Doxorubicin and Vincristine with Methionine Depletion
Contributed to Survival in the Yoshida Sarcoma Bearing Rats

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Abstract. Several anti-cancer agents show increased toxicity if administered with methionine-depleting total parenteral nutrition (Met-deplete TPN). Changes in the cell cycle due to Met-deplete TPN were investigated, and then the enhancement of the anti-tumor effects of serial combinations of doxorubicin (ADM), a drug acting on late S-G2 phase and vincristine (VCR), an antimitotic drug, under Met-deplete TPN was also examined in the tumor-bearing rats. According to the fraction of labeled mitosis, within 3 to 4 days after the introduction of Met-deplete TPN in the ascites type Yoshida sarcoma (YS) -bearing rats, the cell cycle of the tumor cells showed marked delay and the fraction of labeled mitosis decreased to less than 70 %. However, this delay was recovered immediately after methionine infusion, with on increase in the labeled mitotic cell population. In the experiment using solid type YS-bearing rats, ADM was administered intraperitoneally under Met-deplete TPN for 8 days, followed by intraperitoneal VCR administration with methionine-containing TPN for 3 days, and then fed on solid food and water ad libitum until death. This serial combination of Met-deplete TPN with ADM and VCR resulted in marked suppression of the tumor and prolonged survival in comparison to the control groups with a significant difference (p<0.001) (generalized Wilcoxon test).

Anti-methionine cancer chemotherapy, the method of administration of antineoplastics under methionine depletion, has emerged as a possible effective cancer therapy (1, 2, 4, 14, 16). An L-methionine and L-cysteine lacking amino acid solution, namely AO-90, was produced by removing L-methionine from commercial Vuj-N type Pan-Amin S® (Otsuka Pharmaceutical Co. Ltd., Tokyo, Japan) (Table I). By using AO-90 as the only source of protein for total parenteral nutrition (TPN)(3), marked methionine depletion could be obtained in vivo without any particular adverse side effects (6). This parenteral treatment has been proved to enhance the toxicity of several antineoplastic agents such as 5-fluorouracil (5-FU)(9, 11) and nimustine hydrochloride. (8)

It has been reported that the tumor-selective cell cycle arrest occurred in the late S-G2 phase in tissue culture study using methionine-depleting medium (14). However, in our experiments using ascites type hepatoma-bearing rat, cell cycle synchronization could not be demonstrated by Met-deplete TPN in the flow cytometric studies (results not shown), it was also reported that the same cell cycle block was demonstrated in vivo in YS-bearing nude mice experiments using prolonged dietary starvation of methionine (13). Therefore, we made further investigations using the classical method of labeled mitosis wave, an autoradiographic examination using 3H-thymidine, in the same tumor-bearing rat model to identify the influence of Met-deplete TPN on the tumor cell cycle in vivo.

Moreover, in another experiment, the effect of serial administration of doxorubicin (ADM), a drug acting on the late S-G2 phase cells, and vincristine (VCR), an anti-mitotic drug were also investigated.

Materials and Methods

Animals and tumors. Male Donryu rats obtained from the Shizuoka Laboratory Animal Center (Shizuoka, Japan) and YS tumor cells (donated by the Sanyo Institute, Tokyo, Japan) were maintained as ascites tumors by weekly i.p. implantation were used. Seven days after implantation, ascites tumor cells were used in the experiments described below.

Experiment I: Analysis of the influence of met-deplete TPN on the cell cycles of the ascites YS cells in vivo (Figure 1). Twenty-seven 7-week-old, male Donryu rats, weighing about 200g, received i.p. transplantation of 5x106 cells of ascites-type tumor YS (day 0). Apart from rats in the
Table I. Composition of AO-90 and Pan-Amin SR amino acid solutions.

<table>
<thead>
<tr>
<th>Amino acid content</th>
<th>AO-90 (g/100ml)</th>
<th>Pan-Amin SR (g/100ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Arginine</td>
<td>0.66</td>
<td>0.66</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>0.30</td>
<td>0.30</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>0.55</td>
<td>0.55</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>1.23</td>
<td>1.23</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>1.49</td>
<td>1.49</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>-</td>
<td>0.71</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>0.87</td>
<td>0.87</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>0.54</td>
<td>0.54</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>0.18</td>
<td>0.18</td>
</tr>
<tr>
<td>L-Valine</td>
<td>0.61</td>
<td>0.61</td>
</tr>
<tr>
<td>Glycine</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Na (mEq/A)          | <3              | 13                    |
Cl (mEq/A)          | 0               | 155                   |
Total amino acid (g/100ml) | 7.43 | 8.14 |
Total N (g/100ml)   | 1.19            | 1.26                  |

Table II. Composition of TPN solution TPN regimens in the tumor-bearing rat experiment.

<table>
<thead>
<tr>
<th></th>
<th>AO-90 group</th>
<th>Pan-Amin S group</th>
<th>Glucose group</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% Glucose</td>
<td>(ml)</td>
<td>105</td>
<td>105</td>
</tr>
<tr>
<td>AO-90</td>
<td>(ml)</td>
<td>123</td>
<td>-</td>
</tr>
<tr>
<td>Pan-Amin S</td>
<td>(ml)</td>
<td>-</td>
<td>123</td>
</tr>
<tr>
<td>Electrolyte solution</td>
<td>(ml)</td>
<td>22.6</td>
<td>22.6</td>
</tr>
<tr>
<td>Vitamin mixture</td>
<td>(ml)</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Sterile water</td>
<td>(ml)</td>
<td>-</td>
<td>123</td>
</tr>
<tr>
<td>Total volume</td>
<td>(ml)</td>
<td>251</td>
<td>251</td>
</tr>
<tr>
<td>Total calories</td>
<td>(kcal)</td>
<td>247</td>
<td>250</td>
</tr>
<tr>
<td>Total N</td>
<td>(g)</td>
<td>1.46</td>
<td>1.55</td>
</tr>
</tbody>
</table>

Non-protein calories / N | 144 | 135 |

* Amounts in mg per 251 ml of infusion; thiamine HCl 0.5, riboflavin 0.05, pyridoxine 0.1, nicotinamide 1.0, pantenol 0.1, ascorbic acid 2.5, hydroxyecobalamin 0.02.

Freely fed group described below, each rat was cannulated in the vena cava according to the reported method (20), soon after transplantation (day 0), and was kept on TPN for 7 days with one of the four regimens, presented in Table II. The amino acid solutions used in TPN were AO-90 and Pan-Amin S® (Table I). During the TPN period, the rats received no food per os, apart from the Freely fed group. To analyze the effect of net-deplete TPN on the cell cycle of tumor cells, we employed the autoradiographic analytical method of labeled mitosis wave, using 

3H-thymidine (18).

The animal groups were as follows:

a) AO-90 group (n=6): Rats received TPN with AO-90 for 7 days and i.p. pulse injection of 500 mCi/kg BW of 3H-thymidine was administered on day 5. The TPN regimen was shown in Table II.

b) Pan-Amin S group (n=5): The rats received TPN with Pan-Amin S® for 7 days. 3H-thymidine was administered to each rat in the same dose and manner as the AO-90 group. The TPN regimen was shown in Table II.

c) A-P® group (n=3): The rats received AO-90 regimen for 4 days, then TPN was changed to Pan-Amin S regimen from the time of i.p. administration of 500 mCi/kg BW of 3H-thymidine.

d) A-P® group (n=2): Each rat received TPN for 4 days with AO-90 regimen, then changed to Pan-Amin S regimen. 16 hours before the change over, administration of 3H-thymidine was performed.

e) Glucose group (n=2): Rats received TPN for 6 days without any amino acid, 3H-thymidine was administered on day 5.

f) Freely fed group (n=8): All rats were given solid food (Oriental Yeast Co., Ltd., Tokyo) and water ad libitum for 7 days. 3H-thymidine was administered to each rat as in AO-90 and the Pan-Amin S groups.

During TPN, the rats were individually housed in metabolic cages. The microinfusion pump was used for constant administration of each

TPN solution. After the i.p. administration of 3H-thymidine, 0.05 ml acetic fluid was obtained from each rat at every one to two hours for 60 to 72 hours as illustrated in the schema of the experimental protocol (Figure 1).

Experiment 2: Antitumor effect of met-deplete TPN with ADM and VCR administration on the YS-bearing rat. Forty-eight, 7-week-old, male Donryu rats, received transplantation of 1×10^6 YS cells into the adipose tissue on the back (day 0). Thirty-two rats were cannulated into the vena cava immediately after tumor transplantation, as in experiment 1 (on day 0), and started with TPN immediately after tumor transplantation (day 0), they were then divided into two groups namely the AO-90 and Pan-Amin S groups, which were kept on TPN for 8 days. The TPN regimens for each group are shown in Table II. The rats received no food per os during TPN. The rats in both groups were subdivided according to ADM and VCR administrations into 4 groups, namely AO-90+/ADM and VCR) group, AO-90 group, Pan-Amin S+/ADM and VCR) group and Pan-Amin S group. The remaining 16 rats were fed with solid food and water ad libitum (Freely fed group) and were also divided into 2 groups according to the administration of these antineoplastic drugs, namely Freely fed+/ADM and VCR) and Freely fed groups. Each of these 6 experimental groups consisted of 8 rats. Regarding the 3 groups with ADM and VCR administrations, each rat received ADM 3.5 times (day 3, 5 and 7) at a dose of 0.5 mg/kg per one time via i.p. injection.

For 3 days, from day 9 to 11, all 32 rats in the AO-90+/ADM and VCR), AO-90, Pan-Amin S+/ADM & VCR) and the Pan-Amin S groups were given the same methionine-containing TPN solution, the Pan-Amin S, as shown in Table II. There after all rats were fed with solid food and water ad libitum. Each rat in the AO-90+/ADM and VCR), Pan-Amin S+/ADM and VCR), and Freely fed+/ADM and VCR) group received i.p. administration of VCR 3 times (on days 9, 10, and 11) at a dose of 0.05 mg/kg and 2 times (on days 10 and 11) at a dose of 0.1 mg (Figure 2). As a result, there were 6 experimental groups as follows:

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Figure 1. Experimental protocol for the analysis of the influence of Met-Deplete TPN on the AH-109A ascites hepatoma cell kinetics in vivo.

a) AO-90 + (ADM and VCR) group (n = 8): Rats received TPN with AO-90 for 8 days, followed by TPN with Pan-Amin S for 3 days. Each rat received ADM 3 times (day 3, 5 and 7) at a dose of 0.5 mg/kg per one time via i.p. injection. Then VCR was also administered at a dose of 0.05 mg/kg on days 9, 10 and 11, and at a dose of 0.1 mg/kg on days 12 and 13.

b) The AO-90 group (n = 8): Rats were maintained in the same manner as the AO-90 + (ADM and VCR) group except for ADM and VCR.

c) The Pan-Amin S + (ADM and VCR) group (n = 8): Rats received TPN for 11 days with Pan-Amin SR. ADM and VCR were administered at the same dose and manner as in the AO-90 + (ADM and VCR) group.

d) The Pan-Amin S group (n = 8): Rats were maintained in the same manner as in the Pan-Amin S + (ADM and VCR) group without ADM and VCR administration.

e) Freely fed + (ADM and VCR) group (n = 8): The rats were not cannulated, but were fed with solid food and water ad libitum and each rat received ADM and VCR administration at the same dose and manner as in the AO-90 + (ADM and VCR) group.

f) Freely fed group (n = 8): Rats were maintained in the same manner as in the Freely fed + (ADM and VCR) group without ADM and VCR administration.

During TPN, rats, excluding the Freely fed + (ADM and VCR) and the Freely fed groups, were individually housed in metabolic cages and a microinfusion pump was used for constant administration of TPN solutions. From day 12, all rats in all groups were fed with solid food and water ad libitum during the experiments without TPN. The life span of each rat was studied.

The same experiments were repeated 3 times because of the rat number of each group was as small as 8 in one experiment due to the limitations of the metabolic cage and infusion pump numbers.

Statistical analysis. Survival periods were analyzed using the product-limit estimation (15). The generalized Wilcoxon test was applied to evaluate the differences between median survival days (MSD) in the 6 groups. The significance of the differences between the curves were also evaluated with correction using Bonferroni’s test.

Results

Experiment 1: Labeled mitosis waves of YS cells in all the rats in each group. Labeled mitosis waves of all the rats in each group obtained in experiment 1 have been drawn in Figure 3. In the AO-90 group, the labeled mitotic fraction was less than 70%, and the duration of cell cycle was prolonged and could not be calculated. In contrast, the curves of the Pan-Amin S group and the Glucose group showed the same pattern as the Freely fed group, though the duration of cell cycle was prolonged to some degree. Immediately after changing the amino acid solution from AO-90 to Pan-Amin S®, the labeled mitotic fraction began to increase and the labeled mitosis wave pattern was similar to that of the Pan-Amin S group within 16 hours (A-P[0] and A-P[16]).

The life span of each rat in the 6 experimental groups is shown in Table III and Figure 4.

Experiment 2: Survival days of the animals in each group. The MSD of each group is summarized in Table III. Figure 4
shows the survival curves of one experiment according to Kaplan and Meier estimation, (an example from experiment 2) since almost identical results were obtained in the other experiments. No rat in any group had a catheter problem during TPN. All rats in the AO-90+(ADM and VCR) group survived more than 28 days after the initiation of the experiment, while more deaths were found in the other 5 groups within 24 days. Also, in the rats received no antineoplastics, AO-90, Pan-Amin S and Freely-fed groups, all rats died within 16 days. Five rats in the AO-90+(ADM and VCR) group survived more than 20 days, and one of them was sacrificed on day 60. The survival curves of the 6 groups are illustrated in Figure 4.

The MSD of the AO-90+(ADM and VCR) group, AO-90 group, Pan-Amin S+(ADM and VCR) group, Pan-Amin S group, Freely fed+(ADM and VCR) group and Freely fed
Figure 3. Illustration of the experimental protocol of the anticancer effect of Met-Deplete TPN with doxorubicin and vincristine on YS-bearing rats.

Table III. Median survival days of mice rats in the 6 experimental groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Median Survival Day</th>
</tr>
</thead>
<tbody>
<tr>
<td>AO-90 + (ADM &amp; VCR)</td>
<td>31.0</td>
</tr>
<tr>
<td>AO-90</td>
<td>13.0</td>
</tr>
<tr>
<td>Pan-Amin S + (ADM &amp; VCR)</td>
<td>18.5</td>
</tr>
<tr>
<td>Pan-Amin S</td>
<td>11.0</td>
</tr>
<tr>
<td>Freely fed + (ADM &amp; VCR)</td>
<td>20.0</td>
</tr>
<tr>
<td>Freely fed</td>
<td>18.5</td>
</tr>
</tbody>
</table>

* Significantly different at P<0.001 in the log-rank test.
** Significantly different between the value by correction Bonferroni’s test at P<0.05.

Discussion

It has been reported that tumor-selective cell cycle arrest occurs in the late S-G2 phase in medium lacking methionine in tissue culture studies (14). Recently the same cell cycle arrest was also demonstrated in vivo in YS-bearing nude mice experiments by prolonged dietary starvation of methionine (13). However, in our earlier flow-cytometric survey, using tumor-bearing rat experiment, no clear cell cycle arrest could be demonstrated. (data not shown)

Although YS cells were thought to be methionine dependent tumor cells, and the results of the experiment 1 suggested that within 3 to 4 days after the introduction of met-deplete TPN, the cell cycle of the tumor cell was markedly delayed and the fraction of labeled mitosis decreased to less than 70%, a cell cycle block in the late S-G2 phase could not be demonstrated. However, the delay in the cell cycle returned to normal immediately after the methionine infusion, with rapid elevation of the labeled mitotic cell population. We considered this failure to acquire complete late S-G2 phase cell cycle arrest to be due to insufficient methionine depletion, since the ascites methionine concentration rapidly decreased to less than one fourth, as 18.4 μM, during this parenteral treatment in YS-bearing rats, as compared to 87.7 μM of control group rats receiving methionine containing TPN (6). In general, it is difficult to feed the rat more than one week by TPN, so to create more severe methionine depletion we used antimethionine agents such as methioninase. In our opinion, we can demonstrate complete cell cycle block in vivo within a short period of 2 to 3 days using the combination of methioninase, namely purified endotoxin-free L-methioninase (17) and Met-deplete TPN.

The anti-tumor effect of sequential administration of ADM and VCR has been examined in one tumor-bearing rat model in the same manner as earlier reported, during (10) and after Met-deplete TPN (12). Consequently, in YS-bearing rats, the rats treated with methionine infusing TPN with VCR administration after met-deplete TPN with ADM, survived
Figure 4. Life span of the rats in 6 experimental groups.

longer than other control group with a significant difference.

This principle has been presented by Stern and Hoffmann (19), on the basis of their tissue culture studies. They reported that the late S-G2 phase cell cycle arrest caused by methionine depletion could selectively potentiate the sensitivity of the tumor to drugs acting on the late S-G2 phase cells, and antimitotic drugs might be effective after the reversal of the methionine depletion cell-cycle block. We consider that the results presented here are the first demonstration of their principle in vivo with sequential administration of both drugs in the same animal. Therefore, Met-deplete TPN has been shown to be useful not only combined with anticancer agents such as 5-FU (9, 11) and ADM, but also after loading the of anti-mitotic drugs such as VCR with methionine infusion following combined treatment. The use of antineoplastic agents can be made more effective by understanding the associated metabolic changes, including the effect on tumor cell kinetics or thiol depletion (8), during or following Met-deplete TPN. Also, we would like to examine the combined effect of Met-deplete TPN with methioninase to achieve complete late S-G2 block of the cell cycle for the radical cure of cancer.

Clinical trials, including a late phase II multicentric prospective randomized comparative study, yielded better clinical results for gastric cancer patients who received this parenteral treatment with 5-FU and mitomycin C than those who received methionine infusing TPN with the same drugs (21, 22). These therapies could be continued for 2 to 3 weeks without any serious adverse effects in these patients (5). We would like to conduct another clinical trial of met-deplete TPN with ADM and VCR on the basis of the results of the present study.

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