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Gallic acid inhibits gastric cancer cells metastasis and invasive growth via increased expression of RhoB, downregulation of AKT/small GTPase signals and inhibition of NF-κB activity

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ABSTRACT

Our previous study demonstrated the therapeutic potential of gallic acid (GA) for controlling tumor metastasis through its inhibitory effect on the motility of AGS cells. A noteworthy finding in our previous experiment was increased RhoB expression in GA-treated cells. The aim of this study was to evaluate the role of RhoB expression on the inhibitory effects of GA on AGS cells. By applying the transfection of RhoB siRNA into AGS cells and an animal model, we tested the effect of GA on inhibition of tumor growth and RhoB expression. The results confirmed that RhoB-siRNA transfection induced GA to inhibit AGS cells' invasive growth involving blocking the AKT/small GTPase signals pathway and inhibition of NF-κB activity. Finally, we evaluated the effect of GA on AGS cell metastasis by colonization of tumor cells in nude mice. It showed GA inhibited tumor cells growth via the expression of RhoB. These data support the inhibitory effect of GA which was shown to inhibit gastric cancer cell metastasis and invasive growth via increased expression of RhoB, downregulation of AKT/small GTPase signals and inhibition of NF-κB activity. Thus, GA might be a potential agent in treating gastric cancer.

Introduction

Gastric carcinoma (GC), which is the second most common cause of cancer-related death in the world, is responsible for more than 700,000 deaths per year (Parkin et al., 2005). In Asian countries, such as Korea and China, GC is the leading cause of cancer death. Conventional therapies for advanced-stage GC include surgery, chemotherapy, and radiotherapy, but the prognosis for advanced-stage disease remains poor. More than 80% of patients with advanced GC have lymph node metastasis, and the remote lymph nodes, such as the para-aortic nodes, are involved in 20% of GC. Therefore, control of lymph node metastasis is the most important prognostic factor for the treatment of GC (Dent et al., 1988; Maruyama et al., 1989; Yonemura et al., 1991). Radiation therapy or chemotherapy does not significantly affect the length or quality of life of patients with advanced GC (Kelsen, 1994). Thus, novel therapies are needed to target the molecular alterations that lead to GC development as well as progression.

Flavonoids are common constituents of the human diet, present in most fruit and vegetables, and comprise several classes, including flavanones, flavonols, flavones and isoflavone. In several cases, flavonoids have been reported to possess anticancer potential (Lee et al., 2006). Gallic acid (GA), widely distributed in plants and foods, has various biological effects. The anticancer effects of GA on Calu-6 and A549 lung cancer cells in relation to reactive oxygen species and glutathione was reported recently (You and Park, 2010). In addition, GA, the major anticancer compound in *Toona sinensis* leaf extracts, was shown to be cytotoxic to DU145 prostate cancer cells, through generation of reactive oxygen species and mitochondria-mediated apoptosis (Chen et al., 2009).

Our recent study showed that the inhibitory effects of GA on AGS cells may be partly exerted through the Ras/PI3K/AKT signaling

Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; GA, gallic acid; MMP, matrix metalloproteinases; TRITC, Tetramethylrhodamine B isothiocyanate; NF-κB, Nuclear factor-kappa B; I-κB, inhibitor protein-kappa B; DAPI, 4′-6-Diamidino-2-phenylindole.

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pathway (Ho et al., 2010). In addition, the increased protein levels of cytoplasmic IκB, which exert inhibitory effects on the transcriptional factor NF-κB, subsequently decrease MMP-2 and MMP-9 activities, resulting in antimetastatic effects. A noteworthy finding in our previous experiment was the increased RhoB expression in GA-treated cells. Members of the Rho family of small GTPases are key regulators of actin reorganization, cell motility, cell–cell and ECM adhesion as well as of cell cycle progression, gene expression, and apoptosis (Fritz and Kaina, 2006). RhoA, like other GTPase family members, such as Ras, Rac1, and cdc42, promotes oncogenesis, invasion, and metastasis (Khosravi-Far et al., 1995), however, emerging evidence points to a tumor-suppressive role for RhoB (Chen et al., 2000). Although Rho family GTPases RhoA, RhoB and RhoC share more than 85% amino acid sequence identity, they may play distinct roles in tumor progression. RhoA and RhoC have been suggested to have positive effects on tumor progression, but the role of RhoB in cancer, particularly in gastric cancer, remains unclear (Zhou et al., 2011).The aim of this study was to evaluate the role of RhoB expression on the inhibitory effects of GA on AGS cells by applying the transfection of RhoB siRNA into AGS cells. In addition, using an animal model, we tested the effect of GA on inhibition of tumor growth and RhoB expression.

Materials and methods

Materials. Gallic acid, DMSO, SDS, phenylmethylsulfonyl fluoride, bovine serum albumin (BSA), leupeptin, deoxycholic acid, and sodium orthovanadate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phosphate buffer solution (PBS), trypsin-EDTA, and powdered Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco/BRL (Gaithersburg, MD, USA). Antibodies against AKT, PI3K, NF-κB, IκB, JNK/phospho-JNK, Jun/phosphor-Jun, Fos, Ras, Rac1, cdc42, fas, RhoA, and RhoB were obtained from BD Transduction Laboratories (San Diego, CA) and Santa Cruz Biotech (Santa Cruz, CA).

Cell culture. A human gastric carcinoma AGS cell line was obtained from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan, ROC). AGS cells were maintained in F-12 nutrient mixture medium (Gibco/BRL, Gaithersburg, MD). The cells were cultured at 37 °C in 5% CO_2 in medium supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/mL of penicillin and 100 mg/mL of streptomycin).

Transient transfection of RhoB siRNA into AGS cells. For transient transfection of siRNA, we used a StealthTM Select RNAi Set (Cat No. 1299003) purchased from Invitrogen Corporation (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. One day prior to transfection, AGS cells were seeded in six-well dishes (2×10^5 cells/well) in DMEM without antibiotics, and the cells were expected to be 60% confluent the next day. On the day of transfection, 150 pmol of Stealth RNAi was diluted into 50 μL reduced serum medium and mixed gently. Meanwhile, 1 μL of Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) was incubated with 50 μL of reduced serum medium at room temperature. Afterwards, diluted StealthTM RNAi and Lipofectamine™ 2000 were combined by mixing gently and incubated for 15 min at room temperature. This mixture was then added to cells and incubated at 37 °C in a humidified CO_2 incubator. After 72 h, transfected cells were counted and subjected to cell migration assay and Western blot. Control cells were exposed to the transfectant with negative control siRNA (Thermo Scientific, Dharmacon Product, Lafayette, CO; category No: D-001810-10-05) instead of RhoB siRNA.

Immunoblotting. AGS cells were transfected with negative control siRNA or RhoB siRNA then treated with or without 3.5 μM GA for 24 h. The protein levels of RhoB were analyzed by Western blotting. β-actin was used for equal loading. Western blotting was performed according to a previously described method (Shiah et al., 1999). In short, the cell lysates were denatured in a sample buffer containing SDS, and equal amounts of total protein were separated on 8–15% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. After blocking with 5% nonfat dry milk, the membranes were incubated overnight at 4 °C with the primary antibodies as indicated. The following antibodies were used: antibodies against NF-κB, IκB, PI3K, AKT, Ras, cdc42, Rac1, RhoA, and RhoB (Santa Cruz Biotech, Santa Cruz, CA). The following day, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies, and detection was performed using an enhanced chemiluminescence (ECL) reagent (Amersham Life Science, Amersham, UK).

Reverse transcription-polymerase chain reaction for RhoB mRNA. AGS cells were seeded in 6-well culture plates (2×10^5 cells/well) and grown to 80% confluence. Cells were transfected with negative control siRNA or RhoB siRNA then treated with or without 3.5 μM GA for 24 h. Total RNA was isolated, and the mRNA expression was analyzed by RT-PCR. The cells were then lysed in 1 mL TRIzol reagent (Invitrogen, Carlsbad, USA) and RNA extracted following the manufacturer's recommendations. Total cellular RNA was used for reverse transcription of cDNA by a standardized technique (MBI Fermentas, Hanover, USA). Obtain cDNA was amplified using specific primers. RhoB (GenBank accession no. NM_004040.2; 591 bp) forward, 5′-ATGGCGGCCATCCGCAAG AAGC-3′, reverse, 5′-TCATAGCACCTTGCAGCAGTTG-3′; glyceraldehyde-3-phosphate dehydrogenase (GenBank accession no. NM_002046.3; 270 bp), forward, 5′-TTGGTATCGTGGAGGGACTCA-3′, reverse, 5′-TGTC ATCATATTTGGCAGGTT-3′). After pre-denaturation at 95 °C for 15 s, polymerase chain reaction (PCR) was carried out as the following program: Rho B: 94 °C for 15 s, 57 °C for 20 s, then 36 cycles of 72 °C for 15 s and 72 °C for 10 min; GAPDH: 30 cycle of 94 °C for 30 s,

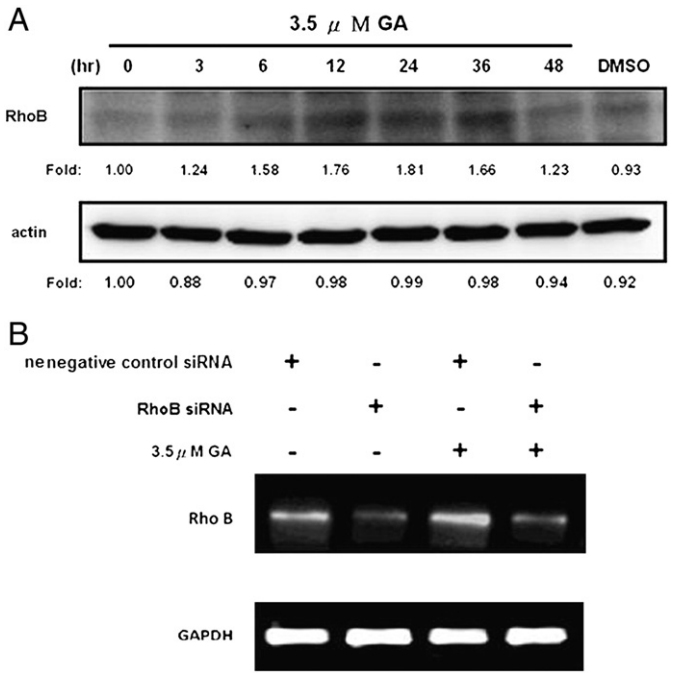

Fig. 1. Immunoblot analysis of the expression of RhoB in AGS cells treated with gallic acid (GA). (A) AGS cells were treated with or without 3.5 μM GA then harvested at different time (0, 3, 6, 12, 24, 36, 48 h). The protein levels of RhoB were analyzed by Western blotting. β-actin was used for equal loading. (B) Effects of gallic acid on RhoB mRNA level in AGS cell. AGS cells were transfected with control siRNA or RhoB siRNA then treated with or without 3.5 μM GA for 24 h. Total RNA was isolated, and the mRNA expression was analyzed by RT-PCR and the GAPDH as the loading control. Values were the average of triplicate experiments.