Inhibitory Effect of a Biscoclaurine Alkaloid, Cepharanthin, on Lung Metastasis of Lewis Lung Carcinoma

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Received January 24, 1991 Accepted March 22, 1991

ABSTRACT—The antimetastatic effect of cepharanthin with or without 5-fluorouracil (5-FU) was examined in an experimental model of lung metastasis induced by Lewis lung carcinoma (3LL) in C57BL/6crSlc mice. Injection of cepharanthin i.p. after removal of the implanted primary tumor inhibited the development of lung metastases. Combination therapy with cepharanthin plus 5-FU inhibited significantly the lung metastases. Lung metastases were inhibited by i.v. injection of peritoneal macrophages activated with cepharanthin. Cepharanthin depressed aniline hydroxylase and aminopyrine demethylase activities of the hepatic microsomal drug-metabolizing system in tumor-bearing mice. Moreover, the concentration of 5-FU in the tissues (lung, liver, kidney, spleen and blood) was increased significantly by coadministration of cepharanthin. A possible mechanism of the inhibition of lung metastases by treatment with cepharanthin may be that this drug acts through macrophage activation and depression of the hepatic microsomal drug-metabolizing system. These findings raise the possibility that combination therapy with cepharanthin plus 5-FU may have clinical value in the prevention of cancer metastasis.

The pathogenesis of metastasis involves many complex biological processes, and its outcome depends on the interaction of tumor cells with their host and is influenced by various factors in the metastatic step (1, 2). The role of host defense in the control of metastasis is not well-understood. It might be possible to eradicate or prevent micrometastases by immunological modulation of the host defense mechanism. Indeed, biological response modifiers such as polysaccharides extracted from plants (3, 4), bacterial cellular components (5), *Corynebacterium parvum* (6), BCG (7) and water extract from herbal medicine (8) that activate the reticuloendothelial system of the host have been found to inhibit develop-

ment of metastases. A pyrimidine antitumor drug, 5-fluorouracil (5-FU) has been proven to be one of the most useful drugs available in clinical oncology. A biscoclaurine alkaloid, cepharanthin, has been shown to affect cell membranes (9–11) and alter the biomembrane movement of Ca^{++} (12). Shiraishi et al. (13) described the effect of cepharanthin on a multidrug resistance KB human cancer cell line. Kato and Suzumura (14) have reported that cepharanthin enhanced the antitumor activity of vincristine. Ono et al. (15) demonstrated that intratumoral administration of cepharanthin significantly inhibited the growth of Sarcoma-180 solid tumor in mice. Recently, Fujimura et al. (16) showed that cepharanthin

overcomes doxorubicin resistance in doxorubicin-resistant murine P388 leukemia cells *in vitro* and *in vivo*. Shimura (17) demonstrated that cepharanthin enhanced phagocytosis and ingestion of polystyrene latex beads into the peritoneal macrophages of mice after i.p. injection. However, the mechanism of the action of cepharanthin on tumors, is still unclear.

Therefore in the present study, we attempted to determine the antimetastatic effect of cepharanthin on Lewis lung carcinoma and the possible mechanisms of its action.

MATERIALS AND METHODS

Animals

Five-week-old females C57BL/6crSlc mice (SPF), weighing 19 to 22 g, were obtained from Shizuoka Laboratory Animal Center (Japan). They were maintained on an CE-2 pellet diet (Clea Japan, Inc., Japan) and water *ad libitum*.

Tumor

Lewis lung carcinoma (3LL) was initially supplied by the Aichi Cancer Center Institute. It was maintained by serial biweekly s.c. passage in C57BL/6crSlc mice. The tumor fragments were stirred in RPMI medium 1640 (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY), containing 0.05% trypsin, at 30°C for 30 min. After rinsing, the viability of the tumor cells was confirmed by the trypan blue dye exclusion method. A suspension of 10^5 viable cells in 0.05 ml of RPMI 1640 was implanted s.c. into the footpad of each mouse.

Drugs

Cepharanthin was kindly provided by Kaken Shoyaku Co., Ltd., Tokyo. The anticancer agent, 5-FU (Kyowa Hakko Kogyo Co., Ltd., Tokyo) was used as a reference drug in some of the experiments. The required concentrations of drugs were dissolved in 0.9% NaCl solution for the study.

Assay of lung metastases

When the implanted tumor in the footpad had become about 4 to 5 mm in diameter 10 days after implantation of tumor cells, the mice were anesthetized by i.p. injection of 1 mg of sodium pentobarbital, and the tumor-bearing leg was then surgically amputated. Mice were killed 14 days after removal of the implanted tumor, and their organs were examined grossly. The gross numbers of metastatic nodules were counted by Wexler's method (18).

Preparation of effector cells

Peritoneal exudate cells (PEC) were collected by washing the peritoneal cavity with 10 ml of RPMI 1640, 4 days after i.p. injection of cepharanthin. PEC were washed 3 times with medium and suspended at a fixed concentration in RPMI 1640 containing 10% fetal calf serum (FCS). For separation of adherent cells, suspensions of PEC were put into plastic flasks coated with serum and incubated at 37°C in a 5% CO₂ incubator to allow macrophages to become attached by the method of Kumagai et al. (19). After incubation for 20 min, nonadherent cells were decanted, and flasks were washed 5 times with medium in an ice bath. Adherent cells were then removed from the flasks by incubation at 4°C in medium containing 0.2% EDTA and 5% FCS, washed, and resuspended in RPMI 1640.

Assay of hepatic drug-metabolizing enzyme

The activity of hepatic drug-metabolizing enzymes was assayed by the production of colored metabolites from aminopyrine and aniline after incubation with microsomes at 37°C in the presence of a NADPH-generating system (20) as described below. For the preparation of microsomes, a 25% liver homogenate in 0.25 M sucrose was centrifuged at $10,000 \times g$ for 20 min at 4°C. The supernatant fraction was aspirated and centrifuged at $105,000 \times g$ for 60 min at 4°C. The microsomal pellet was washed by resuspension in ice-cold 0.15 M KCl-10 mM EDTA, followed by resedimentation at $105,000 \times g$ for 30 min. The final pel-

let was resuspended in an isotonic KCl solution.

For the aniline hydroxylase assay, each incubation mixture consisted of 5 μ M aniline, 2 μ M NADP⁺, 25 μ M glucose-6-phosphate, 3.5 units of glucose-6-phosphate dehydrogenase, 25 μ M magnesium chloride and 1.0 ml of microsomal suspension in a total volume of 5.0 ml buffered at pH 7.4 with 0.1 M potassium phosphate. Reaction was initiated by the addition of microsomes. Incubations conducted in triplicate were carried out for 20 min at 37°C and terminated by the addition of 2 ml of 20% trichloroacetic acid. Precipitated protein was removed by centrifugation, and aliquots of the clear supernatant were assayed for *p*-aminophenol at 630 nm in a spectrophotometer (Beckman Model 34, U.S.A.) according to the method of Kato and Gillette (21).

Aminopyrine demethylase assay was performed by the method of Farquhar et al. (20). Formaldehyde production from aminopyrine was reacted with Nash's reagent and determined by the method of La Du et al. (22) at 412 nm in a spectrophotometer (Hitachi 181 UV-VIS, Japan).

Assay of 5-FU

The assay of 5-FU in the tissue was measured as 5-FU by a bioassay method using an indicator bacterium, *Micrococcus luteus* ATCC 10240 (23).

Statistics

The values are expressed as the means $\pm$ S.E., and the statistical significances were determined with Student's *t*-test. Probabilities of less than 5% ($P < 0.05$) were considered significant.

RESULTS

Inhibition of growth of lung metastases by cepharanthin

The effect of cepharanthin on the growth of lung metastases of 3LL was examined by injecting cepharanthin into mice after removal of the implanted primary tumor. As shown in

Table 1, a single dose of 2.5, 5.0 or 10.0 mg/kg was injected i.p. into mice on the day when the implanted tumor was removed. Significant inhibition of lung metastasis was noted in mice given injections of 10 mg/kg of cepharanthin (Table 1, Exp. 1). Subsequently, a dose of 5.0 or 10.0 mg/kg of cepharanthin was injected i.p. 3 times, once every 2 days, after removal of the tumor. The inhibition of lung metastasis was greatest at a dose of 5.0 mg/kg (Table 1, Exp. 2). These results indicate that there is an optimal dose of cepharanthin for inhibition of lung metastases. A dose of 5.0 mg/kg of cepharanthin was injected i.p. 3 times, before and after removal of the implanted tumor. When cepharanthin was injected i.p. on days 3, 5, 7, 9, 10, 12 and 14 during growth of the implanted tumor, it caused significant inhibition of lung metastases (Table 1, Exp. 3). These results indicate that the inhibition of lung metastases depends on the time.

Additive effects of cepharanthin and 5-FU on lung metastases

The effect of combined therapy with cepharanthin and 5-FU on lung metastases was examined. The development of lung metastases was inhibited by each of these treatments alone, and the effects were additive (Table 2). In a preliminary experiment, the antitumor activity of the reference compound 5-FU at 30 mg/kg/day was most effective in suppressing tumor growth and prevention of lung metastases. With a higher dose of 50 mg/kg, however, the anti-metastatic effect was weaker than that of the control saline-treated group because of 5-FU toxicity (data not shown). An optimal dose of 5.0 mg/kg of cepharanthin and 30 mg/kg of 5-FU were injected i.p., 3 times on days 10, 12 and 14 after tumor implantation; this caused the most significant inhibition of lung metastases, and no metastatic nodules could be seen in 5 out of 10 mice.

Table 1. Inhibition of lung metastases by cepharanthin

Experimental group	Dose (mg/kg)	Day of injection	Lung metastases
			No. of surface nodules
Experiment 1			
Control			41.8 ± 8.8
Cepharanthin	2.5	10	33.4 ± 8.3
	5.0	10	28.9 ± 8.7
	10.0	10	22.1 ± 4.9*
Experiment 2			
Control			47.6 ± 9.9
Cepharanthin	5.0	10, 12, 14	21.9 ± 5.8*
	10.0	10, 12, 14	25.5 ± 6.7*
Experiment 3			
Control			43.3 ± 7.9
Cepharanthin	5.0	3, 5, 7, 9, 10, 12, 14	16.4 ± 4.8*

Each experimental group consisted of 10 mice. Inocula of 10^5 3LL cells were implanted s.c. into one footpad of each mouse on day 0. The implanted primary tumor was surgically removed by amputating the tumor-bearing leg on day 10 after tumor implantation. Cepharanthin was administered i.p. on day 10 (Exp. 1), on days 10, 12 and 14 (Exp. 2) or on days 3, 5, 7, 9, 10, 12 and 14 (Exp. 3). Control mice were given saline and vehicle in the same manner. Lung metastases were estimated 14 days after removal of the implanted tumor. Each value represents means ± S.E. of 10 animals. Statistically significant difference from the control at * $P < 0.05$.

Table 2. Combined effect of cepharanthin and 5-FU on lung metastases

Experimental group	5-FU		Cepharanthin		Lung metastases	
	Dose (mg/kg)	Day of injection	Dose (mg/kg)	Day of injection	No. of surface nodules	Incidence
Control					39.2 ± 7.1	10/10
Cepharanthin			5.0	10, 12, 14	19.1 ± 4.7*	10/10
5-FU	30	10, 12, 14			10.6 ± 3.2*	7/10
5-FU + Cepharanthin	30	10, 12, 14	5.0	10, 12, 14	4.9 ± 2.1**	5/10

Each experimental group consisted of 10 mice. Inocula of 10^5 3LL cells were implanted s.c. into one footpad of each mouse on day 0. The implanted primary tumor was surgically removed by amputating the tumor-bearing leg on day 10 after tumor implantation. Cepharanthin or 5-FU was administered i.p. on days 10, 12 and 14. 5-FU was used as positive control drug. Control mice were given saline and vehicle in the same manner. Lung metastases were estimated 14 days after removal of the implanted tumor. Incidence are given as the ratio of number of mice with metastases/number of mice tested. Each value represents means ± S.E. of 10 animals. * $P < 0.05$ compared with the controls (undergoing amputation only). ** $P < 0.05$, compared with use of 5-FU or cepharanthin by itself.

Table 3. Effect of i.v. transfer of adherent cells from peritoneal exudate cells (PEC) activated with cepharanthin on lung metastases

Experimental group	Lung metastases
	No. of surface nodules
Control	37.9 $\pm$ 5.7
Normal adherent PEC	36.2 $\pm$ 6.3
Cepharanthin-activated nonadherent PEC	30.8 $\pm$ 7.1
Cepharanthin-activated adherent PEC	23.2 $\pm$ 3.8*

Each experimental group consisted of 7 mice. Inocula of 10^5 3LL cells were implanted s.c. into one footpad of each mouse on day 0. The implanted primary tumor was surgically removed by amputating the tumor-bearing leg on day 10 after tumor implantation. PEC were collected from 7 mice per group, 4 days after i.p. injection of cepharanthin (5.0 mg/kg once a day). Cepharanthin-activated adherent and nonadherent cells (1×10^6 /mouse) were injected i.v. on days 10, 12 and 14 after tumor implantation. Normal adherent PEC or control mice were given saline and vehicle in the same manner. Lung metastases were estimated 14 days after removal of the implanted tumor. Each value represents means $\pm$ S.E. of 7 animals. * $P < 0.05$, compared with the controls (undergoing amputation only) and also compared with normal adherent PEC.

Inhibition of i.v. injection of peritoneal macrophages activated with cepharanthin on lung metastases

Peritoneal adherent cells from mice activated with cepharanthin were injected i.v. on days 10, 12 and 14 into mice with lung metastases after tumor implantation. As shown in Table 3, a significant reduction in the number of metastatic nodules was observed in the lungs of mice given injections of peritoneal adherent cells activated with cepharanthin, but not in mice given injections of nonadherent cells treated with cepharanthin. Furthermore, no inhibition of metastases was seen in mice given injections of peritoneal adherent cells taken from normal mice. These results indicate that the transfer of peritoneal macrophages activated with cepharanthin has an inhibitory effect on lung metastases.

Effects of cepharanthin on drug-metabolism in the liver of 3LL-bearing mice

A single dose of cepharanthin (5.0 mg/kg) or 5-FU (30 mg/kg) plus cepharanthin (5.0 mg/kg) was injected i.p. into mice on the day the implanted primary tumor was removed. As shown in Fig. 1, aniline hydroxylase and

Fig 1. Time course of aniline hydroxylase and aminopyrine demethylase activities in hepatic microsomes of Lewis lung carcinoma-bearing mice after a single i.p. injection of cepharanthin or 5-FU plus cepharanthin. Inocula of 10^5 3LL cells were implanted s.c. into one footpad of each mouse on day 0. The implanted tumor was surgically removed by amputating the tumor-bearing leg on day 10 after tumor implantation. Cepharanthin (5.0 mg/kg) or 5-FU (30 mg/kg) was administered i.p. on day 10. Control mice were given saline and vehicle in the same manner. Animals were sacrificed at the indicated time after the injection of drug, and microsomes were prepared from their livers. Each point represents the percentage of the value obtained from the pooled mixture of the livers from 4 mice compared with the control. * $P < 0.05$, compared with the control value. Symbols: (○, △) aniline hydroxylase, (●, ▲) aminopyrine demethylase, (○, ●) cepharanthin, (△, ▲) 5-FU + cepharanthin.

aminopyrine demethylase activities of mice treated with cepharanthin (a single injection of 5 mg/kg) were significantly inhibited compared to the saline-treated controls. The control values were as follows: aniline hydroxylase, 1.41 ± 0.092 ($\mu\text{M/hr/g}$ liver); aminopyrine demethylase, 2.98 ± 0.169 ($\mu\text{M/hr/g}$ liver). Aniline hydroxylase activity was reduced more than aminopyrine demethylase activity. One day after the treatment with cepharanthin plus 5-FU, the activities for aniline hydroxylase and aminopyrine demethylase were reduced to approximately 48–60% of the controls, and they slowly recovered with the elapse of time, but aniline hydroxylase activity in the liver of the mice treated with cepharanthin plus 5-FU remained inhibited even on the 5th day.

To examine whether cepharanthin has a modulating effect on the tissue 5-FU concentration, mice were administered i.p. with cepharanthin (5 mg/kg) or cepharanthin plus 5-FU (30 mg/kg). The concentration of 5-FU in biological samples were measured as 5-FU at 6 hr after the last treatment with the drug. 5-FU was present to a much greater extent in the liver, kidney, spleen, lung, and blood when it was administered in combination with cepharanthin (Table 4).

DISCUSSION

Tumor metastasis is established by a characteristic series of steps involving interaction with the extracellular matrix and basement membranes (24, 25). The inhibition and prevention of metastases are important problems in cancer therapy. It may be possible to inhibit metastasis, which is usually fatal in patients with malignant neoplasms. However, it is difficult to prevent overt metastasis or primary tumor growth. We used 3LL as an experimental model of spontaneous lung micrometastasis. In our model, the influence of the primary tumor on metastasis was excluded by removing the implanted tumor. Thus, this system seemed suitable for investigating the effects of host modulation on micrometastasis. We previously described that a blended Chinese medicine, Shōsaiko-Tō, inhibited the development of lung metastases in this model (8). Ono (26) has observed that oral administration of cepharanthin to tumor-bearing mice resulted in inhibition of 3LL primary tumor growth, preferential suppression of pulmonary metastases, and prolongation of survival time. By itself, cepharanthin was effective in preventing lung micrometastasis in syngeneic mice after the removal of 3LL implanted in the footpad (Table 1).

Table 4. Concentrations of 5-fluorouracil in various tissues from Lewis lung carcinoma-bearing mice after combination with cepharanthin

Tissues	Concentration ($\mu\text{g/ml}$ or g wet weight) of 5-FU in tissues of mice treated with	
	5-fluorouracil (5-FU)	5-FU + Cepharanthin
Blood	0.041 ± 0.007	$0.059 \pm 0.009^*$
Lung	0.58 ± 0.04	$0.91 \pm 0.14^*$
Liver	0.42 ± 0.12	$0.66 \pm 0.24^*$
Kidney	1.24 ± 0.34	$1.72 \pm 0.32^*$
Spleen	2.37 ± 0.98	$3.88 \pm 0.99^*$

Inocula of 10^5 3LL cells were implanted s.c. into one footpad of each mouse on day 0. The implanted tumor was surgically removed by amputating the tumor-bearing leg on day 10 after tumor implantation. Cepharanthin (5.0 mg/kg) or 5-FU (30 mg/kg) was administered i.p. on days 10, 12 and 14. The concentration in tissues were calculated as 5-FU at 6 hr after the last treatment with drug. Each value represents means $\pm$ S.E. of triplicate determinations from 5 mice in a group. * $P < 0.05$, compared with the 5-FU-treated group.

We found that cepharanthin plus 5-FU had additive inhibitory effects in an experimental lung metastasis model (Table 2). Several interpretations of this are possible: 1) The liver drug-metabolizing system was suppressed by treatment with cepharanthin (Fig. 1), resulting in the increase of 5-FU in the tissues. 2) The cytotoxicity of 5-FU was enhanced by the administration of cepharanthin. 3) The tumor cells sterilized by 5-FU might have released abundant antigens, which led to an enhanced immune response. Although the present results do not clarify what mechanism is involved in the combined effect, the experimental results indicate that cepharanthin can serve as an excellent adjuvant to anticancer drugs.

Shimura has been reported that cepharanthin enhanced phagocytosis in the peritoneal macrophages of mice and the third component of the complement system was activated by the i.p. injection of cepharanthin (17). Ito et al. (27) reported that adherent PEC, harvested from mice after i.p. injections of squalene-treated *Nocardia rubra* cell wall skeleton, showed cytolytic activity on tumor target cells in vitro. They also found that this activity was markedly inhibited by treatment with antimacrophage serum and complement or carrageenan. Moreover, Liotta et al. (28) reported that the i.v. transfer of BCG-activated peritoneal exudate cells inhibited lung metastasis in mice that had been implanted with T₂₄₁ fibrosarcomas. Furthermore, Sone et al. (29) demonstrated that after direct stimulation with *Nocardia rubra* cell wall skeleton, alveolar macrophages became tumoricidal against syngeneic, allogeneic and xenogeneic tumor cells in vitro. We have here demonstrated that i.p. injections of adherent PEC activated with cepharanthin inhibit the development of lung micrometastasis in mice. In addition, the antimetastatic effect was not observed when 3LL-bearing mice were injected i.p. with a blocker of immunocompetent cells, carrageenan (2 mg/mouse) on days 10, 12 and 14 after tumor implantation (H. Ito et al., unpublished data). Thus, the anti-

metastatic activity of cepharanthin on 3LL seems to be mediated by the activation of macrophages. In our tests for the toxicity of cepharanthin, the concentrations of cepharanthin used in the present study did not have any side effects such as diarrhea, decrease of body weight, abnormal behavioral changes or decreases of food consumption and water intake. These findings at least indicate that cepharanthin might be a useful agent in the clinic for the prevention of cancer metastasis.

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