Combination of Theanine with Doxorubicin Inhibits Hepatic Metastasis of M5076 Ovarian Sarcoma

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ABSTRACT
Theanine is a peculiar amino acid existing in green tea leaves, which was previously indicated to enhance the antitumor activity of doxorubicin. In the present study, the effect of combination of theanine with doxorubicin against hepatic metastasis of M5076 ovarian sarcoma was investigated. The primary tumor was significantly reduced by the combined treatment on M5076 transplanted (s.c.) mice. The liver weight of control mice increased to twice the normal level because of hepatic metastasis of M5076. In contrast, the injection of doxorubicin alone or theanine plus doxorubicin suppressed the increase in liver weight and inhibited hepatic metastasis. Moreover, the liver weights and metastasis scores demonstrated that theanine enhanced the inhibition of hepatic metastasis induced by doxorubicin. Furthermore, in vitro experiments indicated that theanine increased the intracellular concentration of doxorubicin remaining in M5076 cells. This action suggests that theanine leads the enhancement of the suppressive activity of doxorubicin on hepatic metastasis in vivo. Therefore, it was proved that theanine increased not only the antitumor activity on primary tumor but also the metastasis-suppressive efficacy of doxorubicin. The effect of theanine on the efficacy of antitumor agents is expected to be applicable in clinical cancer chemotherapy.

INTRODUCTION
Metastasis is one of the important factors related to therapeutic efficacy and prognostic survival (1, 2); therefore, the inhibition of metastasis is essential in cancer therapy. However, effective strategies for inhibition of metastasis have not been established to date. In many cases, antitumor agents were administered to clinical patients after operation to prevent tumor metastasis. However, successful suppression of tumor metastasis by means of antitumor agents has not been achieved thus far. Recently, some cytokines were reported to effectively inhibit metastasis and were used for chemotherapy with antitumor agents (3–6). However, these studies have been investigated mostly in animal models; therefore, more research is essential before their clinical use.

Incidentally, the effects of modulators that enhance the efficacy of antitumor agents against tumor metastasis have not been investigated. We reported previously that theanine (Fig. 1), an amino acid specifically existing in green tea (7, 8), enhanced the antitumor activity of DOX (Fig. 2) on Ehrlich ascites carcinoma and M5076 ovarian sarcoma (9, 10). Theanine is a glutamate derivative (7, 8) and exhibits no anticarcinogenesis activity. Combined with DOX, however, theanine increased the DOX concentration in tumor and enhanced the antitumor efficacy of DOX on tumor-bearing mice (9). However, it is not clear whether the effect of the combination with theanine is effective on metastatic cancer.

M5076 is transplantable murine reticulum sarcoma originating in the ovary of C57BL/6 mice and is highly invasive and metastatic (11–13). When M5076 is s.c. transplanted onto mice, a solid tumor arises, spontaneously metastasizes to the liver and lung, and then kills the mice within about 25–30 days (12, 13). In a spontaneous metastasis experiment, combined treatment with theanine and DOX was performed on M5076 tumor-bearing mice. In this study, we demonstrated the enhancing effect of theanine on the inhibitory activity of DOX against the hepatic metastasis of M5076 sarcoma.

MATERIALS AND METHODS
Chemicals. DOX injection, 10 mg/vial (Adriacin), was purchased from Kyowa Fermentation, Inc. (Tokyo, Japan). Theanine (purity, >98%) was purchased from Tokyo Kasei Co., Ltd. (Tokyo, Japan). RPMI 1640 was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). The drugs were dissolved in sterile isotonic saline. The other chemicals used in this study were of the highest purity available.

Animals. Male C57BL/6 and BDF₁ (F₁ from C57BL/6 female and DBA/2 male) mice, 5 weeks of age and weighing 20–25 g, were obtained from Japan SLC, Inc. (Hamamatsu, Japan). The animals were housed in a room maintained at 25 ± 1°C with 55 ± 5% relative humidity and were given free access to regular chow pellets and water. C57BL/6 mice were used for animal passage of M5076 cells, and BDF₁ mice were used for in vivo experiments.

Tumor. M5076 ovarian sarcoma was kindly provided by Dr. T. Tashiro (Japanese Foundation for Cancer Research, Tokyo, Japan). For animal passage, M5076 ovarian sarcoma (1 × 10⁶ cells/animal) were i.p. transplanted into the C57BL/6 mice. The ascites cells were collected on the 14th day after transplantation.

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3 The abbreviation used is: DOX, doxorubicin.
Animal Experiments. M5076 ovarian sarcoma (1 × 10^6 cells/mouse) were s.c. transplanted onto the backs of BDF1 mice. After 21 days, the mice were divided into several groups, each consisting of eight mice. DOX (2.0 mg/kg/day for 4 days) was i.p. injected on the 22nd, 24th, 26th, and 28th days after the transplantation. Theanine (10 mg/kg/day for 4 days) was i.p. administered on the 23rd, 25th, 27th, and 29th days. Control mice were injected with the same volume of sterile isotonic saline. The mice were killed on the 30th day after inoculation by cervical dislocation, and the solid tumor, liver, and lung were immediately removed and weighed. On observation of the livers, the hepatic metastasis was graded, as to the metastasis area ratio in the liver, into five stages: metastasis score 1, <5%; 2, 5–25%; 3, 25–50%; 4, 50–75%; 5, >75%; and 0, normal (0%).

**In Vitro Experiments.** M5076 ovarian sarcoma cells (10^6 cells/animal) were i.p. transplanted into C57BL/6 mice. The ascites were collected on the 14th day after transplantation. The ascites sarcoma cells were washed twice and then resuspended in fresh medium. The cell suspension was then cooled on ice and centrifuged at 150 g for 3 min. The cells were washed and resuspended in fresh medium. The cell suspension was then incubated at 37°C for 120 min in the presence or absence of theanine (1.0 μm).

For determination of the intracellular DOX concentration, the cell suspension was cooled on ice and centrifuged at 150 × g for 3 min. The cells were washed and resuspended in 1.0 ml ice-cold 10 mM phosphate buffer (pH 7.8), and then mixed for 30 s with 5.0 ml of chloroform-methanol (4:1, v/v) and centrifuged at 1200 × g for 15 min. The concentration of DOX in the organic phase was determined with a fluorescence spectrophotometer, HITACHI F2000 (Hitachi Ltd., Tokyo, Japan; excitation wavelength, 470 nm; emission wavelength, 585 nm).

### Table 1 Tumor, liver, and lung weights in M5076 tumor-bearing mice

<table>
<thead>
<tr>
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<th>Tumor (g)</th>
<th>Liver (g)</th>
<th>Lung (g)</th>
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<tbody>
<tr>
<td>Normal</td>
<td>1.44 ± 0.12^a</td>
<td>0.137 ± 0.005^a</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.74 ± 0.58</td>
<td>3.22 ± 0.30</td>
<td>0.196 ± 0.015</td>
</tr>
<tr>
<td>DOX</td>
<td>2.43 ± 0.69</td>
<td>1.84 ± 0.11^b</td>
<td>0.145 ± 0.009^b</td>
</tr>
<tr>
<td>DOX + theanine</td>
<td>2.05 ± 0.20^a</td>
<td>1.64 ± 0.12^b</td>
<td>0.140 ± 0.006^a</td>
</tr>
</tbody>
</table>

^a–c Each value is presented the mean ± SD of eight mice. Significant differences from the level of control group are indicated by: ^a P < 0.001; and ^b P < 0.05. Significant differences from the level of DOX alone group are indicated by: ^c P < 0.05.

**Statistical analysis.** Statistical analysis was performed by Student’s t test and ANOVA.

### RESULTS

**Tumor, Liver, and Lung Weights in M5076 Tumor-bearing Mice.** The weights of the primary tumor, liver, and lung, as the metastatic organs in M5076 bearing mice are shown in Table 1. The tumor weight was not reduced by the injection of DOX alone. In contrast, the combination of theanine with DOX significantly reduced the tumor weight by 30% (P < 0.05 versus control level) compared with the control level. The liver weight of control group was increased to 2.2-fold that of normal mice. The injection of DOX alone significantly reduced to 22% (P < 0.001 versus control level) the increase in liver weight in the control group. Moreover, combined theanine with DOX inhibited the increment of liver weight to 11% (P < 0.001 versus control level; P < 0.05 versus DOX alone level). Similarly, the lung weight of control mice was 1.4-fold of the normal level, whereas DOX administration and combined treatment with theanine and DOX reduced the weights to the normal level.

**Effect of Theanine on the Inhibition of Hepatic Metastasis of M5076 Induced by DOX.** The relation between the metastasis score and relative liver weight (% liver/body weight) in tumor-bearing mice is shown in Fig. 3. The metastasis score was positively correlated with the relative liver weight. The relation coefficient was r^2 = 0.807.

The effect of theanine on the change in the relative liver weight induced by DOX is shown in Fig. 4. The relative liver weight of normal mice was 5.1%, and that of control mice was increased by 1.6-fold. Those of DOX alone and combined treatment groups were reduced.

The hepatic metastasis score in each group is shown in Fig. 5. The metastasis score of the control group was 4.5, and those of DOX alone group and combined treatment group were decreased. Theanine enhanced the inhibitory effect of DOX against hepatic metastasis (P < 0.05).

**Effects of Theanine on the DOX Concentration in M5076 Cells in Vitro.** The intracellular uptakes and retention of DOX in M5076 cells are shown in Fig. 6. Theanine did not change the DOX uptake by M5076 cells. In contrast to that, theanine inhibited the efflux of DOX, and the intracellular concentration of DOX remaining in M5076 cells was increased 1.4-fold (P < 0.01).
DISCUSSION

On M5076 tumor-bearing mice, the action of theanine on the inhibition of hepatic metastasis induced by DOX was investigated. Whereas the primary tumor weight was not changed by injection of DOX alone, combined treatment with theanine and DOX reduced the tumor weight by 30%, and thus theanine increased the antitumor efficacy of DOX. The liver and lung weights of tumor-bearing mice remarkably increased owing to metastasis of M5076, in contrast with those of normal mice. DOX reduced these increases in weights of these organs and inhibited the metastasis. Moreover, the combination of theanine with DOX significantly reduced the increase in liver weight. These results suggested that theanine enhanced the inhibitory effect on hepatic metastasis of DOX.

Fig. 3 The relation between the hepatic metastasis score and relative liver weight. The relative liver weight is expressed as a percentage (liver/body weight).

Fig. 4 Effect of theanine on the change of the relative liver weight induced by DOX. The relative liver weight is expressed as % (liver/body weight). Columns, means of eight mice; bars, SD. Significant differences from the level of DOX alone group are indicated by: **, P < 0.001; and *, P < 0.01.

On observation of the livers, the hepatic metastasis was graded, as to the metastasis area ratio in the liver, into five stages: metastasis score, 1 to 5. An increase in number of metastatic colonies was accompanied by an increase in liver weight. Probably, the liver expanded because of the hepatic metastasis of M5076. At that time, the relation coefficient between the metastasis score and the relative liver weight (% liver/body weight) was $r^2 = 0.807$. Thus, the relative liver weight was demonstrated to be an indicator of hepatic metastasis. The inhibitory effect of the combination of theanine with DOX was evaluated from the changes of the relative liver weight and the metastasis score.

Fig. 5 Effect of the combination of theanine with DOX on hepatic metastasis. Columns, means of eight mice; bars, SD. Significant difference from the level of DOX alone group is indicated by: *, P < 0.01.

Fig. 6 Effect of theanine on the intracellular uptake and retention of DOX in M5076 cells in vitro. The uptake amount for 60 min is represented as the intracellular DOX concentration in uptake system. The retention is represented as the intracellular DOX concentration after incubation for 120 min in retention system. Columns, means of four samples; bars, SD. Significant difference from the level of DOX alone group is indicated by: *, P < 0.01.
or DOX plus theanine-injected groups, the relative liver weights were decreased due to inhibition of hepatic metastasis. In addition, the metastasis scores were decreased in these groups compared with that in the control group; furthermore, the combination with theanine significantly enhanced the metastasis-suppressive efficacy of DOX. Thus, theanine amplified the suppressive efficacy of DOX on hepatic metastasis. Modulators that enhance the antitumor activity of chemotherapeutic agents against primary tumors and which are also effective in inhibiting subsequent metastasis have not been reported to date. Therefore, theanine was found to be a novel biochemical modulator, which enhances both the antitumor activity on primary tumor and also the inhibition of metastasis induced by antitumor agents.

When M5076 was s.c. transplanted onto mice, in all cases, the M5076 metastasized to the liver within 20 days after tumor inoculation (12, 13). At the start of this treatment, tumor metastatic nodules were observed in the livers of all M5076 tumor-bearing mice. Because the injection of DOX plus theanine decreased the hepatic metastasis, it is suggested that DOX inhibited the growth of metastatic tumor in the liver, and then theanine enhanced the antitumor activity of DOX.

For clarifying a part of the action of theanine, the intracellular uptake and retention of DOX in M5076 cells were examined in vitro. Theanine did not affect the uptake of DOX by M5076 cells, whereas theanine increased the remaining intracellular DOX concentration. Thus, theanine reduced the efflux of DOX from M5076 cells and increased the DOX concentration in tumor cells, supporting the enhancement of antitumor efficacy of DOX induced by theanine. Similarly in metastasized tumor, theanine presumably increases the DOX concentration; thus, effective antitumor activity of DOX against metastasis is obtained. Furthermore, during the treatment, tumor cells or minute colonies moved via blood vessels from the primary tumor to the metastatic area. When DOX was injected into mice followed by theanine, DOX was taken up by the tumor cells or colonies, and then theanine inhibited the release of DOX and increased the DOX accumulation in tumor colonies moving via blood vessels. Thus, it is expected that metastatic tumors circulating in vessels are killed by DOX with theanine before they reach the metastatic site. Consequently, the combined drug injection suppressed subsequent metastasis during the treatment.

The inhibition of metastasis by the combination of theanine with DOX was possibly reflected by the reduction in primary tumor weight. However, the finding that pirarubicin, an anthracycline antibiotic, does not inhibit the hepatic metastasis of M5076, although pirarubicin significantly reduces the primary tumor weight (14), suggested that the inhibitory effect against primary tumor does not necessarily involve a suppression of metastasis. From another point of view, because theanine alone has never been found to inhibit tumor metastasis, it is suggested that theanine does not act on some metastatic mechanisms, such as adhesion and invasion. Therefore, theanine was demonstrated to enhance the DOX activity.

In this study, theanine was demonstrated to enhance the inhibition of hepatic metastasis induced by DOX due to increasing the DOX concentration in tumor cells. It was indicated previously that theanine reduces the DOX concentration in the normal liver (9, 10). Thus, theanine appears to increase the DOX concentration only in the hepatic metastasized tumor, i.e., not in normal liver tissue. The transport mechanisms of some anthracycline derivatives in tumor cells were reported to be partly different from those in normal cells (15). Judging from this evidence, it is possible that theanine acts on tumor in a different manner on normal tissues. With theanine, DOX was accumulated selectively in both primary and metastatic tumors, and the effective antitumor activity of DOX is obtained.

In conclusion, the present study revealed that theanine enhances not only the antitumor activity on primary solid tumor but also the metastasis-suppressive efficacy of DOX. Therefore, theanine is effective as a novel modulator that enhances the therapeutic efficacy of antitumor agents. It is expected that this enhancing effect of theanine will be used extensively in clinical cancer chemotherapy.

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