Inhibition of Cellular Proliferation by p53 dependent Apoptosis and G2M Cell Cycle Arrest of *Saussurea lappa* CLARKE in AGS Gastric Cancer Cell Lines

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The root of *Saussurea lappa* includes sesquiterpene lactones such as costunolide and dehydrocostus lactone, and has been shown to be anti-tumorigenic with being used in traditional medicinal therapy in the Eastern Asia. However, the molecular basis of the effects of Saussurea lappa on fate of gastric carcinoma, which incur very frequently in the area, has not been well identified. In this study, the cytostatic effects of *Saussurea lappa* were examined using gastric AGS cancer cells. Cell viability was dramatically reduced by *Saussurea lappa*, in a dose-dependent manner. As time passed after its treatment, apoptotic population was increased and clearly showed G2-arrest. Being consistent, its treatment resulted in maintaining of G1 and S-phase cyclins D1, E, and A even until a significant apoptotic population was observed, for example, at 24 h after treatment. However, G2/M phase cyclin B1 was reduced even at 12 h after treatment. In addition, its treatment increased expression of p53, $p21^{Waf1}$ cyclin dependent kinase inhibitor (CKI), and Bax, resulted in cleavages of procaspase 3 and poly ADP-ribose polymerase (PARP), indicating that such G2 arrest- and apoptosis-related molecules are involved. Therefore, these suggest that extracts of Saussurea lappa root may be a safer and effective reagent to deal with gastric cancers either by traditional herbal therapy or combinational therapy with conventional chemotherapy.

Key words : *Saussurea lappa*, p53, Apoptosis and G2M Cell Cycle Arrest

Introduction

The dried root of Saussurea lappa Clarke has been traditionally used for abdominal pain and tenesmus as a traditional medicine in Korea, China, and Japan. Sesquiterpene lactones including costunolide and dehydrocostus lactone are major components of the root and have been shown to possess various biological activities, including anti-tumor, anti-ulcer, anti-inflammatory, neurocytotoxic and cardiotonic activities[1]. Costunolide has been shown to have preventive effects on intestinal carcinogenesis, via pro-apoptotic effects of costunolide[2,3].

Cell proliferation is a tightly controlled process consisting of multiple checkpoints responsible for regulation of abnormal cell cycle progression. Transitions between G1, S, and G2/M phases are regulated by biochemically-coordinated actions of cyclins, cyclin-dependent kinases (CDKs), CDK inhibitors (CKIs), all of which can in turn be modulated by diverse intracellular signal transduction emanated from extracellular growth cues[4]. So far, the G1 to S phase transition through the restriction (R)point or S phase entry has been shown to be regulated by mitogenic reagents and intact cytoskeletal network integrity and cell adhesion as well[5-9]. In addition to G1-checkpoint, there have been evidenced that cellular systems often adapt G2-check point or arrest to avoid division of cells with abnormal DNA synthesized or damaged[10-12]. One of the molecules involved in the cell growth checkpoint include p53 protein, which induces cell cycle regulators including $p21^{Waf1}$ CKI[12]. In turn, $p21^{Waf1}$ CKI inhibits CDC2-cylinB complex, leading to G2-arrest[11].

However, there has been no clear consensus on the specific link between development of gastric cancer, a major cancer in Asian people, and Saussurea lappa extracts, and the molecular basis of the effects of Saussurea lappa extracts on cell proliferation or apoptosis has not been precisely and fully identified. Our growing knowledge regarding gastric cancer and medicinal herbs can have an ever greater impact on clinical management. Characterization and development of

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reagents to cope with gastric cancer, as efforts for medicinal herbal therapy or combination therapy with conventional chemotherapy, may help medical curersto predict the likelihood of response of gastric cancer patients to herbal medicine therapy or combination therapy, and thus to enhance the survival rate and quality of their lives.

In this study, we have tried to explore how Saussurea lappa extracts influence growth of AGS gastric cancer cells at molecular levels. We found that the extracts of Saussurea lappa root induced apoptosis of gastric cancer cells via activations of pro-apoptotic molecules including Bax, and caspase3 and suppression of anti-apoptotic Bcl_2. In addition, treatment of Saussurea lappa extracts resulted in also G2-growth arrest, presumably which involves p53 and $p21^{Waf1}$ CKI induction and concomitant reduction of cyclin B1.

Materials and Methods

1. Preparation of medicinal herb extracts

The raw extract of Saussurea lappa root was prepared by sonication of dried roots of the plant in 80% ethanol, following by a freeze-drying process of the ethanol extract. The powder form of the extract was dissolved in RPMI 1640 medium (Life Technologies, Inc.) to 10 mg/ml, vortexed at room temperature for 1 min, and incubated at 37°C for 1 hr while rotating before use. This solution was centrifuged at 12,000 rpm for 5 min to remove any insoluble ingredients. The supernatant was passed through a 0.22-μm filter for sterilization and diluted with RPMI 1640 culture medium to final concentrations of 6.25 ~1,000 μg/ml Saussurea lappa extract.

2. Cell Culture

A human gastric cancer cell line, AGS, was purchased from ATCC and grown in RPMI 1640 (Life Technologies, Inc., Rockville, MD) containing 10% FBS (Hyclone Laboratories, Inc., Logan, UT) and 1% gentamicin in a 5% CO_2 humidified atmosphere. Subconfluent monolayers of cells were employed in all experiments.

3. Growth Inhibition Assay

To determine the inhibition effect of Saussurea lappaextracts on proliferation of AGS cells, the percentage of growth inhibition was determined by measuring MTT dye absorbance of viable cells in the absence or presence of Saussurea lappa extracts. Ten thousand cells per well were seeded onto a well of 96-well plates (Nunc, Roskilde, Denmark) for 24 h, treated with various concentrations of Saussurea lappa extracts, and incubated for 3 days at 37℃. Subsequently, 50 μl of MTT (Sigma) at a concentration of 2 mg/ml was added to each well, and cells were incubated for an additional 4 h at 37℃ The supernatant was aspirated, and 150 μl of DMSO were then added to the wells to dissolve any precipitate present. The absorbance was then measured at a wavelength of 570 nm using an ELX800 microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). The IC_{50}'s were calculated assuming the survival rate of untreated cells to be 100%.

4. Annexin V staining

Cells were untreated or treated with Saussurea lappa extracts for 48 h at 100 ug/ml, prior to flow cytometric determination, as explained previously[13].

5. Flow CytometricCell Cycle or DNA Content Analysis

A total of 5×10^5 cells were seeded in 60mm dishes and incubated for 24 h at 37℃. Saussurea lappa extracts at indicated various concentrations was directly added to the dishes and incubated for an additional 24, 48, or 72 hrs. During harvests, both cells detached (probably apoptotic) and adherent were combined, fixed by addition of 4ml 70% ethanol, and stored at -20℃ at least 30 min. Cells were then pelletted, washed twice with ice-cold PBS, incubated in PBS containing 10 g/ml of RNase A (Sigma) for 15 min at 37℃, and stained with 10 g/ml of propidium iodide (PI). The relative DNA content per cell of samples was obtained by measuring the fluorescence of PI that bound stoichiometrically to DNA. The cell cycle was analyzed using a FACStar flow cytometer (Becton Dickinson, San Jose, CA) and a ModFit LT V2.0 computer program.

6. Western Blot Analysis

AGS cells in 100 mm dishes were treated with or without Saussurea lappaextracts for indicated periods. After incubation, cells were washed with ice-cold PBS and lysates were prepared using a lysis buffer containing 20 mM Tris-Cl (pH 7.4), 100 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 5 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM pepstatin A, 0.1 mM antipain, 0.1 mM chymostatin, 0.2 mM leupeptin, 10 μg/ml aprotinin, 0.5 mg/ml soybean trypsin inhibitor, and 1 mM benzamidine. After incubation of the lysates on ice for 30 min, whole cell extracts were cleared by a centrifugation at 13,000 rpm for 20 min. Twenty g of protein were fractionated by SDS-PAGE denaturing gels and transferred onto a nitrocellulose membrane. The membrane was blocked for 1 h in the 20 mM Tris-buffered saline (TBS) buffer containing 5% skim milk and 0.1% Tween 20 and then probed with specific antibodies for indicated molecules. The protein was detected

using chemiluminescence method (Amersham Pharmacia Biotech) followed by autoradiography.

7. Data Analysis

Results shown are representative of at least three independent experiments performed in triplicate and are presented as the means ± standard deviation (SD).

Results

We have had interests in understanding of anticancer activity of Saussurea lappa extracts on a gastric cancer cell line, AGS cells, with special emphasis on its effects on apoptosis and cell cycle arrest pathway.

We first determined the anti-proliferative activity of Saussurea lappa extracts by measuring cell survival rate after its treatment at diverse concentrations ranging from 10ug/ml to 1 mg/ml to AGS cells, using a MTT assay. When cell survival was determined after treatment of Saussurea lappa extracts for 3 days, cells showed a dose-dependent inhibition of cell viability, in which most cells were not vital at 500 ug/ml. The curve fitting of the survival rate showed an IC_{50} of 79ug/ml(Fig. 1).

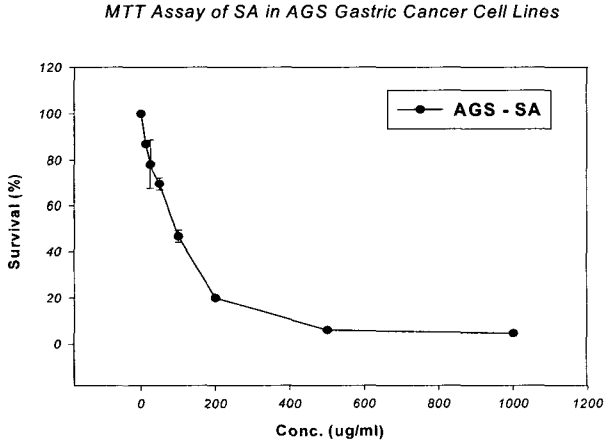
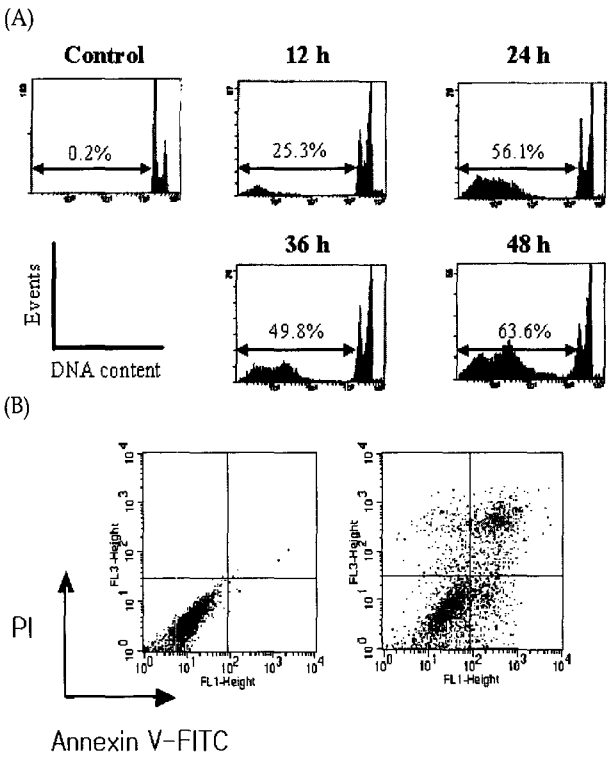


Fig. 1. Saussurea lappa-mediated inhibition of cell viability in gastric AGS cells. AGS cells seeded to wells of 96 well plates in the presence of normal serum containing media at 1.0 x 10^4cells/well. Twenty four hours later, cells were treated with indicated concentrations of Saussurea lappa extracts for further 72 hrs. Then MTT assay was performed as explained in Material and methods. Data shown is representative from three independent experiments.

Therefore, it suggests that Saussurea lappa extracts could induce growth inhibition of gastric cancer cells such as AGS cells, in addition to other type cancer cells previously reported including intestinal carcinoma cells[3] and leukemia[14].

Next we tried to determine if Saussurea lappa extracts induced apoptotic cell death of AGS cells, using flow cytometricquantitation of cell population with subG1 DNA content, as time passed after treatment of Saussurea lappa extracts at 100 ug/ml and propidium iodide staining. A shownin figure 2A, cells in control condition showed no significant apoptotic population, whereas as time passed after the treatment, more cells were becoming rapidly apoptotic. In the condition where the Saussurea lappa extracts was treated for 48 h at 100ug/ml, about 64% cells were apoptotic. In addition, via another approach of annexin V staining, apoptotic population under the same treatment condition was obvious and about 37% (Figure 2B), although the percentage was lower than that by apoptotic population determination via subG1 DNA content analysis probably due to different sensitivities between the two methods. Interestingly, in condition under treatment of Saussurea lappa extracts, cells survived showed more population in G2/M cell cycle phase, indicating that treatment of Saussurea lappa extracts involved G2/M arrest, probably before apoptosis (Fig. 2A). Therefore, we analyzed population of cells at each cell cycle phase among cells survived after treatment of Saussurea lappa extracts (that is, cells with DNA contents of n or 2n). Compared to an untreated control, G1 phase populations showed a clear trend of reduction as time passed, whereas S phase populations was maintained (or slightly increased at best) until 36 h treatment but was declined at 48 h treatment (Fig. 2C). However, in treated conditions, G2/M phase populations were significantly increased as treatment time passed, indicating that cells were undergoing G2-arrest by the treatment (Fig. 2C).



(C)

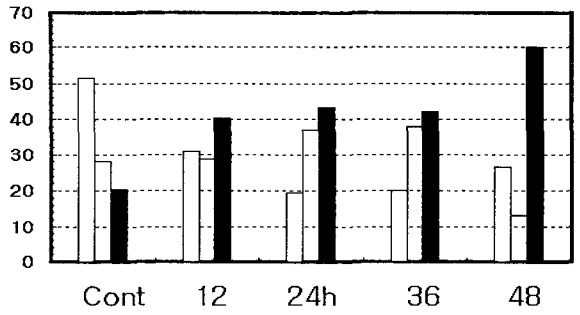


Fig. 2. Treatment of Saussurea lappa extracts induced G2-arrest and apoptosis. (A) Saussurea lappa extracts induced G2-arrest and apoptosis. Cells in 60 mm culture dishes were treated with Saussurea lappa extracts at 100 ug/ml for indicated periods. The treatment was done by a direct addition of Saussurea lappaextract solution into culture media. The solution was made of RPMI1640 culture media as explained in the Materials and methods. After incubation, cells floating and adherent were harvested and combined before PI staining and flow cytometric analysis for subG1 population and cell cycle as explained in the Materials and methods. Shown data are representative from three independent experiments. (B) Apoptotic population determination via annexin V staining after Saussurea lappa treatment. Cells were untreated (left histogram) or treated (right histogram) with Saussurea lappa extracts at 100 ug/ml for 48 h, prior to flow cytometric annexin V staining, as explained previously [13]. Data shown was representative from two independent analyses. (C) Cell cycle analysis by ModFit software was done from normally DNA-containing population in each condition; gating to include cell population with DNA content of n and 2n but not subG1 was performed, prior to analysis of cell cycle population in each condition in (A).

The G2-arrest by Saussurea lappa extracts was confirmed by another approach, directly measuring expression levels of cyclins after treatment of Saussurea lappa extracts. As shown in fig. 3, cyclins D1 and E were not decreased but rather increased by the treatment, indicating that no G1-arrest occurred. Meanwhile, S-phase and G2/M-phase cyclins, A and B1, were generally decreased, indicating again that cells after treatment of Saussurea lappa extracts could not go through mitosis, leading to growth arrest at G2/M phase (Fig. 3).

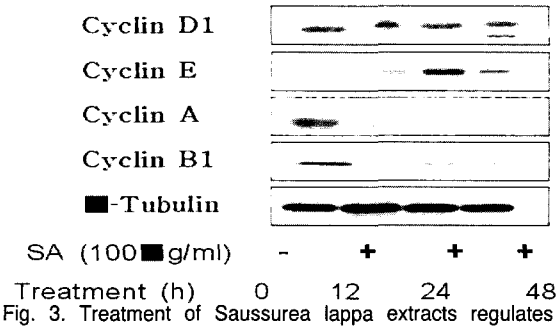


Fig. 3. Treatment of Saussurea lappa extracts regulates cyclins levels. Cells in 100 mm culture dishes were treated with Saussurea lappa extracts at 100 ug/ml for indicated periods. After treatment, cells were washed twice with ice-cold PBS and then lysates were prepared using a RIPA lysis buffer. Lysates normalized to have equal protein amounts were used immunoblots by SDS-PAGE using primary antibodies against indicated molecules, as described in the Materials and methods. The data are representative of at least three isolated experiments.

Previously it was shown that p53 activation and thus induction of $p21^{Waf1}$ CKI is involved in G1 and G2-arrest as well in case of DNA damage. Therefore, we have tried to examine whether both p53 and $p21^{Waf1}$ CKI might be induced by Saussurea lappa treatment. When levels of the both were biochemically measured, it was clearly shown that p53 and $p21^{Waf1}$ CKI were increased as apoptotic population was observed by treatment of Saussurea lappa extracts (Fig. 4 and 2A).

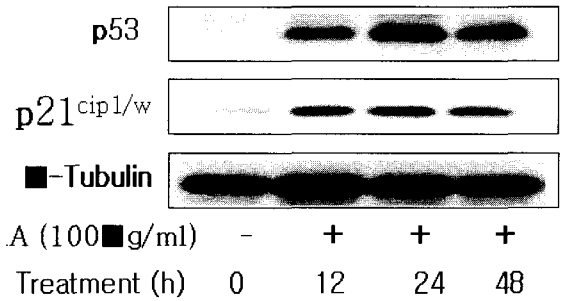


Fig. 4. Treatment of Saussurea lappa extracts induces p53 and $p21^{Waf1}$ CKI. Lysates prepared as explained above were used for Western blots using primary antibodies against indicated molecules. Data shown is representative from three independent observations.

Therefore, it may be suggested that treatment of Saussurea lappa extracts might lead to activation of p53 and then induction of $p21^{Waf1}$ CKI, resulting in G2-arrest and apoptosis. When we next checked levels of certain pro-apoptotic or anti-apoptotic molecules, their levels were well-correlated with the apoptotic trend of AGS cells induced by treatment of Saussurea lappa extracts. That is, the expression level of anti-apoptotic molecules such as Bcl2 was decreased gradually, whereas a pro-apoptotic molecule such as Bax, opposing the action of Bcl2, was increased by treatment of Saussurea lappa extracts (Fig. 5). Furthermore, cleavages of procaspase 3 and PARP were obvious, although we failed to detect active forms of caspase3 and PARP cleavage (at 48 h after treatment), indicating that thereby their activation might target other cellular proteins with leading to apoptosis.

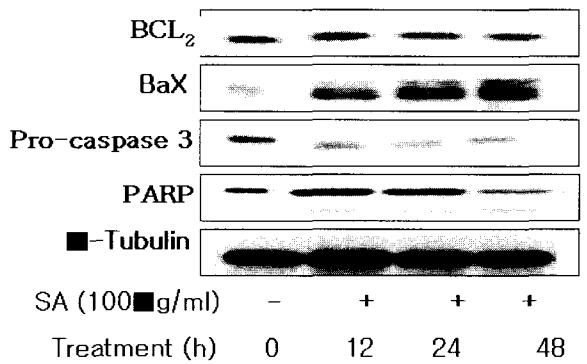


Fig. 5. Treatment of Saussurea lappa extracts resulted in increase or activation of pro-apoptotic molecules and decrease of anti-apoptotic molecules. Lysates prepared as explained above were used for immunoblots using primary antibodies against indicated molecules. Data shown are representative from at least 3 independent experiments.

Taken together, Saussurea lappa extracts, a traditional medicine used in the Eastern Asia frequently with purposes to take care of cancers, was shown to induce G2-arrest of AGS gastric cancer cells probably due to modulation of cyclin levels through p53/p21^{Waf1} CKI induction, and concomitant programmed cell death due to activation of pro-apoptotic molecules and suppression of anti-apoptotic molecules. This indicates that Saussurea lappa extracts can be a candidate for therapeutic reagents at least against gastric cancer.

Discussion

The information generated from this study would help us to develop human clinical trials using medicinal herbs in the future. We observed that treatment of Saussurea lappa extracts induced G2-arrest and apoptosis of AGS gastric cancer cells, probably via induction of p53/p21^{Waf1} CKI, induction and activation of pro-apoptotic molecules, and concomitant suppression of anti-apoptotic molecules, indicating a potency of Saussurea lappa extracts as a traditional anti-tumor therapeutic reagent.

Cell cycle checkpoints are available for cell to be repaired from cellular damages including DNA damages, to dissipate exogenous cellular stress signals, and to look for growth factors outside of cells. In most cases, checkpoints may result in activation of programmed cell death (apoptosis) signaling, if cellular damages are too serious to be properly repaired. Therefore, defects in cell cycle checkpoints and apoptosis would results in tumorigenesis[15]. We observed in cell cycle analysis that treatment of Saussurea lappa extracts resulted in apoptosis and G2/M-arrest as well. Simultaneously we also observed that cyclins D1 and E were increased until 24 h, and S-phase cyclin A was increased until 12 h, although they were gradually declined afterward, indicating a traverse to S-phase cell cycle. However, treatment of Saussurea lappa extracts showed a decrease in the level of G2/M phase cyclin B1 even an earlier time of 12 h after the treatment, indicating that no mitosis might occur. It was also shown that the treatment resulted in induction of p53 and p21^{Waf1} CKI, which are known to be important for G1-arrest and G2-arrest as well. Previously evidences were reported that p53 protein induced cell cycle regulators including p21^{Waf1} CKI[12], which in turn initially inhibits Cdc2-cylinB1 complex and subsequently reduce cyclin B1 and Cdc2 protein levels, leading to G2-arrest [11,16]. In addition to these mechanisms involving p21^{Waf1} CKI, p53-dependent G2 arrest involves transcriptional up-regulation of additional downstream targets including 14-3-3, which modulates the subcellular localization of the cyclin B1/Cdc2 complex[17], and GADD45[18]. In other words, p53 contributes to a sustained G2-arrest through cyclin B1/Cdc2 inhibition by co-localization of the complex with either nuclear p21^{Waf1} CKI or cytoplasmic 14-3-3

In addition to arresting cells at G2-phase, treatment of Saussurea lappa extractsresulted in significant apoptosis as time passed after the treatment. Currently it is not clear whether treatment of Saussurea lappa extracts mighttrigger the death signal through cell membrane-based receptors or not. However, it is clear that apoptosis mediated by the treatment involves activation of caspase(s), as we observed cleavage of procaspase 3. We also observed alteration in levels of Bcl2 and Bax proteins. It is well-known that Bcl2 and Bax family proteins are acting oppositely leading to regulation of activation of caspases[19], and that p53 can regulate induction of the both[20,21]. Bcl_2 is anti-apoptotic and down-regulated by p53, whereas Bax is pro-apoptotic and induced by p53[22-24]. Bax facilitates the release of apoptosis-inducing factor and cytochrome c from the mitochondria, leading to activation of the caspase cascade[25]. Once caspases are activated, diverse cellular proteins are cleaved by their actions, leading to defects in their signaling and/or structural functions and thereby cell death[26]. In this current study, such a relationship is well-consistent in terms of that treatment of Saussurea lappa extracts resulted in induction of p53 and Bax but a concomitant reduction in Bcl_2 expression, leading to apoptosis. Therefore, the cytotoxic effects of Saussurea lappa extracts can be suggested to involve G2-arrest and apoptosis, probably via induction of p53. The p53-mediated G2 checkpoint control and apoptosis are potentially important determinants of tumor sensitivity to DNA damage. Previously it has been demonstrated by many studies showing that, when cells were exposed to combinations of DNA damaging agents and checkpoint-inhibitory drugs, p53 is inactivated and G2 checkpoint control is relaxed, leading to enhanced cytotoxicity. This suggests the possibility that such combinations could be used therapeutically to target tumor cells specifically, since p53 function is compromised or absent in diverse human tumors including gastric cancer[27]. Therefore, observations from studies including this current one may have an important clinical implication as a medicinal herbal therapy to take care of gastric cancers. In addition, combinational therapy with conventional chemotherapy to deal with gastric cancers may be a safer way to extend the survival time of gastric cancer patients and improve qualities of their lives.

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