Costunolide Triggers Apoptosis in Human Leukemia U937 Cells by Depleting Intracellular Thiols

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We have previously demonstrated that costunolide, a biologically active compound that was isolated from the stem bark of *Magnolia sieboldii*, induced apoptosis in human cancer cells. In the present study, we investigated the underlying mechanisms and suggest that costunolide induces apoptosis in human promonocytic leukemia U937 cells by depleting the intracellular thiols. Costunolide treatment rapidly depleted the intracellular reduced glutathione (GSH) and protein thiols, and this preceded the occurrence of apoptosis. Pretreatment with sulfhydryl compounds such as GSH, N-acetyl-L-cysteine, dithiothreitol and 2-mercaptoethanol almost completely blocked the costunolide-induced apoptosis, highlighting the significance of the intracellular thiol level in the process. Furthermore, overexpression of Bcl-2 also significantly attenuated the effects of costunolide. The apoptosis-inducing activity of costunolide is likely to depend on the exomethylene moiety because derivatives in which this group was reduced, such as dihydrocostunolide and saussurea lactone, did not deplete the cellular thiols and showed no apoptotic activity. Taken together, the present study demonstrates that the costunolide-induced apoptosis depends on intracellular thiols contents, which are modulated by Bcl-2.

Key words: Costunolide — Apoptosis — Glutathione — Bcl-2 — Exomethylene

Costunolide, isolated from the stem bark of *Magnolia sieboldii*, is a sesquiterpene lactone that exhibits various biological and immunological actions. It has been reported that some sesquiterpenes induce apoptosis in cancer cells,[1, 2] and costunolide was proven to have a preventive effect against intestinal carcinogenesis,[3] suggesting that costunolide may also have an apoptosis-inducing activity. However, despite the various biological actions of costunolide, its mechanisms of action are still unknown.

Apoptosis is cellular suicide or programmed cell death, which is mediated by activation of evolutionarily conserved intracellular pathways. Recently, the relationship between apoptosis and cancer has been emphasized, and increasing evidence suggests that the processes of neoplastic transformation, progression and metastasis alter the normal apoptotic pathway.[4] Moreover, apoptosis offers some clues about effective anticancer therapy, and many chemotherapeutic agents indeed exert their anti-tumor effects by inducing apoptosis of cancer cells.[5]

In recent years, changes of intracellular redox status during apoptosis have been a target of extensive researches, because redox changes were observed during apoptosis induced by oxidative or non-oxidative stimuli.[6, 7] Reduced glutathione (GSH), the most abundant cellular thiol and the major determinant of the cellular redox equilibrium, has been shown to be an important factor in apoptosis.[8, 9] Kane and collaborators first recognized the importance of thiols as modulators of apoptosis propensity in 1993, and they noted that expression of Bcl-2 in the GT1-7 neural cell line prevented death as a result of GSH depletion.[10] Mirkovic *et al.* have reported that resistance to radiation-induced apoptosis in Bcl-2-expressing cells was reversed by depleting cellular thiols.[11] Under these conditions, Bcl-2 levels remained unchanged, suggesting that Bcl-2 was not directly regulating apoptotic sensitivity, but rather was modulating sensitivity through an intermediary such as GSH.

We have previously demonstrated that costunolide induced apoptosis in HL-60 human leukemia cells.[12, 13] As intracellular thiols play an important role in maintaining cellular redox equilibrium that is pivotal for cell survival, we hypothesized that costunolide may change intracellular redox status and thus trigger apoptosis. In this study, the molecular mechanisms of apoptosis induced by costunolide and the role of Bcl-2 were investigated in human promonocytic leukemia U-937 cells.

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MATERIALS AND METHODS

Materials RPMI 1640 medium, fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Life Technologies, Inc. (Grand Island, NY). RNase, leupeptin, aprotinin, 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), reduced glutathione (GSH), N-ethylmaleimide (NEM), 2-vinylpyridine, N-acetyl-L-cysteine (NAC), 2-mercaptoethanol (2-ME), dithiothreitol (DTT), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), and oxidized glutathione (GSSG) were purchased from Sigma Chemical Co. (St. Louis, MO). Proteinase K was from Wako Pure Chemical Industries (Osaka). Costunolide used for this study was isolated from the stem bark of *Magnolia sieboldii* as previously described.[12, 13]

Cell culture and costunolide treatment Human promonocytic leukemia U937 cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo) and Bcl-2-overexpressing U937$_{bcl-2H}$ were constructed as previously described.[14] The cells were cultured in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin sulfate (100 μg/ml) in an atmosphere of 95% air 5% CO_2 and were seeded in plates after 3 passages. Costunolide was dissolved in dimethylsulfoxide (DMSO), and cells were treated with costunolide for the indicated time period.

Detection and quantification of DNA fragmentation The genomic DNA was prepared for gel electrophoresis as previously described.[12] Electrophoresis was performed in a 1.5% (w/v) agarose gel in 40 mM Tris-acetate buffer (pH 7.4) at 50 V for 1 h. The fragmented DNA was visualized by staining with ethidium bromide after electrophoresis. DNA fragmentation was quantitated as previously reported.[14] In brief, cells were lysed in a solution containing 5 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 0.5% (w/v) Triton X-100 for 20 min on ice. The lysate and supernatant after centrifugation at 27 000g for 20 min were sonicated for 40 s, and the level of DNA in each fraction was measured by a fluorometric method using DAPI. The amount of the fragmented DNA was calculated as the ratio of the amount of DNA in the supernatant to that in the lysate.

Determination of the GSH level Cells were washed twice with phosfate-buffered saline (PBS) and treated with 5% trichloroacetic acid (TCA) to extract cellular GSH. The mixture was centrifuged at 13 000g for 1 min to remove the denatured proteins. GSH was determined by an enzymatic method as previously described.[15] Briefly, 100 μl of the TCA extract was incubated in 1 ml of a reaction mixture containing 0.1 M sodium phosphate buffer (pH 7.5), 5 mM EDTA, 0.6 mM DTNB, 0.2 mM NADPH, 1 unit/ml GSH reductase, and then the increase in absorbance at 412 nm for 2 min was measured. For determination of GSSG, the same DTNB recycling assay was performed after using 2-vinylpyridine to remove the reduced GSH. Briefly, 2 μl of 2-vinylpyridine and 6 μl of triethanolamine were simultaneously mixed with 100 μl of sample, followed by incubation in the dark at room temperature for 1 h before initiation of the recycling assay. The kinetics of the reaction were monitored for 10 min. The increment in absorbance at 412 nm was converted to GSH concentration using a standard curve prepared by using known amounts of GSH.

Measurement of intracellular protein thiols Cells were washed twice with PBS and treated with 5% TCA. Samples were then vortexed and kept on ice for 30 min for complete protein precipitation. After centrifugation, the protein precipitate was washed with the same TCA and then redissolved in 0.1 M Tris-HCl buffer (pH 7.5), containing 5 mM EDTA and 0.5% sodium dodecyl sulfate (SDS). One aliquot of this protein precipitate was reacted with a solution containing 0.1 M sodium phosphate buffer (pH 7.5), 5 μM EDTA, 0.6 mM DTNB, 0.2 mM NADPH, 1 unit/ml GSH reductase, and another aliquot of the protein solution was treated with 5 mM NEM before reaction to obtain the background value for subtraction. The concentration of intracellular protein thiol was expressed as nmol of SH equivalents/mg protein using GSH as a standard.

Chemical transformation of costunolide An excess of NaBH$_4$ (40 mg, 1.06 mmol) was added to 5 ml of MeOH, in which 30 mg of costunolide (0.13 mmol) was dissolved. The mixture was stirred at 0°C for 20 min, then the reaction was quenched with the aqueous NH$_4$Cl solution. The mixture was concentrated to remove MeOH and extracted with Et$_2$O. After having been dried over anhydrous MgSO$_4$, the ether layer was concentrated under reduced pressure to give a solid, which was crystallized from CH$_2$Cl$_2$-Et$_2$O to afford pure dihydrocostunolide (30 mg) in quantitative yield. The structure of dihydrocostunolide was confirmed by nuclear magnetic resonance (NMR) spectroscopy. When neat dihydrocostunolide (18 mg, 0.08 mmol) was heated at 180°C for 3 min, Cope rearrangement occurred to give saussurea lactone and dihydrocostunolide as a mixture. The mixture was purified by column chromatography on silica gel (EtOAc/hexane=1/10) to provide saussurea lactone (3.3 mg, 18%) together with recovered starting material, dihydrocostunolide (4.8 mg, 27%). The NMR spectral data of saussurea lactone was identical with those of authentic saussurea lactone.

Statistical analysis All values are presented as the means±SD. Statistical analyses were performed using Student's *t* test.

RESULTS

Induction of apoptosis by costunolide In our previous studies,[12, 13] we reported that costunolide induced apoptosis in HL-60 human promyelocytic leukemia cells both

concentration- and time-dependently. Essentially identical results were observed when human promonocytic leukemia U937 cells were exposed to costunolide (Fig. 1). The amount of fragmented DNA, quantified by a fluorometric method using DAPI, gradually increased time- and concentration-dependently (Fig. 1A), and a ladder pattern of internucleosomal fragmentation of DNA was apparent when cells were treated with 10 μM costunolide for 2 h (Fig. 1B). Furthermore, the condensation of chromatin in nuclei and the degradation of the nuclei were observed from cells treated with 10 μM costunolide for 4 h (data not shown). These results indicate that costunolide induced apoptosis in U937 cells.

Effect of costunolide on intracellular thiols As certain forms of sesquiterpene lactones were reported to conjugate with sulfhydryl groups and intracellular thiols play critical roles in cell survival, we hypothesized that costunolide induces apoptosis, probably by depleting intracellular thiols. Thus, we next examined the effect of costunolide on the intracellular thiol level (Fig. 2). Costunolide rapidly decreased the intracellular GSH concentration in a time- and concentration-dependent manner, and a statistically significant difference was detected as early as 15 min after the start of incubation when a concentration of 10 μM or higher was used (Fig. 2A). The levels of protein thiols were also decreased dose- and time-dependently under these conditions, and in this case, a significant difference was observed at 1 h (Fig. 2B). The significance of intra-

A

B

Costunolide (10 μM)

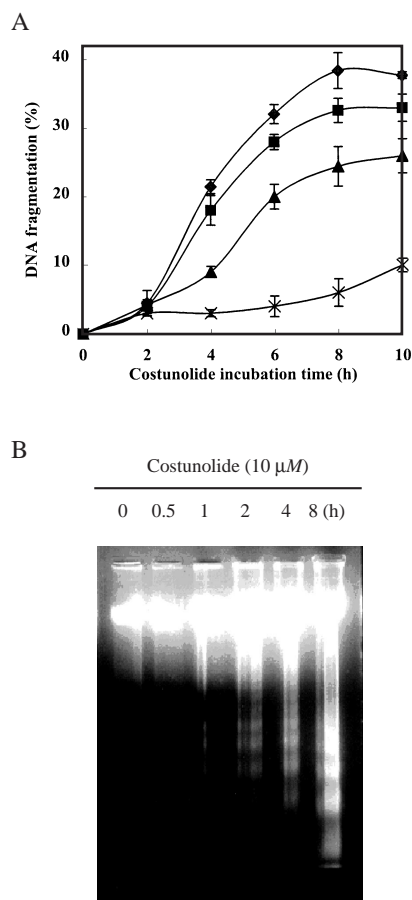0   0.5   1   2   4   8 (h)

Fig. 1.   Effects of costunolide on the induction of apoptosis and DNA fragmentation in U937 cells. (A) U937 cells were treated with 2–10 μM costunolide for the indicated times. The extent (%) of fragmentation was determined using DAPI as described in "Materials and Methods." Data presented are the means±SD of results from three independent experiments. ◆ 10 μM, ■ 5 μM, ▲ 3 μM, × 2 μM. (B) U937 cells were treated with 10 μM costunolide for the indicated times, and DNA fragmentation was analyzed by agarose gel electrophoresis.
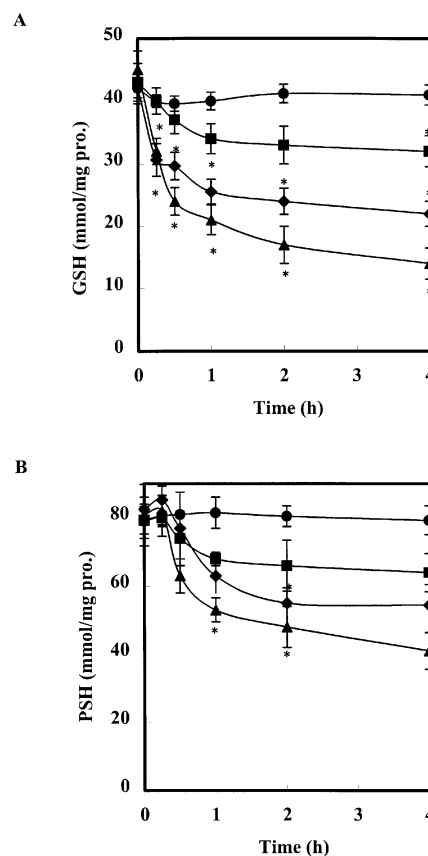
A

B

Fig. 2.   Effects of costunolide on the levels of intracellular GSH and protein thiols. Cells were treated with various concentrations of costunolide for the indicated period and then the levels of intracellular GSH (A) and protein thiols (B) were determined. Data are presented as means±SD of results from three independent experiments. Significant difference compared to the control group (∗ $P<0.01$, Student's t test). ● 0 μM, ■ 5 μM, ◆ 10 μM, ▲ 20 μM.

cellular thiol level for cell survival was further demonstrated when the effect of thiol-containing sulfhydryl compounds on the costunolide-induced apoptosis was examined; pretreatment of U937 cells with GSH (5 mM), NAC (5 mM), DTT (0.2 mM) or 2-ME (0.2 mM) almost completely abrogated the costunolide-induced apoptosis (Fig. 3).

Structural requirement of costunolide for apoptosis The biological effects of sesquiterpene lactones have been attributed to these compounds' ability to deactivate enzymes and other essential proteins by forming covalent bonds with sulfhydryl groups.[16] To test the idea that a methylene moiety conjugated to the lactone carbonyl might be essential for the apoptosis-inducing activity, we synthesized derivatives, which no longer contain a methylene moiety (Fig. 4A). First, we reduced costunolide with NaBH$_4$ to afford dihydrocostunolide. When this compound was further heated at 180°C for 3 min, Cope rearrangement occurred to give saussurea lactone (Fig. 4A). As expected, we found that semi-synthesized dihydrocostunolide and saussurea lactone showed no marked apoptosis-inducing activity up to 20 μM (Fig. 4B) and had no effect on the intracellular thiol level (data not shown). Therefore, these results suggest that the methylene moiety of costunolide is required for conjugation with the intracellular GSH and protein thiols, and that intracellular thiol depletion leads to the induction of apoptosis in U937 human leukemia cells.

Bcl-2 prevents the costunolide-induced intracellular thiol depletion and apoptosis Our observations that the apoptotic propensity of leukemia cells is associated with the intracellular thiol content led us to investigate the role

of Bcl-2 in the costunolide-induced apoptosis, because Bcl-2 is known to modulate the relative viability of cells via the levels of intracellular thiols. To this end, U937 parental cells were transfected with the pRc-RSV expression vector control (U937v) or the pRc-RSV expression vector containing the human *Bcl-2* gene (U937$_{bcl-2H}$) as previously described.[14] These cells were treated with or without 10 μM costunolide for 8 h, and then the extent of DNA fragmentation was measured (Fig. 5A). DNA fragmentation of U937v cells induced by costunolide was approximately 37%, which is almost identical to that of the untransfected U937 cells. In contrast, U937$_{bcl-2H}$ was completely resistant to the costunolide treatment, indicating that apoptosis induced by costunolide in U937 cells was inhibited by the overexpression of Bcl-2 protein. Under these conditions, the Bcl-2 expression level was also monitored (Fig. 5B). The Bcl-2 protein level of U937v cells was unchanged during the first 2 h of cos-
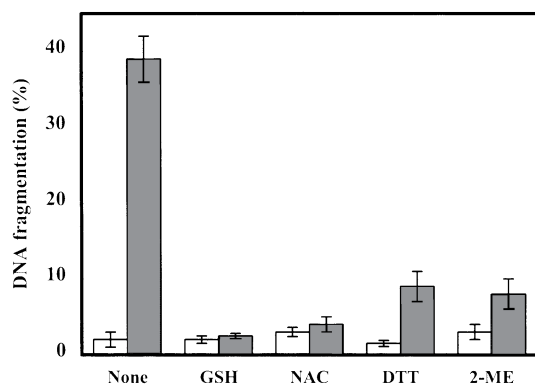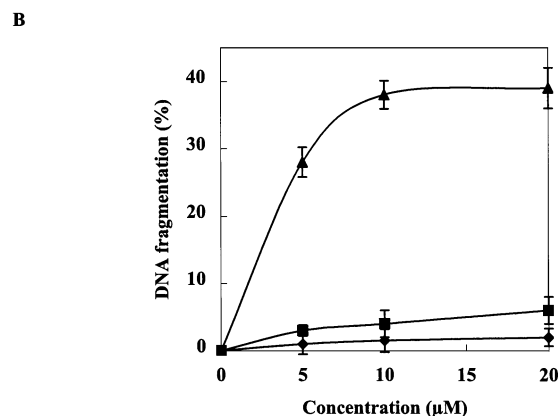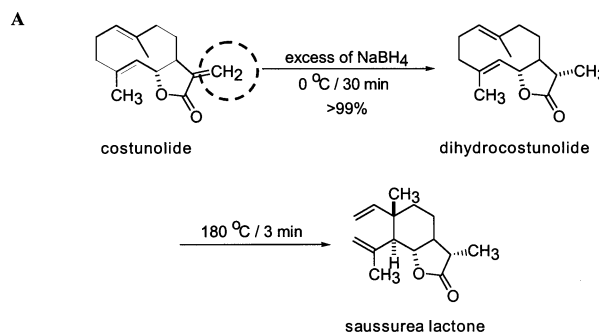
Fig. 4. (A) Costunolide was chemically transformed as described in "Materials and Methods," affording dihydrocostunolide and saussurea lactone that no longer contain the exomethylene moiety. (B) The extent of DNA fragmentation of DNA in cells that had been treated with the indicated concentrations of costunolide (▲), dihydrocostunolide (■) and saussurea lactone (◆) for 8 h was measured.

Fig. 3. Effects of thiol-containing antioxidants on the costunolide-induced DNA fragmentation. U937 cells were pretreated without and with 5 mM GSH, 5 mM NAC, 0.2 mM DTT, and 0.2 mM 2-ME for 1 h, and then challenged with costunolide 10 μM for 8 h. DNA fragmentation was measured using DAPI as described in "Materials and Methods." □ none, ■ costunolide.

tunolide treatment, a period during which intracellular thiol levels fell by more than 50% (Fig. 2), and then began to decline after 4 h. In contrast, the Bcl-2 protein level of U937$_{bcl-2H}$ cells remained constant up to 8 h treatment with costunolide (Fig. 5B).

Furthermore, we examined the intracellular GSH and protein thiol levels in U937, U937v and U937$_{bcl-2H}$ cells as a function of time during costunolide treatment (Fig. 6). The results show that, in contrast to U937 and U937v cells, a slight decrease in GSH and protein thiol level after 10 μM costunolide treatment occurred in U937$_{bcl-2H}$ cells (Fig. 6). Considering all the data, we conclude that costunolide triggered apoptosis by depletion of intracellular thiols that are modulated by Bcl-2.

DISCUSSION

Because of the significance of apoptosis in both physiological and patho-physiological situations, the mechanism by which particular agents trigger apoptosis has become a subject of intensive study. Of the various mechanisms suggested for apoptosis, much attention has been given to intracellular redox status. It has recently been demonstrated that intracellular GSH, the main determinant of intracellular redox status, is depleted before the onset of apoptosis induced by various agents.[17-20] Moreover, artificially depleting intracellular GSH renders cells more sensitive to apoptotic agents.[21,22] To investigate the possible mechanisms involved in costunolide-induced apoptosis, the effect of costunolide on intracellular thiols was then determined in the present study. First, it was found that costunolide rapidly depleted intracellular GSH and protein thiols (Fig. 2). Second, the time-course studies revealed that the intracellular thiol depletion occurred prior to the onset of costunolide-induced apoptosis as determined by DNA fragmentation assay and DNA ladder detection by agarose gel electrophoresis (Figs. 1 and 2). Finally, we demonstrated that the ability of costunolide to induce apoptosis was significantly reduced by restoring the intracellular GSH level with sulfhydryl compounds such as NAC,
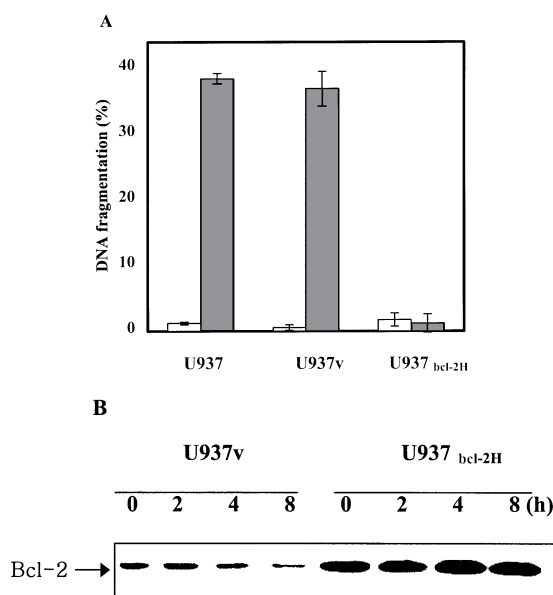

Fig. 5. (A) U937 cells were stably transfected with the expression vector alone (U937v) or the vector containing the human *Bcl-2* gene (U937$_{bcl-2H}$). As a control, untransfected U937 cells (U937) were also used. These cells were treated with 10 μM costunolide for 8 h, and then the extent of DNA fragmentation was measured. Data are presented as the means±SD of results from three independent experiments. (B) The expression level of Bcl-2 of U937v and U937$_{bcl-2H}$ cells were determined by western blot analysis at the indicated time during 10 μM costunolide treatment. □ none, ■ costunolide.
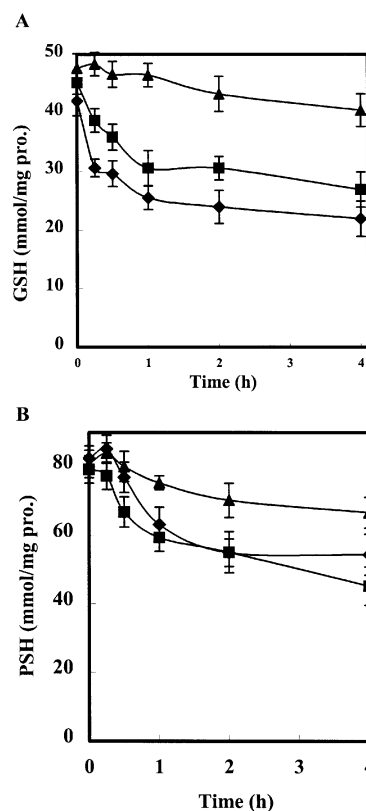

Fig. 6. Effect of the overexpression of Bcl-2 proteins on the concentrations of intracellular GSH and protein thiols. U937 (◆), U937v (■) and U937$_{bcl-2H}$ (▲) cells were treated with 10 μM costunolide for 15 min, 30 min, 1 h, 2 h, and 4 h, respectively. The levels of intracellular GSH (A) and protein thiol (B) were measured. Data are presented as the means±SD of results from three independent experiments.

2-ME, GSH and DTT (Fig. 3). Therefore, these results strongly suggest that the rapid depletion of intracellular thiols might play a critical role in costunolide-induced apoptosis.

In general, intracellular GSH can be depleted because of (i) increased intracellular oxidation of GSH,[6] (ii) stimulated GSH efflux,[17, 20] and (iii) inhibition of GSH synthesis. In an attempt to further understand the underlying mechanisms of the costunolide-induced apoptosis, we measured the intracellular level of GSSG, as well as the concentrations of GSH and GSSG in the culture medium, but there was no apparent difference between the costunolide-treated and -untreated cells (data not shown). Therefore, it is unlikely that the intracellular GSH depletion by costunolide was due to either an increase of oxidation or efflux of GSH. Moreover, costunolide depleted the intracellular thiols even more rapidly than buthionine sulfoxamine (BSO), a specific inhibitor of intracellular GSH synthase; 10 μM costunolide depleted about 27.1% of intracellular GSH within 15 min of incubation (Fig. 2A), whereas BSO depleted only 16.5% of intracellular GSH after 3 h treatment (data not shown), indicating that the *de novo* synthesis of GSH is not a target of costunolide action, either. Costunolide also significantly depleted intracellular protein thiol levels (Fig. 2B), whereas BSO exhibited no obvious effect on the concentration of protein thiols (data not shown). We hypothesized that the α-methylene-γ-lactone moiety of costunolide may be essential for GSH depletion since it is well established that the α-methylene-γ-lactone moiety of sesquiterpenes is able to conjugate with sulfhydryl groups. To test this hypothesis, we synthesized derivatives of costunolide which lack the exomethylene moiety (Fig. 4A). As shown in Fig. 4B, these derivatives almost completely lacked the ability to induce DNA fragmentation. Therefore, our results strongly suggest that rapid depletion of intracellular GSH is most probably the result of rapid binding of the exomethylene moiety of costunolide with intracellular GSH.

In the present study, a rapid depletion of protein thiols was also observed in costunolide-treated cells (Fig. 2). The depletion of protein thiols may also be due to the binding of costunolide with thiol groups. Some recent studies have shown that intracellular proteins such as GSH-S-transferases, nitric oxide synthase, NADPH oxidase and thioredoxin play important roles in maintaining intracellular redox status.[23, 24] Although we did not examine the activities and expression of these enzymes, the rapid depletion of intracellular protein thiols and the subsequent changes in the enzyme activity may also contribute to the costunolide-induced apoptosis.

It was hypothesized several years ago that the Bcl-2 oncogene functions in an antioxidant pathway to block apoptosis.[25] In accordance with this hypothesis, several Bcl-2-expressing cells have been shown to have higher basal levels of GSH.[26] In this study, although we were not able to find any significant difference in the GSH levels between the control and Bcl-2-overexpressing U-937 cells, overexpression of Bcl-2 almost completely blocked the costunolide-induced apoptosis as well as the GSH depletion (Figs. 5 and 6). As shown in Fig. 5B, the Bcl-2 level of U937v cells remained constant up to 2–4 h during the costunolide treatment, and thereafter gradually decreased. Although we cannot completely rule out the possibility that costunolide directly inhibits the Bcl-2 expression via unknown mechanisms, this seems unlikely because costunolide did not inhibit the Bcl-2 level of U937$_{bcl-2H}$ cells. Rather, the reduction in Bcl-2 level observed in the late period of costunolide treatment seems to be a secondary effect of cell death, which was Bcl-2-independently triggered by thiol depletion. The results presented here suggest that costunolide-induced depletion of intracellular thiols unbalances the redox status, and allows reactive oxygen species to mediate a physical signal into the death pathway. This suggestion is supported by our previous report,[12] which showed that costunolide induced ROS generation, thereby inducing mitochondrial permeability transition, and cytochrome *c* release to the cytosol.

Compounds capable of inducing apoptosis of human cancer cells are of interest as potential anti-cancer agents, and many chemotherapeutic agents indeed produce anti-cancer effects by inducing apoptosis of cancer cells.[5] In the present study, we demonstrated that costunolide, a natural compound isolated from the stem bark of *Magnolia sieboldii*, induced apoptosis of human cancer cells, and we are now investigating whether costunolide can be further developed into an anti-cancer agent.

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