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The Novel Triterpenoid CDDO Induces Apoptosis and Differentiation of Human Osteosarcoma Cells by a Caspase-8 Dependent Mechanism

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ABSTRACT

The oleanane triterpenoid 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) is a multifunctional molecule that induces monocytic differentiation of human myeloid leukemia cells and inhibits proliferation of diverse human tumor cell lines. The present studies on human osteosarcoma cells demonstrate that CDDO induces mitochondrial cytochrome *c* release, caspase-3 activation, and internucleosomal DNA fragmentation. Overexpression of the caspase-8 inhibitor CrmA blocked CDDO-induced cytochrome *c* release and apoptosis. By contrast, overexpression of the antiapoptotic Bcl-x_L protein blocked CDDO-induced cytochrome *c* release, but only partly inhibited caspase-3 activation and apoptosis. In concert with these findings, we demonstrate that CDDO: 1) activates caspase-8 and thereby caspase-3 by a cytochrome *c*-independent mechanism and 2) induces cytochrome *c* release by caspase-8-dependent cleavage of Bid. The results also demonstrate that treatment of osteosarcoma cells with CDDO induces differentiation, as assessed by alkaline phosphatase activity and osteocalcin production, and that this response is abrogated in cells that overexpress CrmA. These findings demonstrate that CDDO induces both osteoblastic differentiation and apoptosis by caspase-8-dependent mechanisms.

The synthetic oleanane triterpenoid 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) induces monocytic differentiation of human myeloid leukemia cells, adipogenic differentiation of mouse 3T3-L1 fibroblasts, and nerve growth factor (NGF)-induced neuronal differentiation of rat PC12 cells (Suh et al., 1999). The mechanisms responsible for the differentiating effects of CDDO remain unclear. CDDO inhibits the proliferation of diverse types of human tumor cell lines. CDDO also inhibits the formation of inducible nitric-oxide synthase and cyclooxygenase-2 in macrophages, microglia, and fibroblasts (Suh et al., 1999). Moreover, we recently reported that CDDO induces apoptosis of human myeloid leukemia (Ito et al., 2000).

Mitochondria transduce proapoptotic signals by release of cytochrome *c* into the cytoplasm (Liu et al., 1996; Kluck et al., 1997; Yang et al., 1997). Cytochrome *c* associates with cytoplasmic apoptotic protease activating factor 1 and thereby activates procaspase-9 and caspase-3 (Li et al., 1997; Srinivasula et al., 1998). Other studies have shown that caspase-8 can directly activate caspase-3 (Stennicke et al., 1998). Caspase-8 is activated by stimulation of the Fas receptor, recruitment of FADD/Mort-1 to the receptor and thereby oligomerization and autoprocessing of caspase-8 (Boldin et al., 1996; Muzio et al., 1996). These findings have demonstrated that receptor-mediated apoptosis can be induced by a mitochondria-independent mechanism. Caspase-8 also cleaves Bid, a proapoptotic member of the Bcl-2 family that induces the release of cytochrome *c* (Li et al., 1998; Luo et al., 1998). Bid-induced release of cytochrome *c* thereby amplifies caspase-8-initiated induction of apoptosis.

The present studies demonstrate that CDDO induces apoptosis of osteosarcoma cells and that this response involves caspase-8-dependent cleavage of caspase-3 and Bid. The results also show that CDDO induces differentiation of osteosarcoma by a caspase-8-dependent mechanism.

Experimental Procedures

Cell Culture and Reagents. Saos-2 human osteosarcoma cells (American Type Culture Collection, Manassas, VA) were grown in McCoy's 5a medium supplemented with 10% heat-inactivated fetal bovine serum (Sigma), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Human U2OS osteosarcoma cells were

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ABBREVIATIONS: CDDO, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid; NGF, nerve growth factor; PARP, poly(ADP-ribose) polymerase; *p*NA, *p*-nitroanilide; PKC, protein kinase C; ara-C, 1-β-D-arabinofuranosylcytosine.

grown in Dulbecco's modified Eagle's medium with the same supplements. Stock solutions of 10 mM CDDO were made in dimethyl sulfoxide, and aliquots were frozen at −20°C. Cells were seeded at a density of 1×10^6/100-mm culture dish 24 h before treatment with CDDO.

Isolation of the Cytosolic Fraction. Cytosolic fractions were prepared as described (Kharbanda et al., 1997). Cells were washed twice with PBS and then suspended in ice-cold buffer (20 mM HEPES, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin, aprotinin, and pepstatin A) containing 250 mM sucrose. The cells were disrupted by five strokes in a Dounce homogenizer. After centrifugation of the lysate at 10,000*g* for 5 min at 4°C, the supernatant fraction was centrifuged at 105,000*g* for 30 min at 4°C. The resulting supernatant was used as the soluble cytosolic fraction.

Immunoblot Analyses. Total cell lysates were prepared in lysis buffer containing 1% Nonidet P-40 as described previously (Kaufmann, 1989). Proteins were separated by SDS-10, -12.5, or -15% polyacrylamide gel electrophoresis and then transferred to nitrocellulose filters. After blocking with 5% dried milk in PBS-Tween-20, the filters were incubated with anti-cytochrome *c* (Kirken et al., 1995), anti-Bid (Luo et al., 1998), anti-caspase-9 (PharMingen, San Diego, CA), anti-caspase 3 (anti-CPP32; Transduction Laboratories, Lexington, KY), anti-protein kinase Cδ (PKCδ, Santa Cruz Biotechnology, Santa Cruz, CA), anti-poly(ADP-ribose) polymerase (PARP) (Kauffman et al., 1993), anti-Bcl-x_L (Novartis, East Hanover, NJ) or anti-CrmA. After washing and incubation with horseradish peroxidase-conjugated anti-rabbit (Amersham Pharmacia Biotech, Piscataway, NJ) or anti-mouse (Amersham), the antigen-antibody complexes were visualized by enhanced chemiluminescence (Amersham).

Transient Transfection. Saos-2 cells were transiently transfected by the calcium phosphate method with Bcl-x_L/pE1, CrmA/pE1 or empty vector. At 12 h of transfection, CDDO was added and the cells were incubated for another 24 or 48 h. Total cell lysates or cytosolic fractions were prepared as described and then subjected to immunoblot analysis. Transfection efficiency, as determined with GFP-vector, was reproducibly more than 50% (55–70%).

Assays of Caspase-8 Activity. Caspase-8 activity was measured by spectrophotometric detection (405 nm) of the chromophore *p*-nitroanilide (*p*NA) after cleavage from the labeled substrate IETD-*p*NA (FLICE/Caspase-8 Colorimetric Assay Kit; BioVision Research Products, Palo Alto, CA).

Analysis of DNA Fragmentation. Internucleosomal DNA fragmentation was assessed as described previously (Ito et al., 2000).

Flow Cytometry. DNA content was assessed by staining ethanol-fixed cells with propidium iodide and monitoring by FACScan (Becton Dickinson, Mountain View, CA). Numbers of cells with subG1 DNA content were determined with the MODFIT LT program (Verity Software House, Topsham, ME).

Alkaline Phosphatase Activity. Saos-2 cells were washed with PBS, homogenized in 0.5 M Tris, pH 9.0, containing 0.9% NaCl and 1% Triton X-100 and centrifuged at 12,000*g* for 15 min. Alkaline phosphatase activity in the resultant supernatants was assayed by measuring the release of *p*-nitrophenol from *p*-nitrophenyl phosphate (Sigma Chemical, St. Louis, MO) as described previously (Bretaudiere and Spillman, 1984). Protein content was determined with commercially available kits (Micro/Macro BCA; Pierce Chemical Co., Rockford, IL) to determine specific activity.

Osteocalcin Production. The production of osteocalcin was assessed in culture supernatants using a commercially available radioimmunoassay kit (Human Osteocalcin RIA Kit; Biochemical Technologies, Stoughton, MA) as described previously (Boyan et al., 1998).

Results

CDDO Induces Apoptosis of Osteosarcoma Cells. To determine whether CDDO induces apoptosis of osteosarcoma cells, we treated Saos-2 cells with this agent and then assayed for internucleosomal DNA fragmentation. The results demonstrate that exposure to 5 μM CDDO results in endonucleolytic DNA cleavage (Fig. 1A). Similar results were obtained after CDDO treatment of U2OS osteosarcoma cells (Fig. 1A). In concert with the induction of apoptosis, Saos-2 and U2OS cells also responded to CDDO with cleavage of PARP (Fig. 1B).

CDDO-Induced Apoptosis Involves Activation of the Caspase Cascade. To determine whether CDDO-induced apoptosis involves activation of caspase-3, we assessed cleavage of procaspase-3 by immunoblot analysis. The results demonstrate that caspase-3 is activated at 3 to 6 h of CDDO treatment (Fig. 2A). In concert with this result, the caspase-3 substrates PKCδ and PARP were cleaved with similar kinetics (Fig. 2, B and C). These results demonstrate that CDDO-induced apoptosis involves activation of the caspase-3 cascade.

CDDO-Induced Apoptosis Involves Mitochondrial Cytochrome *c* Release. To determine whether CDDO-induced apoptosis involves the release of cytochrome *c*, we subjected cytosolic preparations to immunoblot analysis with anti-cytochrome *c*. Although exposure of Saos-2 cells to CDDO resulted in increased cytosolic cytochrome *c*, this response was not observed until 18 to 24 h (Fig. 3A). Because the initiator caspase-8 also functions upstream to mitochondria (Boldin et al., 1996; Muzio et al., 1996), we questioned whether CDDO activates caspase-8 by assaying cell lysates for cleavage of IETD-*p*NA. The results demonstrate that induction of caspase-8 is detectable as early as 3 h of CDDO

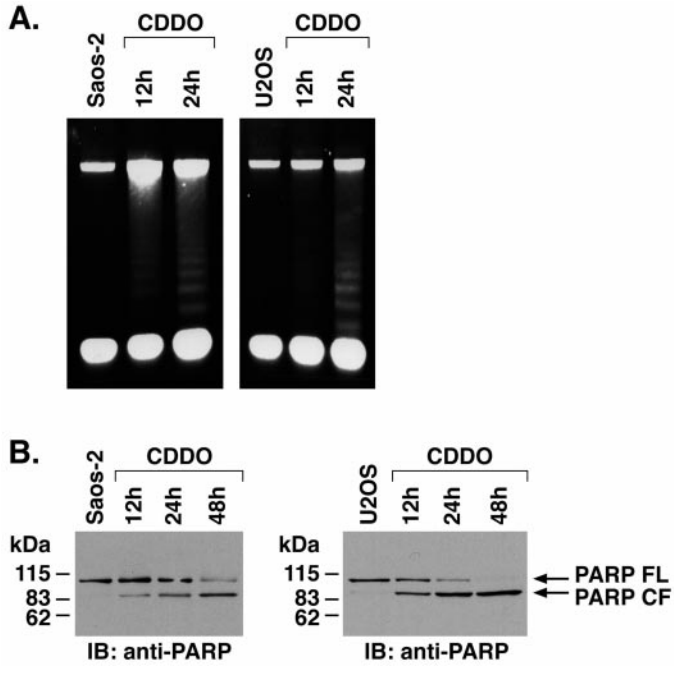

Fig. 1. Induction of apoptosis by CDDO in osteosarcoma cells. Saos-2 and U2OS cells were treated with 5 μM CDDO and harvested at 24 and 48 h. A, DNA fragmentation was monitored by electrophoresis in 1.5% agarose gels. B, total cell lysates were analyzed by immunoblotting with anti-PARP antibody. IB, immunoblot; FL, full length; CF, cleaved fragment.

treatment (Fig. 3B). Because caspase-8 cleaves Bid, and the C-terminal fragment of cleaved Bid translocates to mitochondria and then induces the release of cytochrome *c* (Li et al., 1998; Luo et al., 1998), we also subjected cell lysates to immunoblot analysis with an anti-Bid antibody. The results demonstrate that Bid is cleaved in CDDO-treated Saos-2 cells with kinetics similar to those found for cytochrome *c* release (Fig. 3C). These findings suggest that CDDO induces the release of cytochrome *c* via caspase-8 activation and subsequent Bid cleavage.

Caspase-3 Is Activated Directly by Caspase-8 in Response to CDDO. Because CDDO induces caspase-3 before the release of cytochrome *c*, we hypothesized that direct activation of caspase-3 by caspase-8 might be involved in CDDO-induced apoptosis. To address this issue, we studied Saos-2 cells that transiently overexpress the anti-apoptotic Bcl-x_L protein (Saos-2/Bcl-x_L), the caspase-8 inhibitor CrmA (Saos-2/CrmA), or empty vector (Saos-2/neo). Treatment of Saos-2/CrmA cells, but not Saos-2/Bcl-x_L, with CDDO was associated with a block in caspase-8 activation (Fig. 4A). By contrast, both Bcl-x_L and CrmA blocked the release of cytochrome *c* (Fig. 4B). Moreover, although overexpression of CrmA completely blocked caspase-3 activation, this effect was only partially diminished by overexpression of Bcl-x_L (Fig. 4C). As controls, lysates were analyzed by immunoblotting with anti-Bcl-x_L (Fig. 4D) and anti-CrmA (Fig. 4E). These findings indicate that the cellular response to CDDO involves caspase-8-mediated activation of caspase-3 by a predominantly cytochrome *c*-independent mechanism.

CDDO Induces Apoptosis by a Caspase-8-Mediated Mechanism. To further determine whether caspase-8 activation is necessary for CDDO-induced apoptosis, Saos-2/neo, Saos-2/Bcl-x_L, and Saos-2/CrmA cells were treated with CDDO and then assessed for subG1 DNA content. The results demonstrate that Saos-2/CrmA cells are resistant to CDDO-induced apoptosis (Fig. 5A). By contrast, CDDO-induced apoptotic death was attenuated only in part by Bcl-x_L overexpression (Fig. 5B). These findings provide further sup-

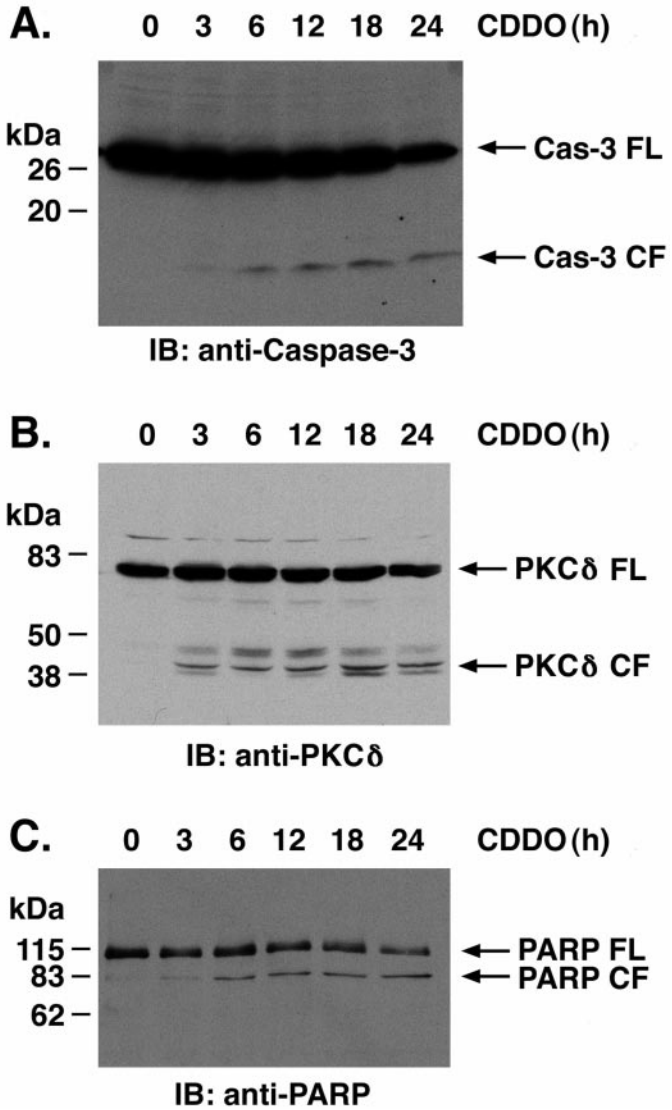

Fig. 2. CDDO induces cleavage of caspase-3 and PKCδ. Saos-2 cells were treated with 5 μM CDDO and harvested at the indicated times. Total cell lysates were analyzed by immunoblotting with anti-caspase-3, anti-PKCδ, or anti-PARP. IB, immunoblot; Cas-3, caspase-3; FL, full length; CF, cleaved fragment.

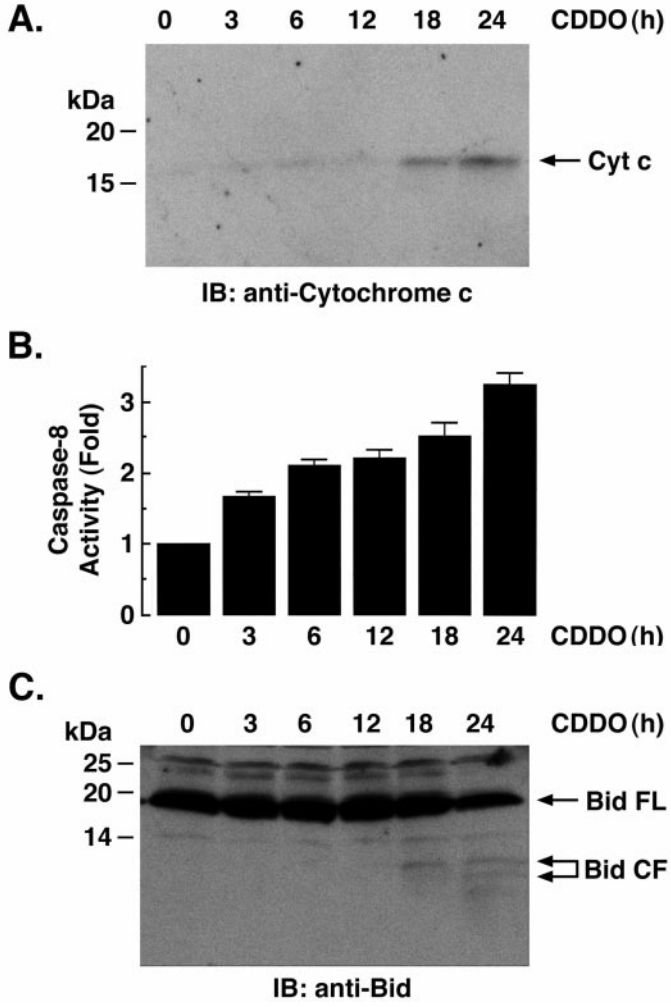

Fig. 3. Cytochrome *c* release, caspase-8 activation, and Bid cleavage in response to CDDO. Saos-2 cells were treated with 5 μM CDDO and harvested at the indicated times. A, cytosolic lysates were analyzed by immunoblotting with anti-cytochrome *c*. B, total cell lysates were assayed for caspase-8 activity. The results are expressed as fold-increase in caspase-8 activity compared with control (mean ± SE of two independent experiments each performed in duplicate). C, total cell lysates were analyzed by immunoblotting with anti-Bid. IB, immunoblot; Cyt c, cytochrome *c*; FL, full length; CF, cleaved fragment.

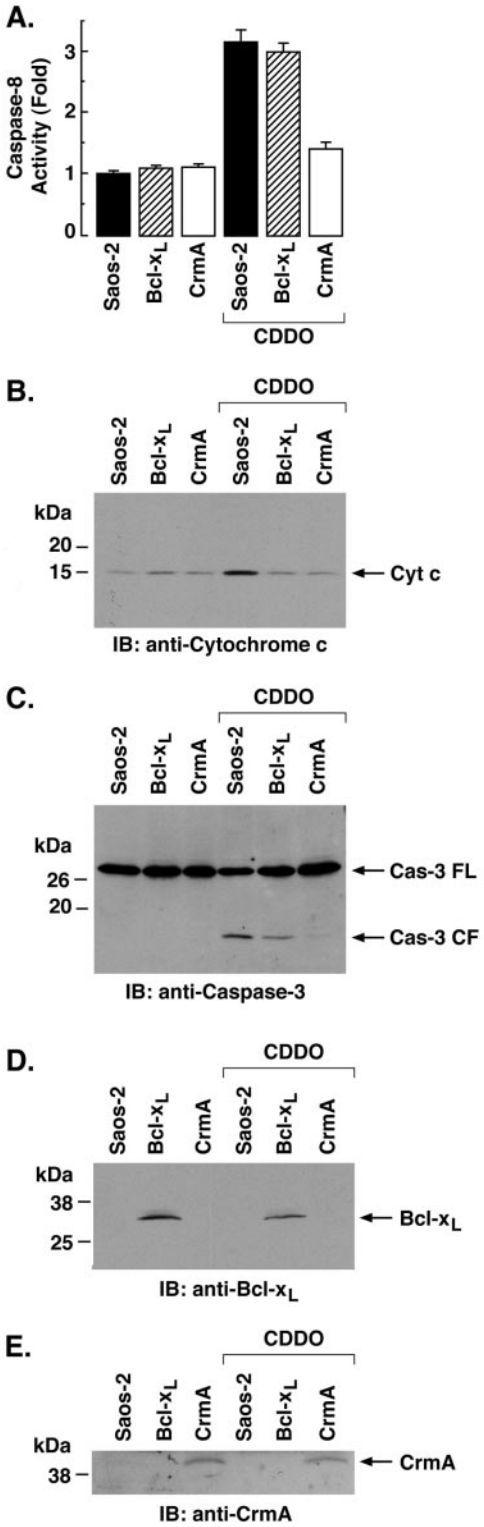

Fig. 4. Effects of Bcl-x_L and CrmA on CDDO-induced caspase-8 activation, cytochrome *c* release, and caspase-3 activation. A, Saos-2/neo (■), Saos-2/Bcl-x_L (▨) and Saos-2/CrmA (□) cells were treated with 5 μM CDDO and harvested at 24 h. Cell lysates were assayed for caspase-8 activity. Results are expressed as the fold increase (mean ± S.E.) for two independent experiments each performed in duplicate. B, cytoplasmic lysates were analyzed by immunoblotting with anti-cytochrome *c*. C, total cell lysates were analyzed by immunoblotting with anti-caspase-3. D and E, total cell lysates were analyzed by immunoblotting with anti-Bcl-x_L and anti-CrmA to assess the levels of expression of transfected Bcl-x_L and CrmA. IB, immunoblot; Cyt c, cytochrome *c*; Cas-3, caspase-3; FL, full length; CF, cleaved fragment.

port for a model in which caspase-8 activation is necessary for CDDO-induced apoptosis in osteosarcoma cells.

CDDO Induces Osteoblastic Differentiation by a Caspase-8-Mediated Mechanism. CDDO induces adipogenic differentiation of mouse 3T3-L1 fibroblasts and contributes to NGF-induced neuronal differentiation of rat PC12 cells (Suh et al., 1999). Therefore, we examined the effects of CDDO on osteosarcoma cells by assessing alkaline phosphatase activity and osteocalcin production as markers of osteoblastic differentiation (Schwartz et al., 2000). Because alkaline phosphatase activity was not detectable in the culture supernatants, these assays were performed on cell lysates. The results demonstrate that CDDO increases alkaline phosphatase activity of Saos-2/neo cells in a dose-dependent manner (Fig. 6A). Similar findings were obtained for osteocalcin production (Fig. 6B). By contrast, there was no detectable effect of CDDO on alkaline phosphatase activity or osteocalcin production in Saos-2/CrmA cells (Fig. 6A). These results suggest that CDDO induces osteoblastic differentiation by a caspase-8-dependent mechanism. Consistent with these results, DNA fragmentation induced by CDDO was also abrogated in Saos-2/CrmA cells (Fig. 6B). These findings collectively demonstrate that CDDO induces both osteoblastic differentiation and apoptosis by caspase-8-dependent mechanisms.

Discussion

Triterpenoids are known to be anti-inflammatory and anticarcinogenic (Nishino et al., 1988; Huang et al., 1994). The synthetic triterpenoid CDDO suppresses the formation of inducible nitric-oxide synthase and cyclooxygenase-2 (Suh et

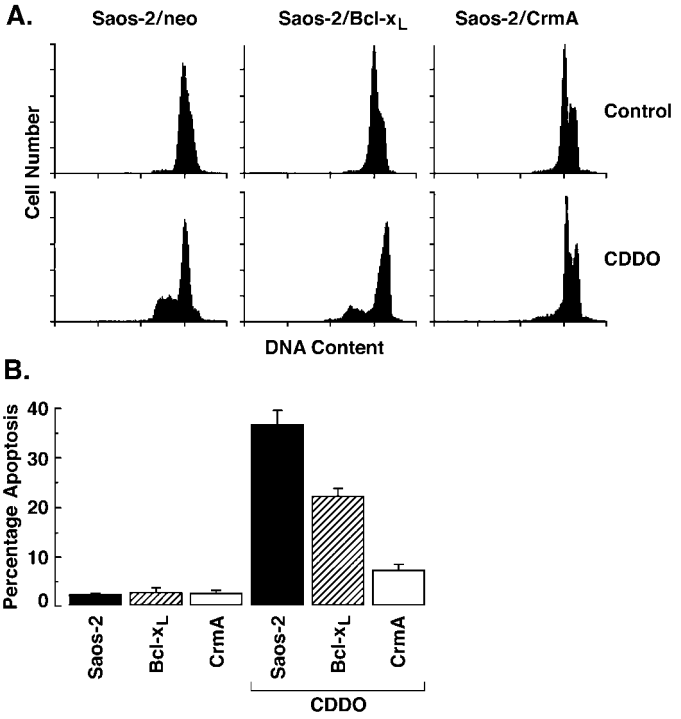

Fig. 5. Effects of Bcl-x_L and CrmA on CDDO-induced apoptosis of osteosarcoma cells. Saos-2/neo, Saos-2/Bcl-x_L and Saos-2/CrmA cells were treated with 5 μM CDDO and harvested at 24 h. The percentage of cells with subG1 DNA was determined by flow cytometry. Results are expressed as the mean ± S.E. of two independent experiments each performed in duplicate.

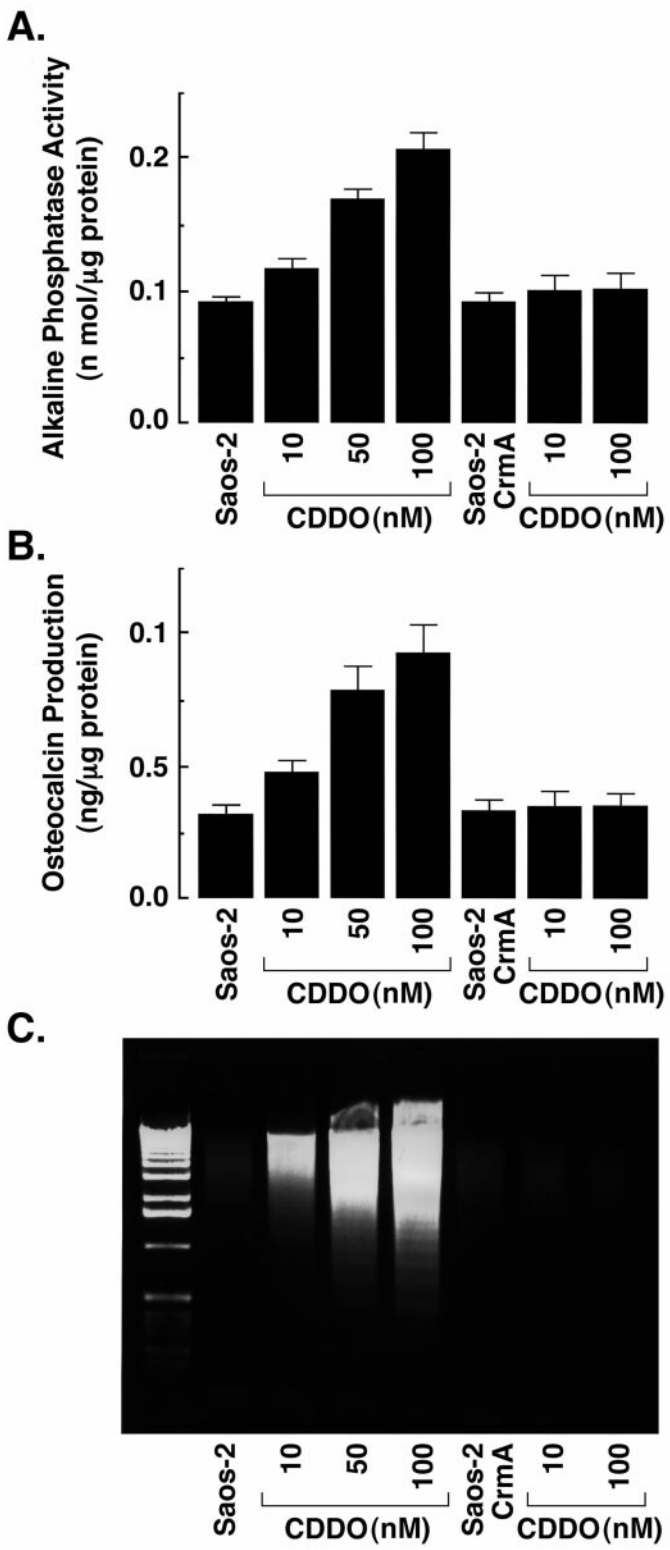

Fig. 6. Effect of CDDO on the alkaline phosphatase activity and DNA fragmentation in Saos-2 cells overexpressing CrmA. Twelve hours after the transfection Saos-2/neo and Saos-2/CrmA cells were treated with 10, 50, or 100 nM CDDO for 48 h. A, the alkaline phosphatase activity is expressed as nanomoles of *p*-NP per microgram of protein per minute. Results are expressed as the mean ± S.E. of two independent experiments, each performed in duplicate. B, culture supernatants were assayed for osteocalcin levels. Results are expressed as the mean ± S.E. of two independent experiments each performed in duplicate. C, DNA fragmentation was monitored by electrophoresis in 1.5% agarose gels.

al., 1999), known enhancers of carcinogenesis, in the cellular response to various inflammatory cytokines (Takahashi et al., 1997; Ambs et al., 1998; Hida et al., 1998; Tsujii et al., 1998). CDDO has also been found to induce differentiation of diverse kinds of cells (Suh et al., 1999). In addition, CDDO has been shown to induce apoptosis of human leukemia cells by a caspase-8-dependent mechanism in which caspase-3 is activated by mitochondria-dependent and -independent pathways (Ito et al., 2000). In this regard, other anticancer drugs, such as cisplatin and etoposide, have been shown to kill cells through caspase-8-mediated pathways (Micheau et al., 1999). The present studies extend the analysis of the biological activities of CDDO by demonstrating that CDDO induces both apoptosis and differentiation of human osteosarcoma cells by a caspase-8-dependent mechanism.

The results show that CDDO induces apoptosis of osteosarcoma cells by caspase-8-mediated cleavage of caspase-3. In this context, CDDO activates caspase-8 and then caspase-3, which precedes Bid cleavage and cytochrome *c* release. Overexpression of the caspase-8 inhibitor CrmA blocked CDDO-induced activation of caspase-3 and apoptosis, whereas these responses to CDDO were diminished only in part by overexpression of Bcl-x_L. These results collectively indicate that caspase-8 is functioning as an initiator caspase in CDDO-induced apoptotic pathway and support a model in which the caspase-8-initiated cascade is amplified by mitochondria signaling (Fig. 7). Overexpression of Bcl-x_L in leukemia cells inhibits 1-β-D-arabinofuranosylcytosine (ara-C) induced caspase-3 activation and apoptosis. As shown in Saos-2 cells, the effects of CDDO on leukemia cell apoptosis were diminished only in part by overexpression of Bcl-x_L. Moreover, CrmA overexpression blocked CDDO-induced but not ara-C-induced caspase-3 activation and apoptosis (Ito et al., 2000). These findings indicate that, in contrast to ara-C, CDDO activates caspase-3 predominantly by a caspase-8-dependent mechanism.

Certain agents have been reported to have both apoptotic and differentiating effects. ara-C incorporates into replicating DNA, inhibits proliferation by functioning as a relative chain terminator (Kufe et al., 1980; Major et al., 1981; Kufe

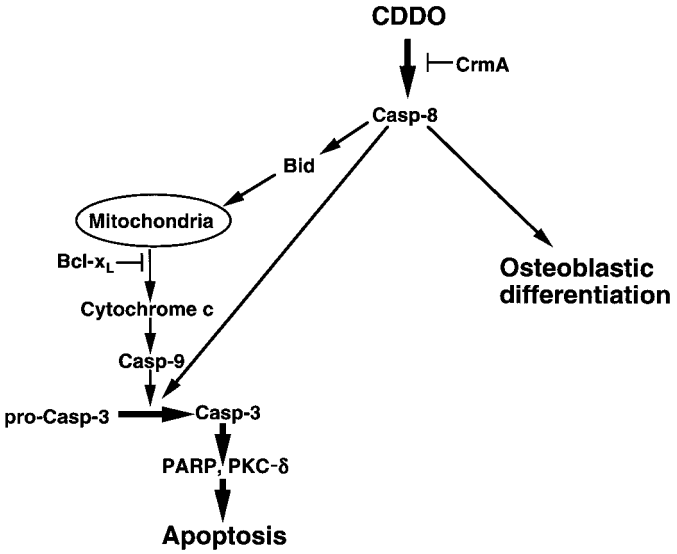

Fig. 7. Schematic representation of CDDO-induced apoptosis and differentiation of osteosarcoma cells.

et al., 1984; Ohno et al., 1988), and induces apoptosis with internucleosomal DNA fragmentation and proteolytic activation of protein kinase Cδ (Gunji et al., 1991; Emoto et al., 1996). In addition, treatment of myeloid leukemia cells with ara-C is associated with induction of a differentiated phenotype (Griffin et al., 1982; Luisi-DeLuca et al., 1984). By contrast, ara-C treatment had little if any effect on induction of osteosarcoma cell differentiation (data not shown). Other studies have shown that 12-tetradecanoylphorbol-13-acetate induces both monocytic differentiation and apoptosis of myeloid leukemia cells by a PKCβ-mediated mechanism (Pandey et al., 2000). Like ara-C and 12-tetradecanoylphorbol-13-acetate, the present work demonstrates that CDDO induces both differentiation and apoptosis of osteosarcoma cells. Our results also show that overexpression of CrmA inhibits both CDDO-induced apoptosis and osteoblastic differentiation. The results further demonstrate that, although CDDO-induced apoptosis is partially abrogated in Saos-2/Bcl-x_L cells, overexpression of Bcl-x_L had no detectable effect on CDDO-induced differentiation (data not shown). These findings thus indicate that CDDO-induced activation of caspase-8 is functional in both apoptosis and differentiation. To our knowledge, this is the first demonstration that caspase-8-mediated signaling is involved in cellular differentiation. The findings that caspase-8 functions in CDDO-induced differentiation provide further support for a model in which differentiation and apoptosis are coordinated through the same pathway.

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