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Antimetastatic Effects of Capsaicin in Murine B16 Melanoma Cell Lines

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ABSTRACT **: The present work was undertaken to evaluate the antimetastatic potential of capsaicin (8-methyl-N-vanillyl-6-nonenamide) by measuring its effects on matrix metalloproteinase activity, cell invasion and lung metastasis. Significant inhibition of matrix metalloproteinase-2 activity by capsaicin (100 μM) was detected by gelatin zymography. *In vitro* invasion assay showed capsaicin (50, 100 μM) reduced tumor cell invasion (28-40%). Capsaicin (i.p., 2.5 mg/kg) inhibited development of lung colonization (58%). These results suggest that capsaicin prevents metastasis in part through suppression of invasion of B16F10 melanoma cells by inhibiting matrix metalloproteinase-2 responsible for degradation of extracellular matrix.**

Key words : **capsaicin, zymography, matrix metalloproteinase, antimetastatic effect**

Introduction

Tumor metastasis is the leading cause of death in cancer patient. The process of tumor metastasis comprises of multiple sequential highly selective steps. Invasion of basement membrane is a crucial step in the complex process leading to metastasis (Hart and Fidler, 1980; Srivastava *et al.*, 1986; Nicolson, 1988; Mundy and Yoneda, 1995). Considerable attempt has been made to develop new devices and drugs to inhibit invasion and metastasis without side effects. Capsaicin (8-methyl N-vanillyl-6-noneamide), the pungent ingredient found in red pepper, has long been used as spices, food additives and drugs (Iwai *et al.*, 1979; Suzuki and Iwai, 1984; Cordell and Araujo, 1993). Recently, a series of studies demonstrated that capsaicin inhibits mutagenicity and DNA binding of some chemical carcinogens, possibly by suppressing their metabolic activation (Jang *et al.*, 1989; Teel, 1991, 1993; Miller *et al.*, 1993; Zang *et al.*, 1993).

Morere *et al.* reported that capsaicin preferentially suppressed the growth of some transformed cells of human origin, including HeLa, ovarian carcinoma, mammary adenocarcinoma, and human promyelocytic leukemia (HL-60) cells in culture (Morre *et al.*, 1993; Morre *et al.*, 1995). They also observed that direct injection of capsaicin into the B16 mouse melanoma transplanted in C57BL/6 mice significantly suppressed the growth of tumors. Moreover, a few studies have discerned that the topical application of capsaicin did not induce the epidermal ornithine decarboxylase activity, suggesting that it lacks tumor promotional activity (Park and Surh, 197; Park *et al.*, 1998). Surh also reported that capsaicin inhibited the papilloma formation when given prior to each topical dose of phorbol ester. In the present study, we determined the ability of capsaicin to inhibit the formation of lung colonization in a mouse (C57BL/6) model system, and also its effect on matrix metalloproteinase (MMP) activity associated with metastasis.

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Materials and Methods

Materials

Capsaicin was purchased from Sigma Chemical Co. (St. Louis, MO). Capsaicin was resuspended in dimethyl sulfoxide (DMSO). Eagles minimal essential medium (MEM), fetal bovine serum, penicillin and streptomycin were purchased from Gibco BRL (Grand Island, NY, USA).

Animals

5-weeks-old male C57BL/6N mice were obtained from Laboratory of Animal Resources of National Institute of Toxicological Research (Seoul, Korea).

Cell line and culture conditions

The murine B16-F10 melanoma cell lines were obtained from Dr. I. J. Fidler (The university of Texas, M.D. Anderson Cancer Center) and were cultured in MEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO_2.

Growth inhibition

The B16-F10 cells were grown to 80% confluence and treated with capsaicin (0, 10, 25, 50, and 100 μM) for 72 hr. The viable cells were determined by (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. The amount of the dye extracted was quantified at 570 nm.

Gelatin zymography

The activities of MMPs in the conditioned media were assayed by the zymography as described previously (Li *et al.*, 1999). The amount of gelatinase (MMP-2 and MMP-9) in the conditioned media was quantified by cell number. The conditioned media of 10^6 cells were analyzed by gelatin-based zymography. After electrophoresis, the gel was rinsed with 2.5% Triton X-100 and incubated for 18 hr at 37°C in incubation buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM $CaCl_2$, 1 μM $ZnCl_2$, and 0.02% NaN_3] with or without capsaicin (100 μM and 200 μM). Zymograph gels were scanned with Kodak DC-120 Digital image scanner (Kodark Distal Science, New York) linked to a computer and quantified.

Invasion assay

B16-F10 melanoma cell invasion was assayed using a 24-well Boyden chamber (Costar, Cambridge, MA) with polycarbonate filters of 8 μm pore size. These filters were precoated on lower side with 0.05 mg/ml cold Matrigel (Collaborative Research, Laxington, KY). A total of 5×10^4 cells in 200 μl MEM containing 50 μM or 100 μM capsaicin were added to the upper chambers in this invasion assay. Chambers were incubated for 6hr in a humidified atmosphere of 5% CO_2 at 37°C. Invasion was determined by counting the cells that migrated to the lower side of the filter with a light microscope ×400. Ten fields were counted for each assay. The percentage of invading cells was calculated by the formula: %invasion = (mean number of cells migrating in test/ mean number of cells migrating in control) × 100.

In vivo analysis of antimetastatic effect of capsacin

To determine the inhibitory effects of capsaicin on metastatic ability using highly metastatic B16-F10 cells *in vivo*, 1×10^6 viable cells/0.2 ml of Hank's balanced salt solution were injected into tail vein of male C57BL/6 mice at 6-7 weeks of age. The animals in the experimental groups were given a solution of capsaicin of three different doses (0.5, 1.25, and 2.5 mg/kg/day). Control animals received buffer instead of capsaicin. The administration of capsaicin solution was started 7 days before the injection of B16-F10 cells and continued for 3 weeks. Mice were killed 4 weeks after inoculation and size of their lungs were measured.

Statistical analysis

The mean of the assays was determined and compared to untreated controls. The values were analyzed by a two-tailed unpaired *t* test between the drug-treated group and the untreated control group. *p* values of $p < 0.05$ were considered to be significant.

Results

We initially examined whether capsaicin (1~100 μM) could affect the growth of the B16-F10 melanoma cell lines. Cells were grown in the presence of various concentration of capsaicin for 72 hr. Capsaicin at concentration of 50 μM or higher significantly inhibited the cell growth (Fig. 1).

Since the number of metastasized lung colonies is dependent on tumor invasiveness, adhesion on the endothelial cells, attachment to the extracellular matrix, and cell growth, the effects of capsaicin on the invasive potential of B16-F10 cells were tested *in vitro* using a Boyden chamber coated with Matrigel. As shown in Fig. 2, capsaicin inhibited the ability of B16-F10 cells to reach the bottom of the transwell through the Matrigel. Compared with the untreated control, capsaicin inhibited the ability of invasion of B16-F10 cells passed through the Matrigel coated filters in dose-dependent.

We also studied the inhibitory effects of capsaicin on MMPs activity using a gelatin zymography. After electrophoresis on the 10% polyacrylamide gel containing gelatin, the gels were incubated in the incubation buffer with 100-200 μM capsaicin or without for 18 hr at 37°C. Gelatin zymography revealed two biologically important bands corresponding to estimated 92-KD

and 72-KD as determined from standard molecular (Fig. 3A). Bands of lower M.W. (85-KD and 66-KD) than two major bands were also present. The level of MMP-9 band was a little changed in the incubation with capsaicin (100-200 μM), while the two forms of MMP2 were markedly decreased in the incubation of 100 or 200 μM capsaicin (Fig. 3B). Furthermore, to determine the effect of capsaicin on the secretion of MMPs by the cells, we treated three different concentrations (50 and 100 μM) of capsaicin in the

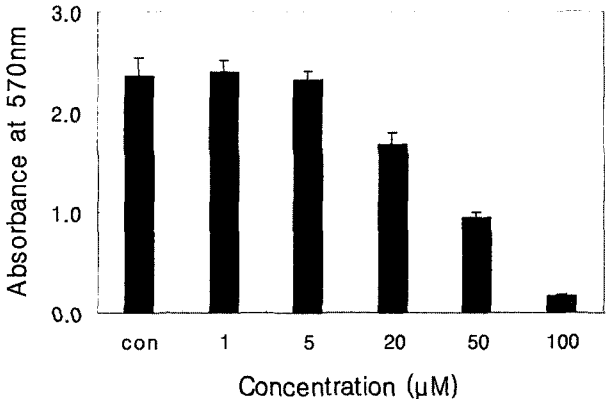

Fig. 1. Effects of capsaicin on the B16-F10 melanoma cell growth following various concentration treatment for 72 h. The number of cells were determined and the results presented are means ± SE of triplicates.

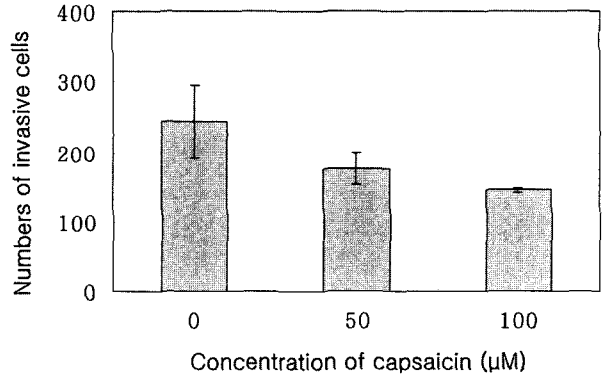

Fig. 2. Effects of capsaicin on the invasion of B16-F10 cells. B16-F10 cells (5×10^4 cells/200 μl MEM) with or without capsaicin were seeded onto matrigel-coated filters on the upper compartment of chamber. Cells were incubated for 6 hr at 37°C. Filters were removed, fixed, stained and the cells that had migrated in the test and the control were counted. All experiments were performed in duplicate. The percentage of invading cells was calculated by the formula: %invasion = (mean number of cells migrating in test/mean number of cells migrating in control) × 100.

B16-F10 cells for 24 hr. Gelatin zymograpy of conditioned media revealed two pro-gelatinases and two active forms but there are no difference between control and capsaicin treated cells (Fig. 3C).

The inhibitory effects of capsaicin on *in vivo* metastastic potential of B16-F10 cells were shown in Fig. 4. Injection of 1×10^6 B16-F10 cells into the tail vein of C57BL/6 mice in the absence of capsaicin (control) resulted in development of many colonies to the lung. Significantly decreased colonies and lung weights were observed in the capsaicin-administrated groups compared with the untreated control (Fig. 4A). Metastasis to the lung was inhibited when the animals were administered with 2.5 mg/kg/day capsaicin (Fig. 4B).

There was no significant difference in body weights among the groups (data not shown), indicating that capsaicin did not cause any severe toxicity.

Discussion

The B16 series of murine melanoma cell lines is a well-characterized model system for the analysis of tumor progression (Fidler, 1975; Hart and Fidler, 1980). We used highly metastatic B16-F10 cell line for this study. *In vitro* invasiveness assay revealed that capsaicin inhibited 28-40% of invasion of this cell line at 50 μM and 100 μM concentration. During the invasion assay, these concentrations were not cytotoxic in the cells. To escape from the primary tumors, cancer cells have to interact with the basement membrane, then induce its lysis and finally migrate through the membrane to invade adjacent tissues. Matrix metalloproteinases (MMPs) are involved in the invasion and metastasis of human cancers by mediating the degradation of extracellular matrix components. Much research attention has been focused on the role of MMPs in tumor invasion and metastasis (Woodhouse *et al.*, 1997; Ellrbroek and Stack, 1999). In an attempt to evaluate the antimetastatic potential of capsaicin, we investigated that the activities of MMPs in B16-F10 were measured by gel zymography. Capsaicin has shown to inhibit the MMP-2 activity (Fig. 3A and B).

Interestingly, capsaicin didn't play an important role in excretion of MMPs while significantly decreased the MMP-2 activity (Fig. 3C). These results indicating capsaicin directly inhibit MMP-2 activity. To understand capsaicin binds the zinc atom on the active site of MMP-2 such as synthetic inhibitor of MMP like Batimastat,

more studies are required. The biological relevance of data obtained from *in vitro* assays should always be confirmed using more physiological *in vivo* assays such as spontaneous or experimental metastatic assay (Price *et al.*, 1990; Garcia *et al.*, 1992). Thus we explored possible effects of capsaicin on experimental metastases in a murine model. *In vivo* study, we report that capsaicin (2.5 mg/kg/day) injected i.p., reduced lung colonization in mice (58%) (Fig. 4). The results presented in this study indicate that capsaicin inhibit the invasion of B16F10 melanoma cells by inhibition of MMP-2, thereby inhibiting lung metastasis.

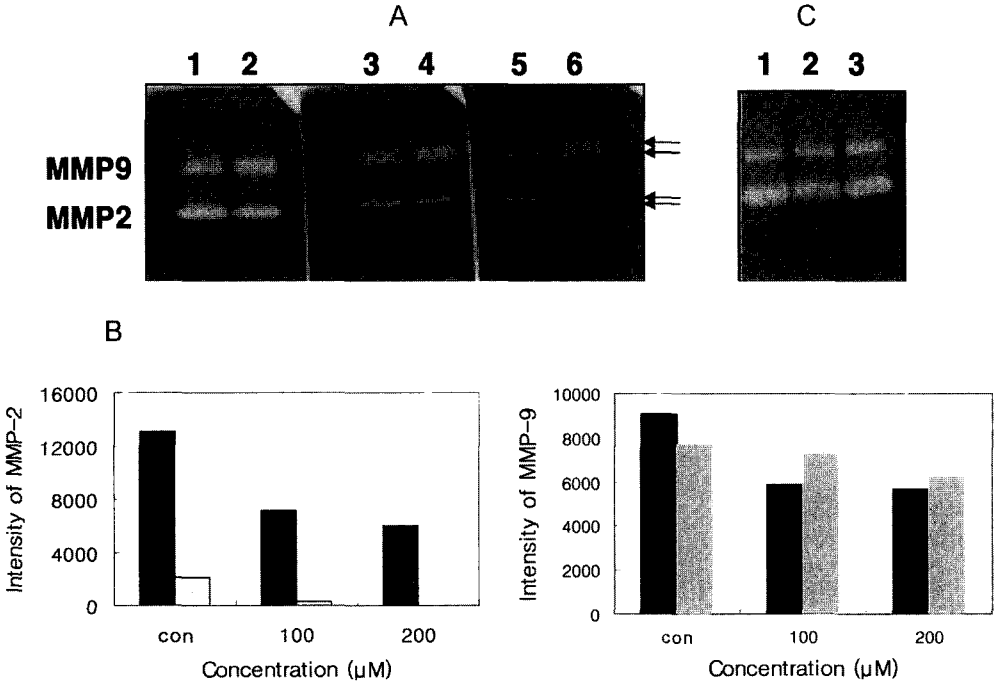

Fig. 3. Zymographic and densitometric analysis of MMPs in B16-F10 cells treated with capsaicin. A shows inhibitory effect of capsaicin on the activity of MMPs (Lane 1 and 2 are untreated control; Lane 3 and 4 are treated with 100 μM capsaicin; Lane 5 and 6 are treated with 200 μM capsaicin). B is densitometric analysis of MMPs in B16-F10 cells. C shows effect of capsaicin on the secretion of MMPs of B16-F10 cells after treatment of capsaicin (Lane 1 is untreated control; Lane 2 and 3 are treated with 50 or 100 μM capsaicin, respectively).

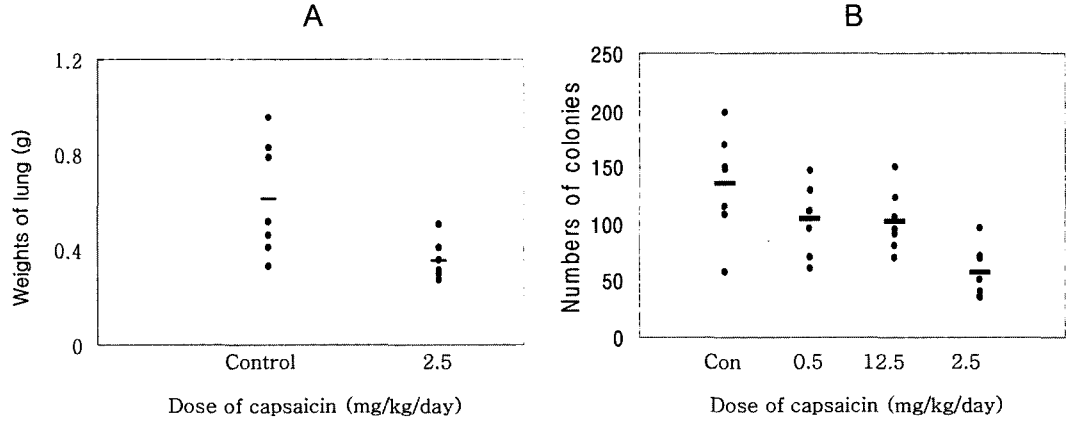

Fig. 4. Antimetastatic effect of capsaicin in the experimental metastasis study. The administration of capsaicin solution was started 7 days before the injection of B16-F10 cells and continued for 3 weeks. Mice were killed 4 weeks after inoculation. Colonies and the size of their lungs were measured. Bar represent average of data. ($p < 0.05$).

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