as the parental clone.

We carried out the staurosporine experiment, based on Susin et al[14], and present the apoptosis data as a percentage of total cells which thus can demonstrate efficacy. Data on the control virus and prodrug alone were shown in our previous publications[1] to have no toxicity at the amounts used.

The Bcl-2-overexpressing clones were selected in high levels of G418 in which the parental cells die. The clones were shown by Western blotting to highly express Bcl-2 with B1S expressing more than BB. Bcl-2 was almost undetectable in the parental cells. ELISA testing confirmed the high levels of Bcl-2 in BB and B1S and very low levels in the parental cells. Bcl-2-overexpression also correlates with staurosporine resistance. Therefore, the properties of the clones are due to the high Bcl-2 expression and not due to the clonal variation.

Cell survival measured by MTT assay

The MTT assay showed that the rAdMET/SeMET gene therapy killed the BB and B1S clones as efficiently as it killed the parental A549 cells (Fig 5). Cell survival curves were almost identical for each clone. The IC_{50} values for the parental and bcl-2-overexpressing clones were similar

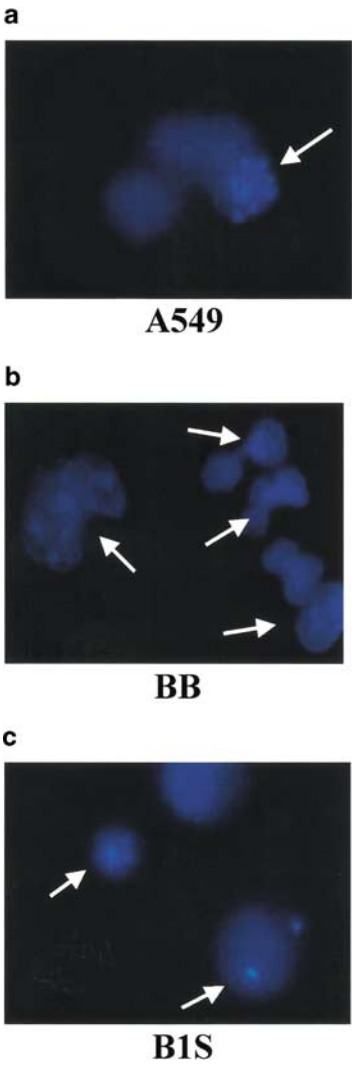

Figure 4 (**a**–**c**). rAdMET/SeMET-induced apoptosis. A549 and bcl-2-overexpressing clones BB and B1S cells were treated with rAdMET/SeMET and stained with Hoechst 33258 as described in the the Materials and methods. (**a**) A549, (**b**) BB, (**c**) B1S; magnification $\times 400$.

in the range of 3.8–5.3 μM. As we previously reported, SeMET alone has an IC_{50} of between 100 and 1,000 μM.[1] Thus, rAdMET/SeMET gene therapy is effective against bcl-2-overexpressing cells.

Release of cytochrome c *from mitochondria to cytosol*

Western blotting of the cytosolic fractions of the cells treated with rAdMET/SeMET showed cytochrome c release into the cytosol in both parental A549 cells and the bcl-2-overexpressing clones, BB and B1S (Fig 6). ELISA also showed that cytochrome *c* release occurred in each clone after rAdMET/SeMET treatment (Fig 7). To confirm that the fractionation technique itself did not damage the mitochondria and cause cytochrome *c* release, we also prepared samples from untreated cells in the same

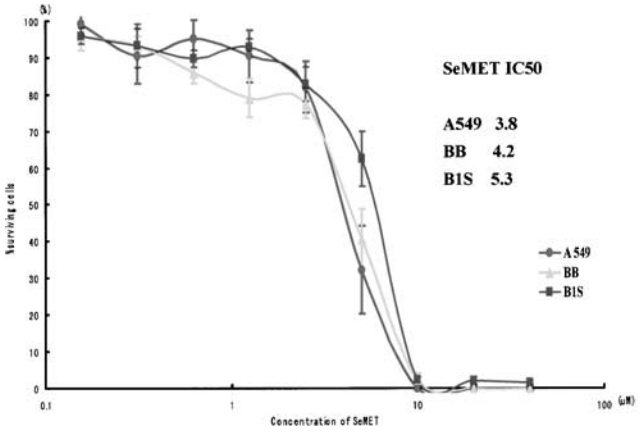

Figure 5 Efficacy of rAdMET/SeMET on survival. A549 and bcl-2-overexpressing BB and B1S cells (5×10^3) were treated with rAdMET and incubated with various concentrations of SeMET (0–20 μM) for 3 days. Cytotoxicity was measured with an MTT assay and a survival curve was calculated. IC_{50} values were determined by the median-effect equation.

way. No cytochrome *c* release was seen in these preparations from any of the clones (Fig 6).

Bystander effect of rAdMET/SeMET

Hamel et al[9] reported that bcl-2 significantly reduced the bystander effect in HSV-tk/GCV therapy. To evaluate the bystander effect of the rAdMET/SeMET gene therapy, we cocultured nontransduced cells with rAdMET-transduced cells of each clone. Our results showed that rAdMET/SeMET gene therapy showed strong bystander effect even against bcl-2-overexpressing BB and B1S clones (Fig 8). Cell survival curves of each clone were essentially identical. This strong bystander effect was thought to be caused by the diffusion to the nontransduced cells of gaseous methylselenol produced from SeMET in the MET-gene-transduced cells. Thus, methylselenol seems to induce apoptois even in the presence of high levels of bcl-2.

SeMET itself is relatively nontoxic to mammalian cells since it cannot be converted to selenol.[16,17] Methylselenol catalyzes oxidation of thiols[18] at ambient pO_2 to generate toxic reactive oxygen species such as superoxide[19,20] and induces apoptosis (Fig 9). These oxidants are thought to activate the mitochondrial permeability transition and cause mitochondrial swelling and loss of membrane potential.[21,22] Subsequently, cytochrome *c* is released into the cytosol and binds to Apaf-1 and activates caspase 9,[23] which in turn activates downstream caspases and induces apoptosis as we previously showed in rAdMET/SeMET-treated cells.[1,24]

Methionine α,γ lyase (methioninase) from *Pseudomonas putida* catalyzes α,γ-elimination reactions of L-methionine,[25,26] and can degrade SeMET to methylselenol, α-ketobutyrate, and ammonia.[27] We have taken advantage of this feature of the MET gene to previously design a

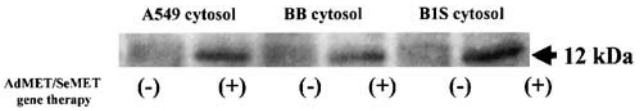

Figure 6 Western blot for cytochrome *c* release. A549 and bcl-2-overexpressing clones BB and B1S (5×10^4 cells) with and without rAdMET/SeMET treatment were harvested. Cytosols were prepared as described in the Materials and methods. Protein (30 μg) was loaded on each lane of a 10–20% Tris-glycine SDS gel. After membrane transfer, signal was detected with a chemiluminescence reagent.[28]

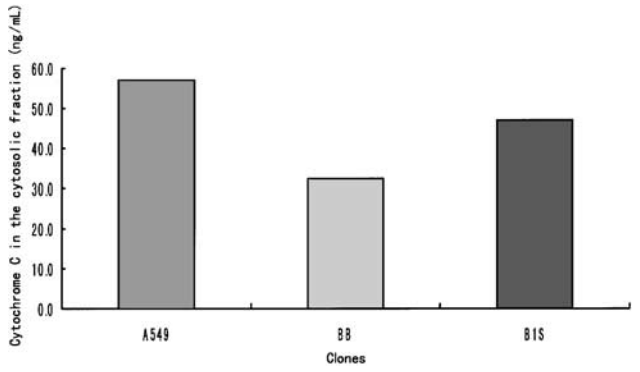

Figure 7 ELISA for cytochrome *c* release. A549 and bcl-2-over-expressing BB and B1S cells (5×10^7) were treated with rAdMET/SeMET. Cytosols were prepared as described in the Materials and methods. A standard curve was prepared with the preknown standards given in the kit. Cytochrome *c* quantitated based on the standard curve.

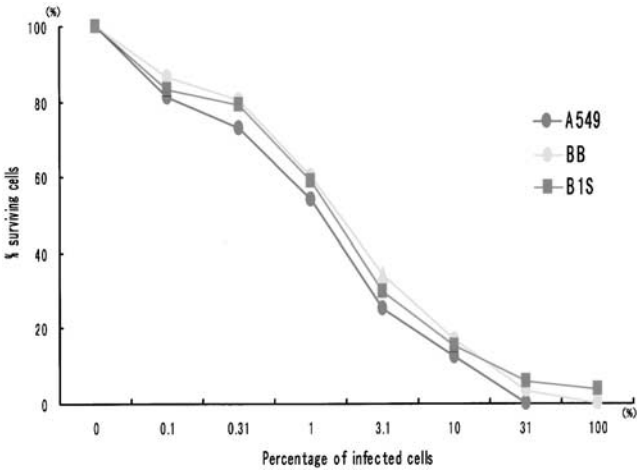

Figure 8 Bystander effect of rAdMET/SeMET. A549 and bcl-2-overexpressing BB and B1S cells were infected with rAdMET (MOI 40) and cocultured with nontransduced cells at different ratios with or without SeMET (20 μM) for 3 days. Cytotoxicity was measured with the MTT assay and survival curves calculated.

gene-directed enzyme prodrug therapy system for cancer therapy.[1]

In the current study, we show that rAdMET/SeMET gene therapy could overcome bcl-2 overexpression and induce apoptosis. We believe the powerful advantages of rAdMET/SeMET treatment are promising for future development of this gene therapy strategy.

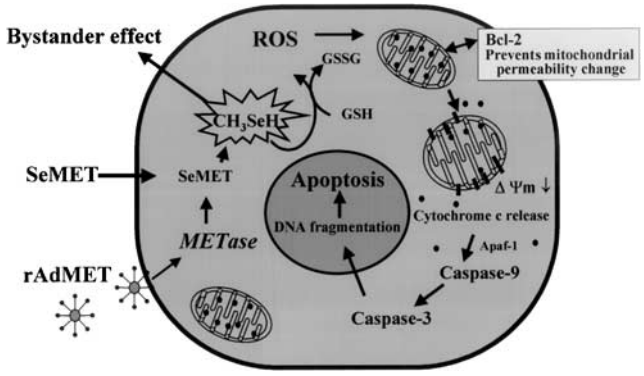

Figure 9 Proposed mechanism of rAdMET/SeMET-induced apoptosis. The rAdMET gene product, methionine α,γ-lyase (MET) converts nontoxic SeMET to methylselenol, which, in contrast, catalyzes oxidation of thiols at ambient pO_2 to generate toxic superoxide. Apoptosis occurs mainly via a mitochondrial pathway.

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