Jpn. J. Cancer Res. 87, 194–199, February 1996

Enhanced Anticancer Effect of Vincristine with Methionine Infusion after Methionine-depleting Total Parenteral Nutrition in Tumor-bearing Rats

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Methionine-depleting total parenteral nutrition (Met(−) TPN), in which an amino acid solution devoid of L-methionine and L-cysteine is infused, is thought to reduce tumor cell growth through acting as a partial late S-G_2 (i.e., late-S and G_2 phases) blocker. The antitumor effect of vincristine (VCR), which acts on mitotic phase cells, was examined with methionine infusion immediately after Met(−) TPN in Yoshida sarcoma (YS)-bearing rats. Rats were given Met(−) TPN for 8 days immediately after inoculation with YS cells (days 0 to 8), which was followed by methionine-containing (Met(+)) regular TPN for 3 days (days 9 to 11) along with intraperitoneal administration of 0.05 mg/kg/day VCR. All rats were then fed solid food and water *ad libitum* until they died, with 0.1 mg/kg VCR administration on days 12 and 13. As controls, a Met(−) TPN only group, Met(+) TPN groups with and without VCR, and freely fed groups with and without VCR were studied. The progression of YS was markedly suppressed by Met(−) TPN with VCR. The median survival time in days was 25 days, significantly longer ($P < 0.001$) (generalized Wilcoxon's tests) by 11 to 14 days than that of any of the other groups. In conclusion, VCR appears to have greater efficacy as an anticancer agent when administered together with methionine after Met(−) TPN.

Key words: Methionine-depleting TPN — Cell cycle — Vincristine — Yoshida sarcoma-bearing rat — Methionine

Methionine has been reported to be indispensable for proliferation of most types of tumor cells, not only *in vitro* but *in vivo*.[1-5] We devised a methionine-depleting total parenteral nutrition (Met(−) TPN) to decrease safely the content of methionine *in vivo* by infusing an amino acid mixture devoid of L-methionine and L-cysteine in total parenteral nutrition (TPN).[6] During 3 weeks of this parenteral treatment in dogs, plasma methionine levels were reduced to 1/3 within 4 days after commencement and then remained at about the same value until the 21st day without notable adverse effects except for a reversible deterioration of the nutritional state.[7] In cancer tissue, the methionine concentration also decreased to 1/3 of that of control groups in a 10-day Met(−) TPN study in tumor-bearing rats, although the anticancer effect was not marked.[8, 9]

We have studied this parenteral treatment as an adjunct to cancer chemotherapy[6] from the viewpoint of amino acid imbalance.[10] The antineoplastic effects of several anticancer agents were enhanced by Met(−) TPN in tumor-bearing animal experiments and in clinical trials on gastrointestinal cancer without serious adverse effects on the host.[6, 11-20] This treatment causes many derangements in tumor metabolism. In tumors growing in rats, the cell cycle of the tumor cells was delayed. Further, the fraction of labeled mitosis was reduced markedly, but increased again immediately after methionine infusion (unpublished results). Both glutathione and glutathione disulfide in hepatic and tumorous tissues also decreased markedly after methionine depletion.[13]

Recently, Guo *et al.* demonstrated, in Yoshida sarcoma (YS)-bearing nude mice, that YS cells were arrested in the late-S and G_2 phases (late S-G_2) of the cell cycle when the animals were fed a methionine-depleted diet.[21] We demonstrated the synergism of doxorubicin, which affects late S-G_2 phase cells, with Met(−) TPN in YS-bearing rats.[18] In an *in vitro* study, Stern and Hoffman showed that when cells entered mitosis following administration of methionine after late S-G_2 cell cycle arrest caused by methionine depletion, the toxicity of antimitotic drugs such as vincristine (VCR) was enhanced selectively.[22]

Therefore in this study, we examined whether such a synergistic anticancer effect of VCR could be observed *in vivo*, when VCR was administered with methionine-containing (Met(+)) TPN immediately after Met(−) TPN in YS-bearing rats.

MATERIALS AND METHODS

Animals and tumor For the experiment described here, male Donryu rats obtained from the Shizuoka Laboratory Animal Center (Shizuoka) were used. YS tumor

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cells (donated by the Sasaki Institute, Tokyo) were maintained as an ascites tumor in the Donryu rats by weekly intraperitoneal implantation. In the experiment, tumor cells, sampled 7 days after transplantation, were inoculated into each rat after determination of the number of viable cells using the trypan blue exclusion test.

Experimental design: Inhibition of YS growth by VCR administration with methionine following Met(−) TPN in YS-bearing rats Forty-eight 7-week-old male Donryu rats, weighing approximately 200 g, were used for the experiments. Rats were transplanted with 1×10^4 YS cells into the dorsal adipose tissue (day 0). A solid-type tumor developed.

Thirty-two rats were cannulated into the vena cava immediately after tumor transplantation according to the method of Steiger *et al.*[23] (on day 0), and were divided into two groups, the Met(−) TPN (AO-90) group and Met(+) TPN (Pan-Amin S) group, which were kept on TPN for 8 days. AO-90 is an amino acid solution which does not contain L-methionine or L-cysteine. Pan-Amin S is a commercial amino acid solution containing L-methionine, from which AO-90 was prepared by removal of L-methionine. Table I summarizes the compositions of AO-90 and Pan-Amin S amino acids solutions. Table II shows the composition of both TPN solutions infused into each group as a daily dose per kg. The rats received no food orally while receiving TPN. The remaining 16 rats were given solid food (Oriental Yeast Co., Ltd., Tokyo) and water *ad libitum* (freely fed groups). From day 9, all rats in both the AO-90 and Pan-Amin S groups were given methionine-containing Pan-Amin S as the nitrogen source for 3 days until day 11, then all rats were given solid food and water *ad libitum* until they died of advanced cancer. Eight rats in each of the AO-90, Pan-Amin S, and freely fed groups were administered VCR intraperitoneally 3 times (on days 9, 10, and 11) at a dose of 0.05 mg/kg and 2 times (on days 12 and 13) at 0.1 mg (Fig. 1). As a result, there were 6 experimental groups as follows:

1. The AO-90+VCR group (n=8): Rats received TPN with AO-90 for 8 days, followed by TPN with Pan-Amin S for 3 days. VCR was 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. From day 12 until they died, animals were given solid food and water *ad libitum.*
2. The AO-90 group (n=8): Rats were maintained in the same manner as the AO-90+VCR group except for VCR.
3. The Pan-Amin S+VCR group (n=8): Rats received TPN for 11 days with Pan-Amin S. VCR was administered at the same dose and in the same manner as in the AO-90+VCR group.

Table I. Compositions of AO-90 and Pan-Amin S Amino Acid Solutions

Amino acid	AO-90	Pan-Amin S
L-arginine	0.66	0.66
L-histidine	0.30	0.30
L-isoleucine	0.55	0.55
L-leucine	1.23	1.23
L-lysine	1.49	1.49
L-methionine	0	0.71
L-phenylalanine	0.87	0.87
L-threonine	0.54	0.54
L-tryptophan	0.18	0.18
L-valine	0.61	0.61
Glycine	1.00	1.00
Na (mEq/liter)	<3	13
Cl (mEq/liter)	0	155
Total amino acid (g/100 ml)	7.43	8.14
Total N (g/100 ml)	1.19	1.26

Table II. TPN Regimens for the YS-bearing Rats

	AO-90 group	Pan-Amin-S group
50% Glucose (ml)	105	105
AO-90 (ml)	123	—
Pan-Amin S (ml)	—	123
Electrolyte sol. (ml)	22.6	22.6
Vitamin mixture[a] (ml)	0.1	0.1
Sterile water (ml)	—	—
Total volume (ml)	251	251
Total calories (kcal)	247	250
Total N (g)	1.46	1.55
Nonprotein calories/N	144	135

a) Amounts in mg per 251 ml of infusate ; thiamine HCl 0.5, riboflavin 0.05, pyridoxine 0.1, nicotinamide 1.0, pantenol 0.1, ascorbic acid 2.5, hydroxycobalamin 0.02.

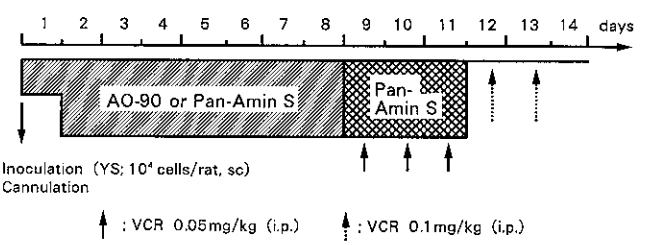

Fig. 1. Experimental protocol. After inoculation of YS, all rats except freely fed+VCR and freely fed groups underwent central venous cannulation with subsequent TPN administration (each regimen is summarized in Table II). TPN was continued for 8 days and followed by methionine-containing TPN solution (Pan-Amin S) for 3 days. VCR (0.05 mg/kg) was administered i.p. 3 times (days 9, 10, 11) and then VCR (0.1 mg/kg) was given i.p. 2 times (days 12, 13).

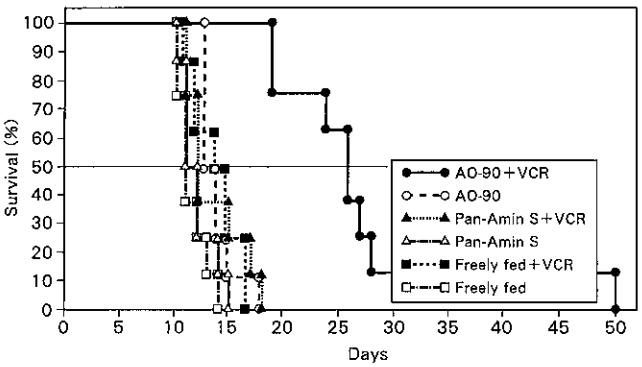

Fig. 2. Survival rates of rats in each experimental group. All rats in the AO-90+VCR group survived more than 18 days and the median survival time in days (MSD) of this group was 25 days. All rats in the other 5 groups died from their tumor within 18 days and the MSD in these groups ranged from 10.67 to 14 days. There were significant differences in the MSDs between the AO-90+VCR group and the other 5 groups at $P<0.001$ (generalized Wilcoxon's test).

Table III. Median Survival Time in Days (MSD) in Each Group

Group	MSD (days)
AO-90+VCR	25.0
AO-90	13.0
Pan-Amin S+VCR	11.6
Pan-Amin S	11.0
Freely fed+VCR	10.67
Freely fed	14.0

* $P<0.001$ (generalized Wilcoxon's test).

4. The Pan-Amin S group (n=8): Rats were maintained in the same manner as in the Pan-Amin S+VCR group, except for VCR administration.
5. Freely fed+VCR group (n=8): No rats were cannulated, but all were given solid food and water *ad libitum*, and on days 9, 10, 11, 12, and 13 VCR was administered at the same dose and in the same manner as in the AO-90+VCR group.
6. Freely fed group (n=8): Rats were maintained in the same manner as in the freely fed+VCR group, except for VCR administration.

During TPN, rats except for the freely fed+VCR and the freely fed groups, were individually housed in metabolic cages and a microinfusion pump was used for constant administration of TPN solutions. Rats in all groups were given solid food (Oriental Yeast Co., Ltd.) and water *ad libitum* during the experiments without TPN. The life span of each rat was observed.

Statistical analysis Survival periods were analyzed using the product-limit estimate developed by Kaplan and Meier.[24] The generalized Wilcoxon's test was applied to evaluate the differences between median survival time in days (MSD) in the 6 groups.

This experiment was repeated three times with almost identical results.

RESULTS

The results of one experiment of the three are presented here, because the results of all three experiments were similar.

Life span in the 6 experimental groups

Survival: No rat in any group had catheter problems during TPN. All rats except in the freely fed groups survived more than 12 days after initiation of the experiment. All rats in the AO-90+VCR group survived more than 18 days, while all rats in the other groups died from the tumor within 18 days after tumor inoculation. In the AO-90+VCR group, 5 rats survived more than 25 days. One rat in this group died of the tumor on day 50. Survival curves of the 6 groups are shown in Fig. 2.

The MSD in the AO-90+VCR group was 25 days, as compared with 13 days in the AO-90 group, 11.6 days in the Pan-Amin S+VCR group, 11 days in the Pan-Amin S group, 10.67 days in the freely fed+VCR group, and 14 days in the freely fed group. There were significant differences between the AO-90+VCR group and each of the other 5 groups at $P<0.001$ (generalized Wilcoxon's test) (Fig. 2, Table III).

DISCUSSION

To lower the plasma and tissue methionine content, we prepared a special unbalanced amino acid solution (AO-90), devoid of sulfur-containing amino acids (i.e., L-methionine and L-cysteine).[6] This solution was infused safely by the TPN method, resulting in methionine depletion. This TPN method has been investigated with regard to the effect on body metabolism and antitumor efficacy.[7-13] Using the parenteral treatment only, we could not obtain increased survival, although the methionine concentration in the cancer tissue was decreased to 1/3 of that of control groups by day-10 Met(−) TPN in tumor-bearing rats.[8,9] However, combined treatment with administration of several anticancer agents including nimustine hydrochloride,[13] actinomycin-D,[11] 5-fluorouracil (5-FU),[15,16] doxorubicin[18] and mitomycin C (MMC)[14] during Met(−) TPN allowed prolonged survival of the tumor-bearing rats.

In the autoradiographic examination using ^{3}H-thymidine on AH-109A ascites hepatoma-bearing rats, the tumor cell cycle in the Met(−) TPN group (AO-90

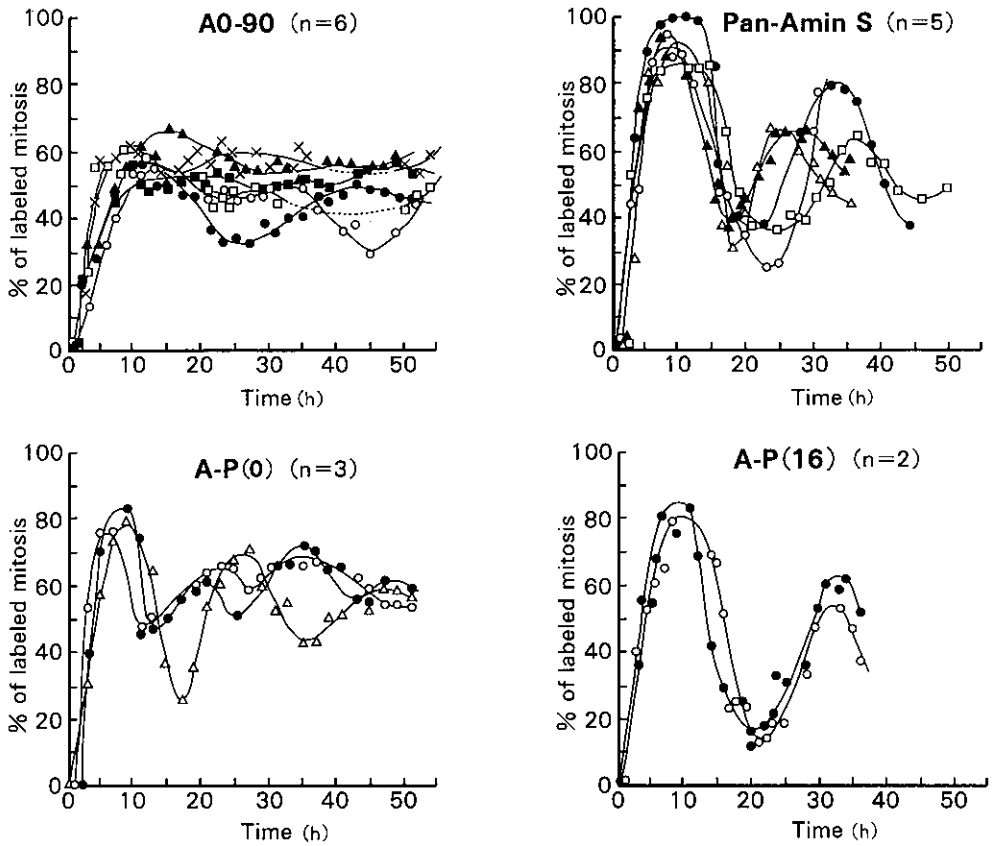

Fig. 3. Labeled mitosis waves of the tumor cells in all the rats in AO-90, Pan-Amin S and A-P groups. The A-P(0) group showed the wave immediately after switching the amino acids from AO-90 to Pan-Amin S and A-P(16) showed the waves 16 h after the exchange. In the AO-90 group, the labeled mitotic fraction was less than 70%, and the duration of the cell cycle could not be calculated. 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 h (A-P(0) and A-P(16)).

group) was markedly delayed and the fraction of labeled mitosis was reduced to less than 70% of the Met(+) control group (Pan-Amin S group). The cell cycle arrest recovered immediately after initiation of infusion of the methionine-containing Pan-Amin S amino acid. The fraction of labeled mitotic phase cells increased within a short period, and the labeled mitosis wave showed the same pattern as that of the Met(+) Pan-Amin S group (Fig. 3) (unpublished data).

Hoffman and Jacobsen reported a tumor-selective late S-G_2 block of the cell cycle of cancer cells in medium lacking methionine in a tissue culture study.[3] Guo *et al.*[21] recently demonstrated that YS cells in mice on a methionine-free diet were arrested in the late S-G_2 phases. This was followed by YS regression. We reported that the anticancer efficacy of doxorubicin, which acts on the late S-G_2 phases cells, was enhanced by Met(−) TPN in YS-bearing rat experiments, with prolongation of the life span of the tumor-bearing animals.[18]

Stern and Hoffman reported that the late S-G_2 phase cell cycle arrest caused in the tumor cells by methionine depletion could selectively potentiate sensitivity of the tumor to anti-mitotic drugs when the methionine depletion-induced cell-cycle block was reversed *in vitro*.[22] We believe that the results presented in this report are the first to demonstrate their principle *in vivo*; i.e., the enhancement of antitumor effects of VCR administration with methionine infusion immediately after Met(−) TPN in YS-bearing rats.

Our experimental model seems to be relevant to the treatment of clinical cases. The findings that rats in the Met(−) TPN+VCR group survived significantly longer than those in any other groups is an important result. In our pilot clinical trial on advanced gastric cancers, we

observed an enhancement of VCR toxicity in the tumor on continuous low-dose intravenous administration for 3 days after Met(−) TPN.[25]

As for side effects, we reported that the toxicity of nimustine hydrochloride was not enhanced by methionine depletion, although the anticancer effect was strengthened.[9, 13] But in this study, we made no assessment of side effects because, due to the small size of the animals, serial blood samplings were impossible. However, there appeared to be no severe side effect in comparison with the control groups because all animals died of cancer, but not other causes, including infection, with prolonged survival in the AO-90+VCR group. Of course, this should be confirmed, but we think that the elongation of the life span was the endpoint of this type of experiment, because the results included not only anticancer effect, but also side effects. We conclude that Met(−) TPN is likely to be useful not only in combined treatment with anticancer agents such as 5-FU and doxorubicin, but also in the case of VCR with methionine infusion after methionine depletion.

In clinical trials in gastrointestinal cancers including phase I, early phase II and late phase II trials, Met(−) TPN combined with 5-FU and/or MMC administration showed a marked anti-tumor effect in advanced gastrointestinal cancer.[6, 12, 14, 17, 19, 20]

In conclusion, not only 5-FU and MMC, but also other anticancer agents including doxorubicin may show marked anticancer effects when combined with Met(−) TPN. After completion of this phase of treatment, VCR should be effective when administered together with methionine. However, the chemosensitizing effect on VCR administered with methionine after Met(−) TPN can not be fully explained and further studies are necessary to elucidate the mechanism and the side effects. Greater efficacy might be achievable by more complete methionine depletion with methioninase.[26, 27]

(Received July 3, 1995/Accepted October 26, 1995)

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