Depletion of Serum Methionine by Methioninase in Mice

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Abstract. Methionine dependence is a tumor-specific metabolic defect found in human cancer cells as well as in fresh human tumor specimens. Methionine dependent tumors cease growing when deprived of methionine, unlike normal cells which can substitute homocysteine for methionine for their growth requirement. We have previously purified a stable, endoxacin-free methioninase from the bacterium, Pseudomonas putida. We demonstrate in this report that purified methioninase can lower the serum levels of methionine in normal and nude mice from 60 μM to approximately 5 μM within 1 hour. The circulating half-life of methioninase is approximately 100 minutes in mice after i.v. injection. The enzyme therefore seems to be a good candidate as an antitumor agent for methionine-dependent tumors.

Methionine dependence, the inability of tumor cells to grow in vitro when methionine is removed from the culture medium and replaced by homocysteine, occurs frequently in malignant cell lines derived from all types of cancers (1-7). Methionine dependence also has been observed in fresh human solid tumor specimens in primary histoculture and therefore seems to occur in actual human cancer (8). Normal cells and tissues tested are methionine independent and grow after methionine is replaced by homocysteine (1, 3, 8).

Under conditions of a limiting methionine source, methionine-dependent tumors arrest in the late-S/G2 phase of the cell cycle (8, 9). Methionine dependence may be due to over-utilization of methionine for transmethylation reactions, resulting in low free-methionine pools and low S-adenosylmethionine/S-adenosylhomocysteine ratios, thereby blocking cell division (10-12).

In vitro treatment protocols were designed by us to exploit the methionine dependence of tumors with methionine starvation and cell cycle-specific chemotherapy, such that in co-cultures of tumor and normal cells only the normal cells could survive (13).

Recently a number of investigators have attempted to exploit methionine dependence of tumors for therapeutic effects in vivo. Poupon et al found for the RMS-J1 rat rhabdomyosarcoma tumor that a methionine-depleted diet lowered the metastatic potential of the tumor while not having significant effects on local tumor growth in rats (14). Goseki et al (15) found that a methionine-free total parenteral nutrition (TPN) mixture for rats bearing the Yoshida sarcoma extended the survival of the rats and slowed tumor growth of the rats, especially with the use of doxorubicin. Kreis, with the use of a methioninase, demonstrated some slowing of the W-256 rat carcinosarcoma growing in rats (16).

We have demonstrated that the Yoshida tumor growing in nude mice can actually regress with prolonged dietary methionine starvation with an extended survival period of the mice compared to tumor-bearing mice on a methionine-containing diet. (Guo, H-Y., Lishko, V.K., Herrera, H., Groce, A. and Hoffman, R.M., unpublished data). However, methionine starvation alone was insufficient to prevent metastases and initial growth of the tumor. A methionine cleaving enzyme could possibly lower methionine levels. A number of investigators have previously attempted purification of methioninases from various sources, but none seem pure or sufficiently stable for use as an injectable anticancer drug (16-22).

We have developed a greatly simplified, high-yield purification procedure that enables large scale production of endoxacin-free methioninase for eventual therapeutic purposes (24).

In order to test the potential antitumor efficacy of methioninase, it is first necessary to determine the ability of the enzyme to lower serum methionine levels and to determine the half life of the enzyme, which is the subject of this report.

Materials and Methods

Purification of methioninase. This procedure involves: 1) a heat step of the cell extract at 60°C for 5 minutes. 2) DEAE Toyopearl ion-exchange chromatography; 3) DEAE Sephadex A50 ion-exchange gel filtration chromatography; 4) affinity chromatography on Actidean to remove the endotoxin bound to the enzyme. The yield for this purification is 80% or more. The methioninase has four subunits of approximate molecular weight 43 KD (24).

**Figure 1.** *In vitro* cleavage of methionine by methioninase in mouse serum. Methioninase was added to dialyzed serum at a concentration of 100 nM. The incubation was carried out for 24 hours in the presence of 0.05 units of methioninase under sterile conditions. Ordinate values represent the amount of methionine cleaved by methioninase.

**Figure 2.** The clearance of methioninase from mouse blood. Methioninase (0.55 units) was injected into the tail vein of the mice. Activity of methioninase was measured in 100 μl of blood at 10, 60, and 210 minutes after injection.

**Methioninase assay.** The assay was carried out in a 1 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. The reaction was stopped by adding 0.5 ml of 4.5% TCA. The suspension was centrifuged at 15,000 X g for 5 minutes. 0.5 ml of supernatant was taken for α-ketobutyrate determination (18). The amount of protein was determined by measuring optical density at 280 nm.

**Animals.** Both athymic nu/nu mice and heterozygote nu/+ mice were used in this study and bred in the animal facilities of Anti Cancer, Inc.

**Diets.** Defined diets were obtained from Teklad (Madison, Wisconsin):
- Methionine-containing diet: #TD 93000
- Methionine-free diet: #TD 92007

**Measurement of serum methionine.** The concentration of methionine in the serum was determined as described by Heinrisson and Meredith (23). 50 μl of serum, with 0.5 nmole of norleucine added as a standard, were deproteinized by filtration through a Millipore Ultrafree-MC filter (NYWL 5.000). The filtrate was evaporated in a SpeedVac concentrator. Amino acid derivation was carried out in 25 μl of a pyridine:acetonitrile:methanol mixture (7:3:1) with 1 μl of phenylisothiocyanate. The sample was incubated for 10 min at room temperature and evaporated to dryness. The precipitate was dissolved in 300 μl of water and a 50 μl aliquot was analyzed by FPLC with a PepRPC (Pharmacia-LKB) column. The elution buffer consisted of solvent A, containing 50 mM ammonium acetate, pH 6.8, and solvent B, containing 50% acetonitrile in 100 mM ammonium acetate, pH 6.8.

**Results and Discussion**

**In vitro depletion of methioninase in mouse serum.** One ml of mouse serum was dialyzed against phosphate buffered saline (PBS) containing 10 μM pyridoxal phosphate. Methioninase was added to a final concentration of 100 μM. The serum was preincubated for 5 min at 37°C, pH 7.4. Fifty μl of purified methioninase containing 0.05 units were added to the mixture. 150 μl aliquots were taken for measurement of α-ketobutyrate in order to determine methioninase cleavage at various time points over 24 hours after addition of enzyme. As can be seen from Figure 1, the production of α-ketobutyrate took place over the 24 hour measurement period, indicating the stability of the enzyme in serum.

**In vivo half-life of methioninase.** After i.v. delivery of three units of methioninase in normal mice, 100 μl of blood were taken from the tail vein starting at 10 minutes and then at 1 hour, 2 hours and 31/2 hours. Methioninase activity was determined in the blood sample. As can be seen in Figure 2, the in vivo half life of the enzyme was 100 minutes. Even after three hours, there was at least 15% of the enzyme still circulating.

**Kinetics of methionine repletion after administration of methioninase.** After injection of 3 units of methioninase, mouse blood was taken at 2, 4, and 6 hours during which time the animals were fed with a methionine free diet. The initial level of methionine was 60 μM, by two hours after injection, 10 μM; by 4 hours, 12 μM; and by 6 hours 13 μM.

Thus, even though the 1/2-life of methioninase is only approximately 100 minutes, the methionine levels remain low for a reasonable period during the six hour measurement period.

**Activity of methioninase in normal mice on a methionine-free diet.** The mice were maintained on a methionine-free diet. As can be seen in Figure 4, the methionine levels in the serum of the mice dropped from 60 μM to 6 μM by 10 days on the methionine-free diet. Gradually the methionine levels increased up to 30 μM by day 30, possibly due to proteolysis. The methionine levels subsequently decreased and reached a plateau at about 8 μM between day -30 and -40. The mice did not exhibit any overt toxic effects except for weight loss of 40-50%. Methioninase was administered on day -60 and decreased the methionine concentration in the serum from 8 μM to approximately 1mM.
Activity of methioninase in nude mice on a methionine-free diet. As can be seen in Figure 5, when nude mice were put on a methionine-free diet the levels of methionine levels decreased from approximately 60 μM to 12 μM by day -3 and rose to 30 μM by day -6. On day -6, 3 units of methioninase were injected i.v. and methionine levels dropped to 5 μM after 1 hr. Methionine levels rose to 30 μM overnight and a subsequent i.v. injection again lowered the methionine levels back to approximately 5 μM. Thus, nude mice could be depleted of serum methionine by methionine-free diet and methioninase. This point is important for future efficacy studies of methioninase with nude mice carrying human tumors.

IP vs IV administration of methioninase. As can be seen in Figure 6, i.v. or i.p. injections of methioninase have a similar effect on the plasma methionine of treated animals. Nude mice kept on a methionine-free diet were injected intravenously or intraperitoneally with 3 units of the enzyme. After 1 hr, the methionine concentration decreased from 38 μM to 12 μM and from 38 to 14 μM, respectively after i.v. and i.p. injections.

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References


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