Postoperative Oral Administration of Uracil and Tegafur Inhibits Progression of Micrometastasis of Human Breast Cancer Cells in Nude Mice

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ABSTRACT

We recently established a metastasis model in nude mice using the MKL-4 cell line, a cotransfectant of the MCF-7 human breast cancer cell line with fgg-4 and lacZ in which micrometastases in several organs can be quantitatively observed. First, to develop a new postoperative metastasis model, we investigated the timing of occurrence of micrometastasis and the influence of tumor removal on the progression of micrometastasis in this model. Micrometastases into lymph nodes and lungs were detected 3 weeks after the cell injections. Tumor removal 3 weeks after the injections significantly enhanced the progression of micrometastasis into lymph nodes and bone. Second, to study the effect of a mixed compound, UFT (a molar ratio of uracil: tegafur of 4:1), which has been widely used in the postsurgical adjuvant setting in Japan, 15 or 20 mg/kg UFT were administered p.o. for 4 weeks to tumor-bearing mice or to mice in which transplanted tumors were resected 3 weeks after the injections. Either dose of UFT significantly inhibited the tumor growth as well as the progression of micrometastasis into lymph nodes, lungs, liver, and brain. In addition, enhanced progression of micrometastasis in all explored organs by the tumor removal was significantly inhibited by the administration of either dose of UFT. In conclusion, this new postsurgical metastasis model may be useful for evaluating the efficacy of agents used in the postoperative adjuvant setting. UFT may be an effective drug for inhibiting the progression of micrometastasis after surgery.

INTRODUCTION

The efficacy of postoperative adjuvant chemotherapy and/or endocrine therapy in reducing the mortality of breast cancer patients has been widely accepted on the basis of the data from many prospective clinical trials (1–3). These agents used in the postoperative adjuvant setting have already been proven to be effective in the treatment of advanced breast cancer. The antitumor activity of these agents is believed to be involved in inhibition of the progression of micrometastasis, which may already exist at the time of surgery. However, evaluation of such an inhibitory effect in animal experimental models is hard because of the difficulty in quantitatively measuring the degree of micrometastasis in animal organs. Sledge et al (4) recently introduced a new postoperative metastasis model in nude mice using a human breast cancer cell line, MDA-MB-435 (5). They reported that a matrix metalloproteinase inhibitor, Batimastat, inhibited the regrowth of primary tumors and reduced the number and volume of macroscopic lung metastases after the primary tumor removal. However, neither any promoting effect of the primary tumor removal nor any inhibitory effect of the metalloproteinase inhibitor on the progression of micrometastasis has yet been described.

We recently established a new metastasis model in nude mice using the MKL-4 cell line, a cotransfectant of the MCF-7 human breast carcinoma cell line with a potent angiogenic factor gene, fgg-4, and a genetic marker gene, bacterial lacZ encoding β-gal.3 Transplantation of MKL-4 cells into the mammary fat pad of female nude mice produces rapid-growing tumors without supplementation of estradiol. In addition, whole-organ staining of excised organs from these nude mice for β-gal activity makes metastatic tumor cells blue, helping us to measure the degree of micrometastasis quantitatively. This staining revealed that MKL-4 cells spontaneously metastasized into the lymph nodes, lungs, liver, brain, and bone (bone metastasis was observed for the first time in this study). This metastasis model should be useful for evaluating the inhibitory effect of certain agents on metastatic activity (6, 7). Our previous experiment using this model revealed that a potent antiangiogenic agent, TNP-470, clearly inhibits angiogenic activity, tumor growth, and...
and spontaneous metastasis into the lymph nodes and distant organs (8).

5-FU is a widely used antimetabolite for the treatment of various cancers. Tegafur is a prodrug of 5-FU which is mainly converted to 5-FU by microsomal P450 enzymes in the liver (9). Fujii and colleagues (10–12) found that the coadministration of uracil and tegafur at a molar ratio of uracil:tegafur of 4:1 enhanced the concentration of 5-FU in the tumor and produced maximal antitumor activity in animal model systems. This mixed compound, UFT (Taiho Pharmaceutical Co. Ltd., Tokyo, Japan), has been widely used in the treatment of breast, gastrointestinal, and head and neck cancers in Japan (13, 14). Because UFT is administered p.o., well tolerated, and devoid of severe neuropenia, it may provide a better quality of life and cost benefit for patients with advanced cancer. Clinical and experimental studies of UFT in the treatment of colorectal, gastric, and breast cancers are underway in Western countries (15–22). Very recently, a pharmacokinetic study showed that p.o. UFT generates a higher peak level of 5-FU than can be achieved with protracted i.v. 5-FU infusion (23). Although UFT has also been used in the postoperative adjuvant setting for several cancers in Japan (24–27), the scientific basis for postoperative UFT administration in reducing recurrence and the mortality of cancer patients is still unclear. Therefore, we decided to establish a new postoperative metastasis model and to clarify the usefulness of postoperative UFT administration in this model.

**MATERIALS AND METHODS**

**Agents and Animals**

Uracil and tegafur, provided by Taiho Pharmaceutical Co. Ltd., were mixed (uracil:tegafur molar ratio of 4:1) and dissolved in 0.5% (w/v) carboxymethyl cellulose at a final tegafur concentration of 1.5 or 2.0 mg/ml. The given dose (15 or 20 mg/kg) of tegafur was expressed as that of UFT. All reagents used in the whole-organ staining for β-gal activity described below were purchased from Sigma Chemical Co. (St. Louis, MO). The reagents used in culture were purchased as follows: Iscove’s modified Dulbecco’s medium from Life Technologies, Inc. (Tokyo, Japan), fetal bovine serum from ICN Biochemicals Japan (Osaka, Japan), and trypsin from Difco Laboratories (Detroit, MI). Female BALB/c-nu/nu mice (4-week old) were purchased from CLEA Japan (Tokyo, Japan).

**Experimental Procedure**

The four consecutive experimental protocols were as follows.

**Experiment 1.** Semiconfluent MKL-4 cells growing in Iscove’s modified Dulbecco’s medium supplemented with 10% fetal bovine serum at 37°C in a 5% CO2-air atmosphere were trypsinized, harvested, and counted in a hemocytometer using trypan blue exclusion. Approximately 1 × 10^7 viable cells/0.1 ml of saline were injected into the right upper mammary fat pad (a single injection/mouse) of 4-week-old female athymic nude mice. In this first experiment, 42 nude mice were given injections of MKL-4 cells. Seven mice were sacrificed by a cervical dislocation every week from 2 to 7 weeks after the cell injections. The right axillary lymph node and lungs were excised from each mouse and stained for β-gal activity to measure the degree of micrometastasis using a dissecting microscope. The weights of the excised primary tumors were measured.

**Experiment 2.** Twenty nude mice were given injections of MKL-4 cells. Because the results of experiment 1 suggested that micrometastases may occur 3 weeks after the cell injections, transplanted MKL-4 tumors were resected in an aseptic condition under ether anesthesia 3 weeks after the injections in the tumor removal group (10 mice). In the control group (10 mice), no surgical maneuver was carried out. All of the mice were sacrificed 7 weeks after the injections. The right axillary lymph node, distal lymph nodes (contralateral axillary or bilateral inguinal lymph nodes), lungs, liver, brain, and lumbar vertebral bone were excised, stained for β-gal activity, and observed. The weights of the excised primary tumors were measured.

**Experiment 3.** Thirty nude mice were given injections of MKL-4 cells. In the treatment groups (10 mice in each group), 15 or 20 mg/kg UFT were given p.o. to the mice six times a week from 3 to 7 weeks after the injections. The given volume of the UFT solution was 10 ml/kg for either dose. In the control group (10 mice), the same volume of the vehicle was given in the same manner. All of the mice were sacrificed 7 weeks after the injections. Weights of the excised primary tumors and mice were measured. The lymph nodes and distant organs were excised, stained for β-gal activity, and observed.

**Experiment 4.** Thirty nude mice were given injections of MKL-4 cells. In both groups as described below, transplanted MKL-4 tumors were resected 3 weeks after the injections. In the treatment groups (10 mice in each group), 15 or 20 mg/kg UFT were given p.o. to the mice six times a week from 3 to 7 weeks after the injections. In the control group (10 mice), the same volume of the vehicle was given in the same manner. All of the mice were sacrificed 7 weeks after the injections. The weights of the mice and right axillary lymph nodes were measured. Then, the lymph nodes and distant organs were excised, stained for β-gal activity, and observed.

All experimental protocols in this study were approved by the Animal Care and Use Committee of Kawasaki Medical School.

**Whole-Organ Staining for β-gal Activity**

The excised organs from the sacrificed nude mice were cut into two pieces except for lungs and immediately fixed for 2 h at room temperature with 2% (v/v) formaldehyde-0.2% (v/v) glutaraldehyde in PBS. The samples were then incubated with 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.02% (v/v) NP40, and 2 mM MgCl2 in PBS at 4°C overnight. Cut surfaces of the organs or whole lungs were observed and microphotographs were taken with an Olympus SZH dissecting microscope (Olympus, Tokyo, Japan). The degrees of micrometastasis in each organ were defined as follows: grade 0, no metastasis observed; grade 1, 1–5 microscopic metastatic foci observed; grade 2, 6–10 microscopic metastatic foci observed; grade 3, more than 10 microscopic metastatic foci observed; and grade 4, macroscopic metastasis (approximately larger than 3 mm) observed. Representative blue-stained metastatic foci are shown in Fig. 1. All of the samples were observed and the grading of metastasis was performed by one of the authors (M. N.) in a blinded manner. Because the sizes of microscopic...
Enous micrometastases may occur 3 weeks after the injections. These findings suggest that lymphatic and hematogenous micrometastases in this model.

**Effect of Tumor Removal on the Progression of Micrometastasis.** On the basis of the data of experiment 1, transplanted MKL-4 tumors were resected 3 weeks after the cell injections in the tumor removal group. The weight of the resected tumors was $0.15 \pm 0.04$ g (mean $\pm$ SD) in this group. In the control group, the weight of the tumors 7 weeks after the injections was $1.20 \pm 0.33$ g. As shown in Table 1, the degree of micrometastasis into the axillary lymph node, distant lymph nodes, or bone was significantly promoted by the removal of primary tumors. Although the degree of micrometastasis into the lungs, liver, or brain in the tumor removal group was slightly higher than that in the control group, the difference was not statistically significant. These findings suggest that tumor removal may enhance the progression of micrometastasis existing before the tumor removal.

**Effect of UFT on Tumor Growth and Progression of Micrometastasis.** p.o. administration of either 15 or 20 mg/kg UFT for 4 weeks from 3 weeks after the cell injections significantly reduced the weight of the primary tumors ($P < 0.01$ in each comparison to the control group). Neither dose of UFT affected the body weight of the mice (Table 2). Moreover, this treatment inhibited the progression of micrometastasis in the tumor-bearing mice. The degree of micrometastasis into the distant lymph nodes (for the 20 mg/kg-treatment group alone), lungs, liver, or brain was significantly reduced by the treatment with either dose of UFT. Micrometastasis into the axillary lymph node or bone was also slightly reduced by the treatment, but this difference was not statistically significant (Table 2). These findings suggest that the p.o. administration of either 15 or 20 mg/kg UFT has a large enough potential to inhibit both the growth of primary tumors and the progression of both lymphatic and hematogenous micrometastases in this model.

**Effect of UFT on the Progression of Micrometastasis after Tumor Removal.** On the basis of the data of experiments 2 and 3, 15 or 20 mg/kg UFT were administered p.o. to the mice in which the primary tumors were resected 3 weeks after the cell injections. The degree of micrometastasis into each explored organ was significantly reduced by the treatment with either dose of UFT (Table 3). In addition, the weight of the axillary lymph nodes in the UFT treatment groups (mean $\pm$ SD, 7.1 $\pm$ 3.1 mg for the 15-mg/kg group and 6.9 $\pm$ 3.1 mg for the 20-mg/kg group) was significantly smaller than that in the tumor removal-alone group ($24.8 \pm 7.8$ mg; $P < 0.01$ in each comparison). Again, no difference between the treatment and control groups was seen in the body weight of the mice. Interestingly, in 1 and 2 mice, respectively, of the 10 mice treated with 15 and 20 mg/kg UFT, no micrometastasis was observed in any explored organs. This inhibitory effect of UFT on the progression of micrometastasis in tumor-resected mice seemed to be more obvious than that in the tumor-bearing mice of experiment 3. These findings suggest that the enhanced progression of micrometastasis by the removal of primary tumors may be effectively inhibited by the postsurgical administration of UFT in this model.
Inhibition of Progression of Micrometastasis by UFT

Fig. 2. The growth curve of MKL-4 transplanted tumors and the relationship between the timing of sacrifice of the mice and the frequency of micrometastases in the axillary lymph node or lungs. •, mean tumor weight. Bars, SD. ○ and △, percentage of mice who are micrometastasis positive in the axillary lymph node and lungs, respectively.

Table 1

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<th>Organ</th>
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<tr>
<td></td>
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<td>0 4 6 0 0</td>
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<tr>
<td>Distant lymph nodes</td>
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<tr>
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<td>0 2 7 1 0</td>
</tr>
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a The grade of micrometastasis in each organ was defined as described in "Materials and Methods."

b The number of mice with a certain grade of metastasis in the explored organ. Ten mice were tested in each group.

c Tumor removal significantly promoted the progression of micrometastasis: P < 0.05.

d Tumor removal significantly promoted the progression of micrometastasis: P < 0.01.

DISCUSSION

Postsurgical adjuvant chemotherapies have been introduced into the treatment of many malignancies. All agents used in the postsurgical adjuvant setting have already been proven to be effective in the treatment of advanced malignancies. The antitumor effect of these agents has been thought to lead to the inhibition of growth of micrometastasis from malignancies and to delay or decrease the occurrence of clinically detectable recurrences. As expected, many clinical trials after the surgical removal of primary breast cancer have demonstrated that some antitumor agents improve disease-free survival and/or the overall survival of the patients (1–3). However, it is difficult to observe how these agents inhibit the progression of micrometastasis in these patients. This is also true in experimental animal models. We recently developed a new spontaneous metastasis model in nude mice using the MCF-7 human breast cancer cell line cotransfected with fgf-4 and lacZ. This model provided us with a quantitative measurement of micrometastasis in both lymph nodes and distant organs (6, 7). Therefore, we decided to develop a new postsurgical metastasis model using this animal model.

First, we studied when micrometastasis occurs in the MKL-4 metastasis model. The results of experiment 1 indicated that both lymphatic and hematogenous micrometastases occur 3 weeks after the cell injections (Fig. 2). Similar findings were also made in our previous study (7). In addition, the frequency, degree, and size of micrometastasis increased as time passed and as the primary tumor grew (not shown in detail). These findings suggest that micrometastasis may occur and progress from 3 weeks onward after the injections. Therefore, we next investigated whether or not removal of the primary tumors 3 weeks after the injections promotes the progression of micrometastasis. As expected, the tumor removal significantly enhanced the progression of micrometastasis into both lymph nodes and distant organs (Table 1). It has been observed that the removal of primary tumors promotes the progression of secondary sites, i.e., micrometastases, in some experimental animal models (28–33). Recently, an antiangiogenic factor, angiostatin secreted from the primary tumors, was reported to be one of the most likely candidates to cause this phenomenon (34, 35). As our purpose in this study was not to elucidate the molecular mechanisms of such a phenomenon, the expression of angiostatin in the primary tumors and the effect of the tumor removal on the degree of neovascularization and apoptosis in the secondary sites was not studied. Otherwise, our primary purpose was accomplished because a new postsurgical metastasis model in which micrometastasis can be measured quantitatively after tumor removal was established.

Interestingly, micrometastasis into bone or bone marrow was frequently observed and its progression was promoted by...
Unpublished data.

Antiproliferative effect

Tumor growth was significantly reduced by p.o. administration of antitumor agents, such as tegafur or tiFF, with other chemotherapeutic agents has been investigated in clinical phase studies for the treatment of gastric cancer (36, 37). In addition, combined use of UFT with other chemotherapeutic agents has been investigated in clinical phase studies for the treatment of breast cancer and gastrointestinal cancer is common in Japan (13, 14). These agents have also been used in postsurgical adjuvant settings. Recently, UFT has been used with leucovorin for the treatment of advanced colorectal and gastric cancer in Western countries because of its fewer adverse effects and comparative antitumor effect with i.v. 5-FU infusion (17, 19–21). In addition, combined use of UFT with other chemotherapeutic agents has been investigated in clinical phase studies for the treatment of gastric cancer (36, 37). In the present study, on the basis of our preliminary study (38), relatively low doses of UFT were administered p.o. Either 15 or 20 mg/kg UFT significantly inhibited the growth of MKL-4 transplanted tumors without affecting the body weight of the mice. The progression of micrometastasis after tumor removal in comparison to that in the control group: \( P < 0.01 \).

The grade of micrometastasis in each organ was defined as described in “Materials and Methods.”

The number of mice with a certain grade of metastasis in the explored organ. Ten mice were tested in each group.

The p.o. administration of UFT significantly inhibited the progression of micrometastasis after tumor removal in comparison to that in the control group: \( P < 0.01 \).

The p.o. administration of UFT significantly inhibited the progression of micrometastasis after tumor removal in comparison to that in the control group: \( P < 0.05 \).

The p.o. administration of UFT significantly inhibited the progression of micrometastasis after tumor removal in comparison to that in the control group: \( P < 0.05 \).
Inhibition of Progression of Micrometastasis by UFT

suggested to be identical to thymidine phosphorylase (40). PD-ECGF has been reported to increase many types of malignant tumors (41). Therefore, there is a possibility that tegafur might influence the activity of PD-ECGF and tumor angiogenesis. Further investigation is needed to elucidate the action mechanisms of UFT on the progression of micrometastasis after tumor removal. However, to the best of our knowledge, this is the first demonstration that p.o. administration of a 5-FU derivative inhibits the progression of micrometastasis after surgery.

To develop a new postoperative metastasis model in which the early events of metastasis, i.e., micrometastases, can be evaluated, we investigated the timing of occurrence of micrometastasis and the effects of the removal of primary tumors on the progression of micrometastasis in the MKL-4 metastasis model. Tumor removal at the time when micrometastasis occurred significantly enhanced the progression of micrometastasis after surgery.

REFERENCES

Postsurgical oral administration of uracil and tegafur inhibits progression of micrometastasis of human breast cancer cells in nude mice.

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