Inhibitory Effects of Evodiamine on in Vitro Invasion and Experimental Lung Metastasis of Murine Colon Cancer Cells

Masaru OGASAWARA,* a, b Toshiyuki MATSUBARA,* and Hideyo SUZUKI a
Toyama Prefectural Institute for Pharmaceutical Research,* 17–1 Nakataikouyama, Kosugi-machi, Imizu-gun, Toyama 939–0363, Japan and Japan Science and Technology Corporation, Domestic Research Fellow,* b 4–1–8 Hon-machi, Kawaguchi, Saitama 332–0012, Japan. Received March 26, 2001; accepted April 23, 2001

We have previously reported that evodiamine had a marked inhibitory activity on tumor cell migration in vitro. To extend our study, the effects of evodiamine on invasion, growth, and metastatic development of colon 26-L5 cells were examined here. Evodiamine inhibited the invasion of tumor cells into Matrigel in a concentration-dependent manner, and achieved 70% inhibition at 10 μg/ml. Treatment of tumor cells with evodiamine for 24 h showed little effect on tumor growth at concentrations of less than 10 μg/ml, whereas an over 48-h treatment resulted in a concentration- and time-dependent inhibition. Pretreatment of tumor cells with 10 μg/ml evodiamine before inoculation into mice caused 70% reduction in their lung metastasis formation. When evodiamine at 10 mg/kg was administered into mice from the 6th day after tumor inoculation, the number of tumor nodules in lungs was decreased by 48% as compared to control. The inhibition rate was equivalent to that produced by cisplatin, a potent anti-cancer drug. Evodiamine did not affect the body weight of mice in the experimental period, whereas cisplatin caused serious weight loss. These results suggest that evodiamine may be regarded as a promising agent in tumor metastasis therapy.

Key words  evodiamine; colon cancer; metastasis; invasion; migration

Tumor metastasis is a major cause of death in cancer patients, and its blockade has been considered to enable cancer patients to survive.1 Thus, it is important to find out promising agents with anti-metastatic activity.

The cascade of cancer metastasis comprises a complex multistep process and tumor invasion into surrounding tissues plays an important role in tumor metastasis.2 Tumor invasion is subdivided into 3 steps, including 1) tumor cell attachment to extracellular matrix components, 2) local degradation of the matrix by tumor cell-associated proteases, and 3) tumor cell locomotion into the region of the matrix modified by proteolysis.3 Therefore, inhibition of any of these steps may result in suppression of tumor invasion and metastasis.

We have recently performed the screening of 75 natural compounds for inhibitory activity on tumor cell migration in vitro and found that evodiamine had a remarkable anti-migratory activity with an IC 50 value of 1.25 μg/ml.4 Evodiamine is one of the main constituents of Evodia Fructus5 and has been shown to possess anti-tumor growth,6 bronchoconstrictive,7 anti-nociceptive,8 vasorelaxant,9 catecholamine-secretory10 and anti-nitric oxide producing11 properties. In contrast, the effects of evodiamine on tumor cell invasion and metastasis remain unclear.

In the present study, we investigated the effects of evodiamine on invasion, growth, and metastatic development of colon 26-L5 cells.

MATERIALS AND METHODS

Materials  Evodiamine (Fig. 1) and cisplatin were purchased from Matsuura Yakugyo Co., Ltd. (Aichi, Japan) and Sigma Chemical Co. (St. Louis, MO, U.S.A.), respectively. Evodiamine and cisplatin were dissolved in dimethyl sulfoxide (DMSO) and phosphate-buffered saline, respectively. In each in vitro treatment, evodiamine was diluted with DMSO, and then the solution was added into cell-suspended medium to be the final concentration of 1% DMSO. No precipitation of evodiamine was observed under a microscope at the final concentrations of less than 10 μg/ml.

Cells and Cell Culture  Murine colon 26-L5 adenocarcinoma cell line, derived from colon 26, was kindly provided by Prof. I. Saiki (Toyama Med. Pharm. Univ., Inst. of Natural Medicine, Toyama, Japan). The cell line is highly liver metastatic compared with the colon 26 parental cell line12 and also shown to metastasize to lungs potently in the experimental metastasis model.13 The cell line was maintained in RPMI-1640 (Gibco, Grand Island, NY, U.S.A.) supplemented with 10% fetal bovine serum (FBS), 2-mercaptoethanol, 100 U/ml penicillin, and 0.1 mg/ml streptomycin.

Animals  Inbred 6-week old female Balb/c mice were purchased from Shizuoka Laboratory Animal Center, Hamamatsu, Japan. All mice were housed in a controlled environment with 12 h light/dark cycle, temperature of 24±2 °C and humidity of 55±10%, and they were given commercial food and tap water ad libitum. After an acclimatization period of 1 week, mice were used in the present study.

Cell Invasion Assay  Tumor cell invasion through reconstituted basement membrane (Matrigel) was assayed according to the methods as reported previously with some modifications.14 In Transwell cell culture chambers (Costar 3422, Cambridge, MA, U.S.A.), filters with 8-μm pore size (Nucleopore, Pleasanton, CA, U.S.A.) were coated with 60 μg of Matrigel (Collaborative Research Inc., Bedford, MA, U.S.A.) on the upper surface and 0.5 μg of fibronectin (Iwaki Glass Co. Ltd., Tokyo, Japan) on the lower surface.

Fig. 1. Chemical Structure of Evodiamine from Evodia rutaecarpa

* To whom correspondence should be addressed. e-mail: masaru.ogasawara@pref.toyama.jp © 2001 Pharmaceutical Society of Japan
Colon 26-L5 cells (2×10^5) suspended in RPMI-1640 containing 0.1% bovine serum albumin were pretreated with various concentrations of evodiamine for 30 min on ice, and then added to the upper compartment. Evodiamine was also added to the lower compartment. Control was vehicle given in the same way. After a 5-h incubation at 37°C, cells were fixed and stained with 0.5% crystal violet for 30 min. Cells that had invaded to the lower surfaces of filters were extracted with 30% acetic acid, and absorbance of cell lysate was measured at 590 nm.

**Cell Proliferation Assay** Colon 26-L5 cells (1×10^4, 5×10^3 or 2×10^3) suspended in RPMI-1640 containing 5% FBS were seeded onto wells of 96 multiwell plates (Falcon 3072, Becton Dickinson, NJ, U.S.A.) with vehicle or various concentrations of evodiamine and then incubated at 37°C for 24 h, 48 h or 72 h, respectively. The culture medium was exchanged to fresh 5% FBS-medium with vehicle or evodiamine after a 48-h incubation. Then, WST-1 solution (Wako pure Chemicals Ind. Ltd., Osaka, Japan) was added into each well and incubated for an additional 2 h. Absorbance of each well was measured at 450 nm.

**Experimental Lung Metastasis Assay** In the evodiamine-pretreatment assay, colon 26-L5 cells (4×10^5) were harvested and treated with 0.1 to 10 μg/ml evodiamine at a density of 4×10^3 cells/200 μl on ice for 30 min, and then the treated cells were injected intravenously into 6 mice per group with the compound. Control was vehicle given in the same way. In some experiments, 200 μl of 10 μg/ml evodiamine was injected through the tail vein of mice just after intravenous injection of tumor cells to clarify the effect of the co-injected evodiamine on host cells. Fourteen d after tumor inoculation, tumor nodules in lungs were counted under a dissection microscope. In the evodiamine-administration assay, colon 26-L5 cells (4×10^5) were inoculated intravenously into 5 mice per group, and then evodiamine or vehicle was administered intraperitoneally 6 consecutive times beginning 6 d after tumor inoculation. Cisplatin was injected intravenously into mice on the 6th day and the 9th day. The number of tumor nodules was determined on the 14th day as described above.

**Statistical Analysis** All data are expressed as mean±S.D. Statistical differences were evaluated by a one-way analysis of variance (ANOVA) followed by Dunnett’s post-hoc test, and p<0.05 was considered significant.

**RESULTS**

**Effect of Evodiamine on Matrigel Invasion by Tumor Cells** We have previously found that evodiamine had a marked inhibitory activity on migration of colon 26-L5 cells in vitro.4) Because the migratory ability of tumor cells is prerequisite for achievement in tumor cell invasion,3) we investigated the effect of evodiamine on invasiveness of colon 26-L5 cells by a Matrigel invasion assay. As shown in Fig. 2, evodiamine suppressed invasion of tumor cells into Matrigel in a concentration-dependent manner and achieved 70% inhibition at 10 μg/ml.

**Effect of Evodiamine on Tumor Cell Proliferation** We examined the effect of evodiamine on proliferation of colon 26-L5 cells in vitro. As shown in Fig. 3, a 24-h exposure of tumor cells to evodiamine exhibited little effect on tumor cell proliferation at concentrations of less than 10 μg/ml, whereas an over 48-h exposure caused a marked suppression in a concentration- and time-dependent manner at the same concentration range. Evodiamine at 100 μg/ml showed an obvious cytotoxicity even in the 24-h treatment.

**Effect of Evodiamine on Lung Metastasis Formation** As evodiamine showed a remarkable inhibitory activity against invasion of colon 26-L5 cells (Fig. 2), we examined the effect of evodiamine on the formation of lung metastasis by colon 26-L5 cells in mice (Fig. 4). Tumor cells were treated with indicated concentrations of evodiamine in vitro before inoculation into mice. The pretreated tumor cells showed the concentration-dependent decrease of their lung metastasis formation with a maximum reduction rate of 70% at 10 μg/ml. In contrast, when tumor cells and evodiamine were injected separately into mice without their pretreatment with the compound, the formation of lung metastasis was not suppressed (Fig. 4, hatched column). These results indicate that the anti-metastatic effect of evodiamine may be ascribed to its direct effect on tumor cells but not on host cells.

**Effect of Evodiamine on Expansion of Lung Micrometastasis** We further investigated the effect of evodiamine on the expansion of lung metastatic foci produced by colon 26-L5 cells in mice. Administration of evodiamine was performed starting 6 d after tumor inoculation. As shown in Fig. 5A, the administration of evodiamine produced 48 and...
DISCUSSION

We have demonstrated in this study that evodiamine could markedly inhibit *in vitro* invasion of colon 26-L5 cells independent of reducing cell viability and their *in vivo* lung metastasis formation and the expansion.

Tumor invasion has been shown to play an essential role in tumor metastasis formation. In fact, some studies have shown that anti-invasive compounds had inhibitory activity on tumor metastasis. These reports lead us to speculate that inhibition of tumor cell invasion by evodiamine may be responsible for the reduction in tumor metastasis formation. On the other hand, how evodiamine affects other metastatic properties of tumor cells, besides tumor invasion, may need to be considered in the experimental metastasis model used in this study, because the model includes various events in achievement of tumor metastasis formation such as platelet aggregation by tumor cells, tumor cell adhesion to endothelial cells and the following modulation of their biological activities, as well as tumor invasion. Therefore, clarifying the exact inhibitory mechanism(s) of evodiamine in tumor metastasis formation needs further studies.

Preventing tumor metastasis expansion as well as its formation is an important and practical approach to curative therapy of cancer patients. We have found here that administration of evodiamine into tumor-bearing mice could inhibit the metastatic expansion potently although the inhibition rate showed a plateau (about 50% inhibition) at 10 mg/kg. Notably, the inhibitory effect of evodiamine was equivalent to that of cisplatin (2 mg/kg). Moreover, evodiamine had little effect on the body weight of mice at its effective dose (10 mg/kg), whereas cisplatin produced a serious loss. Furthermore, when evodiamine at 30 mg/kg was injected into mice 5 consecutive times a week for 2 weeks, levels of GOT, GPT, CRE and cell density of leukocytes in blood of the mice on the 14th day were not affected as compared to normal mice, although a small amount of body weight loss was observed (data not shown). These observations suggest that evodiamine could prevent tumor expansion without inducing serious dysfunction in organs such as liver, kidney and bone marrow.

The anti-expansive effect of evodiamine on tumor micrometastases may be dependent on inhibition of not only tumor invasion into surrounding tissues but also its growth, because evodiamine exhibited a marked inhibitory effect on tumor growth *in vitro* in an over 48-h treatment. Additionally, as an alternative mechanism of evodiamine, angiogenic suppression could be speculated, because some agents effective in inhibiting tumor invasion have been revealed to possess anti-angiogenic activity based on inhibition of endothelial cell invasion. The effects of evodiamine on angiogenesis remains to be investigated.

Although various anti-invasive and -metastatic compounds have been shown, few compounds acting through reduction of tumor cell migration are reported. Moreover, most anti-cancer drugs currently used for chemotherapy are expected to have a marginal inhibitory effect on tumor cell migration. In these points, evodiamine seems to be notable because its tumor preventive action may be ascribed mainly to the regulation of tumor cell migration.

In conclusion, we have demonstrated that evodiamine inhibited tumor cell invasion by mediating suppression of its migration, resulting in the main cause of the tumor prevention in the formation and expansion, and that evodiamine may be regarded as a promising agent in tumor metastasis.
Acknowledgments  We are grateful to Prof. Ikuo Saiki (Toyama Medical Pharmaceutical University, Japan) for valuable suggestions and the critical evaluation of this manuscript. This work was supported by the grant from Japan Science and Technology Corporation.

REFERENCES