

RESEARCH COMMUNICATION

Chios Mastic Gum Extracts as a Potent Antitumor Agent that Inhibits Growth and Induces Apoptosis of Oral Cancer Cells

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Abstract

Purpose: The purpose was to investigate Chios mastic gum (CMG) extract as an potential anti-tumor agent for oral squamous cell carcinoma *in vitro*. **Methods:** We designed a study to examine the effects of CMG extracts on growth of oral squamous cell carcinoma cell line, YD-10B and to determine whether the extracts could induce apoptosis through the activation of caspase-3, using the common chemotherapeutic agent Paclitaxel (Taxol, Bristol-Myers Squibb) as a control. **Results:** MTT assay suggested that both CMG and Taxol inhibited the proliferation of YD-10B cells in a time and dose dependent manner. Moreover, 10 μ g/mL of CMG and 50 μ g/mL of Taxol caused fragmentation of the genomic DNA at 24 hour. Finally, 10 μ g/mL of CMG and 50 μ g/mL of Taxol caused cleavage of procaspase-3 in western blot analysis. **Conclusions:** These results suggest CMG's potential as an anti-tumor agent.

Keywords: Oral squamous cell carcinoma cells - Chios mastic gum - YD-10B cells

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Introduction

Many anticancer drugs exert their cytotoxicity by inhibiting DNA synthesis and cell replication. However, side effects such as bone marrow suppression, gastrointestinal toxicity, and renal damage remain to be addressed (Peterson et al., 1992). One avenue to developing clinically applicable chemotherapeutic agents with fewer complications is to screen traditional medicinal plants, which have been used for thousands of years with a very low number of side effects, for their anticancer activity (Lee et al., 2007; Park et al., 2008; Ricci et al., 2010).

Chios mastic gum (CMG) is a white resin obtained from the trunk and leaves of *Pistacia lentiscus var. Chia*. Mastic gum and its essential oils are natural antimicrobial agents that have been used extensively in Mediterranean and Middle Eastern countries, both as a dietary supplement and herbal remedy, since antiquity (Loizou et al., 2009). Mastic gum is known to have antioxidant (Abdel-Rahman and Soad, 1975), anti-bacterial (Magiatis et al., 1999; Koutsoudaki et al., 2005), cosmetic, and teeth-cleaning properties (Wellmann, 1907), as well as anti-*Helicobacter pylori* properties (Huwez et al., 1998; Marone et al., 2001). Recent studies demonstrate that CMG induces apoptosis of human colon cancer cells through caspase-dependent pathways *in vitro* and cell growth inhibition of prostate cancer cells (Balan et al., 2005; Balan et al., 2007), as well as inhibiting the proliferation of androgen-independent prostate cancer, apparently through modulation of the NF- κ B target gene (He et al., 2007).

We designed an experiment to examine the effects of CMG extracts on the growth of oral squamous cell carcinoma cell line YD-10B (Lee et al., 2005) and to determine whether the extracts could induce apoptosis through the activation of caspase-3, known to be the key mediator of apoptosis. In this study, Paclitaxel (Taxol, Bristol-Myers Squibb) was used as the control.

Paclitaxel, a commonly used chemotherapeutic agent, is a taxane anti-neoplastic agent with a broad spectrum of activity for various cancers (Suzaki et al., 2006) although it has been shown to have major adverse effects such as peripheral neuropathy, myelotoxicity, bradycardia, hypotension, arthralgia, myalgia, granulocytopenia and hypersensitivity (Sekine et al., 1996; Furuse et al., 1997). Paclitaxel can induce apoptosis of NB-1 oral carcinoma cells, which may be mediated by down-regulation of Bcl-2 together with up-regulation of Bax (Nonaka et al., 2006).

Materials and Methods

Materials

Chios mastic gum (CMG) extracts were obtained from Mastic Korea (Seoul, Korea); Paclitaxel (Taxol, Bristol-Myers Squibb, Canada) was used as the control. The following reagents were obtained commercially: Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were from Hyclone (Logan, UT, USA); DNA fragmentation assay kit was from BioVision (CA, USA); ECL Western-blotting reagents was from Amersham (NJ, USA); caspase-3 antibody was from BD (NJ, USA). All

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experiments were performed using the YD-10B cell line, originally established from a tongue SCC, with a doubling time of 25.3 hours.

Cell culture

Human oral squamous carcinoma YD-10B cell lines were derived from our oral cancer research institute. These cell lines had been cultured in a medium consisting of 1:3 mixture of calcium-free Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone) and calcium-free F12 (Gibco) medium supplemented with 10% fetal bovine serum (Hyclone), 29.6 mM sodium bicarbonate, 2×10^{-12} M tri-iodothyronine, 1×10^{-11} M cholera toxin, 0.04 μ g/mL hydrocortisone, 0.5 μ g/mL insulin, 0.5 μ g/mL transferrin, 100 U/mL penicillin, and 100 μ g/mL streptomycin in a humidified atmosphere containing 5% carbon dioxide at 37°C.

Growth inhibition of YD-10B cells by CMG extract and taxol

The cells were plated in a 24-well plate, incubated overnight, then treated with a series of CMG extracts and Taxol at concentrations of 5, 10, 25, 50, and 100 μ g/mL respectively. CGM extracts were dissolved in dimethyl sulfoxide (DMSO) and kept frozen at -20°C until use. The final concentrations of DMSO were less than 0.1% and had no effect on YD-10B cells proliferation in my preliminary studies. The cells were treated for 24 hours and 48 hours, and then treated with 500 μ g/mL of thiazolyl blue tetrazolium bromide (MTT solution) at 37°C with 5% CO₂ for 3 hours. The MTT-formazan crystals were dissolved in the DMSO and the cell viabilities measured using an ELISA reader at 570nm.

DNA fragmentation analysis

The cells were treated with 5, 10, 25, 50, and 100 μ g/mL of CMG extracts and Taxol, and 10 μ g/mL CMG extracts and 50 μ g/mL Taxol for various time points (0, 6, 12, 24, 48h). About 5×10^6 pellet cells were collected in a 1.5ml microcentrifuge tube. Then, following a quick apoptotic DNA ladder detection kit (BioVision, USA) procedure, the cells were lysed with TE lysis buffer, incubated with enzyme, and added with ammonium acetate, isopropanol, and 70% ethanol. The DNA fragments were equally loaded on 1.2% agarose gels containing 0.5 μ g/mL ethidium bromide.

Western blot analysis of caspase-3

The treated YD-10B cells were washed with ice cold PBS buffer and lysed in a cell lysis buffer (Cell Signaling Technology). The protein concentration was determined using the bicinchoninic acid assay with BSA as standard. Proteins (40 μ g) in the cell lysates and tumor samples were separated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for caspase-3 and transferred to immobilon polyvinylidenedifluoride membranes (Millipore Co., Bedford, MA, USA). The membrane was blocked with 5% skim milk in PBST for 1 h at room temperature and incubated with anti-caspase-3 (1:1000) antibody. After washing with TBST buffer three times, the blot was incubated with secondary antibody

and bound antibody was detected by ECL kit (Amersham pharmacia biotech) with chemiluminescence, exposing blots to Hyperfilm.

Results

Chios mastic gum (CMG) extract induced reduction of cell survival.

A MTT assay was initially performed in order to analyze the effects of the CMG extracts on the viability of the YD-10B cells. After treatment of YD-10B cells (0 to 100 μ g/mL) with CMG extracts at 24 h, cell viability was reduced to less than 40% (5 μ g/mL) and 10% (10 μ g/mL), whereas it was reduced to less than 60% (5 μ g/mL) and 50% (10 μ g/mL) after Taxol treatment. At 48 h, the viability of YD-10B cells treated with either agent was remarkably reduced to less than 10%. Although there were no gross differences in cell viability between CMG extracts and Taxol at high concentrations (25, 50, 100 μ g/mL), both agents generally inhibited the growth of YD-10B cells time- and dose-dependently. In particular, CMG extracts showed a greater potential effect at low concentration than Taxol (IC₅₀ [concentration required for 50% cell growth inhibition] = 5.0 μ g/mL at 24 h).

DNA fragmentation analysis

The cell death induced by CMG extracts was examined in terms of DNA fragmentation, the biochemical hallmark of apoptosis. In the result of DNA electrophoresis, 10

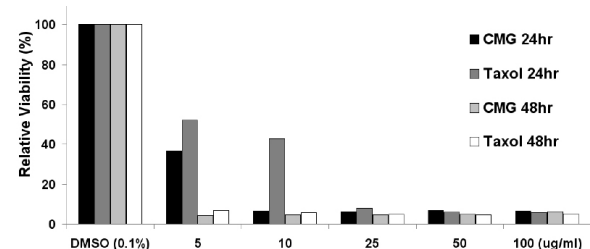


Figure 1. Cytotoxicity and Growth Inhibition Determined by MTT Assay. YD-10B cells were treated with CMG extracts and Taxol (5~100 μ g/ml) for 24 h and 48 h. The viability of YD-10B cells were decreased in dose- and time-dependent manner.

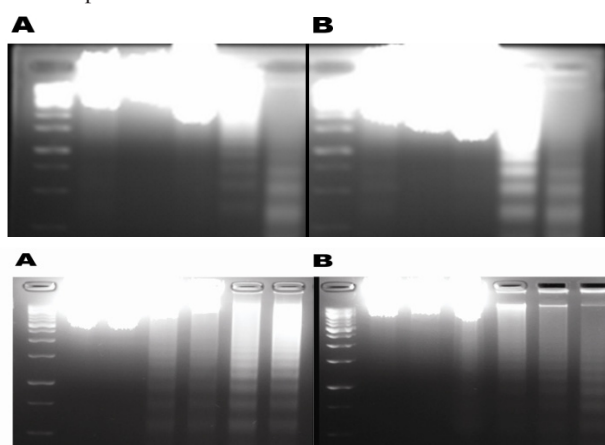


Figure 2. Fragmentation of Internucleosomal DNA. YD-10B cells were treated with CMG extracts (A) and Taxol (5~100 μ g/ml) (B) for 24 h and 48 h. The viability of YD-10B cells were decreased in dose- (above) and time-dependent manner (below)

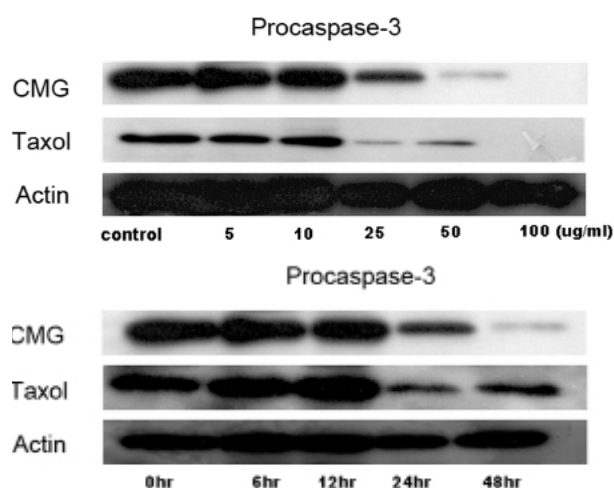


Figure 3. Cleavage of Procaspase-3 YD-10B cells were treated with CMG extracts (A) and Taxol (5~100 µg/ml) (B) for 24 h and 48 h. Cleavage progressed in a dose- (above) and time-dependent (below) manner.

µg/mL of CMG extracts and 50 µg/mL of Taxol caused characteristic DNA fragmentation of the genomic DNA at 24 hr (Fig. 2 above) and 10 µg/mL of CMG extracts and 50 µg/mL of Taxol caused fragmentation of the genomic DNA as early as 24 hr (Fig. 2 below). The experiment was conducted in triplicate with the same results.

Induction of apoptosis through a caspase-3-dependent mechanism

Caspase-3 is believed to be a key protease activated during the early stage of apoptosis. The caspases are activated by a sequential cascade of cleavage of their inactive forms. For instance, active caspase-3 proteolytically cleaves and activates other caspases as well as other relevant target molecules in the cytoplasm or nucleus. The cleavage of procaspase-3 was evaluated using Western blot analysis to determine whether caspase-3 is involved in the apoptosis induced by CMG extracts and Taxol. Dose-dependently, 25 µg/mL of CMG extracts and Taxol caused the cleavage of procaspase-3 (Figure 3 above) and time-dependently, 10 µg/mL of CMG extracts and 50 µg/mL of Taxol caused the cleavage of procaspase-3 as early as 24 hr in western blot analysis (Figure 3 below).

Discussion

Clinically, surgery is considered the primary treatment modality, effective in early lesions. Although a combination of surgery and radiotherapy is effective in more advanced lesions, radiation-only therapy shows a poor survival rate (less than 25%) in inoperable lesions (Kaliora et al., 2007). Concurrent chemo-radiotherapy (CCRT) has recently emerged as a new treatment modality. However, its limitation to specific agents, high cost, and more serious side effects such as healing disorder, oral mucositis, and nausea with vomiting make it difficult to apply clinically (Treister and Sonis, 2007). Thus, extensive research has been carried out in order to develop new natural chemotherapeutic agents with less toxicity and fewer side effects than conventional chemotherapeutic

agents. As mentioned in the introduction, Chios mastic gum (CMG) is a white resin obtained from the trunks and leaves of *Pistacia lentiscus* var. Chia and already known to have anti-*Helicobacter pylori* properties (Huwez et al., 1998; Marone et al., 2001). It induces apoptosis of human colon cancer cells and inhibits growth of prostate cancer cells (Balan et al., 2005; Balan et al., 2007). Kaliora et al. (2007) reported that Crohn's disease patients with mild to moderate activity subjected to mastic treatment seemed to improve clinically and to have better-regulated inflammation and antioxidant status (Kaliora et al., 2007). The study concluded that the use of natural products as a primary treatment in Crohn's disease merited wider support and research, especially considering the harm of long-term corticosteroid use. Further research in larger cohorts is needed to determine the efficacy of natural products such as mastic in treating Crohn's disease.

As a natural chemotherapeutic agent, CMG extracts have recently been reported to have growth inhibitory and apoptotic effects, Balan et al. (2007) finding that CMG induces an anoikis form of cell death in HCT116 colon cancer cells that includes events associated with caspase-dependent pathways and might thus serve as a chemotherapeutic agent for the treatment of human colon and other cancers (Balan et al., 2005; Balan et al., 2007).

Since its approval by the Food and Drug Administration (FDA) for the treatment of advanced ovarian cancer in December 1992, Taxol (Paclitaxel) has emerged as one of the most active anticancer agents in clinic for the therapy of ovarian, breast, non-small cell lung cancer, AIDS-related Kaposi's sarcoma, bladder, prostate, esophageal, head and neck, and cervical and endometrial cancers (Bissery et al., 1991; Fu et al., 2009).

This study was designed to examine the effects of the CMG extracts on the growth of the oral squamous cell carcinoma cell line YD-10B and to determine whether the extracts could induce apoptosis through the activation of caspase-3, which is known to be the key mediator of apoptosis. Commonly used chemotherapeutic agent Paclitaxel (Taxol, Bristol-Myers Squibb) was used as the control.

In MTT assay, both agents showed growth inhibitory effects time- and dose-dependently, with CMG extracts showing greater potential effect than taxol at low concentration (5~10 µg/mL).

DNA fragmentation analysis shows that the growth inhibitory effect of CMG extracts occurs through apoptosis; Western blot analysis shows the apoptosis to be caspase-3 dependent.

Apoptosis, programmed or physiological cell death, plays an important role in embryogenesis, homeostasis, and certain pathologic events. The biochemical hallmark of apoptosis is the appearance of a fragmentation pattern in chromatin which indicates DNA cleavage at the linker regions between nucleosomes. It produces a characteristic pattern of DNA cleavage into 180-bp oligonucleosome units, which generates integer fragments (a DNA 'ladder') when the DNA from apoptotic cells is subjected to conventional gel electrophoresis (Wyllie, 1980). As a result of DNA electrophoresis, 10 µg/mL of CMG extracts and 50 µg/mL of Taxol caused characteristic DNA

fragmentation of the genomic DNA at 24 hr (Fig. 2 above) and 10 µg/mL of CMG extracts and 50 µg/mL of Taxol caused fragmentation of the genomic DNA as early as 24 hr (Fig. 2 below). Such results indicate that both agents induce a growth inhibitory effect through apoptosis and the possibility that CMG extracts have greater potency than Taxol.

The activation of a family of intracellular cysteine proteases, called caspases, plays a key role in the initiation and execution of apoptosis induced by various stimuli (Datta et al., 1997; Liu et al., 1997). Among the several different members of caspases identified in mammalian cells, caspase-3 plays a direct role in the proteolytic cleavage of the cellular proteins responsible for progression to apoptosis (Datta et al., 1997; Liu et al., 1997; D'Amours et al., 1998). It is synthesized as a 33-kDa inactive proenzyme requiring proteolytic activation.

In this study, CMG extracts and Taxol caused the dose- and time-dependent proteolytic cleavage of procaspase-3. Although the detailed mechanism of the induction of apoptosis by CMG extracts has not been determined (Balan et al., 2007), it was hypothesized that CMG extracts induce apoptosis through the activation of caspase-3.

In conclusion, CMG extracts and Taxol inhibited growth and induced apoptosis of YD-10B oral cancer cells in vitro and CMG extracts had greater antitumor potency than Taxol. Although the results reported here are limited to in vitro findings, they may serve as a cornerstone of more extensive in vivo studies; furthermore, the induction of apoptosis by the CMG extracts suggests their use in chemotherapy alongside other anticancer agents.

Acknowledgements

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