Guggulsterone Induces Apoptosis in Colon Cancer Cells and Inhibits Tumor Growth in Murine Colorectal Cancer Xenografts

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Directed by professor Won Ho Kim

The Master's thesis
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degree of Master of Medical Science

Min Ji An
June 2010

This certifies that the Master's thesis
of Min Ji An is approved.

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June 2010

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석사학위 과정을 마무리 하면서 지난 2년이란 시간을 다시 한번 돌이켜보면서 힘든 시간보다 재미있고 즐거웠던 일들이 먼저 떠오르면서 미소 짓게 합니다. 연구 목적과 가설을 토대로 실험해서 나온 결과가 신기하기만 했었고 많은 실수와 실패를 거치면서 만들어진 데이터가 하나의 스토리로 만들어 질 때 그 성취감은 이루어 말할 수 없던 값진 시간들이었습니다. 이제 졸업을 앞둔 저는 제 인생의 한 챕터가 넘겨지고 새 다른 챕터를 맞이하게 됩니다. 저는 이제까지 제 앞에 닫혀진 문을 멍하니 쳐다보면서 누군가 열어주기를 바래었지만 이제 주어진 문을 내 스스로 열려고 노력을 해야 하며 나아가야 할 때인 것 같습니다.

본 연구를 수행하는데 있어서 아낌없는 지도와 조언으로 이끌어 주시며 연구에 대한 뜨거운 열정으로 귀감이 되시는 김원호 교수님께 진심으로 감시 드립니다. 바쁘신 와중에도 항상 귀중한 시간을 내어 소중한 가르침을 주셨고 실험 진행이 잘 되지 않을 때 충분히 수행해 낼 수 있다는 믿음을 저에게 심어주셨습니다.

학위과정을 받으면서 정말 실험자가 가져야 할 마음과 태도, 또한 실험을 하는데 있어서 단순히 하는 것이 아니라 할 것을 미리 노트에 기록하고 그 기록된 계획에 맞추어 진행하고 정리해야 될 것 등 무엇으로도 살 수 없는 아주 귀한 가르침과 제 인생에 있어서 소중한 기회를 허락해 주신 따스하고 열린 마음을 지니신 천재희 교수님께 깊은 감사의 마음을 전합니다.

바쁘신 와중에도 저의 미흡한 논문을 심사하시느라 귀한 시간을 내어주시고 지도해주신 김건홍 교수님께 진심으로 감사 드립니다.

이 학위논문은 제 혼자의 힘으로 쓰여진 것이 아니라 항상 옆에 같이 일하고 시간을 보냈던 동료들의 도움으로 이루어진 결실입니다. 같이 학위과정을 보내면서 도와주고 웃어주었던 동갑내기 선희, 멋지고 항상 우리 랩을 챙겨주시며 언제든 제 편이 되어 주셨던 권

지희 선생님, 랩 일을 챙기고 이끌어가신 김승원 선생님, 깔끔하고 꼼꼼하게 한편으로는 재미있는 얘기로 우리를 즐겁게 해준 이미정 선생님, 항상 저에게 학문 적으로나 인생의 진로에 있어서 많은 도움과 가르침을 주신 박수진 선생님께 이 자리를 빌어 감사의 말을 드립니다.

무엇보다 이 학위를 무사히 마칠 수 있도록 한결 같은 마음으로 지켜봐 주고 응원해준 가족들에게 사랑 한다는 말을 전하고 싶습니다.

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Minji An

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ABSTRACT

Guggulsterone Induces Apoptosis in Colon Cancer Cells and Inhibits Tumor Growth in Murine Colorectal Cancer Xenografts

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(Directed by professor Won Ho Kim)

The plant sterol guggulsterone has recently been shown to have anti-inflammatory properties and anti-tumorigenic potential in certain cancer cell types. In this study, I investigated the anti-tumorigenic effects of guggulsterone and elucidated its molecular mechanisms in HT-29 human colon cancer cells and murine xenografts. In the present study, I determined whether guggulsterone induces apoptosis in HT-29 colon cancer cells in vitro, and the mechanism by which it does so. Furthermore, we carried out *in vivo* studies to examine the effect of guggulsterone in a colon cancer HT-29 xenograft model. Guggulsterone significantly suppressed cell viability and increased apoptosis in HT-29 cells in a dose-dependent manner by activating caspase-3 and caspase-8. Furthermore, guggulsterone decreased cIAP-1, cIAP-2, and Bcl-2 levels and increased the levels of truncated Bid, Fas, p-JNK, and p-c-Jun. The size of HT-29 xenograft tumors in guggulsterone-treated mice was significantly smaller than of the size of tumors in control mice. Immunohistochemical staining showed that Bcl-2 expression in tumor

xenografts was inhibited by guggulsterone treatment. The present study indicates that guggulsterone induces apoptosis in HT-29 human colon cancer cells and inhibits the growth of HT-29 xenograft tumors *in vivo*, suggesting a potential therapeutic use for this compound in the treatment of colorectal cancer.

Key Words: Guggulsterone, colorectal cancer, apoptosis, xenograft

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Ⅰ. INTRODUCTION

Colorectal cancer is one of the leading causes of malignant neoplasm-related mortality worldwide.[1, 2] Although approximately 70-80% of patients are eligible for curative surgical resection at the time of diagnosis, about 50% of all newly diagnosed patients ultimately develop metastatic disease.[3] These patients eventually receive systemic chemotherapy, but despite significant advances in the development of chemotherapeutic agents, none of the existing drugs for the treatment of colorectal cancer have a non-relapsing cure rate. Current palliative chemotherapy aims only to improve survival and quality of life in patients with advanced colorectal cancer.[4] Moreover, orthodox chemotherapy produces many systemic side effects. In light of these facts, more effective and safer therapeutic strategies for advanced or unresectable colorectal cancer are urgently needed.

Dysregulation of apoptosis, a fundamental biological process that regulates a variety of normal physiological processes ranging from development to aging,[5] leads to a variety of human pathologies including cancer and

autoimmune diseases. The apoptotic cascade can be initiated via the death receptor or mitochondrial pathways, but both these pathways converge at the level of caspase-3 activation. Apoptosis depends on the activity of caspase proteases and is accompanied by characteristic morphologic changes such as chromatin compaction, membrane blebbing, and cell shrinkage. Growing evidence of the link between apoptosis and cancer suggests that modulation of the apoptotic pathway could be used for cancer treatment. Interestingly, the cytotoxic effect of most of the currently used chemotherapeutic drugs typically occurs through apoptosis. Therefore, the ability of tumor cells to respond to damage and eventually activate the apoptotic pathway may in part determine the ultimate success of cancer therapy. It is well documented that apoptosis can be induced by a variety of drugs with diverse chemical structures and different mechanisms of action; this is mediated through a variety of signaling pathways that lead to the down-regulation of anti-apoptotic genes and up-regulation of pro-apoptotic genes.

Many pharmaceutical products currently available originate from plant extracts; various phytochemicals have been shown to have anti-tumor properties both *in vivo* and *in vitro*. Clinical trials of herbal medicines have also been conducted in the search for novel chemotherapeutic drugs. Guggulsterone [4,17(20)-pregnadiene-3,16-dione] is a plant polyphenol obtained from the gum resin of the Indian Ayurvedic medicinal plant, *Commiphora mukul*. It has been used for millennia to treat arthritis, obesity, disorders of lipid metabolism, hypothyroidism, and inflammation.[6-8] The active compounds in this extract are the isomers E- and Z-guggulsterone.[9] We reported previously that guggulsterone blocks the nuclear factor-kappa B (NF-κB) signaling pathway by targeting the IκB kinase complex in intestinal epithelial cells; we also reported that guggulsterone attenuates dextran sulfate-induced acute murine colitis.[10] NF-κB activation is considered a pro-survival signal and this transcription factor may be closely linked with carcinogenesis,

in part through its regulation of apoptosis.[11] Several studies have demonstrated that NF-κB plays a critical role in the inhibition of tumor necrosis factor (TNF)-mediated apoptosis.[12-14] Accordingly, there have been several recent reports that guggulsterone exhibits promising anti-tumor effects in some cancer cell types. However, it has not been determined if guggulsterone has anti-tumor effects in colorectal cancer. Based on this, we hypothesized that guggulsterone may mediate anti-colorectal cancer effects by modulating the apoptotic pathway. Thus, the purpose of this study was to investigate the anti-tumor effects of guggulsterone in relation to apoptosis in human colon cancer and to further define its molecular mechanisms in both human colon cancer cells and a murine colon cancer xenograft model.

Ⅱ. MATERIALS AND METHODS

1. Reagents.

Z-guggulsterone (Sigma, St. Louis, MO, USA) was dissolved in DMSO as a 16 mM stock solution and stored at -20^{o}C. For treatment, this solution was diluted in serum free media to give various concentrations of guggulsterone (0-50 μM). DMSO was used as a vehicle control.

2. Antibodies.

The following antibodies were purchased from their respective sources: caspase-9, caspase-3, caspase-8, cIAP-1, cIAP-2, Bak, Bid, Bad, Bcl-2, Fas, p-JNK, and p-c-Jun (Santa Cruz Biotechnology, Santa Cruz, CA, USA); β-actin (Sigma).

3. Cell lines and cell culture.

The human colon cancer cell line HT-29 and the normal intestinal cell line IEC-18 were obtained from the Korean Cell Line Bank (Seoul, Korea) and the American Type Culture Collection (Manassas, VA, USA), respectively. Monolayer cultures of HT-29 and IEC-18 cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100mg/mL streptomycin (Gibco BRL, Grand Island, NY, USA). All cells were grown at 37℃ in a humidified atmosphere containing 5% CO_2.

4. MTT assay.

Cell viability was determined by measuring absorbance using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazonium bromide (MTT, Sigma), representing the percentage of living cells. Briefly, cells were plated in 24-well plates at 5 x 10^4 cells/well and serum-starved for 1 day using serum free media. The cells were then treated with serum free media containing various

concentrations of guggulsterone (0-50 μM). After 24, 48, and 72 h, 50 μL of MTT solution (2 mg/ml) was added to each well and the reaction mixture was incubated at 37°C in a 5% CO_2 atmosphere for 4 h. The MTT solution was removed and 500 μL of DMSO was added. The optical density was measured using a spectrophotometer at 570 nm (SPECTRA MAX 340, Molecular Devices, GMI, USA).

5. Hoechst 33258 staining.

Morphological changes were analyzed by fluorescent microscopy using Hoechst staining. HT-29 and IEC-18 cells were plated in 4-well chamber slides at a density of 2×10^4 cells/well and treated with various concentrations of guggulsterone (0-50 μM) for 24 h and 48 h, respectively. The cells were then washed twice with PBS and fixed using 4% formaldehyde for 15 min. Next, the cells were washed in PBS and incubated in 50 μL of Hoechst 33258 solution (50 ng/mL in PBS; Sigma) for 20 min in the dark. The samples were prepared with mounting media and examined using fluorescence microscopy with a DAPI filter (BX51TR, Olympus, Tokyo, Japan).

6. Annexin V-FITC apoptosis detection.

Cells were plated in 6-well plates at a density of 2×10^5 cells/well and treated with various concentrations of guggulsterone (0-50 μM) for 48 and 72 h. Attached cells were harvested by trypsinization and incubated with annexinV-FITC (BD Biosciences, Franklin Lakes, NJ, USA) and propidium iodide (Sigma) for 15 min at room temperature in the dark. Cells were then analyzed by flow cytometry. The degree of apoptosis was assessed by annexin V-FITC and propidium iodide staining, as per the manufacturer's instructions.

7. Western blot analysis.

Cells were harvested, washed twice with PBS, and resuspended in lysis

buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2 EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 μg/mL leupeptin, and 1 mM PMSF) supplemented with protease inhibitors (Roche Molecular Biochemicals, Mannheim, Germany). A BCA protein assay (Pierce, Rockford, USA) was used for protein quantification. Proteins (25 μg) were separated in 4-12% bis-Tris gels (Invitrogen Life Technologies, Carlsbad, CA, USA) and transferred to PVDF membranes (Invitrogen Life Technologies). Membranes were incubated for 1 h at room temperature with primary antibody, followed by incubation with their respective secondary antibodies. All membranes were developed using the ECL Western Blotting Detection Kit Reagent (Amersham Biosciences, Freiburg, Germany).

8. Xenograft assay.

Five week old male *nu/nu* mice were purchased from Charles River Japan (Yokohama, Japan) and quarantined for 1 week before being used for xenografting. All experiments were conducted under the institutional guidelines established at the Yonsei University College of Medicine (Seoul, South Korea). Eighteen mice that were 6 weeks of age were injected with tumor cells for the xenograft experiments. 1×10^7 HT-29 cells were injected subcutaneously into the flanks of 17 mice. The mice were randomly assigned to one of three groups. Once the tumors reached an approximate volume of 100-150 mm^3, the first group (n = 6) was selected as a control group and received the DMSO vehicle for 2 weeks. The second group (GGS20, n = 6) received daily intraperitoneal injections of guggulsterone (20 mg/kg) and the third group (GGS40, n = 6) received daily intraperitoneal injections of guggulsterone (40 mg/kg) for 2 weeks.

For all mice, tumors were measured in two dimensions using calipers every 2-3 days and the tumor volumes were calculated using the following formula:

volume = $a \times b^2/2$, where *a* is the width at the widest point of the tumor and *b* is the width perpendicular to *a*. Results from individual mice were plotted as average tumor volume versus time.

9. Histology and immunohistochemistry for Bcl-2.

One day after the last injection of guggulsterone or DMSO, the mice were sacrificed and the tumors were removed. The tumors were fixed with formalin and embedded in paraffin. Serial sections (4-μm thick) were cut from each paraffin block and stained with either hematoxylin-eosin (H&E) or Bcl-2 (1:200 dilution; Santa Cruz Biotechnology) using the Dako REAL™ Envision Kit (DakoCytomation, Carpinteria, CA, USA). Images were obtained using a Zeiss LSM 510 confocal microscope.

Ⅲ. RESULTS

1. Guggulsterone inhibits the survival of human colon cancer cells.

We first examined the effect of guggulsterone on the survival of HT-29 cells using the MTT assay. The viability of HT-29 cells was reduced significantly in a concentration-dependent manner by treatment with guggulsterone. Treatment of HT-29 cells with 50 μM guggulsterone for 72 h caused a 70% reduction in cell viability, while no change was observed in the DMSO-treated control (Fig. 1). To test whether guggulsterone was toxic to normal intestinal epithelial cells, we similarly treated IEC-18 cells with guggulsterone; no effect on their growth was found (data not shown).

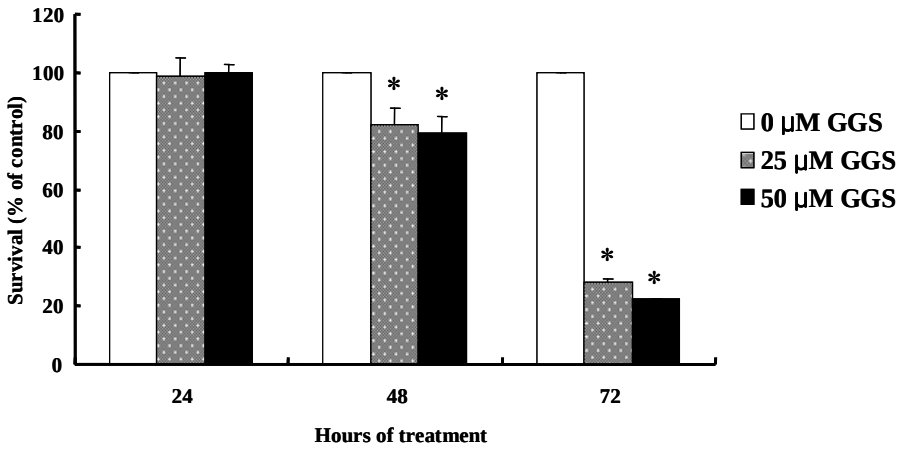

Figure 1. Effects of guggulsterone on the survival of HT-29 colon cancer cells. Exponentially growing cells were treated with various concentrations of guggulsterone (0-50 μM) for 24, 48, and 72 h. Cell survival was determined by the MTT assay and cell viability was determined as a percentage of the corresponding control. Each bar represents the mean ± S.E.M. (n=3 or 6). * = $P<0.05$ relative to untreated.

2. Guggulsterone induces chromatin condensation in colon cancer cells.

Based on the above results, we hypothesized that guggulsterone may induce apoptosis in HT-29 cells. To verify whether the decreased cell viability of HT-29 cells treated with guggulsterone was related to apoptosis, Hoechst staining was used. The Hoechst procedure stains nuclei that contain nicked DNA, a typical feature of cells undergoing apoptosis. Morphological analysis of Hoechst-stained HT-29 cells treated with guggulsterone for 24 and 48 h revealed that they had undergone remarkable morphological changes (Fig. 2A). At these concentrations of guggulsterone, no change was observed in the normal intestinal cell line IEC-18 cells. Fluorescent microscopic observation after Hoechst staining showed that a substantial number of cells treated with guggulsterone acquired apoptotic features, as evidenced by nuclear fragmentation.

3. Guggulsterone increases the number of early apoptotic cells in colon cancer cells.

FACS analysis using Annexin V-FITC staining and propidium iodide accumulation was used to differentiate early apoptotic cells (Annexin V-FITC+ and PI-) from living cells (Annexin V-FITC - and PI-). Compared with the control group, guggulsterone treatment of HT-29 cells increased the number of early apoptotic cells in 30% of cells at 72 h and decreased the number of living cells (Fig. 2B). The Annexin V binding assay thus confirmed the hypothesis that guggulsterone induces apoptosis in human colon cancer cells.

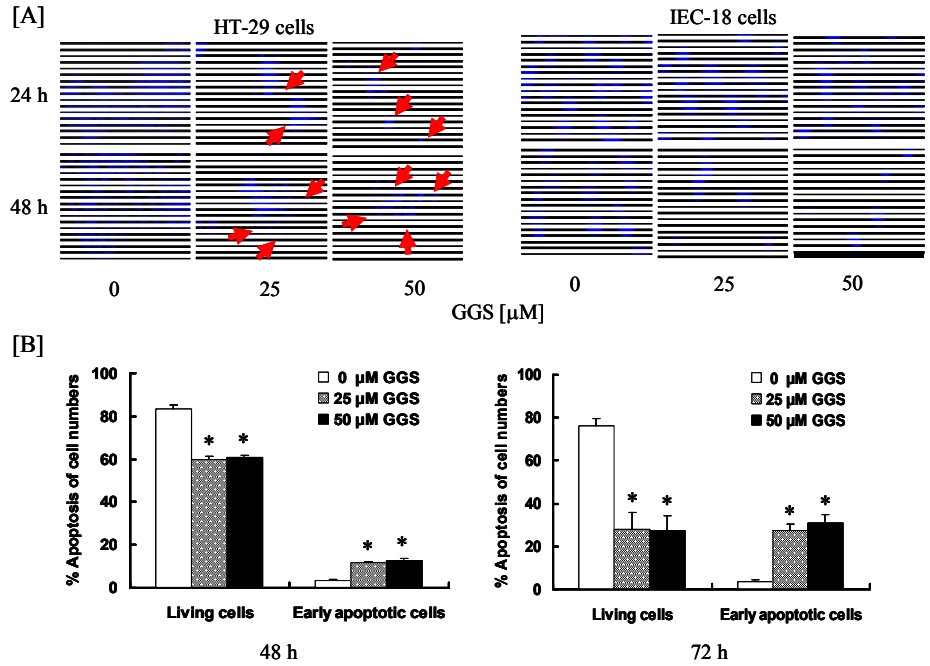

Figure 2. Guggulsterone induces morphologic changes in colon cancer cells, but has no effect on intestinal epithelial cells. HT-29 and IEC-18 cells exposed to guggulsterone (0-50 μM) were examined using fluorescent microscopy after Hoechst staining at 24 and 48 h of treatment (A). Flow cytometry analysis of Annxin V-FITC staining and PI accumulation after exposure of HT-29 cells to guggulsterone for 48 and 72 h (B). The number of living cells and early apoptotic cells is expressed as a percentage of total cell number. Each bar represents the mean ± S.E.M. (n=3 or 6). *= $P<0.05$ relative to untreated.

4. Guggulsterone induces the activation of the mitochondria-dependent apoptosis pathway in colon cancer cells.

Recent studies have identified caspases as important mediators of apoptosis induced by various apoptotic stimuli.[15] To determine whether caspases might

be associated with guggulsterone-induced apoptosis, we measured the caspase-9 and -3 levels in guggulsterone-treated HT-29 cells by western blot analysis. The analysis demonstrated suppressed levels of both pro-caspase-9 and -3 at 24 and 48 h in a dose-dependent manner and increased levels of cleaved caspase-3 only at 48 h (Fig. 3), thus clearly indicating that guggulsterone-induced apoptosis is associated with caspase activation. To investigate guggulsterone's effects on the IAP family of proteins associated with apoptosis, we examined the expression level of two members of the IAP family, cIAP-1 and cIAP-2.[16, 17] The expression of cIAP-1 and cIAP-2 was significantly inhibited by guggulsterone in a concentration-dependent manner at 24 h after treatment (Fig. 3). cIAP-1 expression was still inhibited by guggulsterone at 48 h after treatment. We also examined whether guggulsterone induces apoptosis by modulating the expression of Bcl-2 family members. Guggulsterone increased the level of truncated Bid (an activated form of Bid) at 48 h after treatment, but showed no effect on the expression of Bak. Moreover, guggulsterone decreased the level of Bcl-2 at both 24 h and 48 h after treatment (Fig. 3).

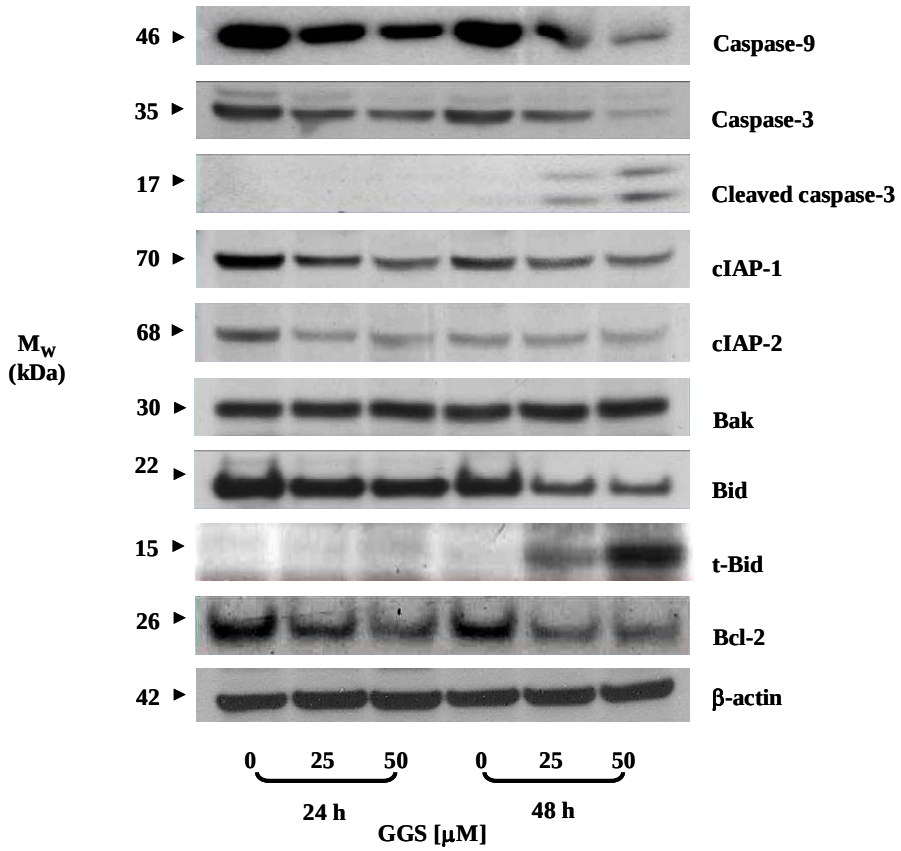

Figure 3. Guggulsterone reduces the protein levels of total caspase-9, increases the levels of caspase-3, and inhibits the protein levels of cIAP-1, cIAP-2, t-Bid, and Bcl-2 in HT-29 cells. Cells were treated with various concentrations of guggulsterone (0-50 μM) for 24 and 48 h. Western blots for each protein were done at least twice using independently prepared lysates.

5. Guggulsterone induces the activation of the extrinsic apoptotic pathway in colon cancer cells.

To initiate the extrinsic apoptotic cascade, Fas binds to its receptor and both recruits and activates the Fas receptor-associated death domain (FADD)

protein, which triggers the caspase-8 pathway, which eventually leads to apoptosis.[18-20] To determine whether guggulsterone increases Fas-induced caspase-8 activation, the expression levels of Fas, caspase-8, and cleaved caspase-8 were measured in colon cancer cells. Western blot analyses showed that guggulsterone increases Fas protein expression and induces the activation of caspase-8 (Fig. 4). As JNK is one of the main upstream kinases involved in phosphorylating the pro-apoptotic NH2-terminal of c-Jun,21 we also examined the functional activity of JNK by analyzing the phosphorylation of c-Jun. Consistent with a time course of JNK activation, phosphorylated c-Jun levels increased in the presence of guggulsterone at 24 and 48 h after treatment. The total level of phosphorylated JNK was increased only at 48 h (Fig. 4). These results demonstrate that guggulsterone potentiates Fas-mediated apoptosis in accordance with the activation of caspase-8.

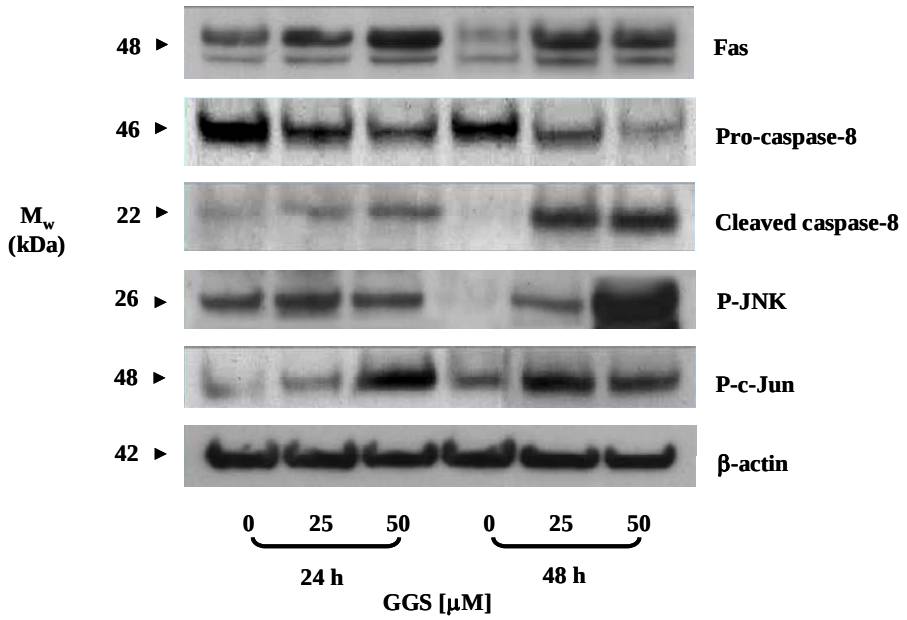

Figure 4. Guggulsterone increases the protein levels of Fas, p-JNK, p-c-Jun,

and caspase-8 in HT-29 cells. Cells were treated with various concentrations of guggulsterone (0-50 μM) for the indicated time. Western blots for each protein were carried out at least twice using independently prepared lysates.

6. Guggulsterone suppresses tumor growth in an HT-29 xenograft model.

To verify the anti-tumor effect of guggulsterone *in vivo*, we examined whether guggulsterone inhibits tumor growth in HT-29 xenografts. Mice implanted with HT-29 cancer cells were treated daily with guggulsterone for 2 weeks. No deaths occurred after 2 weeks. Tumors in guggulsterone-treated mice were visibly smaller than in vehicle-treated mice (Fig. 5A). The average tumor volume of mice treated with guggulsterone (40 mg/kg) was markedly lower (78%) than in control mice (134.5±19.4mm3 vs. 615.6±51.8mm3) (Fig. 5B). Body weights of the control and guggulsterone-treated mice groups were recorded periodically to determine whether guggulsterone administration caused weight loss. The average body weight of guggulsterone-treated mice did not differ from that of the control mice (Fig. 5C). These results clearly indicate that guggulsterone effectively inhibits tumor growth in HT-29 xenografted mice without causing any profound side effects in the mice.

[A]

Control GGS 20 mg/kg GGS 40mg/kg

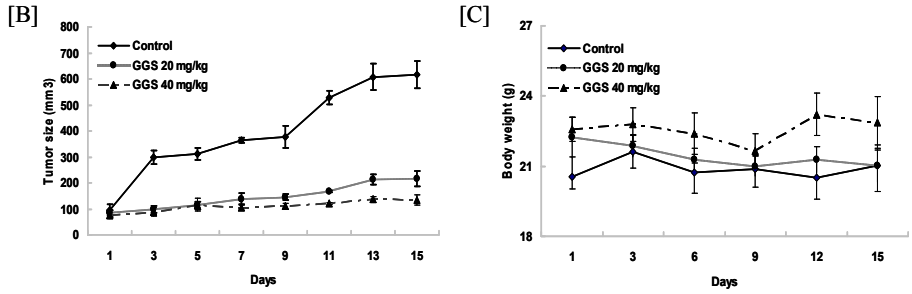

Figure 5. The effect of guggulsterone on the development of HT-29 xenograft mice. Viable HT-29 cells ($1x10^7$) were injected subcutaneously into the flanks of male nude mice. After the tumors reached 100-150 mm^3 in size, the mice received an intraperitoneal injection of guggulsterone (20 and 40 mg/kg) or vehicle (DMSO) daily for 2 weeks [A]. Average tumor volume in vehicle-treated control mice and mice treated with 20 and 40 mg/kg guggulsterone [B]. Average body weight of control and mice treated with 20 and 40 mg/kg guggulsterone [C]. Each bar represents the mean ± S.E.M. (n=6). Means at a time without a common letter differ significantly with $P<0.05$.

7. Guggulsterone down-regulates the expression of Bcl-2 in an HT-29 xenograft model.

To determine the mechanism by which guggulsterone inhibits tumor growth, we further assessed Bcl-2 protein expression in tumor sections from control and guggulsterone-treated mice by immunohistochemical staining. As expected, Bcl-2 was expressed in the cytoplasm of tumor cells, which is known to play an inhibitory role in apoptosis. Tumor tissues from guggulsterone-treated mice showed less Bcl-2 staining than tumor tissues from control group mice (Fig. 6D-F). These data suggest that apoptosis in colon cancer cells can be induced by guggulsterone *in vivo.*

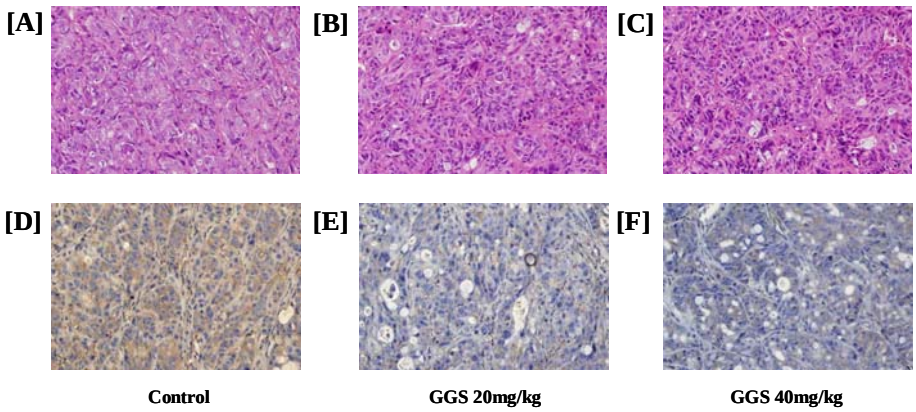

Figure 6. Guggulsterone decreases the levels of Bcl-2 in HT-29 xenograft mice. Viable HT-29 cells ($1x10^7$) were injected subcutaneously into the flanks of male nude mice. After the tumors reached 100-150 mm^3 in size, the mice received an intraperitoneal injection of guggulsterone (20 and 40 mg/kg) or vehicle (DMSO) daily for 2 weeks. After the final treatment, the mice were euthanized and the tumors were removed, fixed with formalin, and embedded in paraffin. Hematoxylin and eosin staining and immunohistochemical staining with Bcl-2 were preformed thereafter. Magnification: x20.

Ⅳ. DISCUSSION

Despite recent advances in understanding the carcinogenesis of colorectal cancer, the increasing incidence and relatively low remission rate of chemotherapy have spurred researchers to develop more effective and safer treatment regimens by adopting novel and innovative approaches. The discovery and use of active medicinal compounds from natural sources has provided alternative treatment choices. Guggulsterone has been reported to have potent anti-tumor activities in some cancer cells, including prostate cancer,[22, 23] lung cancer, acute myeloid leukemia, and breast cancer cells.[24, 25] In addition, recent studies have revealed that guggulsterone inhibits angiogenesis *in vitro* and *in vivo* in human prostate cancer cells[26] and suppresses NF-κB activation in leukemia, breast, and multiple myeloma cells.[16,27] However, it was not previously determined whether guggulsterone modulates the apoptotic pathway in colon cancer cells. Moreover, it was not known whether guggulsterone has *in vivo* anti-cancer effects regardless of cancer type, including colorectal cancer. In the present study, we demonstrated the anti-cancer effect of guggulsterone via the induction of apoptosis in colorectal cancer cells both *in vitro* and *in vivo*. Given the central role of apoptosis in cancer treatment, the ability of guggulsterone to inhibit colorectal cancer supports the clinical utility of this agent as an anti-cancer drug for colorectal cancer. To the authors' knowledge, this is the first report that clearly characterizes the anti-tumor properties of guggulsterone in colon cancer cells and tumor xenografts.

It is important for an effective chemotherapeutic agent to cause irreversible death in cancer cells, i.e., apoptosis. Two pathways regulate the onset of apoptosis. The primary apoptotic cell death pathway, termed the "intrinsic", "mitochondrial-mediated", or "Bcl-2-regulated" pathway, initiates caspase-dependent signals that regulate the role of the Bcl-2 family.[28-31] The Bcl-2 family is composed of pro-apoptotic and anti-apoptotic members. The anti-

apoptotic proteins, including Bcl-2 and Bcl-xL, inhibit apoptotic cell death by preventing the release of mitochondrial cytochrome c. Pro-apoptotic proteins, including Bax and Bid, induce the intrinsic cell death pathway which culminates in apoptosis.[21, 32, 33] The crucial checkpoint in the intrinsic apoptotic pathway is the serial activation of caspases. This is mediated by a mitochondrial protein, Diablo, which is released into the cytosol by depolarization. Diablo exerts a pro-apoptotic role by binding to the inhibitor of apoptosis protein (IAP).[34, 35] In the present study, we first examined whether the anti-tumor effects of guggulsterone are related to apoptosis in human colon cancer cells. We showed that guggulsterone significantly suppressed cell viability in colon cancer cells and found that the decreased cell viability of guggulsterone-treated colon cancer cells was related to apoptosis. Moreover, nuclear chromatin condensation has been demonstrated in HT-29 cells that were treated with guggulsterone. Furthermore, we sought to gain insight into the mechanism of apoptosis induction by guggulsterone. The observation of caspase-3 activation also confirms that the promotion of apoptosis by guggulsterone involves a caspase-dependent pathway. Expression of members of the Bcl-2 family was also examined in guggulsterone-treated cells. We observed down-regulation of Bcl-2 expression; there was no change in the expression of the pro-apoptotic member Bax. These findings suggest that induction of apoptosis in guggulsterone-treated HT-29 cells is associated with a caspase-dependent cascade that involves activation of the mitochondrial pathway initiated by the inhibition of Bcl-2. More tests should be conducted to better characterize guggulsterone-induced apoptotic signaling. Non-transformed intestinal epithelial cells (IEC-18 cells) appear resistant to growth inhibition and apoptotic induction by guggulsterone. The selective activity of guggulsterone towards cancer cells warrants further preclinical and clinical evaluations of its efficacy against colorectal cancer. Further studies are needed to elucidate the mechanistic basis of the differential

responses to guggulsterone of normal cells and cancer cells. The extrinsic (death receptor) pathway is the second apoptotic death pathway and is mediated by members of the tumor necrosis factor receptor (TNF-R) family, including Fas and TNF-R1, which contain the intracellular death domain.[18, 19, 36] Ligands binding to these death receptors trigger the recruitment and activation of caspase-8 through the adaptor protein Fas-associated death domain.[18, 19] Caspase-8 then activates caspase-3 and other executioner caspases that systematically mediate apoptosis.[37, 38] Our results show increased levels of Fas and cleaved caspase-8 in guggulsterone-treated colon cancer cells, demonstrating that guggulsterone potentiates Fas-mediated apoptosis. In certain types of cells, there is cross-talk between the mitochondrial-mediated and the death receptor-mediated apoptotic pathways.[18, 19, 39] Bid is a pro-apoptotic BH3-only member of the Bcl-2 family that is an important component of Fas-induced apoptosis.[40, 41] Bid is cleaved and thereby activated by active caspase-8 to form truncated Bid (tBid).[41] Activated Bid acts on the mitochondria to orchestrate the release of cytochrome c and other cell death events culminating in apoptosis.[42, 43] Our results show that guggulsterone increased the amount of tBid in HT-29 cells in a dose-dependent manner, suggesting that increased cleavage of Bid to produce tBid is one of the reasons for directed apoptosis via the death receptor-mediated pathway. IAPs have also been reported to inhibit apoptosis because they function as direct inhibitors of activated effector caspase-3 and caspase-7. Furthermore, cIAP-1 and cIAP-2 can also inhibit cytochrome c-induced activation of caspase-9.[21, 43] Our results also demonstrated that guggulsterone treatment decreases the level cIAPs which, reciprocally, increases the activity of caspases in colon cancer cells. The c-Jun NH2-terminal kinase (JNK) signaling pathway is essential for apoptosis in response to excitotoxic stress.[44] The JNK signaling pathway may also influence mitochondrial stability and is associated with the Bcl-2 family of apoptotic

regulatory proteins through the phosphorylation and subsequent inactivation of the anti-apoptotic protein Bcl-2.[45-48] Another potential target of JNK signaling is Bid. Our results show that guggulsterone increased the level of phosphorylated c-Jun in HT-29 cells, leading to an increase in the level of phosphorylated JNK. In short, our results demonstrate that guggulsterone can act through both major apoptotic pathways in colon cancer cells.

Consistent with our results, several studies have recently been performed to understand the mechanisms by which guggulsterone inhibits cancer cell growth. For instance, guggulsterone-induced apoptosis was associated with induction of the pro-apoptotic family members Bax and Bak in prostate cancer cells, [22] consistent with our data. Moreover, guggulsterone inhibited cell proliferation and induced apoptosis through the activation of JNK, suppression of Akt (also known as protein kinase B), and down-regulation of anti-apoptotic protein expression in leukemia, head and neck carcinoma, multiple myeloma, lung carcinoma, melanoma, breast carcinoma, and ovarian carcinoma cells.[16, 24]

To test the physiological relevance of *in vitro* guggulsterone-mediated anti-tumor effects *in vivo*, the antitumor effects of guggulsterone were evaluated in HT-29 xenografted nude mice. Guggulsterone significantly inhibited tumor growth of HT-29 cells xenografted into mice without causing mortality, significant weight loss, or other noticeable major side effects. These observations are in agreement with our *in vitro* studies showing that treatment of HT-29 cells with guggulsterone results in a concentration-dependent induction of apoptosis. The promising antitumor activity coupled and lack of toxicity of guggulsterone suggests that it has the potential to be a novel therapeutic agent in colorectal cancer chemotherapy. Moreover, the present study shows that the *in vivo* guggulsterone-mediated suppression of HT-29 xenograft growth is accompanied by inhibition of Bcl-2. Thus, monitoring the expression levels of apoptotic regulatory molecules affected by guggulsterone

may be an alternative strategy for monitoring its effect on the primary stages of colon cancer treatment. Based on the above findings, it is reasonable to conclude that apoptotic induction is an essential event in the guggulsterone-mediated suppression of HT-29 cell growth *in vivo*. However, our observations should be confirmed in further animal studies to more definitely establish the *in vivo* relevance of the *in vitro* findings. Moreover, we used relatively high guggulsterone doses in treating the mice. If guggulsterone could be administered intravenously or into tumor feeding vessels, higher concentrations could be achieved with lower doses. Further dosing studies are also needed if we are to extrapolate our findings to humans.

Ⅴ. CONCLUSION

In conclusion, our data demonstrate that guggulsterone induces activation of the mitochondria-dependent pathway and the extrinsic pathway of apoptosis in colon cancer cells and inhibits the growth of HT-29 xenografts *in vivo*, which suggests that guggulsterone has the potential to become a valuable therapeutic agent for the treatment of colorectal cancer.

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ABSTRACT (IN KOREAN)

Guggulsterone이 대장암세포에서 세포사멸 유도와 마우스 대장암 유발모델에서의 암세포 성장 억제

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안 민 지

Guggulsterone의 항암효능에 대한 몇몇 암세포주를 이용하여 세포증식과 사멸에 대한 효능을 조사한 많은 연구가 있음에도 불구하고, guggulsterone이 인간의 대장암 세포증식 억제능 및 항암제에 관련된 기전연구는 아직 미비하다. 따라서 본 연구에서는 guggulsterone의 대장암 세포증식 억제효과와 각 단계에서 중요한 역할을 하는 biomarkers에 대한 영향을 조사하는 세포사멸의 기전연구인 in vitro 실험과 in vivo 대장암 모델에서 guggulsterone의 암 발생 및 억제효능을 조사하였다. Guggulsterone은 대장암 세포인 HT-29의 세포 증식을 유의적으로 억제하였고, 세포증식을 억제한 guggulsterone은 DNA condensation 증가와 apoptotic cell 수를 증가시켰다. Guggulsterone에 의해 유도된 apoptosis의 기전을 조

사해본 결과 Bcl-2 family는 mitochondria의 막 투과성 조절과 cytochrome *c* 방출을 제어하여 apoptosis를 조절 하는데 pro-apoptotic protein인 Bak에는 변화가 없었지만 truncated Bid를 48시간에 현저히 증가시켰으며, anti-apoptotic protein인 Bcl-2를 감소시켰다. Inhibitor of apoptosis protein (IAP)는 caspase 활성을 억제하여 apoptosis 작용을 억제한다. IAP family 중 cIAP-1에서 농도 의존적으로 단백질 수준이 감소했으며, cIAP-2는 24h에서만 감소하였다. Caspase는 apoptosis의 조절에 중요한 작용을 하는데 guggulsterone의 처리로 농도 의존적으로 pro-caspase-3, 9을 감소시키고 cleaved caspase-3를 증가시켰다. Guggulsterone은 death receptor로 알려진 Fas의 단백질 수중을 증가시켰으며, guggulsterone에 의해 활성화 된 death receptor pathway를 통해 유도 된 totoal caspase-8의 degradation 시키고 cleaved 된 caspase-8을 증가 시켰다. 또한 guggulsterone은 SAPK/JNK pathway에 중요한 역할을 하는 JNK와 c-Jun의 인산화를 증가시켰다. 대장암을 유발시킨 xenograft mouse모델에서는 guggulstetone을 투여하자 tumor 증식이 control과 비교하여 현저히 줄어들었고 면역염색에서는 guggulsterone을 투여한 군이 그렇지 않은 군에 비해 유의하게 Bcl-2발현이 감소함을 확인하였다.

따라서 본 연구의 결과, guggulsterone은 death receptor와 mitochondria-dependent pathway의 이 두 가지 경로를 통하여 인간의 대장암 세포의 apoptosis를 유도하였으며, guggulsterone에 의해 유도된 apoptosis가 xenograft mouse모델에서 tumor의 증식을 억제 하였음을 알 수 있었다.