Abstract. Capsaicin is known to have tumor suppressive effects. However, the molecular mechanisms and targets of capsaicin involved in exerting anticancer activity are complex and remain to be clarified. The aim of the current study was to investigate the effects of capsaicin on human gastric cancer cells (AGS cells) and demonstrate that capsaicin induced apoptosis in AGS cells. Results of the MTT assay and flow cytometry revealed that capsaicin potentially inhibited the proliferation of AGS cells and induced apoptosis in a dose-dependent manner. Cleaved caspase-3 was increased and Bcl-2 was reduced by treatment with capsaicin in AGS cells. Capsaicin treatment decreased the expression of phosphorylated ERK 1/2, p38 MAPK or JNK in AGS cells. The results of this study suggest that capsaicin may serve as an anti-tumorigenic agent in human gastric cancer.

Introduction
Capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide), a vaniloid receptor agonist, is the principal pungent constituent of the plant genus Capsicum that is present in chilli peppers (1). The relationship of capsaicin to genotoxicity, carcinogenicity, and prevention and treatment of cancer has been widely explored (2). This alkaloid compound has been found to inhibit cancer growth and progression in vitro and in vivo and to induce apoptosis in various types of cancer cells (3-8). However, several investigations of the carcinogenic potential of capsaicin in animal models have also been reported (2,8,9). The molecular mechanisms underlying capsaicin-induced apoptosis are cell-type dependent. Despite the cumulative evidence for the tumor suppressive effects of capsaicin, few studies have been undertaken concerning the effects of capsaicin on cell signaling and molecular pathways leading to apoptosis in gastric cancer (10-12).

In the present study, we investigated the effects of capsaicin on human gastric cancer cells and demonstrated that capsaicin induced apoptosis and autophagy in human gastric cancer cells.

Materials and methods

Cell cultures and culture condition. Human gastric carcinoma AGS cells were purchased from the American Type Culture collection (Manassas, VA, USA). The cells were cultured at 37°C in a 5% CO₂ atmosphere with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Capsaicin was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and prepared as an ethanol stock solution, which was then diluted in intracellular solution prior to use.

Measurement of cell proliferation. Cell viability was determined using the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide assay. Briefly, 5x10⁵ cells/well were seeded in 96-well plates and allowed to adhere overnight. The concentrations of capsaicin used in this experiment were 0, 10, 20, 30, 60, 80, 100, 200 and 300 µM. Each group contained three wells. After the samples were treated with capsaicin for 12 h, 10 µl of MTT (0.5 mg/ml; Sigma) was added to each well, and the cells were incubated at 37°C for 4 h. The reaction was then stopped by lysing the cells with 200 µl of dimethyl sulfoxide (DMSO) for 15 min. Quantification measurements (optical density) were obtained at a wavelength of 450 nm using spectrophotometric analysis.

Effect of capsaicin on apoptosis of AGS cells as determined by flow cytometry. To evaluate the impact of capsaicin on apoptosis, FACS analysis was performed. Cell apoptosis was assessed using the Annexin V assay (Annexin V-FITC apoptosis detection kit; BD Pharmingen, San Jose, CA, USA). AGS cells (1x10⁵ cells) were treated with various concentrations (0-300 µM) of capsaicin for 12 h, respectively. Annexin V-FITC and propidium iodide (PI) labeling was then performed according to the manufacturer's instructions. The cells were double-stained with Annexin V-fluorescein isothiocyanate

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(FITC) and PI, followed by flow cytometry. To quantify early apoptotic cells, flow cytometry was used to assess binding of fluorescence-labeled Annexin V to externalized phosphatidylserine. PI uptake was measured to assess cells in the late stages of apoptosis or cells that sustained direct plasma membrane damage.

Western blotting. Total protein was lysed with RIFA buffer (1 M Tris-HCl, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA) with 1 mM phenylmethanesulfonyl fluoride (PMSF) and Halt™ protease inhibitor cocktail (Thermo Scientific, Rockford, IL, USA). Protein concentrations of the cell lysate were quantified using BCA™ protein assay (Thermo Scientific) with bovine serum albumin (BSA) as a standard. Total protein (10-20 µg) was subjected to electrophoresis on 10% SDS-polyacrylamide gels and then transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). The membrane was incubated for 1 h in blocking solution (5% BSA in TBS-Tween 20 buffer) and sequentially blotted with primary antibodies at 4°C overnight. Antibodies against c-Jun N-terminal kinase (JNK), phospho-JNK, extracellular signal-regulated kinase 1/2 (ERK 1/2), phospho-ERK 1/2 (1:1,000 dilution; Cell Signaling Technology, Inc., Danvers, MA, USA), p38 mitogen-activated protein kinase (MAPK), phospho-p38 MAPK, caspase 3, cleaved caspase 3, Bcl-2, and β-actin were purchased from Cell Signaling Technology (Beverly, MA, USA). After rinsing in TST, the membrane was incubated with horseradish peroxidase (HRP)-labeled anti-rabbit or anti-mouse immunoglobulins as the secondary antibody (1:5,000 dilution, Santa Cruz Biotechnology, Inc., Santa Cruz, USA) at room temperature for 1 h. Enhanced chemiluminescence was used to visualize the bands by LAS 3000 (Fuji Film, Tokyo, Japan).

Statistical analysis. The Student’s t-test was used to determine the significance of differences between treatments and respective controls. Values are expressed as means ± SE from at least three independent experiments. P<0.05 was considered to indicate statistical significance. Statistical analysis was assessed using Statistical Package for the Social Sciences (SPSS/PC 20.0, Chicago, IL, USA).

Results

Capsaicin suppresses proliferation in human gastric cancer cells. An MTT assay was used to assess the potential effects of capsaicin on the proliferation of human gastric cancer cells that had been exposed to capsaicin for 12 h at different concentrations. After treatment with capsaicin at concentrations ranging from 10 to 300 µM for 12 h, the cell proliferation was significantly lower than that of the untreated corresponding control groups (P<0.001). Capsaicin decreased the proliferation of the gastric cancer cell line in vitro in a dose-dependent manner (Fig. 1).

Capsaicin induces apoptosis in human gastric cancer cells. Regulation of apoptosis is crucial for the preservation of homeostasis and morphogenesis of human tissue (3). Disturbance of these processes by aberrantly extending cell viability or favoring accumulation of transforming mutation...
is thought to contribute to carcinogenesis (3,4). To evaluate the impact of capsaicin on apoptosis, we performed FACS analysis. Fig. 2 shows flow cytometric plots obtained with Annexin V-FITC/PI assay after a 12-h exposure to different concentrations (0-300 µM). The number of early and late apoptotic cells or directly damaged cells increased subsequent to exposure of the cells to capsaicin (P<0.001, Fig. 3).

Capsaicin-induced apoptosis is associated with the modulation of apoptotic regulatory proteins in human gastric cancer cells. An increase in cleaved caspase-3 expression was detected in the AGS cells following treatment with capsaicin (100 µM) for 12 h (mean ± SE, *P<0.005 and **P<0.05). Protein expression of Bcl-2 was reduced in the AGS cells after treatment with capsaicin (100 µM) for 12 h (P<0.001, Fig. 3).

Discussion

Although previous studies (3-8) have focused on the tumor suppressive effects of capsaicin in human cancer cells, few studies have been undertaken to elucidate capsaicin-induced apoptosis in human gastric cancer cells. Previous results of an XTT assay, DNA fragmentation and flow cytometric analysis revealed that capsaicin (3.5-10 µmol/l) induced apoptotic cell death in an in vitro Korean stomach tumor cell (SNU1) (13). This induction of cell death may be due to a Bcl-2 sensitive apoptotic pathway in a human gastric adenocarcinoma cell line (AGA cells) (12). In this study, cell proliferation significantly decreased in a dose-dependent manner following incubation

**Figure 4.** The effects of capsaicin on apoptotic-related protein expression. An increase in cleaved caspase-3 expression was detected in the AGS cells following treatment with capsaicin (100 µM) for 12 h. Protein expression of Bcl-2 was reduced in the AGS cells after treatment with capsaicin (100 µM) for 12 h (mean ± SE, *P<0.005 and **P<0.05).

**Figure 5.** Capsaicin treatment decreased the expression of phosphorylated ERK 1/2, JNK and p38 MAPK. Cells were incubated with or without capsaicin (100 µM) for 12 h (mean ± SE, *P<0.005 and **P<0.05). The phosphorylation levels of total ERK 1/2, JNK and p38 MAPK were not altered following capsaicin treatment.
of AGS cells with capsaicin for 12 h. The results have also demonstrated that the proportion of early and late apoptotic cells induced by capsaicin increased in a dose-dependent manner. The anticancer effects of capsaicin are both concentration- and time-dependent in vitro (7). Previous findings have shown the effects of capsaicin to be in the low micromolar range and to become maximal at 200-300 µM (2). Our results have shown that a considerable antiproliferative or apoptotic effect of capsaicin occurred at 60-300 µM.

The apoptotic response was evidenced by the activation of caspase-3 generation and the expression of anti-apoptotic markers. The activity of caspase-3 was found to increase with the exposure to capsaicin. In addition, western blotting revealed a reduction of the Bcl-2 gene. Recently, the Bcl-2 gene appears to be a critical regulator of programmed cell death in a variety of physiological and pathological contexts (14). Additionally, the expression of the Bcl-2 protein was reduced when the AGS cells were exposed to higher doses (10-200 µmol/l) of capsaicin, which was similar to results of the present study (12). These results suggest that capsaicin is potentially involved in the modulation of proliferation and apoptosis in human gastric cancer cells.

The ERK 1/2 pathway has been associated with cell proliferation and differentiation (15). Findings of a recent study have demonstrated that exposure to capsaicin clearly inhibited the activation of ERK in breast cancer cell lines including BT-474 and SK BR-3. In human fibrosarcoma cells, capsaicin suppressed the EGF-induced phosphorylation of EGFR, Akt, Raf, ERK1/2 and p38 MAPK (16), while blocking upstream Raf, ERK1/2 and p38 MAPK (16). Findings of a recent study have demonstrated that exposure to capsaicin clearly inhibited the expression of ERK 1/2 phosphorylation reduced the amount of IL-8 produced by AGS cells in response to H. pylori infection (17). In this study, capsaicin suppressed the phosphorylation of ERK 1/2, JNK and p38 MAPK in human gastric cancer cells. Thus, capsaicin may be useful for anti-inflammatory or anti-carcinogenic activity in gastric cancer associated with H. pylori infection.

However, as the pharmacokinetics of capsaicin in human have not yet been investigated, predicting the amount of chili peppers that would need to be consumed to achieve the effective concentration of capsaicin observed is unclear.

Capsaicin reduced cell proliferation and increased apoptosis in a concentration-dependent manner in human gastric cancer cells. Cleaved caspase-3 was increased and Bcl-2 was reduced by treatment with capsaicin in AGS cells. Capsaicin treatment decreased the expression of phosphorylated ERK 1/2, p38 MAPK or JNK in AGS cells.

In conclusion, results of this study suggest that capsaicin may serve as an anti-tumorigenic agent in human gastric cancer. However, the physiological capsaicin dose for the therapeutic or chemopreventive agents is unclear and remains to be determined in future investigations.

References