Recombinant Methioninase Infusion Reduces the Biochemical Endpoint of Serum Methionine with Minimal Toxicity in High-Stage Cancer Patients

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Abstract. The tumor-specific increased minimal requirement for methionine has been shown to be a highly promising therapeutic target. To attack this target we have previously cloned the methioninase gene from Pseudomonas putida and produced recombinant methioninase (rMETHase). A pilot Phase I clinical trial has been carried out to determine rMETHase toxicity, rMETHase pharmacokinetics, and serum MET-depletion in cancer patients. Patients with advanced breast cancer, lung cancer, renal cancer and lymphoma were given a single rMETHase treatment at doses ranging from 5,000 to 20,000 units by i.v. infusion over 6-24 hours. No clinical toxicity was observed in any patient after rMETHase treatment. rMETHase levels reached 0.1 to 0.4 units per ml of serum in the patients which correspond to therapeutic levels in vitro. The lowest serum methionine levels in rMETHase-treated patients were 0.1% of the pre-treatment levels corresponding to approximately 0.1 μM, which also correlates to therapeutic levels in vitro. The results of the rMETHase pilot Phase I clinical trial therefore indicate that i.v. infusion of rMETHase is safe and effectively depletes its biochemical target of serum methionine suggesting potential efficacy in future clinical trials.

The goal of the next generation of cancer chemotherapy is tumor-selective effective therapy. A broad tumor-selective target with high therapeutic potential is the increased minimal methionine requirement of tumor cells relative to normal cells which we have termed methionine dependence [1,2]. The in vitro studies in our laboratory have shown that methionine dependence occurs frequently, if not universally, in many types of human cancer as well as in fresh human tumor specimens but not in normal cells [3-6]. Methionine dependence was found in 20 of 20 tested human tumor cell lines from the NCI Human Tumor Cell Line Screen including kidney, colon, lung, prostate, and melanoma tumor cell lines (Xu, M. et al unpublished data).

Recent results reported by Goseki et al have demonstrated the clinical efficacy of methionine depletion in Phase II clinical trials with a methionine-depleted total parenteral solution (TPN) given to high-stage gastric cancer patients. The methionine-depleted TPN solutions doubled the response and survival rate in combination with 5-FU and mitomycin C as compared to the latter two drugs given with methionine-replete TPN [7-8].

We initially purified endotoxin-free METase from Pseudomonas putida in order to develop anti-methionine chemotherapy targeting the methionine dependence of tumors in vivo [9,11]. The in vivo pre-clinical studies carried out in our laboratory have shown that methioninase effectively inhibited the growth of the Yoshida sarcoma and the human lung tumor H460 in nude mouse-xenograft models [10]. Clinical pharmacokinetics studies showed that native methioninase depleted serum methionine levels more than 100-fold without toxicity [11].

Recently, we have cloned the Pseudomonas putida METase gene into a high expression vector which was transformed into E.coli. With this high expression clone, we have established scale-up protocols which produce high-purity, low-endotoxin recombinant methioninase (rMETHase) [12]. Preclinical studies have shown that rMETHase has high potential as an effective broad-spectrum tumor-selective agent without acute toxicity in nude mice with human tumor xenografts (Tan, Y. et al unpublished data). In vitro studies with rMETHase demonstrated universally lower IC50 values by approximately an order-of-magnitude, in a broad range of human tumor cells compared to five types of normal cells [Xu, M. et al, unpublished data]. We report here that pilot clinical phase I trials of rMETHase infusion in 9 high-stage cancer patients have shown that serum levels of rMETHase reach potential therapeutic levels. rMETHase depleted serum methionine levels to 0.1% of untreated levels without any clinical toxicity. This depleted level of serum methionine also corresponds to a potential therapeutic level.

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Materials and Methods

Production of rMETase.

High expression clone of rMETase and fermentation conditions (12). The pAC-1 rMETase high expression clone established by AntiCancer was used for the production of rMETase. The fermentation procedure for host E.coli cells and the purification protocol for rMETase were as previously described (12). pAC-1 was constructed with the pI7-7 vector containing the T7 RNA polymerase promoter for high expression of the rMETase gene which was cloned from Pseudomonas putida (12). The host E.coli were grown in Terrific Broth and harvested when the OD600 reached 20. The expression level of rMETase reached approximately g/liter of fermentation broth under these conditions.

Purification of rMETase (12). Pre-column treatment. The bacterial pellet was disrupted with a cavitation-type homogenizer (Microfluidics Corp., Newton, MA, model # HC 8000). Heat treatment of the homogenate was carried out at 50°C for one minute.

First column, DEA SE Sepharose FF (pH 7.2) (Pharmacia, Uppsala, Sweden) (12). rMETase was eluted with a linear gradient of 40 to 200 mM potassium chloride in 10 mM potassium phosphate buffer pH 7.2. The fractions containing rMETase were identified by yellow color and activity assay.

Second column, DEAE Sepharose FF (pH 8.3) (12). rMETase was eluted with a linear gradient of 80 to 200 mM potassium phosphate buffer pH 8.3. The fractions containing rMETase were identified by yellow color and activity assay.

Third column, Actidean Etox (Sterogen, Arcadia, CA) (12). To eliminate endotoxin, purified rMETase (10-20 mg protein/ml) was applied on an Actidean Etox column. The protein was eluted with 0.12 M sodium chloride in 10 mM sodium phosphate buffer pH 7.2. The fractions containing rMETase were identified by yellow color and activity assay.

Analysis of rMETase (12).

HPLC: An Hitachi L-6200A Intelligent pump (Hitachi, Ltd, Tokyo, Japan) with a Supelco Proti™ TSK column (G3000 SW XL 30 cm x 7.8 mm) column (Supelco, Bellefonte, PA) was used for all HPLC analysis of rMETase purity. A sample was loaded and eluted with elution solution (0.12 M sodium chloride in 10 mM sodium phosphate buffer, pH 7.2). The protein-containing fractions were identified with a spectrophotometer (Hitachi U2000) at a wavelength of 280 nm.

Electrophoresis (12). Electrophoresis is carried out in 7.5% polyacrylamide-precast plates in 0.2 M Tricine glycine buffer, pH 8.3, both with and without 0.1% SDS.

Potency activity assay (12). The assay was carried out in a one ml volume of 50 mM phosphate buffer pH 8.0, containing 10 μM pyridoxal phosphate and 10 mM methionine for 10 minutes at 37°C with varying amounts of enzyme. and detected with 3-methyl-2-benzothiazolinone hydrazone at OD535. The specific activity was calculated as units/mg protein, with one unit of enzyme defined as the amount that catalyzes the formation of 1 nmol of α-ketobutyrate per minute (12).

Endotoxin assay (12). The endotoxin level was measured with the Limulus Amebocyte Lysate (LAL) test (Bio Whitaker, Walkerville, MD). The concentration of endotoxin was measured at a wavelength of 410 nm.

Serum methionine measurement. Serum methionine measurement was performed by HPLC (Hitachi L-6200A Intelligent pump, Hitachi, Ltd, Tokyo, Japan) after derivatization of serum amino acids with a fluorescein dye reagent, o-phthalaldehyde (OPA) (13). A methionine standard (Sigma) was derivatized with OPA. Serum samples (25 μl) were preincubated by acetonitrile (75 μl). Ten μl of supernatant was mixed with 5 μl of OPA. After 1 minute, 50 μl of 0.1 M sodium acetate (pH 7.0) was added, and a 20 μl sample was loaded on a reversed-phase Supelcoil LC-18DB (particle size 5 μm, 25 cm x 4.8 mm) column at room temperature. Resolution of the amino acid derivatives was accomplished with solution A (tetrahydrofuran/methanol/0.1M sodium acetate pH 7.2, 5:95:500), solution B (methanol). A gradient from 20%-60% solution B was run at a flow rate of 1.5 ml/minute. The eluate was read by a fluorescence spectrophotometer (Hitachi, F1000) at a wavelength of 350-450 nm. The limit of detection was approximately 0.01 μM methionine.

Patients and treatment. Nine patients with advanced stage breast cancer, lung cancer, kidney cancer and lymphoma participated in the ongoing Phase I trial of rMETase (Table 1). Patient selection requirements included no chemotherapy or radiotherapy in the four previous months before this study. The diagnosis of the original cancer and the metastases was confirmed by pathologic analysis. There were no other coexistent medical problems of sufficient magnitude to jeopardize full compliance with this study. Informed consent forms followed IRB approval guidelines.

Formulation for rMETase. rMETase was formulated at a concentration of 10-20 mg/ml in 0.12 M sodium chloride, 10 mM sodium phosphate buffer (pH 7).

The rMETase administered to the patients was more than 99% pure with an endotoxin level of less than 2 EU/ml. Patients

Table 1. Patient characteristics and rMETase toxicity.

<table>
<thead>
<tr>
<th>Name</th>
<th>Diagnosis</th>
<th>Stage</th>
<th>Methionine (μM)</th>
<th>METase</th>
<th>Toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cancer</td>
<td></td>
<td>Base</td>
<td>Lowest</td>
<td>Units</td>
</tr>
<tr>
<td>A.M.E.</td>
<td>Breast cancer</td>
<td>IV</td>
<td>28.6</td>
<td>0.5</td>
<td>10,000</td>
</tr>
<tr>
<td>M.R.</td>
<td>Kidney cancer</td>
<td>III</td>
<td>N/A</td>
<td>N/A</td>
<td>20,000</td>
</tr>
<tr>
<td>O.P.</td>
<td>Breast cancer</td>
<td>III</td>
<td>N/A</td>
<td>N/A</td>
<td>5,000</td>
</tr>
<tr>
<td>P.J.</td>
<td>Lung cancer</td>
<td>IV</td>
<td>N/A</td>
<td>N/A</td>
<td>10,000</td>
</tr>
<tr>
<td>C.M.</td>
<td>Lymphoma</td>
<td>III</td>
<td>N/A</td>
<td>N/A</td>
<td>10,000</td>
</tr>
<tr>
<td>B.O.</td>
<td>Breast cancer</td>
<td>IV</td>
<td>35</td>
<td>0.28</td>
<td>10,000</td>
</tr>
<tr>
<td>R.M.</td>
<td>Lymphoma</td>
<td>III</td>
<td>N/A</td>
<td>N/A</td>
<td>10,000</td>
</tr>
<tr>
<td>L.S.</td>
<td>Gastric cancer</td>
<td>IV</td>
<td>34</td>
<td>1.27</td>
<td>10,000</td>
</tr>
<tr>
<td>S.N.</td>
<td>Breast cancer</td>
<td>IV</td>
<td>34.7</td>
<td>0.1</td>
<td>20,000</td>
</tr>
</tbody>
</table>
Patients (A.M.E. and S.N.) with breast cancer received an 8-hour iv infusion of 10,000 units and 20,000 units of rMETase, respectively. Blood samples were collected before treatment, every four hours during treatment and 24 hours after the start of the infusion. Serum rMETase activities were measured as described in the Materials and Methods. Serum methionine measurement was performed by HPLC after derivatization of serum amino acids with o-phthalaldehyde (OPA) as described in the Materials and Methods.

Figure 1. rMETase pharmacokinetics and serum MET levels in patients. rMETase was measured by activity assay (see text). MET levels were determined by HPLC (see text and Figure 2).

Patient (S.N.) with stage IV breast cancer had an 8-hour iv infusion of 20,000 units of rMETase. Blood samples were collected before treatment, every four hours during treatment and 16 hours after the end of infusion. Serum methionine measurement was performed by HPLC after derivatization of serum amino acids with o-phthalaldehyde (OPA) as described in the Materials and Methods.

Figure 2. Serum methionine measurement was performed by HPLC (Hitachi L-6200A Intelligent pump, Hitachi, Ltd., Tokyo, Japan) after derivatization of serum amino acids with a fluorescein reagent, o-phthalaldehyde (OPA) [13]. A methionine standard (Sigma) was derivatized by OPA. The eluate was read by a fluorescence spectrophotometer (Hitachi, F1090) at wavelengths of 350-450 nm. The limit of detection was approximately 0.1 μM methionine.
received 5,000 units (0.25g) to 20,000 units (1.0g) of rMETase by continuous i.v. infusion for 6-24 hours.

Patient observation and sample collection. All the patients were observed carefully for vital signs, pulse, respiration rate, blood pressure, and temperature were measured every two hours. The blood samples and urine samples were collected before treatment, during treatment every four hours, and at 24 hours. The blood samples were used for rMETase pharmacokinetics (50 μl), serum methionine measurement (25 μl) and laboratory analysis. Laboratory tests and measurements included: a complete blood count with differential, prothrombin time, partial thromboplastin time, electrolytes, protein, albumin, blood urea nitrogen, creatinine, aspartate dehydrogenase, alkaline phosphatase, calcium, uric acid, total and direct bilirubin, serum alanine aminotransferase, and urinalysis. Chest X-ray, electrocardiogram, and pertinent radiographic studies for evaluable/measureable disease were also done. Toxicity was determined according to WHO criteria.

Results and Discussion

Patient characteristics. A total of nine patients were evaluated with rMETase infusion. Of these nine patients, four had stage III or IV breast cancer, one had stage III renal cancer, one had stage IV lung cancer, two had stage III lymphoma, and one had stage IV gastric carcinoma. Their characteristics are listed in Table I.

Toxicity evaluation. None of the nine cases had any observable clinical toxicity during or after a single i.v. infusion of rMETase. Pulse, respiration rates, temperature and blood pressure had almost no change during and after treatment.

Toxicity was evaluated according to standard WHO criteria [14]. The physical examination and the laboratory determination before and after treatment were compared. No acute clinical toxicity was observed in any of the toxicity criteria measured. The patients were treated as described in the protocol of Table I. Blood and urine were collected before treatment, during treatment, and 24 hours after the start of treatment. The results suggest that i.v. infusion of rMETase did not cause any functional damage to major organs. There was no hematological, renal, neurological, or cardiac toxicity in any of the patients.

Pharmacokinetics results. Pharmacokinetic data of single infusion were obtained for rMETase levels in the serum. Typical pharmacokinetics data are shown in Figure 1. Serum rMETase levels achieved were between 0.1 units/ml-0.4 units/ml which correlate with the therapeutic doses of rMETase for cancer cells in vitro (Xu M. et al, unpublished data). These data suggest that the i.v. infusion of rMETase in patients can reach effective doses without toxicity.

Depletion of methionine in the serum. The depletion of serum methionine started within 60 minutes of the infusion, and reached maximum depletion after two hours. Methionine depletion was maintained during the infusion and for at least two hours after the infusion was completed. Methionine was depleted as low as approximately 0.1 μM within two hours (Table I, Figure 2) in patients corresponding to more than a 300-fold depletion. These levels of depleted methionine correspond to therapeutic levels for cancer cells in vitro (Xu M. et al, unpublished data). These results demonstrated that rMETase could essentially completely deplete methionine without any observed clinical toxicity, which is the basis for inhibition of tumor growth. The results justify further clinical trials of this novel and promising agent.

References