Suppression of Mediastinal Metastasis by Uracil-Tegafur or cis-Diamminedichloroplatinum(II) Using a Lymphogenous Metastatic Model in a Human Lung Cancer Cell Line

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ABSTRACT

Purpose and Experimental Design: The extent of lymphatic metastasis is the most important factor in the prognosis for non-small cell lung cancer (NSCLC). Therefore, suppression of lymphatic metastasis provides an improvement in survival time in lung cancer patients. We established a new patient-like model for lung cancer metastasis by orthotopic implantation in severe combined immunodeficiency (SCID) mice and demonstrated the lymphogenous spread histologically using human NSCLC cell lines. The cardinal features of this model are a simple procedure and a similarity to the metastatic form of human lung cancer. The purpose of this study is to assess the inhibitory action of uracil-tegafur (UFT) and cis-diamminedichloroplatinum(II) (CDDP) on lymphatic metastasis and life span prolongation in our lymphogenous metastatic model system using SCID mice.

Results: The inhibition ratios of mediastinal lymph node metastasis were 86.2, 94, and 92.1% for 12 mg/kg body UFT, 17 mg/kg body UFT, and 10 mg/kg body CDDP, respectively. The administration of anticancer drugs prolonged the life span by 4.6 days (17 mg/kg body UFT) and 8 days (10 mg/kg body CDDP) in MST.

Conclusion: We demonstrated that UFT alone and CDDP alone suppressed mediastinal metastasis and prolonged the life span in our lymphogenous metastatic model. Regardless of the administration route and characteristics of anticancer drugs, cytostatic or cytotoxic, our model is capable of evaluating the inhibitory effect of drugs on lymphatic metastasis. This model should make an important contribution to our understanding of the mechanism and selection of drugs for antilymphatic metastasis in lung cancer.

INTRODUCTION

The extent of lymphatic metastasis is the most important influencing factor in the prognosis for NSCLC. The 5-year survival rate after resection for patients with pN2 disease was only 23% (1). Therefore, suppression of lymphatic metastasis provides an improvement in survival in lung cancer patients. Recent clinical studies revealed that some molecules, such as metalloproteinase-2 (2, 3), CD44 variant 6 (4, 5), and vascular endothelial growth factor-C (6, 7), are related to lymphatic metastasis in primary cancer. The close relationship between these molecules and lymphatic metastasis was not examined experimentally, however, because there has been no animal model for lymphatic metastasis in lung cancer yet. We established a new patient-like model of lung cancer metastasis by orthotopic implantation in SCID mice and demonstrated the lymphogenous spread histologically using human NSCLC cell lines (8, 9). The cardinal features of this model are a simple procedure and similar to the metastatic form of human lung cancer.

In this study, we used two anticancer drugs, CDDP and UFT. CDDP is usually administered by the i.v. route and is a cytotoxic agent. UFT is an antineoplastic drug combining uracil and tegafur (fitagrafur; a prodrug of 5-fluorouracil) in a 4:1 molar ratio. It has been widely used in the postsurgical adjuvant setting in Japan. Recently, UFT has been reexamined in Europe and the United States because it offers the advantage of oral administration.

The purpose of this study was to assess the inhibitory action of UFT and CDDP on lymphatic metastasis, the prolongation of the life span in our lymphogenous metastatic model system using SCID mice, and to confirm that our metastatic model is extremely useful for the selection of anticancer drugs to inhibit lymphatic metastasis.

MATERIALS AND METHODS

Chemicals. UFT was provided by Taiho Pharmaceutical Co., Ltd. For in vivo experiments, UFT was suspended in CMC. Matrigel, a basement membrane matrix, was purchased from...
Collaborative Biomedical Products (Bedford, MA). CDDP was purchased from Nippon Kayaku Co.

**Animals.** Male SCID mice (6 weeks of age) with a CB-17 genetic background were purchased from CLEA Japan, Inc. (Tokyo, Japan). These mice had been raised from birth in a specific pathogen-free environment.

**Cells.** Human lung cancer cell line Ma44 (squamous cell carcinoma) was kindly provided by Drs. Masuda and Takata (Osaka Prefectural Habikino Hospital, Osaka, Japan; Ref. 9). An Ma44-3 cell line was cloned using the limiting dilution method in our laboratory. The Ma44-3 cell line was cultured in RPMI 1640 with 10% heat-inactivated fetal bovine serum (BioWhittaker, Walkersville, MD) and maintained at 37°C in a humidified incubator with 5% CO₂ in air.

**Orthotopic Intrapulmonary Implantation Procedure.** As shown in our previous studies (8, 9), Ma44-3 cells were harvested for implantation at 70–80% confluence using 1 mmol/l EDTA (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in PBS (Nissui Pharmaceutical Co., Ltd., Toyo, Japan). The cells were washed in RPMI 1640 and resuspended to a final concentration of 2.0 × 10⁶ cells/ml in RPMI 1640 containing 0.1% BSA (Boehringer Mannheim, Mannheim, Germany). The mice were fully anesthetized by ether inhalation. The experimental mice were placed in the right lateral decubitus position with all four limbs restrained. A 1-cm transverse incision was made in the left lateral skin just below the inferior border of the scapula of the SCID mouse. The muscles were separated from the ribs by sharp dissection, and the intercostal muscles were exposed. The left lung was visible through the intercostal muscles. A 30-gauge needle was inserted ~5 mm into the lung through the intercostal muscle, and an inoculum of 2.0 × 10⁶ tumor cells/ml with 400 μg/ml Matrigel (Collaborative Biomedical Products, Bedford, MA) was then dispersed into the left lung in a final volume of 10 μl (2.0 × 10⁴ cells) of medium. The procedure required ~1 min for completion and was performed easily. The skin incision was closed with 3-0 silk.

**Assessment of the Inhibitory Effect on Tumor Growth and Toxicity of UFT and CDDP in the s.c. Implantation Model.** To determine the optimum dosage of UFT or CDDP, we used a s.c. implantation model. Ma44-3 tumor cells (2.0 × 10⁶) were injected s.c. into the right groin of the mice. Twelve mice were divided into four groups (n = 3). The mice were p.o. administered 0, 12, 17, or 24 mg/kg body of UFT for 7 consecutive days beginning on day 5 after implantation. The control group (0 mg/kg body weight) received only normal saline. As a separate group, six mice were i.p. administered 10 mg/kg body weight of CDDP on day 5 after implantation. In our previous study, mediastinal lymph node metastasis was detected histologically on day 5 and detected macroscopically on day 9. The survival time of mice was 14–18 days (9). The period of administration of UFT was therefore determined to be days 5–11, and the time of CDDP administration was determined to be on day 5 after implantation. The mice were sacrificed on day 14 by ether inhalation and cervical dislocation. The major organs (bilateral lungs, heart, liver, kidneys, adrenal glands, and mediastinal tissues) were removed and fixed in 10% formalin and embedded in paraffin. Five-μm histological sections were made from the mediastinal tissues at 300-μm intervals. The paraffin sections were stained with H&E and examined by microscope. To quantify the volume of mediastinal lymph nodes metastasized by Ma44-3 cells, we selected the slice that had the largest tumor foci in the mediastinal tissues in a low power field (×20) and calculated the area occupied by Ma44-3 tumor cells by NIH image.

**Survival of Orthotopically Implanted Mice Treated with UFT and CDDP.** To examine the life-prolonging activity of UFT or CDDP in this lymphogenous metastatic model system, 26 mice with tumor cells orthotopically implanted were divided into two groups (n = 13). These mice were p.o. administered 0 or 17 mg/kg body of UFT for 7 consecutive days from day 5 after implantation. Twenty mice with tumor cells orthotopically implanted were divided into two groups (n = 10). These mice were i.p. administered 0 or 10 mg/kg body of CDDP on day 5 after implantation.

All of the experimental protocols for this study were approved by the Institutional Animal Care and Use Committee of the University of Tokushima School of Medicine.

**Statistical Analysis.** A one-factor ANOVA and Dunnett’s t test were used for statistical analysis. The Kaplan-Meier method was used to construct curves for overall survival, and a log-rank test was used to determine survival rates.

**RESULTS**

**Changes in the Body Weights of the Mice Treated with UFT.** The changes in the body weights of the mice treated with 0, 12, 17, and 24 mg/kg body of UFT are shown in Fig. 1. All 9 mice treated with 0, 12, and 17 mg/kg body of UFT survived until day 21. Although 1 of 3 mice treated with 24 mg/kg body of UFT survived until day 21, the other 2 mice died on day 14. The body weights of the mice treated with 0, 12, 17, and 24 mg/kg of UFT were 28.6 ± 0.5 SD, 26.1 ± 0.53 SD, and 25.9 ± 0.9 SD on days 21 and 18.7 ± 0.6 SD on day 14, respectively. The body weights of the mice treated with 24 mg/kg body were significantly lower than those of the other three groups on day 14 (P < 0.001). There was no significant
weight loss on day 21 among the other three groups. Although UFT exhibited toxicity at a dose of 24 mg/kg body weight, no toxicity was observed at doses of 12 or 17 mg/kg body weight.

Changes in the Body Weights of the Mice Treated with CDDP. The changes in the body weights of the mice treated with 0, 3, 7, and 10 mg/kg body weight of CDDP are shown in Fig. 1b. All mice treated with 0, 3, 7, and 10 mg/kg body weight of CDDP survived until day 28. However, all mice treated with 15 and 20 mg/kg of CDDP died within 5 days after treatment. The body weights of the mice treated with 7 and 10 mg/kg of CDDP decreased temporarily after CDDP treatment but recovered within 10 days. The body weights of the mice treated with 0 and 3 mg/kg CDDP increased normally. There was no significant difference between the control group (0 mg/kg body weight of CDDP) and the 3, 7, and 10 mg/kg body weight CDDP groups.

Effect of UFT on Tumor Growth Produced by s.c. Implantation of Ma44-3 Cells. The changes in the mean volume of s.c. tumors are shown in Fig. 2a. The volumes of the s.c. tumors treated with 0, 12, 17, and 24 mg/kg body of UFT were 7061 ± 2577 SD, 2353 ± 841 SD, and 1140 ± 275 SD on day 21 and 0 mm³ on day 14, respectively. The growth of s.c. tumors on day 21 treated with 12, 17, and 24 mg/kg UFT was significantly suppressed as compared with tumors treated without UFT (P < 0.01). The inhibitory effect of UFT was dose dependent in the three treated groups, but the difference was not statistically significant.

Effect of CDDP on Tumor Growth Produced by s.c. Implantation of Ma44-3 Cells. The changes in the mean volume of s.c. tumors are shown in Fig. 2b. The volumes of the s.c. tumors treated with 0, 3, 7, and 10 mg/kg body weight of CDDP were 1043 ± 756, 1314 ± 636, 418 ± 216, and 383 ± 89 mm³ on day 21, respectively. The growth of s.c. tumors on day 21 treated with 7 and 10 mg/kg body weight of CDDP was suppressed as compared with 0 and 3 mg/kg body weight of CDDP.
CDDP were 1043 ± 756 SD, 1314 ± 636 SD, 418 ± 216 SD, and 383 ± 89 SD mm³ on day 21, respectively. The growth of s.c. tumors in mice treated with 7 and 10 mg/kg body weight of CDDP was substantially suppressed as compared with that of tumors in those treated with 0 and 3 mg/kg body weight of CDDP, but the inhibitory effect of CDDP was not statistically significant.

**Inhibitory Effect of UFT and CDDP on Mediastinal Lymph Node Metastasis.** The effective doses of UFT were determined from the s.c. implantation experiment to be 12 or 17 mg/kg. The effective doses of CDDP were determined from the s.c. implantation experiment to be 7 or 10 mg/kg. Ma44-3 tumor cells were injected into a lung of 27 mice. Twenty-one mice were p.o. administrated 0, 12, or 17 mg/kg body of UFT during days 5–11 after implantation (n = 7), and 6 mice were i.p. administered 10 mg/kg body weight of CDDP on day 5 after implantation.

In the control group, mediastinal lymph node metastasis was observed in 6 of 7 mice (86%). Two of them had bulky lymph nodes of the mediastinum (Fig. 3a), two showed metastases macroscopically, and two showed metastases microscopically. In the 12 mg/kg UFT group, 1 of the 7 mice showed mediastinal metastasis macroscopically, and 2 of the 7 mice showed mediastinal lymph node metastasis microscopically. Macroscopic or microscopic metastases were observed in 3 of 7 mice (43%). In the 17 mg/kg UFT group, mediastinal lymph node metastases were observed microscopically (Fig. 3b) in 5 of 7 mice (71%). In the CDDP-treated group, 5 of 6 mice (83%) showed mediastinal lymph node metastases microscopically.

The mean area of mediastinal metastases calculated by NIH image for the 0, 12, and 17 mg/kg body UFT-treated groups and the CDDP-treated group had values of 119.9 ± 73.4 SD, 16.9 ± 32.3 SD, 6.8 ± 10.6 SD, and 9.5 ± 13.1 SD pixels/field, respectively (Fig. 4). Bulky or macroscopically observed lymph node swelling was >50 pixels/field. However, single lymph node swelling does not always involve tumor cells. In fact, three mice with single moderate swelling did not have metastasis. Exact histological observation is thus extremely important.

The inhibition ratios of mediastinal lymph node metastasis were 86.2% for 12 mg/kg UFT, 94% for 17 mg/kg UFT, and 92.1% for CDDP as compared with the 0 mg/kg control group. Mice treated with UFT and CDDP showed an inhibitory effect on mediastinal lymph node metastasis, and this effect was statistically significant (P < 0.01). No marked loss of body weight was observed in the UFT- and CDDP-treated groups (data not shown).

**Life-prolonging Activity of UFT (Fig. 5a).** We next compared the life-prolonging activity in the mice treated with 17 mg/kg UFT with that in the mice without UFT in the lymphogenous metastatic model system (n = 13; Fig. 5a). In the 0 mg/kg UFT (control) group, the first death occurred on day 12, and the MST was 15.8 days. In the 17 mg/kg UFT group, however, the first death occurred on day 15, and MST was 21.4 days. The mice treated with UFT showed a marked prolongation of survival as compared with the mice not treated with UFT (P < 0.01).

**Life-prolonging Activity of CDDP (Fig. 5b).** We performed separate experiments for mediastinal metastasis and compared the life-prolonging activity in the mice treated with 10 mg/kg body of CDDP with that in mice without CDDP (n = 10; Fig. 5b). In the control group, the first death occurred on day 15, and MST was 17.2 days. In the CDDP-treated group, however, the first death occurred on day 20, and MST was 25.2 days. The mice treated with CDDP showed a marked prolongation of survival as compared with the mice not treated with CDDP (P < 0.01).

**DISCUSSION**

The status of lymphatic metastasis is a major factor for prognosis and assignment of treatment in patients with NSCLC. The survival curves of NSCLC patients with surgical treatment reflect the extent of lymphatic metastasis. Mountain (1) reported that the 5-year survival rates for the N₀, N₁, and N₂ diseases are 38–67, 25–55, 23%, respectively. Even in small size cancer (tumors of 3.0 cm or less in diameter) and peripheral NSCLC
Suppression of Mediastinal Metastasis by UFT or CDDP

Efficacy on mediastinal metastasis can be considered from two aspects. One is the effect on existing metastatic foci, and the second is the preventive effect on metastasis, which depends on combination regimens have a modest but significant increase in survival as compared with patients who do not receive cisplatin. In Phase III Eastern Cooperative Oncology Group trials, CDDP-based regimens are more effective than those that do not contain cisplatin (20, 21). UFT, which exhibits a cytostatic effect against tumor cells, has been used widely in the treatment of breast, gastrointestinal, head and neck, and lung cancers in Japan. Wada et al. (22) reported an adjuvant trial of 323 patients with all stages of completely resected NSCLC. They compared the life-prolonging effect of a single dose of UFT, concomitant UFT with CDDP plus Vindesine, and no treatment (control). The 5-year survival rates of the single-dose UFT group (64.1%) and the UFT plus CDDP and Vindesine group (60.6%) were significantly high as compared with the control group (49.0%). UFT could be an effective therapeutic opinion for adjuvant chemotherapy for NSCLC. In addition, because UFT is administered p.o. with CMC control (broken line; n = 13) or with UFT 17 mg/kg (solid line; n = 13). The median survival of UFT 17 mg/kg was 21.4 days, compared with control (15.8 days). The difference in survival between controls and treatment group was statistically significant (P < 0.01). b, Kaplan-Meier survival curves for mice administrated i.p. with saline control (broken line; n = 10) or with CDDP 10 mg/kg (solid line; n = 10). The median survival of CDDP 10 mg/kg was 25.2 days, compared with control (17.2 days). The difference in survival between controls and treatment group was statistically significant (P < 0.01).

In this study, we examined the inhibitory effect on lymphatic metastasis of two anticancer drugs. UFT is a representative drug for oral administration and a cytostatic agent. CDDP is a representative drug for i.v. administration and a cytotoxic agent, which has been used recently as a key drug in many combination chemotherapeutic regimens in lung cancer. It was reported that the antitumor effect for NSCLC was 8.4% with UFT alone (17) and 12% (standard dose) to 14% (high dose) with CDDP alone (18). Eagan et al. (19) first used CDDP in combination with other agents in a series of Phase II trials. Most Phase II studies indicate that patients treated with CDDP-based treatment group was statistically significant (P = 0.05).

Fig. 4 The mean area of mediastinal metastases calculated by NIH image microscopically (×20). Mice received injections of Ma44-3 cells into lung and were given UFT (12 or 17 mg/kg; n = 7) or 10 mg/kg CDDP i.p. (n = 6). The mean area of 0 mg/kg UFT-treated group, 12 mg/kg UFT, 17 mg/kg UFT- and CDDP-treated group had value of 119.9 ± 73.4, 16.9 ± 32.3, 6.8 ± 10.6, and 9.5 ± 13.1 pixels/field, respectively; bars, SD. Mice treated with UFT and CDDP showed an inhibitory effect on mediastinal metastasis, and this was statistically significant (P < 0.05).

Fig. 5 a, Kaplan-Meier survival curves for mice administrated p.o. with CMC control (broken line; n = 13) or with UFT 17 mg/kg (solid line; n = 13). The median survival of UFT 17 mg/kg was 21.4 days, compared with control (15.8 days). The difference in survival between controls and treatment group was statistically significant (P < 0.01). b, Kaplan-Meier survival curves for mice administrated i.p. with saline control (broken line; n = 10) or with CDDP 10 mg/kg (solid line; n = 10). The median survival of CDDP 10 mg/kg was 25.2 days, compared with control (17.2 days). The difference in survival between controls and treatment group was statistically significant (P < 0.01).
for antilymphatic metastasis in lung cancer.

to our understanding of the mechanism and selection of drugs capable of evaluating the inhibitory effect of lymphatic metastasis of anticancer drugs, cytostatic or cytotoxic, our model is capable of dichotomy of administration routes, oral or i.p. (i.v.), and characteristics of mice in our lymphogenous metastatic model. Regardless of suppression of mediastinal metastasis and prolonged the life spans of mice in our lymphogenous metastatic model. Furthermore, micrometastasis of the mediastinum in mice treated with CDDP on day 14 ( metastatic lesions in the mediastinum are detected microscopically) is similar to that in mice without UFT on day 5. The period of the administration of UFT is almost the same as that of the prolonged survival of mice treated with UFT. These results suggest that the administration of UFT for 7 days inhibited the lymphatic metastatic activity or the enlargement of the metastatic lesions in the mediastinum, and that the lymphatic metastatic activity recovered, or the metastatic lesions began to grow again after the administration of UFT was discontinued. Recent studies have reported that UFT suppresses tumor growth, tumor hematogenous metastasis, tumor angiogenesis (23, 24), the in vitro invasion activity of Matrigel, and cell detachment from the primary tumor (25). We think that the inhibition of lymphatic metastasis in this study is attributable to multiple functions of UFT, such as its antitumor growth, antiangiogenesis, anti-invasion, and anti-intravasation functions. Although further examination is needed to elucidate the mechanisms of inhibition of lymphatic metastasis, there is no doubt that UFT functions cause tumor dormancy.

Furthermore, micrometastasis of the mediastinum in mice treated with CDDP on day 14 is also similar to that in mice not treated with CDDP on day 5. The mice treated with CDDP showed survival prolongation of 8 days as compared with the mice without CDDP treatment. CDDP is effective in inhibiting the development of secondary metastatic growth (26). These findings suggest that treatment with CDDP delays the progress of lymphatic metastasis of lung cancer.

It is well known that CDDP inhibits DNA synthesis and the division of tumor cells. Recently, it was reported that CDDP inhibited tumor angiogenesis directly (27). However, it was reported previously that CDDP lacked antiangiogenic activity (28). It therefore remains to be elucidated in detail whether CDDP has the ability to conduct antitumor angiogenic activity.

In conclusion, we show that UFT alone and CDDP alone suppressed mediastinal metastasis and prolonged the life spans of mice in our lymphogenous metastatic model. Regardless of the administration routes, oral or i.p. (i.v.), and characteristics of anticancer drugs, cytostatic or cytotoxic, our model is capable of evaluating the inhibitory effect of lymphatic metastasis for drugs. This model should make an important contribution to our understanding of the mechanism and selection of drugs for antilymphatic metastasis in lung cancer.

ACKNOWLEDGMENTS

We are grateful to Kiyomi Aihara for preparing the slides for microscopy.

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