Survival efficacy of the combination of the methioninase gene and methioninase in a lung cancer orthotopic model

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We have previously demonstrated the antitumor efficacy of recombinant methioninase (rMETase) derived from Pseudomonas putida. To enhance the efficacy of rMETase, we have constructed the pLGFPMETSN retrovirus encoding the P. putida methioninase (MET) gene fused with the green fluorescent protein (GFP) gene, pLGFPMETSN or control vector pLGFPSN was introduced into the human lung cancer cell line H460. The methionine level of H460-GFP-MET cells was reduced to 33% of that of H460-GFP cells. rMETase (0.08 U/mL) in the medium resulted in 10% survival of H460-GFP-MET cells compared with untreated controls in vitro. In contrast, rMETase-treated H460-GFP cells survived at 90% of control. Tissue fragments harvested from subcutaneous tumors of H460-GFP-MET or H460-MET were implanted by surgical orthotopic implantation into the lungs of nude mice. A suboptimal dose of rMETase was administered intraperitoneally daily to mice in each group. Overall survival of rMETase-treated animals with H460-GFP-MET tumors was significantly longer than either rMETase-treated or untreated animals with H460-GFP tumors (P<.05 in log-rank test). In two repeat experiments, rMETase-treated animals with H460-GFP-MET tumors had a 30-day survival of 80% and 83%, respectively. Untreated animals with H460-GFP-MET tumors had a 30-day survival of 40% and 58%, respectively. rMETase-treated animals with H460-GFP tumors had a 30-day survival of 0% and 33%, respectively. Untreated animals with H460-GFP tumors had a 30-day survival of 0% and 33%, respectively. The retrovirus-mediated gene transfer of METase decreased the intracellular methionine level of tumor cells and consequently enhanced the efficacy of treatment with the rMETase protein. We have thus demonstrated a new strategy of combination tumor therapy with the gene and gene product of MET. Cancer Gene Therapy (2000) 7, 332–338

Key words: Methionine dependence; recombinant methioninase; methioninase gene; retrovirus; green fluorescent protein; fusion gene.

A number of different strategies for cancer gene therapy have been developed. For example, 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 delivery4 has been demonstrated. However, this strategy is limited to 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.3 This prodrug approach activates ganciclovir by TK-mediated phosphorylation. The retroviral vector encoding HSV-TK is selectively expressed in dividing cells such as those in the tumor. Tumor regression has been shown even when only a small number of cells have been transfected by retroviral HSV-TK in what is called a bystander effect.6–9 Viral transfer of the cytosine deaminase gene, which confers fluorocytosine sensitivity, is also being used in gene therapy experiments.6,10,11 Other gene therapy strategies for cancer include the retinoblastoma gene,12 the E1a gene,13 and the ras gene.14 Tissue-specific promoters are being used to target the expression of therapeutic genes to tumor cells.15–17 Other approaches to gene therapy have included manipulating the immune system with genes such as interleukin-2.5 Autologous tumor cells transduced with a cytokine gene are being used as vaccines.5,18–22

This report describes a new approach to gene therapy that targets the elevated methionine dependence of tumor cells33 with the methioninase (MET) gene derived from Pseudomonas putida. The elevated methionine requirement for cell growth has been shown to be present in the majority of cancer cells compared with normal cells.35–32 There have been several therapeutic strategies developed to target the methionine depen-
was added, and 20 µL of sample were loaded on a reversed-phase Supercil LC-18-DB (Supelco, Bellefonte, PA) column at room temperature. The amino acid derivatives were resolved with solution A (tetrahydrofuran/methanol/0.1 M sodium acetate (pH 7.2); 5/95/900) and solution B (methanol). A gradient from 20% to 60% was run at a flow rate of 1.5 mL/minute. The eluate was read with a fluorescence spectrophotometer (Hitachi F1000) at 350–450 nm.

**In vitro efficacy of rMETase**

The 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide (MTT) assay was used to determine the in vitro growth inhibition of tumor cells by rMETase treatment. Cells (2000 cells/well) were plated in 96-well culture plates in 100 µL of culture medium. After 24 hours, various concentrations of rMETase (100 µL) were added to the wells. After 3 days of incubation with rMETase, cytotoxicity was measured with the MTT assay. MTT was used at a concentration of 0.2 µg/µL with incubation for 2 hours. The resulting formazan product was solubilized in dimethylsulfoxide and absorbance was measured at 540 nm in a BioRad Benchmark Microplate Reader (BioRad Laboratories, Hercules, Calif) using 1-cm pathlength plastic spectrophotometer cells. Growth inhibition was expressed as the percentage of absorbance of treated cultures with respect to untreated cells.

**In vivo evaluation of the efficacy of rMETase**

Fragments (1 mm in diameter) derived from H460-GFP or H460-GFP-MET tumors growing subcutaneously in nude mice were implanted by surgical orthotopic implantation (SOI) into the left lungs of nude mice. From days 2–13 postoperation, 100 U of rMETase were administered intraperitoneally twice a day. At day 17 postoperation, four to five mice in each group were chosen randomly and sacrificed. The primary tumor weights were measured and the incidence of metastasis in relevant organs was evaluated under fluorescence microscopy of fresh tissue. Statistical significance for tumor weights was determined with the Student’s t test. The remaining 10–12 mice in each group were monitored for survival. This experiment was repeated one time. Differences in survival were compared at 30 days. Overall survival was analyzed with the log-rank test.

**RESULTS AND DISCUSSION**

**Transduction of H460 with GFP-MET or GFP genes**

H460 cells transduced with either the GFP-MET or GFP genes were able to grow in vitro. High-expression clones containing these vectors were isolated and selected by the extent of their GFP fluorescence. The GFP fluorescence was evenly distributed in cells with the GFP gene. In contrast, in GFP-MET cells, the fluorescence was localized only in the cytoplasm and not in the nuclei. The reason for localization of GFP-MET expression only in the cytoplasm remains unclear. There were no significant differences in the cell growth rates between GFP cells and GFP-MET cells as determined by comparing their doubling time in vitro (data not shown).

**METase activity and methionine levels**

The METase activity of GFP-MET cells was 33 mU/mg protein (Table 1). METase activity was not detectable in the H460 parent cells or in H460-GFP cells. Pure rMETase has a specific activity of ~20 U/mg protein, which means that the GFP-MET cells expressed METase at ~0.17% of their total protein. The free methionine level of H460-GFP-MET was determined to be ~33% of that of H460-GFP cells or H460 parent cells (Table 1).

**In vitro efficacy of rMETase**

The efficacy of rMETase in vitro was determined with the MTT assay. H460-GFP-MET cells were more sensitive to low concentrations of rMETase compared with H460-GFP cells or H460 cells (Fig 2). In the presence of 0.08 U/mL rMETase, the survival of H460-GFP-MET cells was 10% compared with the untreated cells. In contrast, H460 and H460-GFP cells were only slightly affected by this concentration of rMETase (Fig 2). These results indicated that the MET gene increased the methionine requirement of these tumor cells, making them more sensitive to rMETase.

**Table 1. MET Activity and Free Methionine Levels of MET-Transduced H460 Lung Tumor Cells in Vitro**

<table>
<thead>
<tr>
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<th>MET activity</th>
<th>Free methionine level</th>
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<tbody>
<tr>
<td>H460</td>
<td>0.75 mU/mg protein</td>
<td>30.2 nmol/mg protein</td>
</tr>
<tr>
<td>H460-GFP</td>
<td>1.5 mU/mg protein</td>
<td>33.0 nmol/mg protein</td>
</tr>
<tr>
<td>H460-GFP-MET</td>
<td>33 mU/mg protein</td>
<td>10.1 nmol/mg protein</td>
</tr>
</tbody>
</table>

*Methioninase activity was measured with 3-methyl-2-benzothiazoline hydrazone. Cellular free methionine levels were measured by OPA-derivitized amino acids separated by high performance liquid chromatography. Please see Materials and Methods for a description of procedures.*

**Figure 2. In vitro efficacy of rMETase on H460-GFP-MET cells, H460-GFP cells, and H460 parent cells. Cytotoxicity was assayed with the MTT assay. The IC50 was 0.07 U/mL for GFP-MET cells, 0.115 U/mL for H460 cells, and 0.098 U/mL for GFP cells. In the presence of 0.08 U/mL of rMETase, GFP-MET cell survival was only 10% compared with untreated cells. In contrast, the survival of H460 parental cells and H460-GFP cells were only slightly sensitive to this concentration of rMETase.**

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Figure 3. Orthotopic growth and metastases as visualized by GFP: Lymph node metastases of the left lung hilus of an H460-GFP-MET tumor. A: Bright field microscopy. Arrows indicate lymph node metastases. B: Same field viewed under fluorescence with GFP cube. Primary tumor and lymph nodes are brightly visualized. Arrows indicate lymph node metastases. C: Early stage contralateral lung metastasis of an H460-GFP tumor. Tumor embolus in a small vessel (small arrow) and a small tumor colony (large arrow) are visualized under fluorescence with GFP cube.

rMETase 50% inhibitory concentrations (IC$_{50}$) than a series of four H460-GFP clones ($P < .005$) (data not shown). Whether the strategy of combination therapy with the MET gene and rMETase protein would work with other tumor cell lines is open to question at this time. The toxicity of the MET gene in normal cells has not yet been demonstrated. However, we have described the efficacy and toxicity of rMETase in a series of human tumor cell lines and normal cell strains. This study included seven different tumor types for a total of $>20$ tumor cell lines. The tumor selectivity of rMETase was shown to be very large in this study, with the IC$_{50}$ of the tumor cells lower than that of the normal cells by as much as one order of magnitude. It is thus surmised that the MET gene could have a similar broad range of tumor efficacy and degree of tumor selectivity. This remains to be tested definitively in future experiments in vivo.

We have thus demonstrated a new strategy of combination tumor therapy with the MET gene and its gene product. Future experiments will involve direct in vivo administration of the MET gene both with and without rMETase to tumor-bearing animals.

Table 3. In Vivo Efficacy of the MET Gene and rMETase Protein on the 30-Day Survival Rate of Mice Implanted with H460 Lung Tumors

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
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<tbody>
<tr>
<td>GFP tumor, untreated</td>
<td>0%</td>
<td>33.33%</td>
</tr>
<tr>
<td>GFP tumor, rMETase-treated</td>
<td>0%**</td>
<td>33.33%**</td>
</tr>
<tr>
<td>GFP-MET tumor, untreated</td>
<td>40%*</td>
<td>58.33%†</td>
</tr>
<tr>
<td>GFP-MET tumor, rMETase-treated</td>
<td>80%*</td>
<td>83.33%†</td>
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100 U of rMETase were administered intraperitoneally twice a day as described for Table 2. A total of 10–12 mice in each group were monitored for survival. Differences in survival at 30 days were analyzed with the $\chi^2$ test.

*, $P < .05$; **, $P < .005$; †, $P = .08$; all compared with GFP-MET tumor, rMETase-treated.


