Boswellic acids trigger apoptosis via a pathway dependent on caspase-8 activation but independent on Fas/Fas ligand interaction in colon cancer HT-29 cells

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Boswellic acids are the effective components of gum resin of *Boswellia serrata*, which has anti-inflammatory properties. Recent studies on brain tumors and leukemic cells indicate that boswellic acids may have antiproliferative and apoptotic effects with the mechanisms being not studied in detail. We studied their antiproliferative and apoptotic effects on colon cancer cells and the pathway leading to apoptosis. HT-29 cells were treated with β-boswellic acid (BA), keto-β-boswellic acid (K-BA) and acetyl-keto-β-boswellic acid (AK-BA), respectively. Apoptosis was determined by flow cytometry, by cytoplasmic DNA–histone complex and the activity of caspase-3. The cleavage of poly-(ADP-ribose)-polymerase (PARP) and expression of Fas were examined by western blot. Specific caspase inhibitors, polyclonal Fas antibody, and antagonistic Fas antibody ZB4 were employed to elucidate apoptotic pathways. DNA synthesis and cell viability were examined. Both K-BA and AK-BA increased cytoplasmic DNA–histone complex dose-dependently and increased pre-G1 peak in flow cytometer analysis, with the effects of AK-BA being stronger than K-BA. BA only increased the formation of DNA–histone complex at a high concentration. K-BA and AK-BA increased caspase-8, caspase-9 and caspase-3 activities accompanied by cleavage of PARP. The effects of AK-BA on formation of cytoplasmic DNA histone and on caspase-3 activation were 3.7- and 3.4-fold, respectively, more effective than those induced by camptothecin. The apoptosis induced by AK-BA was inhibited completely by caspase-8 inhibitor and partially by caspase-9 inhibitor. ZB4 blocked exogenous Fas ligand-induced apoptosis, but had no effect on AK-BA-induced apoptosis. AK-BA had no significant effect on expression of Fas. Apart from apoptotic effect, these acids also inhibited [3H]thymidine incorporation and cell viability to different extent. In conclusion, boswellic acids, particularly AK-BA and K-BA have antiproliferative and apoptotic effects in human HT-29 cells. The apoptotic effect is mediated via a pathway dependent on caspase-8 activation but independent of Fas/Fasl interaction.

Abbreviations: AK-BA, acetyl-keto-β-boswellic acid; BA, β-boswellic acid; COX, cyclooxygenase; DMSO, dimethyl sulfoxide; K-BA, keto-β-boswellic acid; MAP, mitogen activated protein; PARP, poly-(ADP-ribose)-polymerase.
and 5% CO₂. BA, K-BA and AK-BA were dissolved in ethanol and camptothecin in dimethyl sulfoxide (DMSO) as 100 mM stock solutions and stored at −20°C. The cells were harvested at ~80% confluence and subcultured in RPMI-1640 medium. After incubation overnight, the cells were treated with the agents tested. Medium containing 0.1% ethanol or DMSO was used as a control.

**Apoptosis detection by ELISA**

Several methods were used to detect apoptosis. The first one is based on a quantification of the enrichment of mono- and oligo-nucleosomes in the cytoplasm by Cell Death Detection ELISA kit. Briefly, 1×10⁶ cells in 100 mL of RPMI-1640 medium were incubated with boswellic acids or camptothecin for 24 h. The cells were lysed and centrifuged. The supernatant containing the cytoplasmic histone-associated DNA fragments was transferred to a microplate coated with streptavidin, and then reacted with a mixture of the anti-histone antibodies labeled with biotin, and anti-DNA antibodies coupled with peroxidase. The substrate of peroxidase was thereafter added and development of the color was read photometrically at 405 nm against 490 nm as background. The specific enrichment of mono- and oligo-nucleosomes released into the cytoplasm was expressed as enrichment factor compared with the control.

**Assay apoptosis and cell cycle phase distribution by flow cytometry**

Cells (3×10⁶) were treated with 100 mM BA, K-BA or AK-BA for 24 h. The cells were then resuspended in ice-cold 70% ethanol and stored at −20°C until analysis. The cellular DNA was stained with PBS containing 100 µg/mL propidium iodide, 0.6% Nonidet P-40 and 100 µg/mL RNase A. Flow cytometric analysis was performed in an Ortho Cytoron Absolute Flow Cytometer (Ortho Raritan, NJ) as described previously (16). The cell cycle phase distribution was analyzed using the Multi2D and MultiCycle software program.

**Assay activities of caspase-3, caspase-8 and caspase-9**

Caspase activities were assayed using their specific substrates as described (17). Briefly, 3×10⁶ cells were incubated with RPMI-1640 medium containing boswellic acids or camptothecin for 24 h. The cells were lysed in 80 µL of ice-cold lysis buffer containing 50 mM HEPES, 100 mM NaCl, 0.1% CHAPS, 1 mM DTT and 0.1 mM EDTA, pH 7.4 and put on ice for 5 min, followed by centrifugation at 10 000 g for 15 min at 4°C. Twenty microliters of the supernatant was added to 100 µL assay buffer containing 50 mM HEPES, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 0.1 mM EDTA, 10% glycerol, pH 7.4. Thirteen microliters of caspase substrate were then added in the reaction system to final concentration of 0.2 mM. The cleavage of the substrate was followed spectrophotometrically at 405 nm and the activities were calculated according to the instruction of the manufacturer.

**Western blot**

For identification of PARP, 3×10⁶ cells were mixed with 100 µL 25 mM Tris–HCl buffer pH 8.0, containing 50 mM glucose, 10 mM EDTA, and 1 mM PMSF, and 50 µL of 50 mM Tris–HCl buffer, pH 6.8, containing 6 M urea, 6% 2-mercaptoethanol, 3% SDS and 0.003% bromophenol blue. The extract was sonicated for 60 s and incubated for 15 min at 65°C before loading. Fifty micromolars of cellular proteins were subject to 10% PAGE, followed by transferring to nitrocellulose membrane electrophoretically. After washing and blocking, the membrane was incubated with PARP antibody (1:2000) for 2 h, and then with biotinylated goat anti-rabbit antibody for 1.5 h. The second antibody was bound to a streptavidin-biotinylated alkaline phosphatase (AP) complex. Color development was proceeded with a Bio-Rad AP conjugate substrates kit (Hercules, CA). For identification of caspase-3, the cells were lysed in 50 mM Tris buffer containing 2 mM EDTA, 10 mM taurocholate, 1 mM µg/ml of leupeptin, 1 µg/ml of aprotinin, 1 mM PMSF and 0.5 mM DTT, followed by sonication for 10 s. Fifty micromolars of protein were subject to 10% SDS–PAGE and transferred to nitrocellulose membrane in a similar way as for PARP. After blocking with 5% dry milk for 4 h, the membrane was then blotted with anti-caspase antibody (1:250). After washing, the second antibody conjugated with alkaline phosphatase was added and the color development procedure was the same as described for PARP. To confirm that an equal amount of proteins was loaded, a parallel western blot was performed in both cases mentioned above. The membranes were blotted with anti-β-actin at 1:10 dilution, according to the instructions of the company.

**Treatment with caspase inhibitors and antagonistic Fas antibody**

To observe the effects of specific caspase inhibitors on boswellic acid-induced apoptosis, Z-DEVD-FMK, Z-IETD-FMK and Z-LEHD-FMK were dissolved in DMSO as 20 mM stock solution and added to the culture medium to a final concentration of 50 µM. After 2 h pre-incubation, AK-BA was added to a final concentration of 100 µM, followed by an additional incubation for 24 h. For the study with antagonistic Fas antibody, the cells were pre-incubated with ZB4 (3 µg/ml) for 2 h. AK-BA or SuperFas ligand was added to a final concentration of 100 µM and 1 µg/ml, respectively, followed by a further incubation for 24 h. The changes of AK-BA induced apoptosis in the presence of these inhibitors were determined as described.

**Assay of cell viability and DNA synthesis**

Cell viability was assayed by determination of the cleavage of tetrazolium salt (WST-1) to formazan by mitochondrial dehydrogenases. The amount of formazan formed is proportional to the viable cell numbers. The cells (2×10⁷) in 200 µL medium were incubated with boswellic acids of different concentrations in a 96 well microplate for 24 h and 20 µL WST-1 was then added in each well. After incubation for 1 h, the production of formazan was read photometrically at 405 nm against 620 nm as a background. The results were expressed as percentage of the control.

The DNA synthesis was measured by [3H]thymidine incorporation. The cells were treated with boswellic acids of different concentrations for 24 h in a microplate (2×10⁵ cells/well in 200 µL medium). After incubation, 0.5 µCi of [3H]thymidine (diluted in 10 µl medium) was added to each well followed by 4 h incubation. The cells were washed with cold PBS three times and then incubated with 10% trichloroacetic acid for 20 min at 4°C. After fixing with methanol for 10 min, the cells were lysed by addition of 200 µl lysis buffer containing 0.25 M sodium hydroxide and 0.1% sodium dodecyl sulfate. An aliquot of 50 µl of the lysate was taken for liquid scintillation counting. DNA synthesis was expressed as percentage of control.

**Statistical analysis**

The results were presented as mean ± standard deviation. The data were analyzed by non-paired Student t-test and P < 0.05 was considered statistically significant.

**Results**

**Apoptosis detected by ELISA**

As shown in Figure 1, both K-BA and AK-BA dose dependently increased the cytoplasmic DNA–histone complex in HT-29 cells. The effect of AK-BA was stronger than that of K-BA, and the effect of BA occurred only at the highest concentration. Incubation with 100 µM BA and K-BA for 24 h increased the levels of cytoplasmic DNA–histone complex to ~2- and 3-fold of control, whereas incubation with 50 µM AK-BA increased the enrichment factor to 6-fold of the control. Further increasing the concentration of AK-BA to 100 µM did not enhance its apoptotic effect significantly.

**Apoptosis measured by flow cytometry**

After incubation with K-BA and A-K-BA of 100 µM for 24 h, the number of apoptotic cells increased significantly as...
Boswellic acid triggers apoptosis in HT 29 cells

Fig. 2. Apoptotic effect of boswellic acids on HT29 cells assayed by flow cytometry. HT-29 cells were treated with 100 µM BA, K-BA or AK-BA for 24 h. DNA was stained with propidium iodide. The figure shows the profile of control cells (A), BA-treated cells (B), K-BA-treated cells (C) and A-K-BA-treated cells (D).

Fig. 3. Changes in the activities of caspase-3, caspase-8 and caspase-9 in HT-29 cells after treatment with boswellic acids. The cells were treated with 100 µM K-BA and AK-BA for 24 h. The cytosolic fraction was extracted and caspase activities were measured. Experiments were performed in triplicate in three separate experiments and results are expressed as mean ± standard deviation. *P < 0.05, **P < 0.01, ***P < 0.001 compared with control.

Fig. 4. Cleavage of PARP detected by western blot. HT-29 cells were treated with 100 µM AK-BA for 24 h. Fifty micrograms of cell-free extract were subjected to 10% SDS–PAGE and the proteins were transferred to nitrocellulose membrane electrophoretically. The membrane was blotted with anti-PARP antibody first and then a secondary antibody coupled with alkaline phosphatase. Color development was proceeded with a Bio-Rad amplified alkaline phosphatase Immun-Blot kit (upper panel). In the lower panel, a parallel experiment was performed and the membrane was blotted with anti-actin antibody.

Cleavage of PARP
It is well known that PARP, the zinc-dependent DNA binding protein that specifically recognizes DNA strand breaks, is cleaved by caspase-3, and the cleavage of PARP is regarded as a specific marker of apoptosis (18). To further confirm the apoptotic effects of boswellic acids in HT29 cells, a western blot was performed to examine the cleavage of PARP by AK-BA. After treating the cells with 100 µM AK-BA, the content of the intact form (113 kDa) of PARP was decreased and its 89 kDa cleaved fragment was increased (Figure 4, upper panel). No changes could be identified when the samples were blotted by anti-actin (Figure 4, lower panel).

Comparison of the apoptotic effect of AK-BA with camptothecin
To evaluate the efficacy of the effects of boswellic acid on HT29 cells, the formation of DNA–histone complex and the activation of caspase-3 induced by AK-BA were compared with those induced by camptothecin. As shown in Figure 5, at 50 µM, both effects of AK-BA were ~3-fold greater than those induced by camptothecin.

Effect of caspase inhibitors and antagonistic Fas antibody on boswellic acid-induced apoptosis
In order to elucidate which pathway was predominant for boswellic acid-induced apoptosis, specific caspase inhibitors and antagonistic Fas antibody ZB4 were employed. As shown in the upper panel of Figure 6, both caspase-3 and caspase-8 inhibitors completely blocked AK-BA-induced apoptosis while the inhibitor of caspase-9 only partially inhibited it. As caspase-8 is closely linked to a signal transduction pathway initiated by interaction of Fas/FasL, we examined whether activation of Fas would be important in mediating the effect of boswellic acids. As shown in the lower panel of Figure 6, pre-treatment with ZB4 did no have any inhibitory effect on boswellic acid-induced apoptosis, although it did completely block the apoptosis induced by exogenous Fas ligand. We then further studied whether the expression of Fas could be increased by boswellic acids. As shown in the upper panel of Figure 7, stimulating the cells with AK-BA or BA at 50 or 100 µM for 24 h had no significant effect on the levels of Fas in the cells. The negative effect were strengthened by the similar levels of β-actin in the samples as demonstrated in the lower panel of Figure 7.

demonstrated by the sub-G1 peak (Figure 2C and D). The apoptotic effect of AK-BA was stronger than that of K-BA, as AK-BA induced a much larger proportion of sub-G1 cells. Flow cytometry did not show a significant increase of sub-G1 peak for BA (Figure 2B) at the same concentration.

Changes in caspase activities
The changes of three types of caspase activities are shown in Figure 3. After treatment with 100 µM K-BA and AK-BA, the activity of caspase-3 increased ~300 and 250%, respectively, compared with control. They also increased the activities of caspase-8 by 88 and 275%, respectively, and those of caspase-9 by 137 and 262%, respectively. BA at 100 µM concentration did not show significant stimulatory effect on the activities of all three kinds of caspases.
Fig. 5. Comparison of the effects of AK-BA on apoptosis and caspase-3 activation with camptothecin. The cells were treated with 50 µM AK-BA or 50 µM camptothecin for 24 h. The formation of DNA–histone complex (upper panel) and the activity of caspase-3 (lower panel) were determined. Experiments were performed in triplicate in three separate experiments and results are mean ± standard deviation. **P < 0.01, ***P < 0.001 compared with control.

Inhibition of DNA synthesis and cell viability by boswellic acids

Figure 8 demonstrated that BA, K-BA and AK-BA inhibited the [3H]thymidine incorporation in a dose-dependent manner (upper panel) with the effect of AK-BA being strongest, followed by K-BA and BA. At 50 µM, AK-BA inhibited DNA biosynthesis by 53%, whereas K-BA and BA decreased it by 33 and 25%, respectively. When the cell viability was determined (Figure 8, lower panel), AK-BA strongly inhibited cell viability in a dose-dependent manner. At 100 µM AK-BA reduced the cell viability by 50%, whereas BA and K-BA only slightly inhibited the viability by 15%. These effects are not statistically significant.

Discussion

In the present study, we found that boswellic acids have antiproliferative and apoptotic effects on HT29 colon cancer cells. The apoptotic effect of AK-BA was demonstrated by the increased cytoplasmic DNA–histone complex, and the increased sub-G1 peak by flow cytometric analysis. The apoptotic effect was also confirmed by the activation of caspase-3,
the executor of apoptosis, and cleavage of PARP, another specific marker of apoptosis.

Apoptotic effect is considered to be one of the important properties for many anticancer drugs. Apoptosis occurs via two major different activation pathways in principle (19,20). One pathway involves changes in mitochondrial transmembrane potential, leading to the release of cytochrome c. Cytochrome c then binds the apoptosis activating factor 1 and procaspase-9, resulting in the activation of caspase-9 by proteolytic cleavage. The other pathway starts with death receptor ligation or Fas/FasL interaction, followed by oligomerization of the receptor, recruitment of Fas-associated death domain protein (FADD), and activation of caspase-8 (21). Both caspase-9 and caspase-8 are defined as initiator caspases and can in turn activate caspase-3, the executor of apoptosis (22,23). Recently, it was found that the cross communication exists between the two pathways, as caspase-8 may activate caspase-9 via Bid, a member of bcl-2 family (24). Although the apoptotic effect of boswellic acids were reported previously in glioma and leukemic cells, the caspase cascade activated by the acids leading to apoptosis has not been studied (9,11). In the present work, we demonstrated for the first time that the activities of caspase-3, caspase-8 and caspase-9 were activated by boswellic acids, indicating that both death receptor-related apoptotic pathway and the mitochondria-related pathway were activated. When specific inhibitors were employed, however, we found that either specific caspase-8 or caspase-3 inhibitor completely blocked the boswellic acid-induced apoptosis, while the caspase-9 inhibitor only partially inhibited it. These findings imply that apoptosis induced by boswellic acids in HT-29 cells is initiated by a pathway involving the activation of caspase-8, leading to the activation of caspase-3 and the cleavage of PARP. The activation of caspase-9 is probably a secondary consequence of the activation of caspase-8 through the cross communication between the two apoptotic pathways (25,26).

It is well known that activation of caspase-8 is closely linked to the death receptor Fas (also known as APO-1 or CD95) that belongs to the tumor necrosis factor/nerve growth factor receptor family (26,27). In colon cancer, the Fas signaling pathway via Fas/FasL interaction was indicated to be involved in liver metastasis, counterattack of tumor-immune conflict and cytotoxic drug-induced apoptosis, although its exact role was still controversial (28–30). In the present study, we found that Fas/FasL interaction was not critical in boswellic acid-induced apoptosis, because antagonistic Fas antibody ZB4 failed to inhibit boswellic acid-induced apoptosis at a dose that completely blocked the apoptosis induced by FasL. The effect of boswellic acids may neither affect the levels of Fas, since western blot failed to identify any increase in Fas after BA and AK-BA stimulation. It is therefore reasonable to propose that boswellic acid induced apoptosis in HT29 cells is mediated by mechanisms downstream of Fas/FasL interaction, such as Fas clustering, recruitment of FADD, or enhancing the cleavage of procaspase-8. These possibilities have been recently indicated in studies with several other anticancer drugs (31–33).

Boswellic acids are inhibitors of 5-lipoxygenase, and the inhibitory effect has been demonstrated in both intact cells and in the cell-free system (3,5,34). It was reported that MK886, the inhibitor of 5-lipoxygenase activating protein triggered apoptosis in both prostate and breast cancer cells (35,36). Thus, the inhibitory effects of boswellic acid on 5-lipoxygenase may be responsible for its apoptotic properties. However, in some leukemic cell lines such as HL-60 and CCRF-CEM cells, which do not express 5-lipoxygenase mRNA constitutively, apoptotic and antiproliferative effect of boswellic acids could still be observed, indicating that the blockade of 5-lipoxygenase may not be the only mechanism underlying its apoptotic effect (37). Boswellic acids were reported to inhibit topoisomerases (37,38), and to increase the intracellular calcium and activity of mitogen activated protein (MAP) kinase in human isolated leukocyte (39). Calcium mobilization has been reported to be linked to death receptor and caspase-8 activation (40,41). Putting together, to find out the relationship between 5-lipoxygenase inhibition, topoisomerase inhibition,
catalyze calcium mobilization, MAP kinase activation and caspase-8 activation are interesting topics for further investigation.

The anticancer effects of boswellic acids varied with the type of the acids. K-BA and AK-BA had significant effects on apoptosis. BA seems only effective in the inhibition of DNA biosynthesis. In terms of either antiproliferative or apoptotic effects among the boswellic acids tested, AK-BA was the most effective one, followed by K-BA and BA. Considering the structure of various boswellic acids, the keto group might be important for apoptotic properties of the acids and the presence of acetyl group may strongly enhance its apoptotic effects.

Our findings that boswellic acids have antiproliferative and apoptotic effects in colon cancer cells may have clinical implications. The chemopreventive effect of NSAIDs such as sulindac on colon cancer has been well documented in the past two decades (42,43). However, blockade of COX by NSAIDs, especially the blockade of COX-1, could cause multiple severe side effects such as the bleeding in the intestinal tract and impairment of renal function (44,45). In contrast, multiple severe side effects such as the bleeding in the intestinal tract and impairment of renal function (44,45). In contrast, boswellic acids did not inhibit COX and their clinical safety was demonstrated in the treatment of Crohn’s disease, ulcerative colitis and bronchial asthma (4,12,14,46). Because sulindac on colon cancer has been well documented in the chemopreventive effect of NSAIDs such as apoptosis in colon carcinoma cells. Finally, AK-BA was found to be ~3-fold more effective than K-BA in a high dose. In addition, we reported recently that K-BA and AK-BA also induced apoptosis in HepG2 cells (47), a cell line that is better differentiated than HT29 cells (48). Thus, boswellic acid may affect cells of different degrees of differentiation. Finally, AK-BA was found to be ~3-fold more effective than camptothecin, a compound that has a similar inhibitory effect on topoisomerase as boswellic acid. Derivatives of camptothecin are normally prescribed in colorectal cancer cases that have not responded to standard chemotherapy treatment. Judged from these aspects above, whether boswellic acids can be promising candidates for the chemoprevention of colon cancer might be an interesting topic for further investigation.

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Boswellic acid triggers apoptosis in HT 29 cells

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