Inhibition by Evodiamine of Hepatocyte Growth Factor-Induced Invasion and Migration of Tumor Cells

Masaru OGASAWARA* and Hideyo SUZUKI

Toyama Prefectural Institute for Pharmaceutical Research; 17–1 Nakataikouyama, Kosugi-machi, Imizu-gun, Toyama 939–0363, Japan. Received September 4, 2003; accepted December 16, 2003

Tumor cell motility plays a crucial role in the establishment of tumor metastasis and is affected by a variety of host-derived factors during the event. Hepatocyte growth factor (HGF) is one of these factors and stimulates tumor cell migration remarkably. We previously reported that evodiamine has a marked inhibitory activity on tumor cell invasion and migration in vitro. In this study, the effects of evodiamine on HGF-induced invasion and migration of tumor cell lines, colon 26-L5 carcinoma, B16-F10 melanoma and Lewis lung carcinoma (LLC) were examined. HGF promoted invasive activity of tumor cell lines with maximal induction of 1.8 times at 30 ng/ml for colon 26-L5 and LLC cells, and 2.0 times at 10 ng/ml for B16-F10 cells. Evodiamine inhibited the HGF-stimulated tumor cell invasion and migration in a concentration-dependent manner, and achieved complete suppression at 30 μM in all of the cell lines tested. When tumor cells were seeded on fibronectin-coated plates with evodiamine, their spreading on the plate was obviously inhibited, while their adhesiveness to fibronectin was unaffected. Evodiamine showed a marginal effect on tumor cell growth in a 24-h incubation, although it exhibited a marked inhibition in an over 48-h incubation. These results suggest that evodiamine suppressed HGF-stimulated invasion and migration of tumor cells partly through inhibition of cell spreading.

Key words evodiamine; migration; hepatocyte growth factor; tumor; spreading

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

One key event in the formation of tumor metastasis is cell motility. A number of host-derived factors have been shown to modulate the motile activity of tumor cells. Hepatocyte growth factor (HGF) is one such factor and has been shown to stimulate the invasion and metastasis as well as migration in a variety of tumor cells.2–4) Clinical investigations also indicate the importance of HGF in tumor metastasis by the findings that a significantly elevated serum level of HGF is observed in the patients with lung,5) prostate,6) gastric,7) and breast cancer.8) From these observations, regulation of HGF activity is recognized as a possible step in tumor metastasis therapy, however, little has been reported about compounds having anti-HGF activity.

We recently reported that evodiamine has remarkable inhibitory activity on in vitro invasion and migration of tumor cells.9)10) Evodiamine is one of the main constituents of Evodiae Fructus11) and has been shown to possess anti-tumor growth,12,13) anti-gastric emptying and gastrointestinal tani- sit,14) anti-aldosterone releasing,15) anti-obesity,16) bronchoconstrictive,17) anti-nociceptive,18,19) catecholamine-secrecyry,20) vasorelaxant,21) and anti-nitric oxide producing22) properties. With regard to the growth inhibition by evodiamine, Fei et al. have clearly demonstrated the molecular mechanisms by which evodiamine increases the expression of the apoptosis inducer Bax and decreases the apoptosis suppressor Bcl-2 in mitochondria, triggering the activation of caspase-3 and DNA fragmentation.15) Moreover, the existence of unknown apoptotic pathways is speculated in the evodiamine-induced apoptosis. In contrast, no reports has been seen on the effects of evodiamine on HGF activity.

In the present study, we investigated the effects of evodiamine on HGF-induced invasion and migration of tumor cell lines, colon 26-L5 carcinoma, B16-F10 melanoma, and Lewis lung carcinoma.

MATERIALS AND METHODS

Materials Evodiamine and hepatocyte growth factor (HGF) were purchased from Matsuura Yakugyo Co., Ltd. (Aichi, Japan) and Funakoshi Co., Ltd. (Tokyo, Japan), respectively. Evodiamine and HGF were dissolved in dimethyl sulfoxide (DMSO) and distilled water, respectively. In each in vitro treatment, evodiamine was diluted with DMSO, and then the solution was added into cell-suspended medium to make a final concentration of 1% DMSO.

Cells and Cell Culture Murine colon 26-L5 adenocarcinoma was kindly provided by Prof. I. Saiki (Toyama Med. Pharm. Univ., Inst. of Natural Medicine, Toyama, Japan). Murine B16-F10 melanoma was kindly provided by Dr. S. Wakuzawa (Hokuriku Univ., Kanazawa, Japan). Lewis lung carcinoma (LLC) was purchased from the RIKEN Cell Bank. Colon 26-L5 cells and B16-F10 cells were maintained in RPMI-1640 supplemented with 10% fetal calf serum (FCS), 2-mercaptoethanol, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. LLC was maintained in DMEM with 10% FCS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin.

Cell Invasion and Migration Assay Tumor cell invasion through a reconstituted basement membrane (Matrigel) was assayed according to the methods reported previously.23) Briefly, in transwell cell culture chambers, filters of 8 μm pore size were coated with Matrigel on the upper surface and 0.5 μg of fibronectin on the lower surface. Volumes of Matrigel were 50 μg for colon 26-L5 cells, and 30 μg for LLC and B16-F10 cells. Tumor cells (2×10⁶) suspended in medium containing 0.1% bovine serum albumin (BSA) 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. The control was the vehicle given in the same way. HGF was applied to the lower compartment as a chemoattractant. After a

* To whom correspondence should be addressed. e-mail: masaru.ogasawara@pref.toyama.lg.jp © 2004 Pharmaceutical Society of Japan
6-h incubation at 37°C, cells were fixed and stained with crystal violet. In a cell migration assay, tumor cells were pre-treated with evodiamine as described above, and then incubated for 3 to 5 h in the presence of HGF in the chambers with filters coated with fibronectin on the lower surface. Cells that had invaded or migrated to the lower surfaces of filters were extracted, and absorbance of the cell lysate was measured at 590 nm.

**Cell Adhesion and Spreading Assay** Cell attachment to fibronectin substratum was assayed using a method reported previously.24) Wells of a 96-well plate were coated with fibronectin (0.5 μg/well) and incubated for 24 h. The plates were then washed and remaining binding sites were blocked with 1% BSA for 30 min. Tumor cells (4×10^4) suspended in serum-free medium were pretreated with various concentrations of evodiamine or vehicle for 30 min on ice, and then seeded into the wells with or without HGF. After incubation at 37°C, unbound cells were removed by aspiration and cells remaining in the wells were stained with crystal violet. Stained cells were lysed and absorbance of the cell lysate was measured at 590 nm. In a cell spreading assay, evodiamine (30 μM) or vehicle-treated tumor cells were incubated for 1 h with 30 ng/ml HGF in the fibronectin-coated microwell plate prepared as described above. Then, tumor cells were fixed, stained with Giemsa’s solution, and observed under a microscope. In some experiments, evodiamine and vehicle were applied to tumor cells adequately spreading on the plate after a 1-h incubation with HGF, and then the cells were incubated for 0.5 h. Morphological appearance of tumor cells was estimated as described above.

**Cell Proliferation Assay** Colon 26-L5 and B16-F10 cells (1×10^4, 5×10^3 or 2×10^3) suspended in RPMI-1640 containing 5% FCS and LLC cells (2×10^4, 1×10^4 or 4×10^3) suspended in DMEM containing 5% FCS were seeded onto wells of 96-well plates with vehicle or various concentrations of evodiamine, and then incubated at 37°C for 24 h, 48 h or 72 h, respectively. Subsequently, WST-1 solution (Wako Pure Chemicals Ind., Ltd., Osaka, Japan) was added to each well and incubated for an additional 3 to 4 h. Absorbance of each well was measured at 450 nm.

**Statistical Analysis** All data are expressed as mean±S.D. Statistical differences were evaluated by two-tailed t-test, and *p*<0.05 was considered significant.

**RESULTS AND DISCUSSION**

Tumor metastasis is composed of a sequential step that involves dissociation of tumor cells from the primary site, translocation to distant organs through vessels, invasion into surrounding tissues of those organs, and growth at the metastatic site.25) During these processes, cell motility is recognized as a prerequisite property for tumor cells and is revealed to be affected by a variety of growth factors including hepatocyte growth factor (HGF).2—4)

We have examined here whether tumor cell invasion and migration induced by HGF is regulated by evodiamine. To basically determine the concentration of HGF for maximal induction of tumor invasion, we investigated the effects of HGF on invasion of colon 26-L5, B16-F10, and Lewis lung carcinoma (LLC) cells at increasing concentrations. As shown in Fig. 1A, HGF induced invasion of tumor cell lines in a concentration-dependent manner. The induction ratio reached 1.8 fold at 30 ng/ml HGF for colon 26-L5 cells and LLC cells, and 2.0 times at 10 ng/ml for B16-F10 cells. Next,
we examined the effects of evodiamine on tumor invasion and migration in the presence of an effective dose of HGF (Figs. 1B–D, Fig. 2). Evodiamine reduced HGF-stimulated invasiveness of colon 26-L5, B16-F10 and LLC cells in a concentration-dependent manner and achieved 100% suppression of HGF activity at 30 μM in any of these cell lines. Enhancement by HGF of the cell migration was also inhibited completely by evodiamine at 30 μM (Fig. 2). In the transwell chamber assay used in this study, cell attachment to fibronectin substratum is the first action in cell migration. To elucidate the mechanism of anti-HGF activity of evodiamine, we examined the effect of evodiamine on tumor cell adhesion to fibronectin in the presence or absence of HGF (Fig. 3). Tumor cells were adhered to the fibronectin-coated plate in a time-dependent manner during a 30-min incubation and ratios of adhered cells at 5-min incubation to those at 30-min were determined as 1.6 on colon 26-L5, 2.1 on B16-F10 and 1.2 on LLC cells; and HGF had no effect on the adhesiveness of tumor cells during the 30-min incubation (data not shown). As shown in Fig. 3, evodiamine did not affect adhesiveness of tumor cells with or without HGF at 20-min incubation. This result indicates that the anti-HGF activity of evodiamine is not due to reduction of cell adhesiveness. Since post action of the cell attachment to fibronectin in the cell migration is cell spreading, we examined whether evodiamine influences cell spreading on the fibronectin-coated plate without affecting cell viability (Fig. 4). When tumor cells were plated and incubated for 60 min, the microscopic investigation revealed that colon 26-L5 cells were spindled and B16-F10 cells were extended with plasma membrane like lamellipodium protrusions at the cell periphery (Figs. 4A, D); on the other hand, tumor cells incubated with evodiamine remained round (Figs. 4B, E). When spreading cells of colon 26-L5 shown in Fig. 4A were treated with evodiamine for 30 min, the microscopic appearance became retracted (Fig. 4C), whereas the extending B16-F10 cells shown in Fig. 4D were morphologically unchanged in the same treatment (Fig. 4F). These results suggest that the mechanism of the anti-HGF effects of evodiamine may be partly the repression of cell spreading, not the cytotoxicity. To further explore this, the effects of evodiamine on cell growth were examined (Fig. 5). A 24-h exposure with evodiamine showed a marginal inhibition in colon 26-L5 and LLC cells, whereas exposure over 48-h caused a marked inhibition in time- and concentration-dependent manner. B16-F10 cells showed a time-dependent but a bell-shaped concentration–responsive curve under 72-h exposure. In any tumor cells tested, the inhibition rates under 30 μM of evodiamine were less than 30% in a 24-h incubation. Considering the evodiamine-treatment period of 1 h in a cell spreading assay, these results are thought to...
support the above speculation that the attenuation by evodiamine of the cellular morphological changes is not caused by the decrease of cell viability. Fei et al. have recently demonstrated that evodiamine induces around 60% cell growth inhibition in a 24-h incubation at 30 μM using HeLa cells and the concentration-dependent inhibition of cell growth of A375-S2 melanoma. These differences in the efficacy of evodiamine between the report by Fei et al. and the present study led us to speculate that the growth-inhibitory effects of evodiamine may depend on the kind of tumor cells involved. LLC cells exhibited too little morphological change to evaluate microscopically (data not shown).

A few studies have previously demonstrated other inhibitors for HGF activity. NK4, a four-kringle fragment of HGF, shows an antagonistic effect on HGF binding to the receptor, resulting in inhibition of HGF-induced tumor cell migration. IL-12 and γ-linolenic acid inhibit HGF-induced tumor migration via up-regulation of cell-surface E-cadherin. IL-4 is also shown to inhibit HGF-induced tumor migration but its mechanism is unknown. In contrast, evodiamine suppressed HGF-induced tumor migration by acting to repress the cell spreading. These results suggest that evodiamine may have a novel inhibitory mechanism against HGF activity.

Cellular shape is maintained by the cytoskeletal organization and the reorganization of the cytoskeleton produces the driving force for cell migration. We observed in this study that cellular morphological change is attenuated by pretreatment with evodiamine before seeding on the culture plate in colon 26-L5 and B16-F10 cells, whereas it is attenuated only in colon 26-L5 cells when treated with evodiamine after cell spreading. These observations indicate that the target molecules of evodiamine play a similar role in the cell spreading but a different role in the post-spreading state between colon 26-L5 and B16-F10 cells. Ballestrem et al. have recently demonstrated that a migrating cell exhibits the formation of lamellipodia via actin polymerization at the leading edges and the retraction of the rear by microtubular reorganization. Further investigations of evodiamine on this cytoskeletal regulation seem to clarify its mechanism of action.

In this study, we have demonstrated that evodiamine remarkably inhibits HGF-induced invasion and migration of tumor cells. Although the underlying mechanism of the effects of evodiamine remains unclear, inhibition of cell spreading may be involved.

REFERENCES