Methionine-Depletion Modulates the Efficacy of 5-Fluorouracil in Human Gastric Cancer in Nude Mice

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Abstract. Human tumors are generally methionine (MET)-dependent in that their growth is inhibited by MET-depletion down to levels that will still allow normal cell growth. The differential effect of methionine depletion on tumor and normal cells has suggested that methionine depletion may be able to modulate many and possibly all classes of cancer drugs. In this report, we determined if MET-depletion could modulate 5-fluorouracil (5-FU) efficacy on the human gastric cancer xenograft, SC-1-NU in nude mice. The tumor-bearing mice were treated with a MET-free diet and intraperitoneal administration of 5-FU at a dose of 30 mg/kg given for four cycles. MET depletion enhanced the antitumor activity of 5-FU by approximately two-fold with statistical significance of p<0.05. The MET-free diet increased intratumoral thymidylate synthetase inhibition early after 5-FU administration; Therefore, MET-depletion was thought to increase the 5-FU antitumor activity by modulating intratumoral folate metabolism. The data in this report suggest the high clinical potential of methionine depletion, combined with 5-FU and leucovorin on refractory tumors such as stomach cancer.

Methionine (MET)-dependence is the inability of tumor cells to grow or survive under the conditions of MET-depletion which still allow normal cells to grow or survive (1-7,17-20, 37, 38). MET-dependence has been observed in many types of human malignant tumors in vitro (1,2) and in vivo (38). Under conditions of MET-depletion, MET-dependent cancer cells, unlike normal cells, cannot divide and arrest in the late-G2 phase of the cell cycle. The tumor-selective late-G2 arrest due to MET-depletion has been observed in vitro (4), and in vivo (5). This tumor-specific cell cycle arrest was exploited in combination with chemotherapeutic agents in vitro to selectively eliminate tumor cells from cocultures with normal cells (6). The differential effect of methionine depletion on tumor and normal cells suggested that methionine depletion may be a universal modulator of other anticancer agents (20, 37).

5-fluorouracil (5-FU) is the most widely used agent against gastric carcinoma, and several regimens of combination chemotherapy with 5-FU have been studied in clinical trials. More effective regimens are needed since gastric carcinoma is relatively resistant to 5-FU. Leucovorin has been shown to be a modulator of 5-FU by raising levels of 5,10-methylene-tetrahydrofolate (21-35). Since methionine depletion has been shown to increase the levels of methionine synthase and release 5-methyltetrahydrofolate for interconversion to other folates (36), it was thought that methionine depletion may modulate the efficacy of 5-FU. We therefore took advantage of tumor-specific methionine metabolism to determine if 5-FU efficacy on a human gastric carcinoma xenograft could be modulated by methionine depletion.

Materials and Methods

Mice. Male BALB/c nu/nu mice originating from the Central Institute for Experimental Animals (Kawasaki, Japan), were purchased from CLEA, Tokyo, Japan. Mice aged 6-8 weeks and weighing 20-22 g were used for this study.

Tumor. A human gastric cancer xenograft, SC-1-NU that was established at the 2nd Department of Surgery of Nagoya University, kindly supplied by Dr. M. Yamauchi, was used in this study.

Transplantation. Tumors in the exponential growth phase in nude mice were resected aseptically, necrotic tissues were cut away, and the remaining viable tumor tissue was cut into pieces about 3 to 4 mm in diameter in Hanks' balanced salt solution. One block was transplanted subcutaneously to either side of the backs of nude mice under ether anesthesia.

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Figure 1. Methionine-free diet modulates 5-fluorouracil efficacy on the human gastric cancer xenograft, SC-1-NU.

a) shows the effect of MET-alone relative to unincinated control. The methionine (MET)-free diet was given for 6 days each week followed by the control diet given on the seventh day.

b) shows the effect of 5-FU alone and the combination of 5-FU and the MET-free diet relative to the MET-free diet alone. Mice treated with the combination chemotherapy groups were treated with intraperitoneal 5-FU at a dose of 30 mg/kg for 4 weeks on the fourth day after each initiation of MET-depletion. In the 5-FU-alone group, 5-FU was administered freely.

Diet. The defined diets, TD 93030 (MET-containing diet) and TD 92077 (MET-free diet) were purchased from TEKLAD (Madison, WI) (5,5). Since normal cells can synthesize MET from homocysteine, the MET-free diet was also depleted of homocysteine and choline to allow extensive depletion of MET in the murine serum. Tumor-bearing mice were allocated into groups consisting of five mice each when the estimated tumor weight reached 100-200 mg. Mice in the control and 5-FU-alone group were fed the MET-containing diet freely everyday. Mice in the MET-depleted group and combination chemotherapy group were fed the MET-free diet freely for six consecutive days each week followed by one day each week when the MET-containing diet was given freely.

Drug. Commerically available 5-fluorouracil (5-FU) was purchased from Kyowa Hakko Kogyo Co., Ltd., Tokyo. Mice were treated with 5-FU intraperitoneally at a dose of 30 mg/kg/week for 4 weeks on the fourth day after the initiation of MET-depletion. In the 5-FU-alone group, 5-FU was administered at the same time and the same dose as the combination group.

Tumor Measurement. The length and width of the tumors were measured with sliding calipers 2 times a week. The body weight of the mice was measured at the same time. The tumor weight was estimated according to the following formula: Tumor weight (mg) = length (mm) x width (mm) / 2 (8).

Evaluation of drug activity. Antitumor efficacy was evaluated by the T/C ratio (%), where T was the actual tumor weight of the treated group and C was the actual tumor weight of the control group at the time of sacrifice. Statistical analysis was performed by the Student’s t-test.

Assay of thymidylate synthetase and its degree of inhibition. After the mice were fed the control or MET-free diet freely for four days, both groups were treated with 30 mg of 5-FU per kg and sacrificed 0, 1, 2, 4, 6, and 12 hours after administration. MET-free feeding was continued in the MET-free alone group. The tumors were resected and frozen in liquid nitrogen as quickly as possible. Thymidylate synthetase (TS) was assayed with the method of Speers et al (10-11) with modifications previously reported elsewhere (12). In brief, an excess amount of [3H]fluorodeoxyuridine monophosphate ([3H]-FdUMP) was added to the samples to determine TS by binding assay. Approximately 200 ng of tumor tissue in 200 μl Tri-HCl, 10 mM 2-mercaptoethanol, 100 mM NaF and 15 mM cytidine monophosphate (pH 7.4) were homogenized with a Polytron, sonicated five times for 20 seconds, and centrifuged at 25,000 g for 60 minutes. Part of the cytosol fraction was stored at 4°C for the assay of free TS and the rest was incubated with 600 μM NH₄HCO₃, 100 mM 2-mercaptoethanol, 100 mM NaF and 15 mM cytidine monophosphate (pH 8.0, Buffer A) at 25°C for 4 hours to release FdUMP from the TS for the assay of total TS.

A volume of 0.05 ml of cytosol fraction was incubated with 0.05 ml of Buffer A, consisting of 0.05 ml of 10 mg/ml bovine serum albumin, 0.01 ml of coenzyme solution (10 mM tetrahydrofolic acid, 67 mM formaldehyde, 15 mM sodium ascorbate) and 0.05 ml of [3H]-FdUMP (in 5 mM potassium phosphate buffer; pH 7.4) at 30°C for 20 minutes. After 1 ml of charcoal suspension (3% activated charcoal, 0.5% BSA, 0.05% dextran T-70 in 0.1 N HCl) had been added to the samples, they were centrifuged at 2,000 g for 20 minutes. 0.8 ml of the supernatant was used to estimate radioactivity with a liquid scintillation counter (LSC-903, Aloka). Free TS was calculated from the measurement as TStotal = (TStotal x TS/TP divisor) x 0.13 x 0.87, where TS is free TS, TStotal is apparent free TS and TStotal is total TS. This
Table I. Modulation of 5-FU by methionine depletion.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean actual tumor weight in mg at end of experiments</th>
<th>SD(^a)</th>
<th>T/C(^b)</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt. 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methionine (+)</td>
<td>2234.9 ± 636.1</td>
<td>29.1</td>
<td>--</td>
<td>6</td>
</tr>
<tr>
<td>Methionine (−)(^d)</td>
<td>1409.6 ± 363.9</td>
<td>63.1</td>
<td>64.3</td>
<td>9</td>
</tr>
<tr>
<td>Expt. II</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methionine (−)(^d)</td>
<td>2623.2 ± 1630.3</td>
<td>39.3</td>
<td>--</td>
<td>7</td>
</tr>
<tr>
<td>5-FU 30 mg/kg(^e)</td>
<td>1397.6 ± 1024.8</td>
<td>53.2</td>
<td>53.2</td>
<td>8</td>
</tr>
<tr>
<td>Methionine (−) + 5-FU(^d)</td>
<td>672.3 ± 285.1</td>
<td>25.6</td>
<td>25.6</td>
<td>4</td>
</tr>
</tbody>
</table>

\(a\) Standard deviation.
\(b\) Treated/control of mean tumor weight.
\(c\) Number of tumors.
\(d\) Mice were fed the methionine (MET)-free diet freely for six consecutive days each week followed by one day when the MET-containing diet was given freely each week.
\(e\) 5-fluorouracil (5-FU) was administered intraperitoneally at a dose of 30 mg/kg for 4 weeks on the fourth day after each initiation of MET-depletion.
\(f\) Undetectable

The SC-1-NU gastric tumor growing s.c. in nude mice was treated as described in the Materials and Methods.

equation was used because 13% of TS combined with FdUMP is dissociated and re-combined with [\(^3\)H] FdUMP during incubation at 30°C for 20 minutes (10). TS inhibition rate (TSIR) was calculated from the measurement as TSIR (% = [(TStotal-TSfree)/TStotal] x 100).

Statistical analysis utilized the paired t-test.

Results

The MET-depletion diet partially suppressed the growth of SC-1-NU. The T/C ratio of the actual tumor weight was 63.1% in the MET-depleted group with a statistically significant difference relative to control at \(p<0.01\) (Figure 1A, Table I).

Tumor growth was remarkably inhibited by the combination of MET-depletion and 5-FU. A statistically significant difference was observed in the actual tumor weight between MET depletion alone and the combination chemotherapy at \(p<0.05\). The T/C ratio was 25.6% and 53.2% in the groups treated with 5-FU with and without the MET-free diet, respectively, compared to the MET-free diet alone.

The time-dependent change of thymidylate synthetase (TS) in the SC-1-NU tumor is shown in Table II. In untreated tumors, total TS (TStotal) was 25.8 pmol/g-tissue, free TS (TSfree) was 23.8 pmol/g-tissue, and the TS inhibition rate (TSIR) was 8.5%. MET-depletion slightly suppressed TStotal to 18.5 pmol/g-tissue. TSIR was 6.5% which was not increased by MET-depletion alone, since TSfree was 17.3 pmol/g-tissue. 5-FU alone suppressed TSfree from 1 to 12 hours after drug administration, resulting in TSIR of 86.0% to 65.8%. This increased TS inhibition was more remarkable in the combination of 5-FU and MET-depletion where TSfree was undetectable 1 hour after 5-FU treatment. TSIRs of the combination group were higher than those of 5-FU alone group with a statistically significant difference of \(p<0.05\) with the paired t-test. This inhibition was observed at the early phase of 5-FU treatment.

Discussion

Methionine (MET)-dependence is a tumor-specific biochemical defect expressed by the inability or decreased ability of tumors to grow or survive under conditions of MET-depletion that still allow normal cells to grow or survive. Many reports have shown that MET-dependence occurs in human tumors of all types in vitro and in vivo (1-7). Recently, we have reported that human cancer xenograft growth is significantly inhibited when the nude-mouse hosts are fed a MET-free diet (3) or are treated with methioninase (38). We have also
demonstrated that MET depletion can significantly modulate the efficacy of cisplatin against the MX-1 human breast carcinoma xenograft when grown in nude mice (13). In previous studies, the body weight of the methionine-depletion treated mice was found to be maintainable by once-per-week administration of a methionine-containing diet (3, 13). In the present report, a similar feeding schedule of MET-depletion was conducted which resulted in low toxicity of tumor-bearing nude mice in terms of death rate and body-weight loss.

MET starvation leads to depleted MET levels in cells, modifies methylation reactions, lowers glutathione levels, alters folate distribution and leads to a tumor-selective cell cycle arrest in late-S/G2 (1-7). In the present study, we have demonstrated the modulating activity of methionine depletion on the efficacy of 5-FU on a human gastric cancer xenograft, SC-1-NU which was statistically significant. When thymidylate synthetase was assessed, MET-depletion was found to have increased the intratumor TSIR with statistical significance, contributing to an incremental increase in FU antitumor activity.

Scalon reported that treatment of cisplatin inhibited MET-uptake and decreased intracellular MET-concentration. Consequently, 5-formyltetrahydrofolate (leucovorin) and 5,10-methyleneetetrahydrofolate levels increased due to accelerated endogenous MET synthesis (36) allowing the formation of a ternary complex with 5,10-methyleneetrahydrofolate which enhances the inhibition of thymidylate synthetase (14). The data in this report suggest that MET-starvation of the tumor-bearing mice enhanced the antitumor effect of 5-FU on SC-1-NU by increasing the intratumoral TSIR.

Hibino et al reported the potentiation of the antitumor effect of 5-FU on the Yoshida sarcoma by MET-free intravenous amino acid solution (AO-90) in rats (15). When the Yoshida sarcoma-bearing rats were treated with AO-90, the serum MET level decreased, intratumor folate content increased, and TS inhibition was elevated with statistically significant differences. The results are consistent with the present report in which a human tumor xenograft was used. MET-free total parenteral nutrition enhanced the antitumor effect of 5-FU and mitomycin C against advanced gastric cancer in the clinic (16,17). These results suggested that MET-depletion therapy is a promising strategy in anticancer chemotherapy.

Several regimens of 5-FU and leucovorin have been used in clinical cancer therapy (21-24,34,35). However, their antitumor activity remains moderate (21-24,34,35). The results of the present study suggest that the antitumor activity of the regimens of combined 5-FU and leucovorin might be improved by methionine depletion. Increased synthesis of methionine within the tumor cells, resulting from the depletion of methionine, probably resulted in elevated intracellular concentrations of 5,10-methyleneetetrahydrofolate.

The optimal modulation of 5-FU at the biochemical level requires large intracellular concentrations of 5,10-methyleneetetrahydrofolate since the stability of the ternary complex with TS increases with increasing concentrations of 5,10-methyleneetetrahydrofolate (21-24). However, the intracellular increase of 5,10-methyleneetetrahydrofolate after leucovorin administration has been moderate in the tumor models tested (21-24). 5,10-methyleneetetrahydrofolate enters the reduced folate active pool through formation of tetrahydrofolate during synthesis of methionine from homocysteine via methionine synthetase which uses 5-methyltetrahydrofolate as a cofactor (21-24).

Thus, depletion of methionine plus administration of leucovorin should result in a great increase in the endogenous production of 5,10-methyleneetetrahydrofolate whereby FdUMP could significantly inhibit TS (21-24).

Future studies will focus on MET depletion with the enzyme methioninase (18, 19, 20) to modulate 5-FU and leucovorin as well as other classes of chemotherapeutic agents in order to develop a new approach to the treatment of cancer.

References


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