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Effects of a Compound Extract from *Agrimonia pilosa* Ledeb, *Grifola umbellata* (pers.) Pilat, and *Gambogia* on Human Gastric Carcinoma MGC-803 Cells

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Abstract Three traditional Chinese medicines, *Agrimonia pilosa* Ledeb, *Grifola umbellata* (pers.) Pilat, and *Gambogia*, are combined to form a compound extract, AGC. In this study, the *in vitro* and *in vivo* inhibitory effects of AGC on human gastric carcinoma MGC-803 cells were demonstrated, and the molecular mechanisms underlying these effects are investigated. Our results indicate that AGC inhibited MGC-803 cell growth in a dose-dependent manner as measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, with an IC_{50} of about 6.045±0.69 μg/mL. *In vivo*, AGC inhibited growth of human gastric carcinoma in xenograft tumors in nude mice, and the inhibitory rate reached 55.2% at 300 mg/kg. The pro-apoptotic activity of AGC was attributed to its ability to decrease the expression of Bcl-2 and Pro-caspase3 and increase the expression of Bax. These results demonstrate that AGC can effectively induce programmed cell death and may be a promising anti-tumor drug in human gastric carcinoma.

Keywords: *Agrimonia pilosa* Ledeb, *Grifola umbellata* (pers.) Pilat, *Gambogia*, human gastric carcinoma MGC-803 cell, apoptosis

Introduction

The use of naturally occurring substances as medicinal drugs has made significant contributions to the treatment of human disease. Traditional Chinese medicine is based on an overall analysis of symptoms and signs, as well as the physical conditions of the patient. Nowadays, practitioners of traditional Chinese medicine routinely use herbs to treat human gastric carcinoma; specifically, *Agrimonia pilosa* Ledeb, *Grifola umbellata* (pers.) Pilat, and *Gambogia* are commonly used in Chinese herbal prescriptions.

A. pilosa was widely used as a haemostatic agent (stypticum) in China thousands of years ago, while recently other beneficial effects of this traditional herb have been demonstrated. Wang *et al.* (1) reported that extract of *A. pilosa* could reduce blood glucose level in normal mice as well as in urophthisis mice. Cao (2) and Murayama *et al.* (3) found that water soluble extracts of *A. pilosa* could stimulate secretion of interleukin (IL)-2 in tumor-bearing mice and human peripheral blood mononuclear cells. Wang *et al.* (4) reported that extracts of *A. pilosa* could reduce blood pressure in rabbits. *A. pilosa* has been shown to possess remarkable anti-mite activities towards *Demodex folliculorum* (5) as well anti-tumor effects. Wang *et al.* (6) revealed that *A. pilosa* was able to produce significant inhibition of MGC-803, SPC-A1, and Hela tumors in xenograft nude mice models. Agrimoniin, tannin of *A. pilosa*, has shown great potency in inhibiting MM2 tumor cell growth *in vivo* and *in vitro* as well as in enhancing the host immune response through its actions on tumor cells and the immune system (7-9). Gao *et al.* (10) also reported that decocted extracts of *A. pilosa* induced apoptosis in HL-60 cells *in vitro*.

G. umbellata is one of the most widely used traditional Chinese medicinal mushrooms, and has also been referred to as *Polyporus umbellatus* Fries or 'chuling' in other scientific reports (11,12). The fruiting bodies of *G. umbellata* are commonly used for treating edema and promoting diuresis (13). It has been reported that *G. umbellata* possesses significant anti-tumor effects. Miyazaki *et al.* (14) reported that a water-soluble glucan from *G. umbellate* markedly inhibited the growth of subcutaneously implanted S_{180} in mice. Ohsawa *et al.* (11) reported that the constituents of *G. umbellata* showed cytotoxic activity on L1210 cell proliferation. You *et al.* (15) used the extracts from the *G. umbellata* fruiting-body for the treatment of experimental liver cancer and demonstrated its potential use as an anticancer agent.

Gambogia is a dry resin secreted from *Garcinia hanburyi* Hook.f. In ancient China, it was used as an antitoxic, haemostatic agent (stypticum) and a pesticide. The main active components of *Gambogia* are gambogic acid, neogambogic acid, morellic acid, and isomorellic acid (16). Recently, there were many reports demonstrating its tumor inhibitory effects in experimental tumor models (17-19).

In an effort to generate a Chinese medicine with potent anti-tumor effects and minimal side-effects, the extract AGC was formulated. The raw materials are consisted with 54.1% *A. pilosa*, 43.3% *G. umbellate*, and 2.6% *Gambogia*, which is got from Chinese traditional popular prescription. In the extract AGC, the effective component is gambogic

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acid and the content is not lower than 0.625%. In this study, the anti-tumor effects of this extract both *in vitro* and *in vivo* on human gastric carcinoma MGC-803 cells were investigated and some of the potential molecular mechanisms underlying its effects were revealed.

Materials and Methods

Reagents 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and was dissolved in 0.01 M phosphate buffered solution (PBS). Primary antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), and IRDyeTM 800 conjugated anti-goat and anti-rabbit secondary antibodies were obtained from Rockland Inc. (Philadelphia, PA, USA).

Cell culture Human gastric carcinoma cell line, MGC-803, was purchased from the Cell Bank of the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were maintained in RPMI-1640 medium (Lot No. 20050205; Gibco Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated calf serum (Sijiqing, Hangzhou, China), 100 U/mL penicillin G, and 100 U/mL streptomycin, pH 7.4 in an incubator (Thermo Forma, Waltham, MA, USA) with a humidified atmosphere of 95% oxygen and 5% CO_2 at 37°C.

Colorimetric MTT assay The viability of cells was measured by MTT assay as previously described (20) where IC_{50} was estimated as the concentration causing 50% inhibition of cell growth.

Cell morphological assessment MGC-803 cells were cultured in RPMI-1640 till mid-log confluence. AGC was added at 2, 20, or 200 μg/mL to the culture media and incubated for another 6 hr. At the end of incubation, the morphology of cells was assessed using an inverted light microscope. All floating and attached cells were harvested with 0.02%(w/v) ethylenediamine tetraacetic acid (EDTA) and 0.25%(w/v) trypsin (Gibco Invitrogen). The cell suspension was fixed with ice cold 4% paraformaldehyde for 20 min and then washed with ice-cold PBS. The cells were permeabilized with 0.03% Triton X-100 and washed with ice-cold PBS, stained with diamidino-phenyl-indole (DAPI) (Santa Cruz Technology) and observed under a fluorescence microscope (Olympus IX51; Tokyo, Japan) with a peak excitation wave length of 340 nm.

Western blot analysis for Bax, Bcl-2, and Pro-caspase3 proteins After MGC-803 cells were incubated with 2 or 20 μg/mL AGC for 6 hr, whole cell lysate was prepared. Western blot analysis for Bax, Bcl-2, and Pro-caspase3 proteins was performed as previously described (21). Immunoreactive protein bands were detected with an Odyssey scanning system (LI-COR Inc., Lincoln, MT, USA).

Human gastric carcinoma graft tumor model All animal experiments were carried out according to the National Institutes of Health 'guide for the care and use of laboratory animals' (Publication No. 85-23, revised 1985). Forty-six-week old female BALB/c nude mice, with a body weight of approximately 20 g, were used in our experiments. Human gastric carcinoma cells, MGC-803, were injected (1×10^6 resuspended in PBS) subcutaneously on the dorsal skin of nude mice. When tumors reached a diameter of 3-5 mm, the mice were randomly grouped (8 mice/group) and intragastric AGC (75, 150, or 300 mg/kg) or PBS alone was administered 3 times/week for 28 days. Tumor size was measured twice/week and tumor volume was determined according to the equation: tumor size= $\text{width}^2\times\text{length}\times0.5$.

Statistical analysis All experiments were performed in triplicate and results are shown as mean±stamdard deviation (SD). Statistical analyses were performed using an unpaired, two-tailed Student' *t*-test. Proability <0.05 and $p<0.01$ were considered statistically significant and very statistically significant respectively.

Results and Discussion

Differential growth inhibition of AGC on MGC-803 cells Exponentially growing MGC-803 cells were cultured continuously in the absence or presence of different concentrations of AGC. The effects of AGC on cell growth were assessed using the MTT assay at 48 hr post-treatment. As shown in Fig. 1, AGC treatment inhibited the growth of MGC-803 cells. The degree of growth inhibition depended on the concentration of treatment. The IC_{50} value was 6.05 ±0.69 μg/mL.

Cell morphological assessment Figure 2A shows the morphological cellular changes observed by light microscopy following treatment with different concentrations of AGC for 6 hr. Following incubation with AGC, the cellular morphology of MGC-803 cells was severely distorted; cells disadhered from the plate and some turned necrotic. The untreated cells displayed regular morphology with distinct cytoskeleton.

The nuclear changes of MGC-803 cells were assessed under fluorescence microscopy (Fig. 2B). Untreated MGC-803 cells stained with equally intensity of DAPI and demonstrated homogeneously distributed chromatin within their nuclei. After AGC treatment, these cells displayed chromatin condensation and nucleolus condensation (pyknosis), which is seen as bright DAPI fluorescence that is suggestive of an early apoptotic event (21). And the apoptosis-inducing effects of AGC also appeared in AO/EB staining cells, in which early apoptotic cells, late apoptotic cells and dead cells can be discriminated (22) (data not shown).

These data demonstrate the cytotoxic effects of AGC *in vitro* clearly.

Western blot analysis for Bax, Bcl-2, and Pro-caspase3 proteins In our attempts at assessing the possible molecular mechanisms underlying AGC activity, Western blotting was used. The results showed a decreased Bcl-2 expression while Bax expression was increased in MGC-803 cells following AGC treatment for 6 hr (Fig. 3). From these results, we conclude that AGC may inhibit Bcl-2 to trigger apoptotic signaling that results in a growth inhibition of

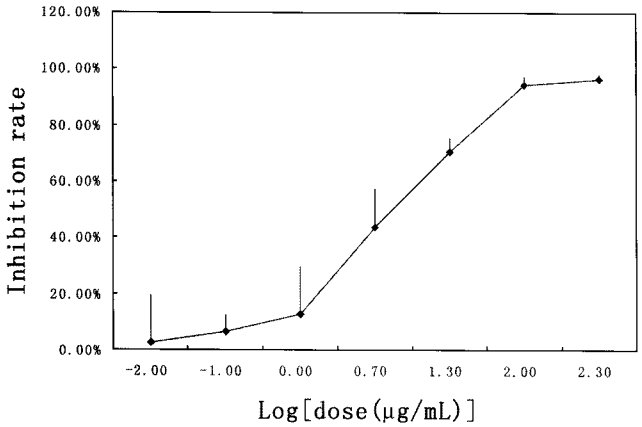

Fig. 1. Inhibitory effect of AGC on MGC-803 cells growth. The inhibitory effects of ACG on human gastric carcinoma MGC-803 cells MTT asssy was detected by MTT assay. A dose-dependent inhibition of AGC on the MGC-803 cell proliferation is shown at 48 hr, with an IC_{50} of 6.045±0.69 μg/mL.

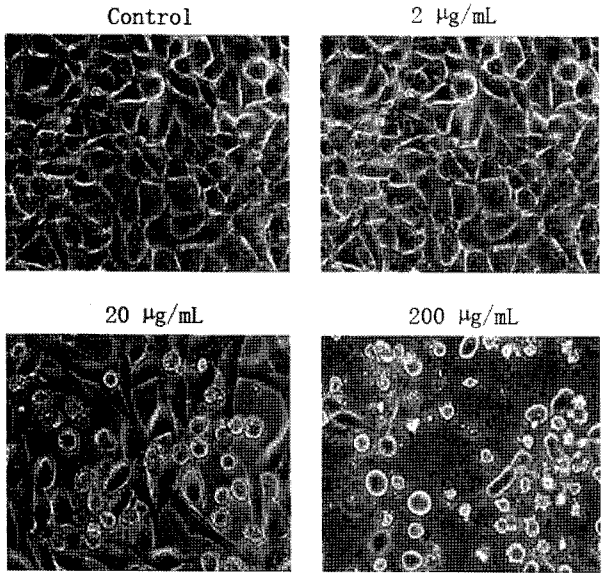

Fig. 2A. Morphologic changes of cells by light microscope (400×). Morphological changes of cells were found by microscopy following treatment with 3 concentrations of AGC for 6 hr (After incubation, MGC-803 cells demonstrated severe cellular morphological alterations. A greater population of cells was noted to become round (disadhesion), floating and necrotic with increasing AGC concentration. The untreated cells are shown with regular morphology and distinct cytoskeleton).

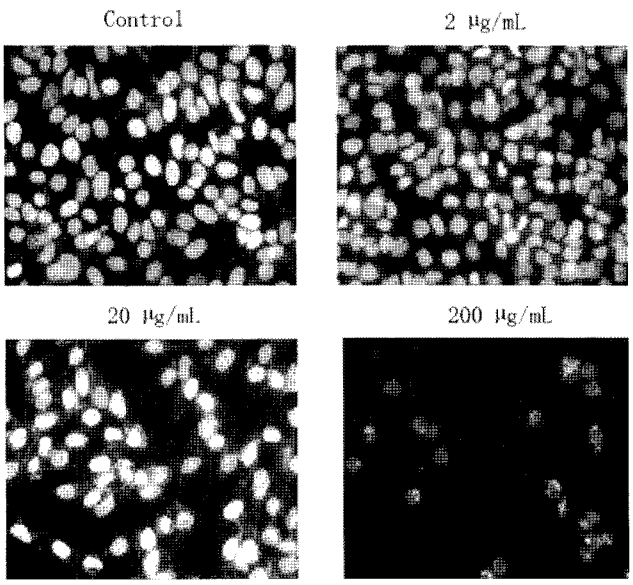

Fig. 2B. Nucleolus morphological changes by DAPI staining (200×). MGC-803 DAPI staining was used to visualize the cell nucleus. Untreated MGC-803 cells were stained evenly with DAPI and showed uniform blue fluorescence with homogeneous chromatin distribution within the nucleus. After AGC treatment for 6 hr, bright fluorescence was observed, representing chromatin condensation and nucleolus condensation (pyknosis), an early indicator suggestive of apoptosis.

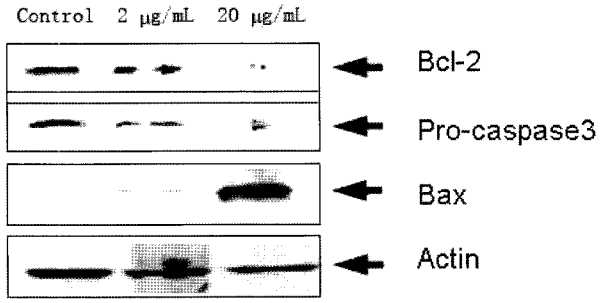

Fig. 3. Effects of AGC on the expression of Bcl-2, Bax, and Pro-caspase3 proteins. MGC-803 cells were incubated with 2 or 20 μg/mL AGC for 6 hr, and western blot analysis for Bax, Bcl-2. and Pro-caspase3 proteins was performed. The expression of Bcl-2 protein decreased while the expression of Bax protein increased and activated caspase-3 was observed.

MGC-803. The Bcl-2 family of proteins can be divided into 2 groups: suppressors of apoptosis (e.g., Bcl-2, Bcl-XL, and Mcl-1) and activators of apoptosis (e.g., Bax, Bok, Hrk, and Bad). Pathological increases in the amounts of one or more of the apoptosis-suppressing proteins have been observed in several types of cancers, although the precise mechanisms that result in their increased expression are poorly understood (23). Previous studies indicated that an increase in pro-apoptotic Bcl-2 family proteins and a decrease in anti-apoptotic Bcl-2 family proteins are involved in the process of apoptosis (24). Thus, the ratio of Bc1-2/Bax proteins might be critical factors for a cell's threshold for apoptosis. In our results, the dramatically decrease of Bc1-2/Bax ratio following AGC treatment was found.

The increased cleavage of Pro-caspase3 to its active form in MGC-803 cells following treatment with different doses of AGC was observed (Fig. 3). Caspases (cysteine aspartate-specific proteases) are a family of intracellular proteins involved in the initiation and execution of apoptosis, amongst which caspase-3 is a key central player. Activation of the execution caspases is often referred to as the apoptotic commitment point in the signaling cascade, representing the point at which the cell irreversibly commits to cell death. Caspase-3 is synthesized as pro-caspase that is then proteolytically processed, at critical aspartate residues, to its active form (25). A previous report

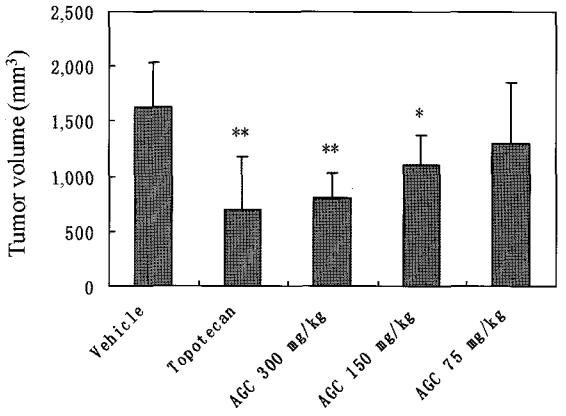

Fig. 4. Inhibitory effect of AGC on xenograft tumor growth. The inhibitory effect of AGC *in vivo* was investigated by xenografted tumors in nude mice with MGC-803 cells. Treatment of tumor-bearing mice with AGC dosages of 75, 150, or 300 mg/kg resulted in 29.1, 46.7, and 55.2% inhibition of tumor growth, respectively, which showed great inhibitory effect of AGC *in vivo* in a dose-dependent manner. Data=mean±SD; Each data was tested with control group data.*$p<0.05$, **$p<0.01$.

shows that caspase-activating proteins such as cytochrome c are released from the mitochondria into the cytosol, where they then bind to caspase-9, a component of the upstream caspase cascade (26). Step by step, the upstream cascade complexes activate components downstream, leading to apoptosis initiation by caspase-3 (27). In the apoptotic processes in some cells, cytochrome c is believed to be released from the mitochondrial membrane through permeability transition pore (PTP), and the loss of mitochondrial membrane potential ($\Delta\Psi m$) indicates the opening of PTP. However, in some other cases, the release of cytochrome c is induced by BH3-only Bcl-2 family proteins without the loss of $\Delta\Psi m$ (28,29). The precise mechanisms conferring the ability of AGC to initiate apoptosis and the process through which cytochrome c is released from the mitochondria are questions for our future analyses.

AGC inhibited xenograft tumor growth To determine whether AGC could inhibit human gastric cancer *in vivo*, the ability of AGC to suppress the growth of established xenograft MGC-803 tumors in nude mice was investigated. Topotecan (topoisomerase 1 inhibitor), a well established chemotherapeutic agent, was administered at 2 mg/kg body weight induced significant inhibition (65.7%) of tumor growth in this nude mouse gastric tumor model (Fig. 4). Treatment of tumor-bearing mice with an AGC dosage of 75, 150, and 300 mg/kg resulted in 29.1, 46.7, and 55.2% inhibition of tumor growth, respectively, compared with vehicle control. These data demonstrate the cytotoxic effects of AGC *in vivo*.

To summarize, our experiments demonstrated that AGC inhibits MGC-803 cell growth *in vitro* and *in vivo*, and induces morphological cellular changes suggestive of apoptosis while decreasing the ratio of Bcl-2/Bax and increasing cleaved caspase3. These findings suggest that AGC might be a promising novel anti-cancer drug in the management of human gastric carcinoma.

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