β -D-Xylopyranosyl-(1 $\rightarrow$ 3)- β -D-glucuronopyranosyl Echinocystic Acid Isolated from the Roots of *Codonopsis lanceolata* Induces Caspase-Dependent Apoptosis in Human Acute Promyelocytic Leukemia HL-60 Cells

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Received October 12, 2004; accepted December 11, 2004

We previously demonstrated that β -D-xylopyranosyl-(1 $\rightarrow$ 3)- β -D-glucuronopyranosyl echinocystic acid (codonoposide 1c), a biologically active compound isolated from the roots of *Codonopsis lanceolata*, is cytotoxic to cancer cells. In the present study, we investigated the effects of codonoposide 1c on the induction of apoptosis, and its putative action pathway in HL-60 human promyelocytic leukemia cells. Codonoposide 1c-treated HL-60 cells displayed several features of apoptosis, including DNA fragmentation, formation of DNA ladders by agarose gel electrophoresis, and externalization of annexin-V targeted phosphatidylserine (PS) residues. We observed that codonoposide 1c caused activation of caspase-8, caspase-9, and caspase-3. A broad caspase inhibitor (z-VAD-fmk), caspase-8 inhibitor (z-IETD-fmk), and caspase-3 inhibitor (z-DEVD-fmk) almost completely suppressed codonoposide 1c-induced DNA fragmentation. We further found that codonoposide 1c induces mitochondrial translocation of Bid from cytosol, reduction of cytosolic Bax, and cytochrome c release from mitochondria. Interestingly, codonoposide 1c also triggered the mitochondrial release of Smac/DIABLO (second mitochondria-derived activator of caspases/direct inhibitor of apoptosis-binding protein with a low isoelectric point) into cytosol, and a reduction in X-linked inhibitor of apoptosis protein (XIAP). Taken together, our data indicate that codonoposide 1c is a potent inducer of apoptosis and facilitates its activity *via* Bid cleavage and translocation to mitochondria, Bax reduction in cytosol, release of cytochrome c and Smac/DIABLO into the cytosol, and subsequently caspase activation, providing a potential mechanism for the cytotoxic activity of codonoposide 1c.

Key words codonoposide 1c; apoptosis; caspase; X-linked inhibitor apoptosis protein (XIAP); Smac/DIABLO

Apoptosis is a highly organized physiologic mechanism involving the destruction of injured or abnormal cells, and is often referred to as programmed cell death.^{1,2)} Disordered apoptosis is important in many disease processes including oncologic and rheumatologic phenomena, and thus the accurate control of apoptosis is important not just to the normal development of an organism, but also to its health and growth.^{3,4)} Therefore, various kinds of agents that can effectively induce apoptosis offer promising strategies for the treatment of cancer. The death circuitry in mammalian cells has two major apoptotic pathways. One is a receptor-mediated pathway involving Fas and other members of the tumor necrosis factor (TNF) receptor family that activate caspase-8,⁵⁾ whereas the other involves cytochrome c, Apaf-1, and caspase-9.⁶⁾ Over the last few years, substantial evidence has been found to suggest that several mitochondrial events are essential for programmed cell death.⁷⁾ One of the early crucial steps in the process of apoptosis is the release of cytochrome c through the outer mitochondrial membrane into the cytosol. In the cytosol, cytochrome c unleashes the activation of caspases, which are cysteine proteases with aspartate specificity.⁸⁾

Recent studies have shown that some members of the human inhibitor of apoptosis protein (IAP) family potently inhibit caspases by direct binding.^{9,10)} Six human IAP-like proteins have been identified and designated as XIAP, cIAP1, cIAP2, NIAP, survivin, and Bruce. XIAP is the most potent of the IAP-related proteins.¹⁰⁾ Recent studies have identified

another important regulator of apoptosis, Smac (second mitochondria-derived activator of caspase) or DIABLO (direct inhibitor of apoptosis-binding protein with a low isoelectric point), which is released from mitochondria into the cytosol during apoptosis and functions by eliminating the inhibitory effects of IAPs on caspases.^{11,12)}

Chemical agents with strong apoptosis-inducing activity but minimal toxicity are expected to have potential utility as anticancer drugs. Thus, as a part of our screening program to evaluate the chemopreventive potential effects of natural compounds, we investigated the effects of codonoposide 1c on cancer cells, and its molecular mechanism of apoptosis induction in HL-60 cells. Here, we report that codonoposide 1c induces apoptosis through Bid cleavage and translocation to mitochondria, Bax reduction in cytosol, cytochrome c release, Smac/DIABLO release, and caspase-8, caspase-9, and caspase-3 activation. To the best of our knowledge, this is the first report to show that codonoposide 1c induces apoptosis through a caspase-dependent mechanism, suggesting a candidate as an apoptosis-inducing cancer chemopreventive agent.

MATERIALS AND METHODS

Materials The β -D-xylopyranosyl-(1 $\rightarrow$ 3)- β -D-glucuronopyranosyl echinocystic acid (Fig. 1) used for this study was isolated from *C. lanceolata* (Campanulaceae) as previously described.¹³⁾ The physicochemical data of codonopo-

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Fig. 1. Chemical Structure of Codonoposide 1c Isolated from the Root of *Codonopsis lanceolata*

side 1c were defined as follows: mp 267–270 °C, IR (KBr): $\nu_{\max}$ (cm^{-1}) = 3400 (OH), 2948 (CH), 1704 (C=O), 1608, 1089, 1041 (glycosidic C–O); $^1\text{H-NMR}$ (500 MHz, pyridine- d_5): (0.82 (3H, s, H-24), 0.87 (3H, s, H-25), 0.91 (3H, s, H-26), 1.03 (3H, s, H-30), 1.10 (3H, s, H-27), 1.27 (3H, s, H-29), 1.76 (3H, s, H-23), 3.33 (1H, dd-like, H-3), 5.18 (1H, br s, H-16), 5.58 (1H, br s, H-12), sugar moieties— δ 5.07 (1H, d, $J=6.8$ Hz, H-1'), 5.10 (1H, d, $J=7.4$ Hz, H-1''). The codonoposide 1c isolated found to be >96% pure was by HPLC. RPMI 1640 medium, fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Life Technologies Inc. (Grand Island, NY, U.S.A.). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tertzolium bromide (MTT), RNase, leupeptin, aprotinin, phenylmethylsulfonylfluoride (PMSF), 4',6-diamidino-2-phenylindole-dihydrochloride (DAPI) and propidium iodide (PI) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Antibodies for caspase-3, caspase-8, caspase-9, cytochrome c, Bid, Bax, Bcl-2, Bcl-xL, Smac/DIABLO, and β -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Antibody for XIAP was purchased from Cell Signaling Technology (Beverly, MA, U.S.A.). z-VAD-fmk, z-IETD-fmk, and z-DEVD-fmk were purchased from A.G. Scientific Inc. (San Diego, CA, U.S.A.).

Cell Culture HL-60 human promyelocytic leukemia cells were obtained from the Korean Cell Line Bank (KCLB). The cells were cultured in RPMI 1640 medium with 10% FBS in a CO_2 incubator at 37 °C in the presence or absence of chemicals.

Detection and Quantification of DNA Fragmentation 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 $27000\times g$ for 20 min were sonicated for 40 s, and the level of DNA in each fraction was measured with 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. Genomic DNA was prepared for gel electrophoresis as previously described.¹⁴ 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.

Apoptosis Assessment by Annexin-V Staining At the end of the various treatment periods, the cells were washed twice with PBS and resuspended in 100 μl of staining solu-

tion containing annexin-V fluorescein in HEPES buffer. The cell suspensions were incubated for 15 min at room temperature, followed by flow cytometer analysis of 20000 cells in each group. Annexin-V binds to those cells that express phosphatidylserine on the outer layer of the cell membrane. Data analysis was performed with the standard Cell Quest software.

Preparation of Cytosolic and Mitochondrial Fractionation and Western Blot Analysis HL-60 cells (2.5×10^7) were collected by centrifugation at $200\times g$ for 10 min at 4 °C. The cells were washed twice with ice-cold PBS, pH 7.2, followed by centrifugation at $200\times g$ for 5 min. The cell pellet was then resuspended in ice-cold cell extraction buffer (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl_2 , 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, PMSF 100 μM , protease inhibitor cocktail) for 30 min on ice. Cells were then homogenized with a glass Dounce B-type pestle (80 strokes) and subjected to centrifugation at $1000\times g$ to remove unbroken cells, pellet nuclei, and heavy membranes. The postnuclear supernatant was centrifuged at $14000\times g$ for 30 min to yield a pellet mitochondria-enriched heavy membrane fraction, and the resulting supernatant was further centrifuged at $100000\times g$ to obtain the cytosolic fraction. Supernatants were then frozen in aliquots at -70 °C until required. The mitochondria-rich fraction was washed once with extraction buffer, followed by a final resuspension in lysis buffer [150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1% NP40, 0.25% sodium deoxycholate, and 1 mM EGTA] containing protease inhibitors. Approximately 40 μg of cytosolic or mitochondrial protein extracts were separated using 15% SDS-polyacrylamide gels and transferred to nitrocellulose. The blots were incubated with monoclonal anti-cytochrome c, anti-Bax, anti-Bcl-2, anti-Bcl-xL, polyclonal anti-Bid, and anti-caspase-3, -8, and -9 antibodies followed by enhanced chemiluminescence (ECL)-based detection (Amersham Pharmacia Biotech).

Immunoprecipitation Samples of the total protein (100 μg) were incubated with the anti-Smac/DIABLO, caspase-3, or caspase-9 polyclonal antibody for 2 h at 4 °C, followed by incubation with 20 μl of the protein A/G-Sepharose beads (Sigma, St. Louis, MO, U.S.A.) for 1 h. The protein complexes were washed 4 times with an immunoprecipitation buffer [50 mM Tris-HCl, pH 7.4, 0.5% NP-40, 150 mM NaCl, 50 mM NaF, 0.2 mM sodium orthovanadate, 1 mM dithiothreitol (DTT), 20 $\mu\text{g}/\text{ml}$ aprotinin, 20 $\mu\text{g}/\text{ml}$ leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF)], and released from the beads by boiling in $2\times$ SDS sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 10% β -mercaptoethanol, 2% glycerol, 0.02% bromophenol blue) for 5 min; the reaction mixture was then resolved by a 12% SDS-PAGE gel, transferred onto a nitrocellulose membrane by electroblotting and probed with the anti-XIAP monoclonal antibody. The blot was developed using an ECL kit.

Data Analysis Data are reported as the mean $\pm$ S.D. of three independent determinations. All experiments were done at least three times, with three or more independent observations each time. Statistical analysis was performed using Student's *t*-test.

RESULTS

Induction of Apoptosis by Codonoposide 1c In a previous study,¹³⁾ we reported that codonoposide 1c is cytotoxic to HL-60 human promyelocytic leukemia cells with an IC_{50} of $30 \mu M$. Essentially identical results were observed when HL-60 cells were exposed to codonoposide 1c (data not shown). This cytotoxicity was attributable to the induction of apoptosis, as demonstrated by the following biochemical features. The amount of fragmented DNA, quantified using a fluorometric method using DAPI, gradually increased in a time- and concentration-dependent manner (Fig. 2A), and a ladder pattern of internucleosomal fragmentation of DNA was apparent when cells were treated with 20 or $40 \mu M$ codonoposide 1c for 8 h (Fig. 2B). To further characterize codonoposide 1c-induced apoptosis, we performed cytofluorometric analysis using annexin V-FITC, which stains phosphatidylserine (PS) residues. As indicated by Fig. 2C, the percentage of annexin V positive cells increased up to 8 h after treatment with $40 \mu M$ codonoposide 1c. At 8 h, the percentage of annexin V positive cells (early stage apoptotic cells) reached about 95%. These results indicate that codonoposide 1c induced apoptotic cell death in HL-60 cells.

Requirement of Caspase Activities during Codonoposide 1c-Induced Apoptosis Since it has been suggested that cytosolic aspartate-specific proteases, called caspases, are responsible for the intentional disassembly of a cell into apoptotic bodies,¹⁴⁾ we investigated the involvement of caspase activation in codonoposide 1c-induced apoptosis in HL-60 cells by Western blotting to analyze the activation of various caspases. Treatment of cells with $40 \mu M$ codonoposide 1c caused the proteolytic cleavages of procaspase-8, procaspase-9, and procaspase-3 in a time-dependent manner (Fig. 3A). To determine whether the activations of caspase-8 and caspase-3 are required for the induction of cell death by codonoposide 1c, we pretreated HL-60 cells with various caspase inhibitors. As shown in Fig. 3B, z-VAD-fmk (a broad caspase inhibitor), z-IETD-fmk (a caspase-8 inhibitor) and z-DEVD-fmk (a caspase-3 inhibitor) were able to markedly attenuate codonoposide 1c-stimulated DNA fragmentation, suggesting that codonoposide 1c-induced apoptotic cell death is largely dependent on caspase activation.

Effect of Codonoposide 1c on the Protein Levels of Bcl-2 Family To evaluate the contribution of the mitochondrial pathway to the induction of apoptosis by codonoposide 1c, we examined changes in cytochrome c release into the cytosol in codonoposide 1c-treated HL-60 leukemia cells. Figure 4 shows that codonoposide 1c treatment significantly increased the level of cytosolic cytochrome c, whereas its level in mitochondria concomitantly decreased. Caspase-8 has been reported to cleave Bid after chemotherapeutic agents treatment,¹⁵⁾ and the truncated Bid (tBid) then triggers the mitochondrial release of cytochrome c into the cytosol and activates caspase-9. To investigate whether caspase-8 activation caused by codonoposide 1c precedes cytochrome c release, we examined the kinetics of Bid cleavage after codonoposide 1c treatment. Figure 4 shows that codonoposide 1c caused time-dependent Bid cleavage, which coincided with changes in caspase-8 activation in HL-60 cells, suggesting that Bid is involved in cytochrome c release after

Fig. 2. Effect of Codonoposide 1c on the Induction of Apoptosis in HL-60 Cells

A, HL-60 cells were treated with various concentration of codonoposide 1c (x, control; ▲, $20 \mu M$; ■, $40 \mu M$; ◆, $60 \mu M$) for the indicated times. The extent (%) of fragmentation was determined using DAPI as described in Materials and Methods. Data presented are means $\pm$ S.D. of three independent experiments. B, HL-60 cells were treated with codonoposide 1c at 20 or $40 \mu M$ for 8 h and total genomic DNA was extracted and resolved on 2% agarose gel. Apoptotic DNA fragmentation was visualized by ethidium bromide staining. C, Contour plots for HL-60 cells treated with codonoposide 1c at $40 \mu M$ for the indicated times and then stained with annexin-V-FITC, which specifically detects exposed phosphatidyl serine residues at the cell surface. Cells appearing in the right (R) half show positive annexin V-FITC staining that indicates phosphatidylserine exposure on the cell surface and the left (L) half shows negative annexin V-FITC staining.

codonoposide 1c treatment. Because it has been shown that tBid, which translocates to mitochondria during apoptosis, is able to trigger a change in Bax conformation,¹⁶⁾ cytochrome c release, and subsequent caspase activation, we further investigated whether codonoposide 1c treatment induces the mitochondrial translocation of Bax. Figure 4 shows that codonoposide 1c treatment ($40 \mu M$) reduces the level of Bax in cytosol. However, the levels of anti-apoptotic Bcl-2 and Bcl-xL were not influenced. These results suggest that codonoposide 1c induces the translocation of Bid protein to mitochondria and that this is followed by the release of cy-

Fig. 3. Induction of Caspase-8, -9, and -3 Activities during Codonoposide 1c-Induced Apoptosis

A, Codonoposide 1c caused the cleavage of procaspase-8, -9, and -3. HL-60 cells were treated with codonoposide 1c (40 μM) for the indicated times. After treatment, whole fractions were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted with caspase-8-, caspase-9-, and caspase-3-specific antibodies. β-Actin was used as an internal control. B, Effect of caspase inhibitors (z-DEVD-fmk, z-IETD-fmk, and z-VAD-fmk) on apoptosis in response to codonoposide 1c-induced DNA fragmentation. HL-60 cells were pretreated with or without z-DEVD-fmk 50 μM, z-IETD-fmk 50 μM, and z-VAD-fmk 50 μM for 1 h, and then challenged with codonoposide 1c 40 μM for 8 h. DNA fragmentation was measured using DAPI as described in Materials and Methods. □, none; ■, codonoposide 1c. Data presented are the means ± S.D. of results from three independent experiments.

tochrome c into the cytosol.

Involvement of Smac/DIABLO in Codonoposide 1c-Induced Apoptosis by Binding with XIAP In addition to cytochrome c, an additional proapoptotic factor, Smac/DIABLO, has been suggested to act as an apoptosis promoter by neutralizing caspase inhibition by IAPs.¹⁷⁾ As shown in Fig. 5A, we examined the codonoposide 1c-triggered release of Smac/DIABLO into the cytosol in HL-60 cells. Codonoposide 1c treatment significantly increased the level of Smac/DIABLO in the cytosol at 2 h. Moreover, the extent of Smac/DIABLO release was enhanced for 8 h after treatment. We next examined changes in XIAP by Western blot analysis. Codonoposide 1c reduced the cellular protein levels of XIAP (Fig. 5A) in a time-dependent manner. Smac/DIABLO is suggested to act as an apoptosis promoter by interacting with XIAP, preventing these proteins from binding and inhibiting caspases.¹⁸⁾ To clarify whether Smac/DIABLO released in response to codonoposide 1c is able to dissociate XIAP from caspase-3 and caspase-9, we immunoprecipitated Smac/DIABLO, caspase-3, and caspase-9 (Fig. 5B), respectively, and detected bound XIAP by Western blot analysis.

Fig. 4. Effect of Codonoposide 1c on Protein Levels of Bcl-2 Family Proteins in HL-60 Cells

Cells were harvested at the indicated times after incubation with codonoposide 1c at 40 μM. Mitochondrial (M) and cytosolic (C) fractions were prepared as described in Materials and Methods. An equal amount of protein from the mitochondrial or cytosolic fractions of each sample was separated on 15% SDS-PAGE gel and then transferred to nitrocellulose membranes and immunoblotted with the indicated antibodies, cytochrome c, Bax, Bid, Bcl-2, and Bcl-xL. β-Actin was used as an internal control.

Fig. 5. Effect of Codonoposide 1c on the Release of Stimulation of Smac/DIABLO and the Binding of XIAP with Smac/DIABLO, Caspase-3, or Caspase-9 in HL-60 Cells

A, Effects of codonoposide 1c on Smac/DIABLO and XIAP expression in HL-60 cells. Cells were harvested at the indicated times after incubation with codonoposide 1c at 40 μM. An equal amount of protein of each sample was separated on 15% SDS-PAGE gel and then transferred to nitrocellulose membranes and immunoblotted with the indicated antibodies Smac/DIABLO and XIAP. β-Actin was used as an internal control. B, Codonoposide 1c-induced binding between XIAP and Smac/DIABLO, caspase-3, or caspase-9 in HL-60 cells. Lysates from control and treated cells were immunoprecipitated (IP) with anti-Smac/DIABLO, caspase-3, and caspase-9. Immune complexes were analyzed by immunoblotting (WB) with anti-XIAP antibody.

Figure 5B demonstrates that in untreated cells, XIAP is associated with caspase-3 and caspase-9 but less with Smac/DIABLO. Codonoposide 1c treatment led to an association of XIAP with Smac/DIABLO and a dissociation of XIAP from caspase-9 and caspase-3. Thus, XIAP reduction possibly

leads to a diminution of its caspase inhibitory activity, resulting in the upregulation of caspase-9 and -3 activities in codonoposide 1c-treated HL-60 cells. These results indicate that the release of Smac/DIABLO from mitochondria to the cytosol activates caspases by neutralizing the inhibitory activity of XIAP, thus leading to apoptotic induction in codonoposide 1c-treated cells.

DISCUSSION

Natural products have been used in traditional and folk medicine for therapeutic purposes. They are generally non-toxic compared to synthetic chemical compounds, and thus provide important sources of promising leads for the development of novel therapeutic drugs. In this regard, the search for new chemopreventive and anti-tumor agents that are more effective and less toxic has created a great deal of interest in phytochemicals. In particular, saponins are compounds of natural origin and are known to have cytotoxic properties. Recently, we found that the linkage of sugar moieties to saponin is required for biological activity, and that the kinds of sugar moieties are also important for biological degradation in plants or in human intestinal bacteria.^{13,19} Although it has been recently reported that codonoposide 1c (IC₅₀ 30 μ M) showed significant cytotoxicity,¹³ the molecular mechanisms involved and its apoptosis-inducing effect have not been elucidated. The present results demonstrate for the first time that codonoposide 1c can induce apoptosis in human leukemia cells. This effect of codonoposide 1c was confirmed by DNA fragmentation assay, DNA ladder with agarose gel electrophoresis, and the loss of membrane asymmetry (Fig. 2).

The mechanisms of apoptotic activation have been studied intensively in different physiological and pathological conditions.^{20,21} Many genes participate in the regulation of apoptosis, and the activation of caspase is a central effector mechanism of the apoptosis that occurs in response to death-inducing signals from cell surface receptors, mitochondria, or endoplasmic reticulum stress.^{22,23} The data presented here indicate that following the exposure of HL-60 cells to codonoposide 1c, caspase-8, -9 and -3 activities are markedly increased in a time-dependent manner. Moreover, pretreatment with the broad caspase inhibitor z-VAD-fmk, the specific caspase-8 inhibitor z-IETD-fmk, and the specific caspase-3 inhibitor z-DEVD-fmk completely prevented codonoposide 1c-induced DNA fragmentation (Fig. 3B), suggesting that the apoptosis induced by codonoposide 1c involves a caspase-8 and caspase-3-mediated mechanism.

It is becoming increasingly clear that mitochondria play critical roles in the process that leads to cell death. Bcl-2-family proteins are either anti-apoptotic (Bcl-2 and Bcl-xL) or pro-apoptotic (Bax and Bak).²⁴ Anti-apoptotic family members like Bcl-2 are localized mainly in mitochondrial membranes where they block membrane permeabilization.²⁵ Proapoptotic members like Bax are translocated from the cytosol to mitochondria facilitating membrane permeabilization. Bid, as a proapoptotic member, is cleaved by caspase-3 or caspase-8 and then the truncated Bid (tBid), which is translocated from the cytosol to mitochondria. tBid then promotes Bax to facilitate membrane permeabilization. One function by which these proteins affect cell death is the regulation of mitochondrial cytochrome c release, whereas Bax

and tBid promotes cytochrome c release, Bcl-2 prevents cytochrome c release in response to apoptotic substances. Whilst investigating how the impact of codonoposide 1c on mitochondria is mediated, we found that codonoposide 1c causes the truncation of Bid and may stimulate the translocation of Bax from the cytosol to mitochondria and cytochrome c release into the cytosol. Mitochondrial membrane potential is the most informative measure of the changes associated with cytochrome c release. However, such measurements in apoptotic cells have produced conflicting results, with reports of no reduction in potential until after cytochrome c release, a decrease in membrane potential associated with cytochrome c release,²⁶ and an initial increase in potential followed by cytochrome c release without a loss of membrane potential²⁷; however, in the present study, codonoposide 1c induced no reduction in $\Delta\psi_m$ prior to the release of cytochrome c (data not shown).

Since codonoposide 1c exerts an effect on mitochondria, we investigated whether codonoposide 1c stimulates the release of Smac/DIABLO from mitochondria into the cytosol. Smac/DIABLO, a mitochondrial protein, functions as a XIAP neutralizer by binding to XIAP. The binding of Smac/DIABLO to XIAPs could presumably prevent their interaction with caspase, and therefore prevent their inhibition of caspase activation. We found that Smac/DIABLO is released from mitochondria at 2 h after codonoposide 1c treatment along with cytochrome c. Although released cytochrome c contributes to the formation of the apoptosome and thereby to the initiation of the caspase-9-dependent caspase cascade, Smac/DIABLO promotes caspase activity by binding to the XIAP in a manner that displaces caspases from XIAP. Since codonoposide 1c induced the release of Smac into the cytosol, we investigated whether XIAP interacts with Smac/DIABLO during codonoposide 1c-induced apoptosis, by immunoprecipitation. As shown in Fig. 5B, codonoposide-1c treatment induced an interaction between XIAP and Smac/DIABLO. We also observed that codonoposide 1c down-regulated XIAP expression in HL-60 cells (Fig. 5A), indicating that XIAP down-regulation may contribute to the activation of caspase in the codonoposide 1c-induced apoptotic pathway. Furthermore, we observed that caspase-3 and -9 were displaced from XIAP in codonoposide 1c-treated HL-60 cells (Fig. 5B). Taken together, these findings suggest that the caspase inhibitory activity of XIAP is diminished by its interaction with Smac/DIABLO and that this eventually promotes caspase activity.

Compounds capable of inducing the apoptosis of human cancer cells receives a great deal of attention due to their potential use as anti-cancer agents. Accordingly, many chemopreventive agents have been found to exert anti-cancer effects by inducing cancer cell apoptosis.²⁸ In the present study, we found that codonoposide 1c, a natural compound isolated from the root of *Codonopsis lanceolata*, induces apoptosis of human promyelocytic leukemia cells, and we are now investigating whether codonoposide 1c can be further developed as a chemopreventive agent.

Acknowledgments This work was supported by a grant from the Korean Science & Engineering Foundation (No. R13-2002-020-01002-0).

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