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Original Article

Effects of Costunolide Derived from *Saussurea lappa* Clarke on Apoptosis in AGS Stomach Cancer Cell Lines

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Costunolide is an active sesquiterpene lactone isolated from the root of *Saussurea lappa* Clarke and is known to exhibit a variety of biological activities, including anti-carcinogenic and anti-inflammatory effects. Nevertheless, the pharmacological pathways of costunolide have not yet been fully elucidated. In this study, its cytotoxic effects were examined using AGS gastric cancer cells. Its treatment resulted in apoptosis in a dose- and time-dependent manner. The effects were attributed to the regulation of pro-apoptotic molecules and suppression of anti-apoptotic molecules. These results suggest that costunolide may be a candidate to deal with gastric cancers by chemopreventive agents.

Key Words : Costunolide, apoptosis, cell proliferation

Introduction

Costunolide is a well-known sesquiterpene lactone contained in *Saussurea lappa*, having been traditionally used for abdominal pain and tenesmus as a traditional medicine in Korea, China, and Japan. Sesquiterpene lactones1 have shown to possess various biological and pharmacological activities including antimicrobial[2-5)], anti-inflammatory6, and antiulcer effects[7)].

Recent studies reported that costunolide exhibited cytotoxic effects on various human cancer cells, including carcinoma and leukemia cells[8)], was considered a potential chemopreventive agent for colon tumorigenesis[9,10)], and inhibited angiogenic response by blocking the angiogenic factor signaling pathway[11)].

Cell proliferation is a tightly controlled process consisting of multiple checkpoints responsible for the 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), and CDK inhibitors (CKIs), all of which can in turn be modulated by diverse intracellular signals transduced from extracellular growth cues[12)].

In this study, we tried to explore how costunolide influences cell proliferation, programmed cell death and the cell cycle in AGS gastric cancer cells at the molecular level. We found that treatment with costunolide in AGS gastric cancer cells induced apoptosis in a p53-dependent and caspase-dependent manner via activation of pro-apoptotic molecules including Bax, caspase

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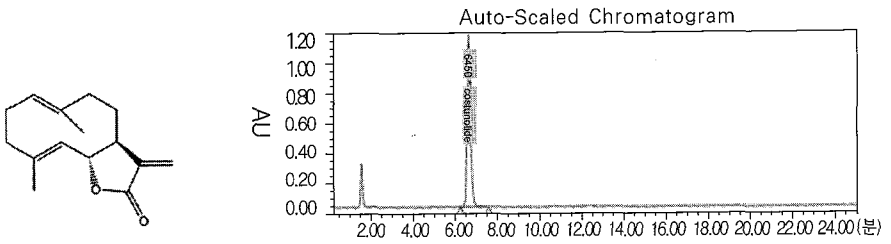

Fig. 1. The Structure and identification of costunolide
(a) The chemical structure of costunolide.
(b) The HPLC histogram of the costunolide purchased from Wako with a peak identified.

3, and suppression of anti-apoptotic Bcl_2. However, contrary to our expectations, we didn't confirm cell cycle arrest in G1 or G2 phase. Differently from RIE cells, we showed that cellular accumulation in G1 or G2 phase, in AGS cells, was more distinct at the 10 μ M than 5 μ M concentration of costunolide.

Materials and Methods

1. Materials

Costunolide, an active sesquiterpene lactone of medicinal herbs, *Saussurea lappa*, (Fig. 1A) was purchased from Wako Pure Chemicals Ind. Co. (Japan, Cat. No. 032-13731). For confirmation of the compound, HPLC analysis was performed with Breeze™ HPLC systems with a Waters 2487 Dual Wavelength Absorbance Detector (Waters Corp., Milford, MA). For all assay purposes, the compound was dissolved in dimethyl sulfoxide, and the control mixtures contained sol vent equivalent to the drug-treated mixtures.

2. Cell culture

A human gastric cancer cell line (AGS) purchased from ATCC or a normal epithelial cell line from rat intestine (RIE) was grown in RPMI 1640 or D MEM-high glucose (Life Technologies, Inc., Rockville, MD) containing 10% FBS (Hyclone

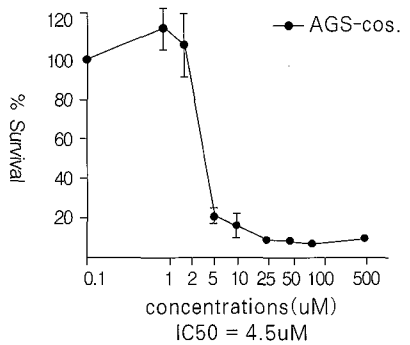

Fig. 2. Cytotoxic effects by the treatment of costunolide on the AGS gastric cells AGS cells were seeded to wells of 96 well plates in the presence of normal culture media at 1.0 × 104 cells/well. Twenty four hours later, cells were treated with indicated concentrations of costunolide for additional 72 hrs. Then MTT assay was performed as explained in Material and Methods. Data shown is representative from three independent experiments, in which each condition was in triplicate and also shown in log scale. Data are shown in mean standard deviation (SD).

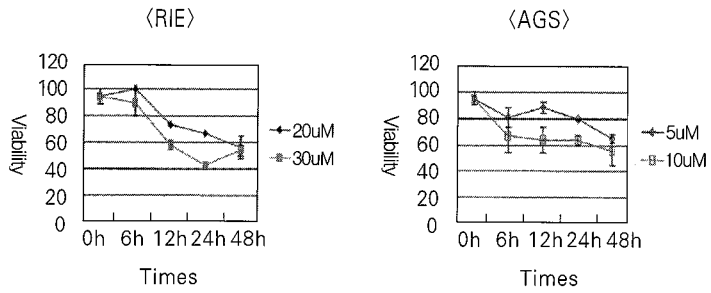

Fig. 3. Effects of costunolide on cellular growth in RIE and AGS cells RIE and AGS cells were treated with DMSO (as control) and 20, 30 μM in RIE and 5, 10 μM of costunolide in AGS for the indicated times. The viable cells were determined by trypan blue dye exclusion assay. Data presented as means±SD of three independent experiments

Laboratories, Inc., Logan, UT) and 1% gentamicin in a 5% CO_2 humidified atmosphere. Sub-confluent monolayers of cells were used in all experiments.

3. Cell viability

To determine the inhibition effect of costunolide on proliferation of cells, cell viability was analyzed by measuring MTT dye absorbance of viable cells in the absence or presence of costunolide, and viability was also assessed by morphology analysis using the trypan blue exclusion assay and an inverted phase-contrast microscope (Leitz, Wetzlar, Germany).

In MTT assay, ten thousand cells per well were seeded into 96-well plates (Nunc, Roskilde, Denmark) for 24 hrs, treated with various concentrations of costunolide, and incubated for 2 days at 37℃. Subsequently, 50 ㎕ of MTT (Sigma) at a concentration of 2 ㎎/㎖ was added to each well, and cells were incubated for an additional 4 hrs at 37℃. The supernatant was aspirated, 150 ㎕ of DMSO was then added to the wells, and then absorbance at a wavelength of 570 nm was

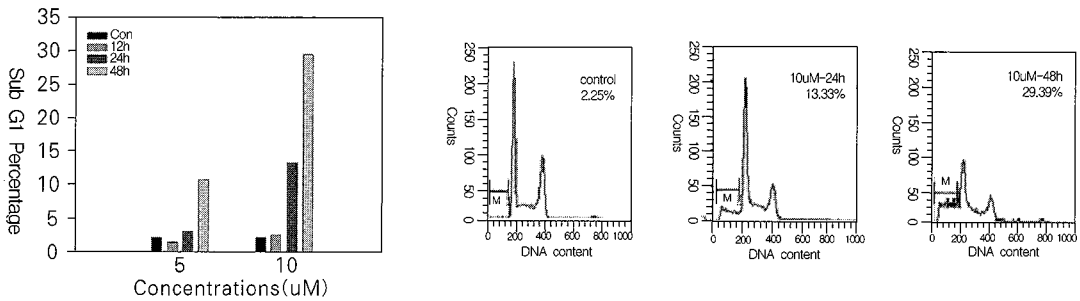

Fig. 4. Treatment with costunolide induced apoptosis in Flow cytometric analysis-Costunolide-induced sub-G1 population. Cells in 60 mm culture dishes were treated with 10 μM costunolide for indicated periods. The treatment was done by a direct addition of costunolide solution into culture media. After incubation, cells floating and adherent were collected and combined before PI staining and flow cytometric analysis for DNA contents, as explained in Materials and Methods. Shown data are representative from three independent experiments. (A) histogram of sub-G1 population at various times. (B) FACS diagram of sub-G1 population at various times.

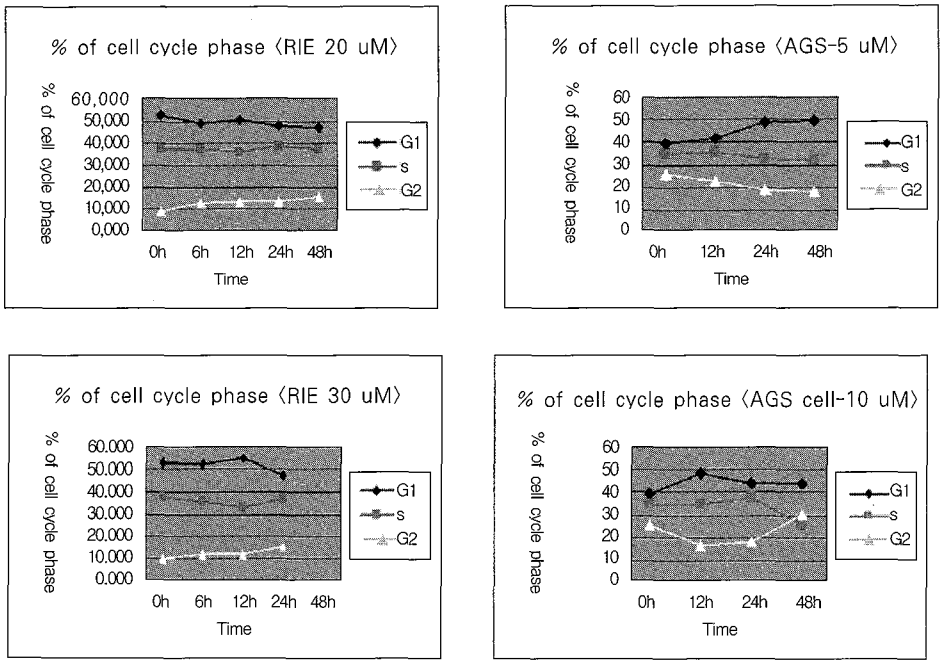

Fig. 5. Non-sub-G1 DNA contents induced by the treatment with costunolide-Cell cycle analysis by ModFit software was done using cells with non-sub-G1 DNA contents (i.e., normally DNA-containing population) in each condition; gating to include cell population with DNA content of n and 2n but sub-G1 was not performed, prior to analysis of cell cycle population

measured using an ELX800 microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). The IC50 was calculated by setting the viability of untreated cells at 100%, expecting that cell death in control cells would be negligible.

In trypan blue exclusion assay, cells in the exponential growth phase were plated at 4×10^4 cells/well in 24-well plates, then after attachment overnight, the medium was changed and the cells were exposed to drugs or an equivalent amount of dimethyl sulfoxide (0.1% v/v). After treatment with costunolide for 0 hr, 6 hrs, 12 hrs, 24 hrs, and 48 hrs in RIE (20 μ M, 30 μ M) and AGS (5 μ M, 10 μ M), attached cells were trypsinized and incubated in a 0.5% trypan blue solution for 10 min at room temperature. Blue labeled cells were considered as nonviable whereas unlabeled cells were regarded as viable.

4. Flow Cytometric Cell Cycle or DNA Content Analysis

A total of 5×10^5 cells were seeded in 60 mm dishes and incubated for 24 hrs at 37℃. Costunolide at the various concentrations indicated was directly added to the dishes and incubated for an additional 24, 48, or 72 hrs. After the incubation, both detached (probably apoptotic) and adherent cells were combined, fixed by addition of 4 ㎖ of 70% ethanol, and stored at -20℃ for at least 30 min. Cells were then pelletted, washed twice with ice-cold PBS, incubated in PBS containing 10 ㎍/㎖ of RNase A (Sigma) for 15 min at 37℃,

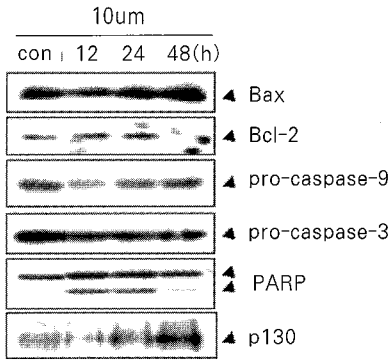

Fig. 6. Treatment with costunolide induced apoptosis in Western blot analysis
Treatment with costunolide resulted in an increase or activation of pro-apoptotic molecules and a decrease of anti-apoptotic molecules. Lysates prepared as explained above were used for immunoblots using primary antibodies against the indicated molecules.

and stained with 10 ㎍/㎖ of propidium iodide (PI). The relative DNA content per cell of the samples was obtained by measuring the fluorescence of PI that bound to DNA. The cell cycle was analyzed using a FACStar flow cytometer (Becton Dickinson, San Jose, CA) and ModFit LT V2.0 software.

5. Western Blot Analysis

AGS cells in 100 mm dishes were treated with or without costunolide for the indicated periods. After incubation, cells were washed with ice-cold PBS and lysates were prepared by 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 ㎍/㎖ aprotinin, 0.5 ㎍/㎖ soybean trypsin inhibitor, and 1 mM benzamidine. After incubat-

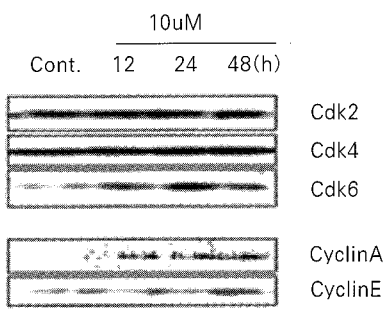

Fig. 7. Treatment with costunolide doesn't regulate cyclin levels
Costunolide treatment of AGS cells didn't affect CDK's cyclin's expression, which means costunolide didn't affect G1 or G2 cell cycle accumulation. Cells in 100 mm culture dishes were treated with 10 μM costunolide for indicated periods. 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 for immunoblots by SDS-PAGE using primary antibodies against the indicated molecules, as described in Materials and Methods.

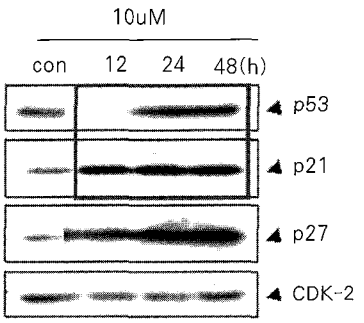

Fig. 8. Treatment with costunolide induced CKIs expressions
Treatment with costunolide resulted in an increase or activation of p53, p21 and p27 CKIs. Up-regulation in p27Kip1 CKI level was especially strongly observed at 10 μM costunolide treatment for 24, and 48 hrs. Lysates prepared as explained above were used for immunoblots using primary antibodies against the indicated molecules.

ing the lysates on ice for 30 min, whole cell extracts were cleared by centrifugation at 13,000 rpm for 20 min. 20 μg of protein were resolved by SDS-PAGE denaturing gels and transferred onto a nitrocellulose membrane. The membrane was blocked for 1 hr in 20 mM Tris-buffered saline (TBS) buffer containing 5% skim milk and 0.1% Tween 20 and then probed with specific antibodies for the indicated molecules. Antibodies against Bax, Bcl_2, pro-caspase 9, pro-caspase 3, PARP, p130, and p53, p21, p27, CDK2 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and Cox 1, Cox 2 from Promega (Madison, WI). The protein was visualized by using an ECL chemiluminescence method (Amersham Pharmacia Biotech), followed by autora-diography.

6. Statistical analysis

Statistical analysis was performed by using Mann-Whitney test or Kruskall-Wallis test.

Results

1. HPLC analysis of costunolide

Our interest lay in examining the anticancer activity of costunolide on a gastric cancer cell line, AGS cells, with special emphasis on the effects of the compound on apoptosis and cell

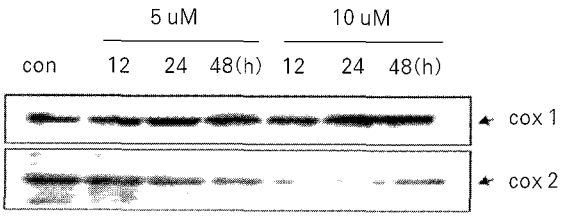

Fig. 9. Treatment with costunolide inhibited Cox 2 expression levels in Western blot analysis
Strong expressions of Cox 2 in AGS cells by treatment with 10 μM of costunolide had a relationship with inflammation. Lysates normalized to have equal protein amounts were used for immunoblots by SDS-PAGE using primary antibodies against the indicated molecules, as described in Materials and Methods

cycle related pathways. We first checked whether the compound purchased from Wako (Japan) really included sesquiterpene lactones, costunolide, by HPLC analysis. As shown in the Fig. 1B, the compound showed two peaks and we confirmed that the high peak was indeed costunolide.

2. Effects on cell viability

We evaluated the effects of costunolide on the growth of AGS human gastric cancer cells. Fig. 2 shows the numbers of viable and non viable cells after 48 hrs treatment with costunolide. The inhibition of cell growth was generally related to the concentration used, so we first checked how costunolide affected the viability of AGS cells, by quantitating the viable cells after treating cells with various concentrations ranging from 1 μ M to 500 μ M using MTT assay. There was a dose-dependent inhibition of cell proliferation. The apparent IC_{50} was determined to be 4.5 μ M (Fig. 2) when cell viability was determined after treatment with costunolide.

Meanwhile, parallel treatment of the compound to a normal epithelial cell line, rat intestine epithelial (RIE) cells, showed much weaker effects on inhibition of viability with an IC_{50} at about 30 μ M, but AGS cells showed the same inhibition of viability at a dose of 10 μ M (Fig. 3A and 3B) in the trypan blue exclusion assay. Therefore, costunolide could induce growth inhibition of gastric cancer cells such as AGS cells. In addition, this has already been reported for other types of cancer cells, including intestinal carcinoma cells[13] and leukemia[14].

3. Cell cycle analysis

We tried to determine if costunolide induced apoptotic cell death of AGS cells. Flow cytometric measurements of cells with sub-G1 DNA content were performed at various times after treatment with 5 μ M and 10 μ M of costunolide. As shown in Fig. 4A, untreated cells did not show any significant apoptosis at either dose, whereas cells were becoming rapidly apoptotic with time after treatment with the compound at both doses, and the effect of 10 μ M was more apoptotic than 5 μ M. When cells were treated with 10 μ M costunolide for 24 hrs and 48 hrs, about 13.3% and 29.3% cells were apoptotic independently (Fig. 4B).

Previously we reported that treated with *Saussurea lappa*, which has costunolide as an active compound, there were more cells in the G2/M cell cycle phase than in the untreated control[15]. Therefore, we thought that costunolide also might involve G2/M arrest, probably before apoptosis, so we analyzed the cell cycle phase of adherent cells after treatment with costunolide (i.e., cells with non-sub-G1 DNA contents) at different time points and doses using RIE and AGS cells. Compared to the untreated control, two cells in the G1 and G2 phase didn't show clear reduction trends over time in both doses in RIE and AGS cells. However, in AGS cells, only 5 μ M treatment cells with costunolide increased G1 phase time dependently (Fig. 5C), but not in 10 μ M treatment cells (Fig. 5D). S phase was not reduced specifically in RIE and AGS cells, but only in the 10 μ M of costunolide treatment group the S phase significantly decreased and G2 phase mildly increased, which showed that there were not any particular changes in any groups. This predominant population in the G2/M cell cycle phase showed mildly only at 48 hrs after treatment with costunolide, indicating that it was not certain cells underwent G2-arrest by this treatment (Fig. 5D).

4. Western blot analysis

When we checked levels of certain pro- apoptotic or anti-apoptotic molecules, their levels correlated

well with the apoptotic trend of AGS cells induced by treatment with costunolide. That is, the expression level of pro-apoptotic molecules, such as Bax, increased significantly at 48 hrs, whereas an anti-apoptotic molecule such as Bcl2, which opposes the action of Bax, also significantly decreased at 48 hrs by treatment with the compound. Furthermore, cleavage of procaspase 3, and loss of intact poly (ADP- ribose) polymerase (PARP) were obvious. Nevertheless, we failed to detect the cleaved products of pro-caspase 3 and PARP, probably due to extensive apoptosis leading to degradation of their cleaved products indicating that their activation could thereby lead to apoptosis.

We also studied the Rb2/p130, which have been identified more recently and are now considered structurally and functionally related to the RB protein. In our study, Rb2/p130 strongly expressed 48 hrs treatment of costunolide, in accordance with the recently report that Rb2/p130 negatively modulate the cell cycle (Fig. 6).

We checked the expressions of cell cycle related proteins of cyclins and cyclin-dependent kinases and could not find any clues to cellular accumulation in specific phases in cyclin E, A and CDK 2, 4, and 6 (Fig. 7). Our results did show that p53 activation and thus induction of $p21^{Waf1}$ are involved in both apoptosis and G1-arrest as well as in response to DNA damage, and expression of $p27^{Kip1}$ is involved specifically in G1-arrest. Therefore, we tried to examine whether p53, p21Waf1 and $p27^{Kip1}$ CKI might be induced by costunolide treatment. When the levels of the three CKIs were biochemically measured, it was clear that p53, p21Waf1 and $p27^{Kip1}$ CKI were increased by treatment with costunolide at 24 hrs time points that were coincident with the induction of apoptosis and G1-arrest. Therefore, costunolide might lead to activation of p53 and then induction of p21Waf1, and $p27^{Kip1}$ CKIs, resulting in apoptosis and G1-arrest (Fig. 8).

In Fig. 9, we checked Cox 2 expressions to investigate the relationship of inflammation. In this study, we found that 10 μ M costunolide treatment in AGS cells significantly inhibited the expression of Cox 2, but not in 5 μ M treatment. This result means that the inflammation-related pathway could have some effect of the inhibition of cellular proliferation in AGS cells. Further studies on this action could be investigated in another experiment, but were not this time.

Discussion

Sesquiterpene lactones have received considerable attention in pharmacological research, due to their anti-neoplastic and anti-inflammatory activity [16,17]. Costunolide has been demonstrated to inhibit the proliferation of various cultured human tumor cells, i.e. lung, ovary, colon and melanoma cancer cells[8], and has been taken into consideration for chemoprevention of colon carcinogenesis[9,10].

In this study, we tried to characterize the mechanism of costunolide's effect and evaluated the antiproliferative effect of costunolide using the AGS gastric cancer cells. *Saussurea lappa*, which has costunolide as main active compound, induced G2/M arrest and apoptosis in AGS gastric cancer cells in the previous results, so we tried to confirm whether G2/M arrest originated with costunolide or not.

The present study demonstrates that costunolide significantly inhibits the proliferative activity of AGS gastric cancer cells in a low cytotoxic fashion, which is worthy of attention, since the discovery of chemopreventive agents with low

cytotoxicity is an important research aim. However, the molecular mechanisms underlying the antiproliferative activity of costunolide are not well understood in AGS gastric cancer cells.

We observed that treatment of costunolide induced apoptosis of AGS gastric cancer cells, probably by both p53-dependent and caspase-dependent pathways involving induction of p53/p21^{Waf1} CKI, induction and activation of pro-apoptotic molecules, and concomitantly suppression of anti-apoptotic molecules. Meanwhile, the compound caused much less significant cytotoxic effects on normal rat intestinal epithelial (RIE) cells, indicating tumor cell specific effects. These results may suggest costunolide as a candidate for anti-tumor therapeutic reagent.

Cell cycle checkpoints are available to allow cellular repair when cells incur damage, including DNA damage, as well as to dissipate exogenous cellular stress signals, and to look for extracellular growth factors. In most cases, checkpoints may result in the activation of programmed cell death (apoptosis) signaling, if the cellular damages are too serious to be properly repaired. Therefore, defects in cell cycle checkpoints and apoptosis would results in tumorigenesis[18].

We observed in the cell cycle analysis that treatment with costunolide resulted in apoptosis but not a particular sure trend in G1 or G2/M-arrest. It was shown that the compound treatment resulted in the induction of p53 and p21^{Waf1} CKIs and p27^{Kip1} CKI (Fig. 8), which are known to be important for G1-arrest, but these results were not in accord with cyclin and CDK changes in FACS analysis.

Cells treated with costunolide induced significant apoptosis over time after the treatment. Currently, it is not clear whether treatment of cells with costunolide might trigger the death signal through cell membrane-based receptors or not. However, it is clear that apoptosis mediated by the treatment involves activation of caspases, as we observed the loss of intact procaspase 3 and cleavage of PARP. We also observed an alteration in the levels of Bcl2 and Bax proteins.

It is well-known that Bcl2 and Bax family members have opposing actions, thereby regulating the activation of caspases[19] (Fig. 6). p53 can regulate the induction of the both pro- and anti-apoptotic factors[20,21]. Bcl2 is anti-apoptotic and down-regulated by p53, whereas Bax is pro-apoptotic and induced by p53[22-24]. Bax facilitates the release of the apoptosis-inducing factor and cytochrome C from the mitochondria, leading to the activation of the caspase cascade[25].

Once caspases are activated, various cellular proteins, including PARP, are cleaved by their actions, leading to defects in their signaling and/or structural functions, thereby resulting in cell death[26].

In this current study, such a signaling mechanism appears to be related with the induction of p53, over-expression of Bax, and activation of caspase 3. Therefore, the cytotoxic effects of costunolide appear to involve an apoptosis, probably via induction of p53 and caspases.

The G1 checkpoint controls mediated via p53/p21-dependent eventual apoptosis are potentially important determinants of tumor sensitivity to DNA damage. Previously it has been demonstrated in many studies that when cells were exposed to a combination of DNA damaging agents and checkpoint-inhibitory drugs, p53 is inactivated and the G1 checkpoint control is relaxed, leading to enhanced cytotoxic effects. Therefore, observations from studies including this current one may have important clinical implications for a chemoprevention approach to treat gastric cancers with fewer side effects on normal epithelial cells.

The RB family, RB, p107 and Rb2/p130, negatively modulate the transition between the G1 and S phases, using mechanisms mostly related to inactivation of transcription factors, such as those of the E2F family, that promote the cell entrance into the S phase[27,28].

In our study, RB2/p130 was significantly over-expressed after 24 hrs, hence we could assume that costunolide treatment in AGS cells could modulate and accumulate the cells in G1 phase, but we didn't obtain concurrent results by FASC analysis or Western blot analysis.

Since up-regulation in $p27^{Kip1}$ CKI level was strongly observed at 10 μ M costunolide treatment for 24, 48 hrs, we investigated the effect of costunolide on CDK 2, 4, 6 kinases and cyclin E and A expressions. As shown in Fig. 7, the costunolide treatment of AGS cells didn't affect CDK's and cyclin's expression, which means costunolide didn't affect G1 or G2 cell cycle accumulation.

Strong expressions of Cox 2 in AGS cells by the treatment of 10 μ M of costunolide also means that this compound had a relationship with inflammation; recently some research has reported that Cox 2 inhibit the MAPK pathways in the upstream29, but we didn't confirm where this compound acts (Fig. 9). Further studies on this action could be investigated in another experiment, but were not this time.

Taken together, the costunolide induced concomitant programmed cell death by activating pro-apoptotic molecules and suppressing anti-apoptotic molecules and might be assumed to induce the G1-arrest of AGS gastric cancer cells, probably by modulating levels through $p53/p21^{Waf1}$, $p27^{Kip1}$ CKI induction, and Rb2/p130 expression, but we didn't confirm that. These results which induce apoptosis strongly indicate that costunolide can be a candidate therapeutic reagent against gastric cancer.

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