# Anti-tumor Effect of L-methionine–Deprived Total Parenteral Nutrition with 5-Fluorouracil Administration on Yoshida Sarcoma-bearing Rats

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**L-methionine–deprived total parenteral nutrition (methionine-deprived TPN), infusing amino acid solution devoid of L-methionine and L-cysteine by the method of TPN as an only protein source, showed enhancement of the effect of several anti-cancer agents. In this study the combined effect of the methionine-deprived TPN with administration of 5-fluorouracil (5-FU) was examined in Yoshida Sarcoma (YS)-bearing rats, from aspects of effects on the tumor metastasis and the host animal's life span, in the following four groups treated with: methionine-deprived TPN with administration of 5-FU, methionine-deprived TPN without administration of 5-FU, L-methionine–contained TPN plus 5-FU, and L-methionine–contained TPN without 5-FU. In the first experiment, TPN was continued for 8 days in the four groups, and the anti-cancer effect of methionine-deprived TPN and administration of 5-FU based on both the growth of the primary tumor at the implanted site and the tumor metastasis was studied from the view point of pathologic findings of animals killed immediately after these treatments. In experiment 2 the survival period was examined after these treatments for 10 days with subsequent oral feeding until death. The results were as follows: proliferation of YS, transplanted subcutaneously, was markedly suppressed; particularly hematogenous metastasis, characteristic in YS, was prominently blunted, then obtained an apparent longer survival period in rats treated with the methionine-deprived TPN with administration of 5-FU.**

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L-METHIONINE, A sulfur-containing amino acid, is essential for methylation in the synthesis of RNA, DNA, protein, and other biochemical substances. In tissue culture studies, it was demonstrated that the various malignant tumor cells were unable to proliferate in a medium deprived of L-methionine.[1-5] Kreis et al.[4] reported that tumor growth was suppressed in rats implanted with Walker carcinosarcoma with transient decrease of methionine level in the body by enzyme. In our previous experiments, we were able to decrease safely the level of methionine in the body using an amino acid mixture lacking in L-methionine and L-cysteine in total parenteral nutrition (methionine-deprived TPN),[6] and this parenteral therapy has been studied as an adjunct to cancer chemotherapy.[7-11] In an experiment with dogs that were subjected to this therapy for 3 weeks, methionine starvation was admitted well enough, without particular adverse side effects, except a decline in the nutritional state to some degree, by this treatment.[6] Furthermore, in the experiments with rat-bearing AH-109A tumor (ascites type), it was demonstrated that methionine-deprived TPN showed an apparent anti-tumor effect.[11] On the other hand, in the experiment of Sato, in lung carcinoma-(solid type) bearing rats, the anti-cancer effect was not so apparent, although L-methionine levels both in tumor and hepatic tissue were markedly reduced.[12] However methionine-deprived TPN with several anti-cancer agents enhanced the anti-tumor effect of almost all of these agents and this enhancement was followed by the suppression of proliferation of Sato lung carcinoma (SLC), without serious adverse side effects in the host animals.[13,14] Therefore it can be proposed that this unbalanced amino-acid solution may be substantially related to the metabolism of various cancer agents, resulting in a synergistic effect when used with suitable anti-cancer agents. Thus this therapy involves as a mechanism, a remarkable delay in turnover of the tumor cell[15] and a marked decline of thiol levels in hepatic and tumorous tissue.[13]

Recently Breillout et al.[16] reported that a methionine-deprived diet suppressed metastasis of a transplantable tumor in mice. Methionine is the precursor of S-adenosyl methionine, which is major methyl group donor in transmethylation; methylation of cell membrane phospholipids

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and DNA is related to metastatic ability of tumor cells. Accordingly we studied the effect of a combination therapy with methionine-deprived TPN and 5-fluorouracil (5-FU) on the metastasis of Yoshida sarcoma (YS), which metastasizes through the blood stream when administered subcutaneously. In conclusion hematogenous metastasis of the tumor was suppressed and, as a result, a marked elongation of life span was observed in the host rats.

## Materials and Methods

### *Experiment 1*

The growth inhibitory effect of combination therapy of methionine-deprived TPN and administration of 5-FU on YS was examined.

Six-week-old male Donryu rats, weighing 163 ± 7.0 g, obtained from Shizuoka Laboratory Animal Center (Shizuoka, Japan) were used for the experiment. Rats were transplanted with $1 \times 10^6$ cells of YS to dorsal adipose tissue (day 0), which made the solid-type tumor. The tumor cells (obtained from Sasaki Institute, Tokyo, Japan) were maintained as ascites tumor in Donryu rats by weekly intraperitoneal implantation. On the seventh day of transplantation, tumor cells were sampled to confirm the number of viable cells using the trypan blue exclusion test. Rats were cannulated to the vena cava immediately after tumor transplantation according to the method of Steiger et al.,[17] and were maintained on TPN with one of the four regimens combined with or without 5-FU. The amino acid solutions used in TPN were AO-90, which does not contain L-methionine and L-cysteine (Table 1) and commercial Pan-Amin S®, from which the AO-90 was prepared by removing L-methionine. The rats were not allowed to take any foods orally during the TPN period. The animal groups were as follows:

1. The AO-90 plus 5-FU group (n = 6) received AO-90 for 8 days. Total parenteral nutrition regimens were shown in Table 2. 5-Fluorouracil was mixed with the TPN solution and infused for 6 days (from day 2 to 7) at a dose of 15 mg/kg/day (90 mg/kg total).
2. The AO-90 group (n = 5) received TPN with AO-90 for 8 days without 5-FU administration.
3. The Pan-Amin S plus 5-FU group (n = 6) received TPN for 8 days with Pan-Amin S®, which contains L-methionine. 5-fluorouracil was infused in the same dose and manner as in the AO-90 plus 5-FU group.
4. The Pan-Amin S group (n = 5) received TPN for 8 days with Pan-Amin S® without 5-FU.

TABLE 1. *Compositions of Amino Acid Solutions*

| Amino Acid | AO-90 (g/dL) | Pan-Amin S® (g/dL) |
|---|---|---|
| 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 | — | 0.71 |
| 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 |
| Total amino acids (g/dL) | 7.43 | 8.14 |
| Total N (g/L) | 11.9 | 12.6 |

TABLE 2. *Composition of TPN Solution (amount daily/kg)*

| TPN Solution | Pan-Amin S | AO-90 |
|---|---|---|
| 50% Glucose (mL) | 105 | 105 |
| Pan-Amin S (mL) | 123 | — |
| AO-90 (mL) | — | 123 |
| Electrolyte sol. (mL) | 25.6 | 25.6 |
| Sohvita* (mL) | 0.1 | 0.1 |
| Total volume (mL) | 254 | 254 |
| Glucose (g) | 52.5 | 52.5 |
| Amino acids (g) | 10 | 9.14 |
| Na (mEq) | 19.0 | 19.0 |
| K (mEq) | 2.6 | 2.6 |
| Ca (mEq) | 1.1 | 1.1 |
| Cl (mEq) | 19.0 | 19.0 |
| Mg (mEq) | 1.1 | 1.1 |
| $HPO_4$ (mEq) | 2.6 | 2.6 |
| Total calories (Kcal) | 250 | 247 |
| Total N (g) | 1.55 | 1.46 |
| Nonprotein calories/N | 135 | 144 |

* Amounts per 254 mL of infusate: thiamine HCl, 0.125 mg; riboflavin sodium phosphate, 0.125 mg; pyridoxine HCl, 0.075 mg; cyancobalamin, 0.75 μg; nicotinamide, 0.5 mg; folic acid, 0.025 mg; biotin, 5 μg; ascorbic acid, 2.5 mg; panthenol, 0.3 mg; retinol palmitate, 62.5 IU; cholecalciferol, 5 IU; tocopherol acetate, 0.375 mg; menatetrenone, 0.05 mg.
TPN, total parenteral nutrition.

During TPN the rats were housed individually in metabolic cages. A microinfusion pump was used for constant administration of TPN solution. At the end of the experiment (day 8), rats were killed and the tumor-implanted area was extracted to measure the tumor and carcass weight. Then major organs, including the liver and lungs, were histopathologically examined for the presence of tumor cells.

### *Experiment 2*

Life span of YS-bearing rats administered with the methionine-deprived TPN combined with the anti-cancer agent 5-FU was studied.

To examine the synergistic effect of the methionine-deprived TPN toward 5-FU, 8-week-old male Donryu rats (Shizuoka Laboratory Animal Center) weighing 257 ± 13 g were transplanted with $1 \times 10^4$ cells of YS into the dorsal adipose tissue as in the experiment 1, and then cannulated vena cava for TPN procedure (day 0). Exper-

imental design is shown in Figure 2. There was slight variation in both the dosage of 5-FU and a section of the administration method, but otherwise the procedure was the same as that for experiment 1. The groups were AO-90 plus 5-FU (n = 6), AO-90 (n = 5), Pan-Amin S plus 5-FU (n = 6), and Pan-Amin S (n = 7). The AO-90 plus 5-FU and the Pan-Amin S® plus 5-FU received 10 mg/kg/day of 5-FU (80 mg/kg total) mixed in TPN solution for 8 days, and also given 10 mg/kg/day intraperitoneally, twice, on days 10 and 11. These tumor-bearing rats were, as in experiment 1, housed individually in metabolic cages and maintained on TPN for 10 days. After TPN the animals were fed a laboratory chow (Oriental Yeast Co., Ltd., Tokyo, Japan) and water *ad libitum* to survey their life span. Then all of the rats were studied, pathohistologically, at necropsy to determine tumor metastasis and cause of death.

We started with six rats in each group of experiment 1 and seven rats in experiment 2. Because those animals that had a problem of solution leakage from the catheter were excluded from the study, the number of rats in each group decreased.

To examine the tumor extension in rats when they were killed (experiment 1) or at death (experiment 2), all animals were surveyed pathologically and the following criteria were used.

(−): No evidence of metastasis with histologic examination.
(±): With histologic examination only a small amount of tumor cells were observed.
(+): With histologic examination small foci of tumorous formation of cancer cells were demonstrated in the examined organs.
(++): With histologic examination, the apparent tumor metastasis-forming masses were demonstrated, but no more than one half of the organ tissue.
(+++): With pathologic examination marked metastasis of the examined organs was demonstrated and was thought to be due to the metastasized organ's failure.

## Results

### *Experiment 1*

*Tumor weight (Fig. 1).* Tumor weight, carcass weight, and tumor weight:carcass weight ratio on day 8 (at the end of the experiment) were 0.69 ± 0.14 g, 153 ± 7.0 g, and 0.5% ± 0.1%, respectively, for the AO-90 plus 5-FU group, 1.62 ± 0.51 g, 153 ± 8.0 g, and 0.7% ± 0.37% for the AO-90 group, 1.64 ± 0.40 g, 174 ± 13 g, and 0.93% ± 0.21% for the Pan-Amin S plus 5-FU group, and 3.13 ± 0.50 g, 174 ± 10 g, and 1.79% ± 0.24% for the Pan-Amin S group. Tumor weight and tumor:carcass weight ratio were significantly lower in the AO-90 plus 5-FU group than other groups at $p < 0.01$ by Student's t test.

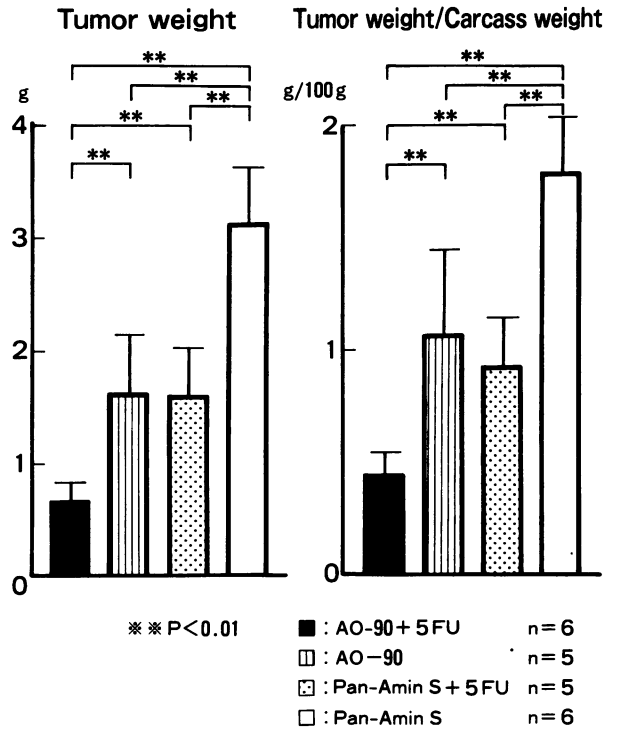


FIG. 1. Illustration of the results of experiment 1 (tumor weight and tumor weight/carcass weight shown in each group).

*Pathohistologic findings (extension of tumorous lesions).* Pathologic findings at the end of the experiment are shown in Table 3 with the above-mentioned criteria.

In the AO-90 plus 5-FU group, only one rat showed hematogenous liver metastasis, and in the AO-90 group, only one rat had metastasis in the liver, the lungs, and the kidneys. Other rats in these two groups showed no hematogenous metastasis with histopathologic examination. On the other hand, in the Pan-Amin S plus 5-FU and the Pan-Amin S groups, all rats developed severe hematogenous metastasis in several organs.

### *Experiment 2*

*Survival days (Fig. 2).* Mean survival days were 45.2 ± 17.6 for the AO-90 plus 5-FU group (two rats were killed on day 60), 30.4 ± 18.0 days for the AO-90 group (one rat was killed on day 60), 21.3 ± 8.4 days for the Pan-amin S plus 5-FU group, and 15.9 ± 3.8 days for the Pan-Amin S group. The AO-90 plus 5-FU group had a significantly longer survival period than the Pan-Amin S plus 5-FU ($p < 0.05$) and the Pan-Amin S ($p < 0.01$) (Wilcoxon's rank sum test) groups.

*Histologic findings at autopsy (Table 4).* In Table 4 the tumor extension of each rat at death was summarized following the above-mentioned pathohistologic findings with the five criteria, which were also used in experiment 1, with other findings such as tumor weight, survival pe-

TABLE 3. *Pathologic Findings of Tumor Metastasis in Each Rat of Four Groups at Autopsy*

| Animal No. | Group | Metastasis | | | | |
|---|---|---|---|---|---|---|
| | | Liver | Kidney | Lung | Heart | Spleen |
| 1 | | − | − | − | − | − |
| 2 | | ++ | − | − | − | − |
| 3 | AO-90 + 5-FU | − | − | − | − | − |
| 4 | | − | − | − | − | − |
| 5 | | − | − | − | − | − |
| 6 | | − | − | − | − | − |
| 7 | | − | − | − | − | − |
| 8 | | ± | − | − | − | − |
| 10 | AO-90 | − | − | − | − | − |
| 11 | | ± | − | ± | − | − |
| 12 | | + | + | + | − | − |
| 13 | | ++ | + | + | − | ± |
| 15 | | ++ | + | − | − | − |
| 16 | Pan-Amin S + 5-FU | ++ | − | + | − | − |
| 17 | | + | − | ++ | + | − |
| 18 | | + | + | + | − | − |
| 19 | | +++ | − | ++ | − | ++ |
| 20 | | +++ | − | +++ | + | ++ |
| 21 | Pan-Amin S | ++ | − | ++ | − | ± |
| 22 | | ++ | ± | ++ | − | − |
| 23 | | ± | − | ++ | − | − |
| 24 | | ± | − | ++ | − | + |

−, ±, +, ++, +++: cf. text.

riod, and so on. With the examination of the tumorous area in death cases, two rats (33.3%) in the AO-90 plus 5-FU group the implanted tumor was decreased in severe necrosis and completely diminished from the implanted site, while only three rats (50%) had slight hematogenous metastasis. The cause of death in this group was organ failure: death induced by respiratory and hepatic failure attributable to hematogenous metastasis in two rats (33.3%); the other two rats (33.3%) probably died of voluminous bleeding body fluid loss or infection from the necrotic lesion of the remnant tumor of the implanted site.

The cause of death seen in the AO-90 group was organ failure resulting from tumor metastasis in three rats (60%) and bleeding from primary tumor lesion in one rat (20%), while the tumor in one rat (20%) was eradicated.

In the Pan-Amin S and the Pan-Amin S plus 5-FU groups, one rat (8.3%) died of body fluid loss, bleeding from primary tumor, and accompanying infection. The other 11 rats (91.7%) were thought to have died of organ failure due to severe hepatic and pulmonary hematogenous metastasis of the tumor.

## Discussion

Increase or deprivation of certain amino acids, *i.e.*, amino acid imbalance, has been reported to induce the inhibitory effect to tumorous proliferation. In particular methionine is documented to be essential for growth of tumor cells. Many tumor cells, including L5178 murine leukemic cells, are difficult to proliferate in a methionine-free medium in comparison with the normal fibroblast.[1-5] In addition, when the L-methionine level was forced to decrease transiently by the enzyme such as L-methioninase in rats transplanted with Walker carcinosarcoma, the proliferation of tumor cells was blunted.[4]

In our recent studies, a special unbalanced amino-acid solution (AO-90), devoid of all the sulfur-containing amino acids, was prepared by eliminating L-methionine and L-cysteine. This solution was infused safely by TPN method to develop methionine starvation in the body, together with minimizing deterioration of nutritional condition.[6] This TPN method was investigated with regard to the influence on body metabolism and anti-tumor effect.[7-13]

Because 50% of nontumor-bearing rats died after the 3-week AO-90 regimen, we administered the TPN solution for 8 days in the experiments. In our experiments

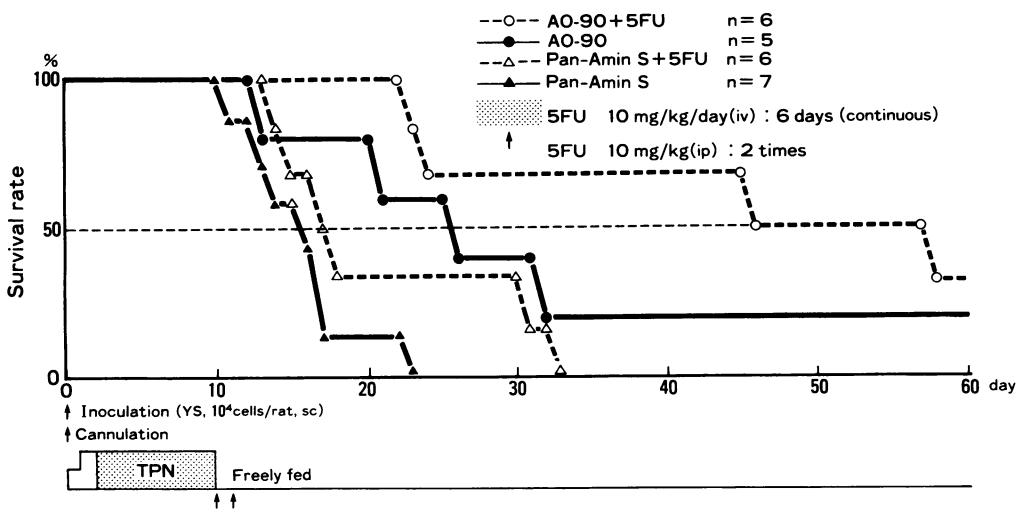


FIG. 2. Illustration of the protocol of experiment 2 and its results (survival rates and days of the rats are shown in each group).

TABLE 4. *Pathologic Findings of Tumor Metastasis at Autopsy, Tumor Weight, Survival Days, Cause of Death and Other Findings in Each Animal of Four Groups*

| Group | Animal No. | Metastasis | | | | | Tumor Weight (g) | Survival Days | Other Findings | Cause of Death |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Liver | Kidney | Lung | Heart | Spleen | | | | |
| | 1 | − | − | − | − | − | None* | <60 | | None |
| | 2 | +++ | +++ | ++ | − | − | 18.6† | 23 | Peritoneal dissemination | Meta§ |
| AO-90 + 5-FU | 3 | − | − | − | − | − | 82.4† | 46 | | Tumor‖ |
| | 4 | − | − | + | − | − | 79 | 58 | | Tumor |
| | 6 | ± | − | − | − | − | None* | ≥60 | | None |
| | 7 | + | +++ | +++ | +++ | − | 16.3 | 24 | Pleuritis | Meta |
| | 8 | − | − | − | − | − | None† | >60 | | None |
| | 10 | +++ | +++ | ++ | − | − | 2.0 | 13 | | Meta |
| AO-90 | 11 | − | − | − | − | − | 24.1 | 32 | Peritoneal dissemination | Tumor |
| | 12 | +++ | +++ | +++ | − | − | 25 | 26 | Pleuritis | Meta |
| | 14 | ++ | ++ | +++ | − | − | 17.6 | 21 | Pleuritis | Meta |
| | 16 | − | ++ | +++ | − | +++ | 8.2‡ | 33 | Peritoneal dissemination | Meta |
| | 17 | +++ | +++ | +++ | ++ | − | 8.1 | 15 | | Meta |
| Pan-Amin S + 5-FU | 18 | ++ | +++ | +++ | − | − | 11.9 | 17 | | Meta |
| | 19 | ++ | +++ | +++ | ++ | − | 10.9 | 18 | | Meta |
| | 20 | | | | | | 48.6 | 31 | Peritoneal dissemination | Meta |
| | 21 | +++ | +++ | +++ | + | − | 12.8 | 14 | | Meta |
| | 22 | + | − | − | − | − | 24.1 | 23 | | Tumor |
| | 23 | ++ | +++ | +++ | +++ | − | 7.2 | 16 | | Meta |
| | 24 | ++ | ++ | +++ | ++ | − | 5.4 | 14 | | Meta |
| Pan-Amin S | 25 | − | − | +++ | − | − | 13.7 | 17 | | Meta |
| | 26 | − | − | +++ | − | − | 13.9 | 17 | | Meta |
| | 27 | +++ | ++ | +++ | ++ | − | 3.1 | 11 | | Meta |
| | 28 | +++ | +++ | +++ | − | − | 7.1 | 13 | | Meta |

−, ±, +, ++, +++: cf. text.

* Tumor in the transplanted area was fall in severe necrosis and diminished from the host animal at autopsy.

† Showed complete regression of the tumor.

‡ Most part of the tumor was fall in necrosis macroscopically.

§ Meta, pathologically diagnosed to be dead of the metastasized lesion.

‖ Tumor, pathologically diagnosed to be dead of bleeding, loss of body fluid and/or infection from the transplanted area's tumor.

the growth of YS did not depend on nutritional regimen to the host animals, whether it was TPN or chow feeding, when calorie intake was limited to not more than 250 kcal/kg. Accordingly we did not include a group of orally fed tumor-bearing rats as another control group. The inoculum size of the tumor cell was different in experiment 1 from that of experiment 2. In general $10^6$ YS cells are inoculated in this kind of experiment. In experiment 1 we inoculated this number of tumor cells. However this amount is too large to demonstrate a life-expanding effect of the regimen because the animals inoculated with this amount of tumor cells die quickly. For this reason we inoculated $10^4$ cells in experiment 2.

As a result proliferation of YS was markedly suppressed by the methionine-deprived TPN. Hematogenous metastasis, which is characteristic to the tumor, was prominently blunted as well. These effects of the regimen apparently resulted in a longer survival period in rats treated with the methionine-deprived TPN with administration of 5-FU.

As we previously reported,[13] nimustine hydrochloride to which SLC is sensitive, exerted the increased anti-tumor effect in rats because of a marked decline of the thiol level in tumorous tissue as well as in the liver.

And in the rats bearing AH-109A tumor cells, the metabolism of nucleic acid in the tumor was altered, as demonstrated by enlargement of nuclear size with accumulation of RNA granular (probably by inhibition of r-RNA processing).[11] Furthermore, in the same rat model, the cell cycle of the tumor has been found to be delayed markedly, as seen from the analysis of fraction-labeled mitosis curve.[15]

However it appears that these observations cannot satisfactorily explain the synergistic increase of anti-tumor effect of cancer agents by concurrent use of methionine-deprived TPN. In fact the effect of methionine-deprived TPN itself was relatively prominent in the AH-109A tumor[11] but unexpectedly was not effective on solid SLC tumors.[12] For example life span was lengthened in the AH-109A-bearing rats when actinomycin D was combined,[8] whereas longer survival time was not achieved in the SLC-bearing rats, although anti-tumor effects were clearly recognized.

In the present study with YS cells that easily induce metastatic organ failure, followed by death with extensive hematogenous metastasis, methionine-deprived TPN was demonstrated to reduce markedly the degree of metastasis, resulting in lengthened survival with combined use with 5-FU.

Breillout et al.[16] reported that a methionine-deprived

diet inhibited metastatic spread of Lewis lung carcinoma and rhabdomyosarcoma tumor cells grafted into animals. They could hypothesize that the metastatic process is easily disturbed by a methionine-deprived diet, affecting the cell membrane phospholipid and DNA methylation. In the same way, AO-90 might inhibit metastatic processes. And the metastases appear to be more receptive of this therapy than do primary tumors.[18] But the chemosensitizing effect to the 5-FU by AO-90 is not fully explainable.

To elucidate the mechanism, it is necessary to make a further fundamental examination. As to possible unfavorable influences by the therapy which concern much in the study, only slight decline of serum protein level was noted in dogs rendered to a 3 week TPN as well as in the clinical pilot trial and phase I study.[8,9,14]

Now it is very necessary to examine further the effect of this unbalanced amino acid solution in combination with various anti-cancer agents and also to elucidate the involving mechanism from the various points. We add herein that clinically effective cases have been seen in advanced gastrointestinal cancer patients.

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