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β-Hydroxyisovalerylshikonin Inhibits the Cell Growth of Various Cancer Cell Lines and Induces Apoptosis in Leukemia HL-60 Cells through a Mechanism Different from Those of Fas and Etoposide

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β-Hydroxyisovalerylshikonin (β-HIVS), which was isolated from the plant, *Lithospermium radix*, inhibited the growth of various lines of cancer cells derived from human solid tumors at low concentrations between 10^{-8} and 10^{-6} M. When HL-60 cells were treated with 10^{-6} M β-HIVS for 3 h, characteristic features of apoptosis, such as DNA fragmentation, nuclear fragmentation, and activation of caspase-3-like activity, were observed. The most characteristic features of the effect of β-HIVS were the remarkable morphological changes induced upon treatment of HL-60 cells with β-HIVS, as visualized on the staining of actin filaments with phalloidin labeled with tetramethylrhodamine B isothiocyanate. Moreover, activation of MAP kinases, such as ERK2, JNK and p38, was detected after treatment with 10^{-6} M β-HIVS preceding the appearance of the characteristics of apoptosis, and the features of the activation of these MAP kinases were quite different from those of Fas and anticancer drug-induced apoptosis. The activation of JNK by β-HIVS was not inhibited by inhibitors of caspases, suggesting that JNK is located either upstream or independent of the caspase signaling pathway. β-HIVS did not inhibit the activity of topoisomerase II. These results indicate that β-HIVS induces apoptosis in HL-60 cells through a mechanism unlike those reported for anti-Fas antibodies and etoposide.

Key words: apoptosis, JNK, leukemia cells, MAP kinase, shikonin.

Cancer cells generally do not differentiate or undergo apoptosis. However, even cancer cells can be induced to differentiate and undergo apoptosis by certain chemical reagents. Most inducers of differentiation, such as camptothecin (*1-3*) and VP16 (*1, 2, 4*), also induce apoptosis in cancer cells. Moreover, various chemotherapeutic agents that are used to treat cancers, such as cisplatin (*1, 4, 5*), adriamycin (*6*), and taxol (*7*), have been reported to have apoptosis-inducing activity. Therefore, chemical agents with strong differentiation-inducing or apoptosis-inducing activity but minimal toxicity are expected to have potential for use as anticancer drugs.

We have identified a variety of inducers of differentiation and apoptosis in cancer cells. These inducers include bufalin (*8, 9*); isoprenoid compounds, such as geranylgeranylacetone (*10*), geranylgeraniol (*11*), and vitamin K_2 (*12*); as well as inhibitors of topoisomerase, such as camptothecin (*13*) and VP16 (*14*). In the present study, we screened various natural products as novel inducers of apoptosis in human leukemia HL-60 cells and we found that β-hydroxyisovalerylshikonin (β-HIVS) had potent apoptosis-inducing activity. Shikonin, a pigment isolated from the Chinese plant, *Lithospermum erythrorhizon*, was reported to exhibit antitumor activity when murine tumors were inoculated intraperitoneally with sarcoma 180 ascites cells (*15*). Recently, Fujii *et al.* reported that shikonin at concentrations above 10^{-5} M inhibited topoisomerase II by inducing the topoisomerase II-mediated cleavage of DNA *in vitro* (*16*). β-Lapachone, a naturally occurring quinone, also inhibits topoisomerases I and II (*17, 18*). We found that, among various derivatives of shikonin, β-HIVS had the strongest apoptosis-inducing activity in HL-60 cells. β-HIVS inhibited the growth of various lines of human cancer cells at concentrations from 10^{-8} to 10^{-6} M. Using HL-60 cells, we demonstrated that the inhibition of growth was due to the induction of apoptosis and that the signal transduction pathway of β-HIVS was quite different from those reported to date for Fas/APO-1/CD95 and anticancer drugs such as etoposide. The characteristics of apoptosis induced by β-HIVS in HL-60 cells are described in the present paper.

MATERIALS AND METHODS

Materials—Human cancer cells were provided by the Japanese Cell Research Resources Bank. Phalloidin labeled with tetramethylrhodamine B isothiocyanate (TRITC), and myelin basic protein (MBP) were purchased from Sigma Chemical (St. Louis, MO).

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Benzyloxycarbonyl-Asp-$CH_2OC(O)$-2,6,-dichlorobenzene (Z-AspCH_2DCB), benzyloxy-carbonyl-Asp-Glu-Val-Asp-fluoromethylketone (Z-DEVD-FMK), and the fluorescent substrates, MCA-YVADAPK(dnp)-NH_2 and MCA-DEVDAPK(dnp)-NH_2, were purchased from the Peptide Institute (Osaka). [γ-^{32}P]ATP was obtained from Amersham (Buckinghamshire, United Kingdom). Polyclonal antibodies against p38 (C20) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Isolation of Shikonin and Its Derivatives—Shikonin and its derivatives were isolated from *Lithospermum radix*. In brief, mature *L. radix* plants were cut into small pieces and extracted with ether, and then the solvent was evaporated off in an evaporator. Shikonin was purified by silica gel thin-layer chromatography after hydrolysis of the derivatives with 2% NaOH. For isolation the derivatives of shikonin, extracts were applied to plates of silica gel (Kieselgel 60; Merck, Berlin, Germany) that were developed with a mixture of *n*-hexane, acetone and formic acid (70:30:1, v/v). Four spots were detected and scraped off. Then the silica gel was extracted with ether and centrifuged, and the supernatant was collected. After complete evaporation of the ether, each sample was dissolved in ethanol. The structures of shikonin and its derivatives were determined by NMR spectroscopy (*19*).

XTT Assay—A suspension of cells was treated with a sample compound. After culture for 18 h at 37°C, a solution of 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide (XTT) and phenazine methosulfate (PMS) was added, and then the culture was continued for an additional 4 h. The absorbance at 540 nm was measured on a 96-well plate with a Micro plate reader (Toyo Soda Kogyo, Tokyo).

Analysis of DNA Fragmentation—Cellular DNA was extracted as reported previously (*9*), and electrophoresis was performed on 1% agarose gels in 40 mM Tris-acetate buffer (pH 7.4) at 50 V. After electrophoresis, DNA was visualized by staining with ethidium bromide.

Preparation of Cell Lysates, and Assaying of Caspase and Protein Kinase Activities—The preparation of cell lysates and assaying of caspase activity were performed with specific fluorescent substrates as described by Enari *et al.* (*20*). Cell lysates for the assaying of kinase activities were prepared as described previously (*9*). Extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) activities were measured as described previously (*9, 21*). p38 activity was measured by immunoprecipitation of the proteins in aliquots of cell lysates containing 400 μg of protein with p38-specific antibodies and protein G-Sepharose (Pharmacia Biotech Japan) for 1 h at 4°C. After washing, the phosphorylation reaction was performed in 12 μl kinase buffer [50 mM Tris-HCl, pH 7.5, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), and 10 mM $MgCl_2$] containing 50 μM [γ-^{32}P]ATP (1 μCi/assay) and 2 mg/ml MBP as the substrate for 10 min at 30°C. The reaction was stopped by the addition of 10×Laemmli buffer. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels (13.5% polyacrylamide) were subjected to autoradiography and labeled proteins were quantified with a Bio-imaging Analyzer (Fuji, Tokyo). The in-gel kinase assay was conducted as described previously (*21*) using a SDS polyacrylamide gel containing 1 mg/ml of GST-c-Jun (1-79).

Staining with Phalloidin-TRITC—Cells were fixed for 5 min in 3.7% formaldehyde in phosphate-buffered saline (PBS) at room temperature, washed twice with PBS and then extracted with piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) buffer (0.1 M PIPES, pH 6.9, 4% PEG 6000, EGTA, and 0.1% Triton X-100). After three washes with PBS, the cells were incubated with 3 μM phalloidin-TRITC in PBS for 30 min at room temperature. Then observations were made under a fluorescence microscope or a confocal laser-scanning microscope (Bio-Rad, Hercules, CA).

Immunoblotting Analysis—Cells were extracted with the lysis buffer as described previously (*21*). SDS-PAGE (10% polyacrylamide) was performed with 50 μg of total protein per sample. The protein bands were transferred to a PVDF membrane (Millipore, Bedford, MA), and then the membrane was incubated with primary antibodies and horseradish peroxidase-linked second antibodies. Immunoblots were developed with electro-chemiluminescence (ECL) reagents (DuPont NEN Research Products, Boston, MA).

RESULTS

Effects of Shikonin and Its Derivatives on Human Cancer Cells—Figure 1, A and B, shows the structures of shikonin and its derivatives that were separated by thin-layer chromatography. The structures were confirmed by nuclear magnetic resonance spectroscopy. The basic structure of shikonin and its derivatives is that of a naphthoquinone. Human leukemia HL-60 cells were treated with shikonin or its derivatives for 18 h, and then cell viability was determined by the Trypan blue exclusion test (Fig. 1C). Shikonin and its derivatives had a lethal effect on HL-60 cells. Among shikonin and its derivatives, β-HIVS was the most cytotoxic, the concentration needed to kill 50% of HL-60 cells in 18 h being 4.1×10^{-7} M. Neither naphthoquinone nor another derivative of naphthoquinone, vitamin K_2, at 10^{-6} M was effective in inducing the cell death of HL-60 cells. In terms of cell death-inducing activity, β-HIVS was about 10 times stronger than shikonin. Therefore, β-HIVS was used in all further experiments. We examined the effects of β-HIVS on the growth of various human cancer cells. Cell growth was monitored in terms of reduction of XTT, and the concentrations for 50% growth-inhibition (IC_{50}) are shown in Table I. From this Table, it can be seen that β-HIVS strongly inhibited the growth of all lines of cells derived from human solid tumors that we examined. The values of IC_{50} ranged from 10^{-8}-10^{-6} M for the various lines of cancer cells.

Induction of DNA Fragmentation by β-HIVS—To determine whether the features of the cell death induced by β-HIVS were those of apoptosis or necrosis, we analyzed the internucleosomal fragmentation of DNA in HL-60 cells that had been treated with 10^{-6} M β-HIVS for various times. As shown in Fig. 2, treatment of HL-60 cells with 10^{-6} M β-HIVS for more than 3 h resulted in the ladder pattern of DNA fragments that is characteristic of apoptosis.

Morphological Changes Induced by β-HIVS—We noticed that HL-60 cells underwent marked morphological changes upon treatment with β-HIVS. We analyzed these changes by confocal laser-scanning microscopy after staining of the cells with TRITC-labeled phalloidin, which binds

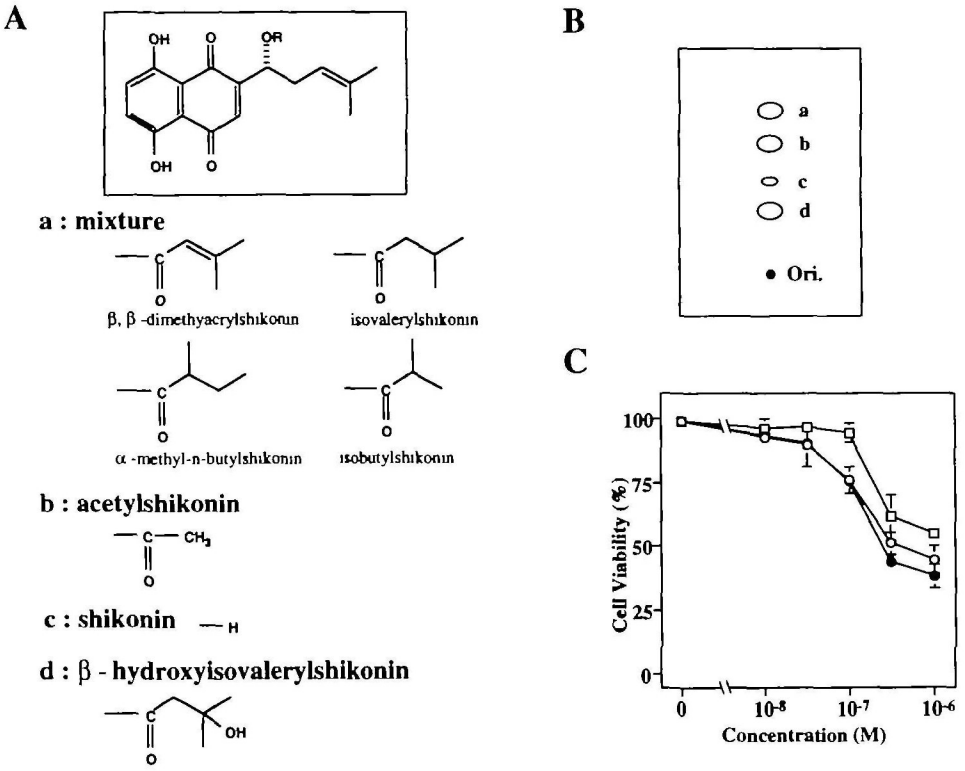

Fig. 1. **Chemical structures of shikonin and its derivatives, and their effects on the viability of human leukemia HL-60 cells.** (A) Structures of shikonin and its derivatives. Upper panel, basic structure of naphthoquinone. See (B) for sources of a through d. (B) Separation by TLC as described under "MATERIALS AND METHODS." Ori, the origin on the TLC plate; spot a, mixture of derivatives of shikonin, namely, β,β-dimethylacrylshikonin, isovalerylshikonin, α-methyl-*n*-butylshikonin and isobutylshikonin; spot b, acetylshikonin; spot c, shikonin; spot d, β-hydroxyisovalerylshikonin (β-HIVS). The structures were confirmed by NMR spectroscopy. (C) HL-60 cells were treated with shikonin or its derivatives for 18 h at various concentrations. The percentage of viable cells was determined by counting cells that excluded Trypan blue. ○, acetylshikonin; ●, β-HIVS; □, shikonin. The data are represented as the means±SD of the results from three independent experiments.

TABLE I. **Inhibitory effects of β-HIVS on the growth of human cancer cells.** Cells were treated with β-HIVS for 4 days. IC_{50} indicates concentrations for 50% inhibition of growth. The percentage viability was determined by the XTT assay, as described under "MATERIALS AND METHODS."

Cells line	IC_{50}(M)
HL-60 (acute promyelocytic leukemia)	$3.5\pm1.2\times10^{-8}$
U937 (monocyte-like, histiocytic lymphoma)	$2.8\pm0.1\times10^{-7}$
VMRC-MELG (colon, malignant melanoma)	$5.5\pm0.5\times10^{-8}$
COLO320DM (colon, adenocarcinoma)	$4.1\pm0.5\times10^{-6}$
AZ-521 (gastric cancer)	$1.5\pm0.2\times10^{-6}$
MIA Paca-2 (pancreatic cancer)	$2.0\pm0.2\times10^{-6}$

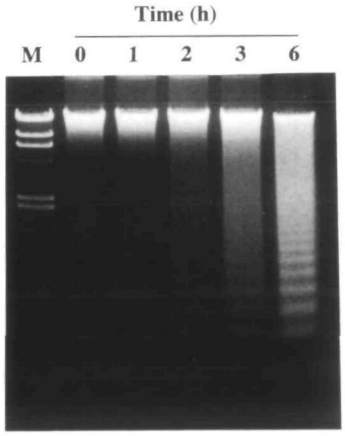

Fig. 2. **DNA fragmentation induced in HL-60 cells by β-HIVS.** HL-60 cells were treated with 10^{-6} M β-HIVS for the indicated times, and then DNA fragmentation was analyzed by agarose gel electrophoresis and staining with ethidium bromide.

to actin filaments and emits red fluorescence. As shown in Fig. 3, actin was dispersed in HL-60 cells before treatment with β-HIVS. On treatment with 10^{-6} M β-HIVS for 3 h, the distribution of actin in HL-60 cells changed markedly and many constrictions were induced on the cell surface (Fig. 3). In order to determine whether or not the polymerization of actin plays a role in the induction of apoptosis by β-HIVS, we treated HL-60 cells with β-HIVS in the presence and absence of cytochalasin B, which inhibits the binding of monomers of G-actin to F-actin. The nuclei of HL-60 cells that had been treated with β-HIVS were condensed and fragmented in both the presence and absence of cytochalasin B (results not shown). These results indicated that the polymerization of G-actin was not needed for the induction of apoptosis by β-HIVS, even though changes in the distribution of actin were induced by β-HIVS.

Induction of Caspase-3-Like Activity by β-HIVS—In order to determine which caspase is activated during the apoptosis induced by β-HIVS, we used peptide substrates coupled to the fluorescent 4-methoxycoumarin-4-yl-acetyl (MCA) group and its quenching 2,4-dinitrophenyl (dnp) group. When HL-60 cells were treated with 10^{-6} M β-HIVS for 2 h, caspase-3-like activity increased markedly with time, but no caspase-1-like activity was detected (Fig. 4A). The pattern of the increases in caspase-3-like activity was well correlated with that of the β-HIVS-induced fragmentation of DNA. To confirm the involvement of caspases in β-HIVS-induced apoptosis, we used two types of inhibitor, benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone (Z-DEVD-FMK) and benzyloxycarbonyl-Asp-CH_2OCO)-2,6-dichlorobenzene (Z-Asp-CH_2DCB). Z-

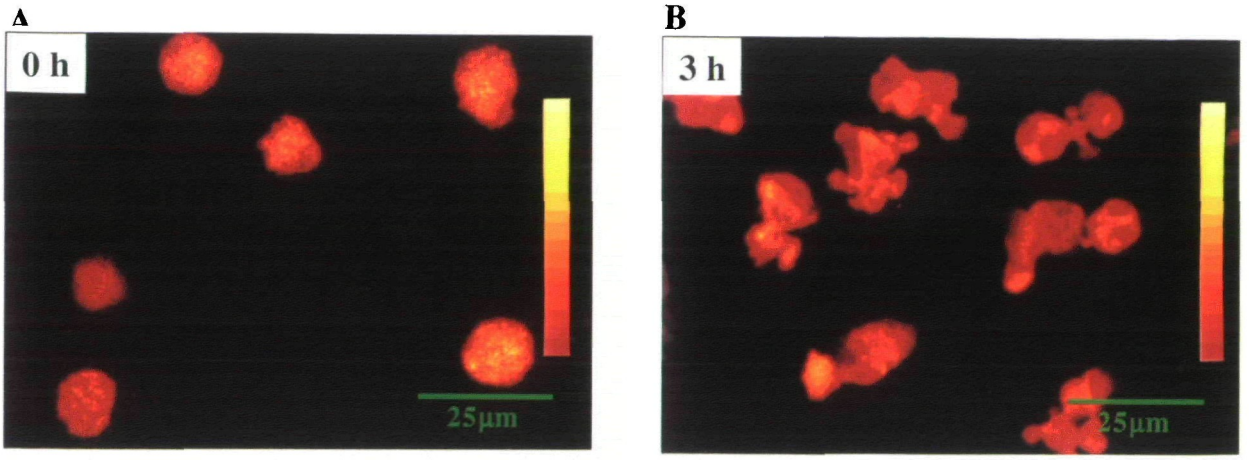

Fig. 3. **Effect of β-HIVS on the cytoskeleton in HL-60 cells.** HL-60 cells that had been treated with 10^{-6} M β-HIVS were stained with phalloidin labeled with TRITC, and the examined under a confocal laser-scanning microscope. A, untreated HL-60 cells; B, after treatment with β-HIVS for 3 h. Scale bar, 25 μm. The concentration of actin decreased in the following order: yellow > red > black.

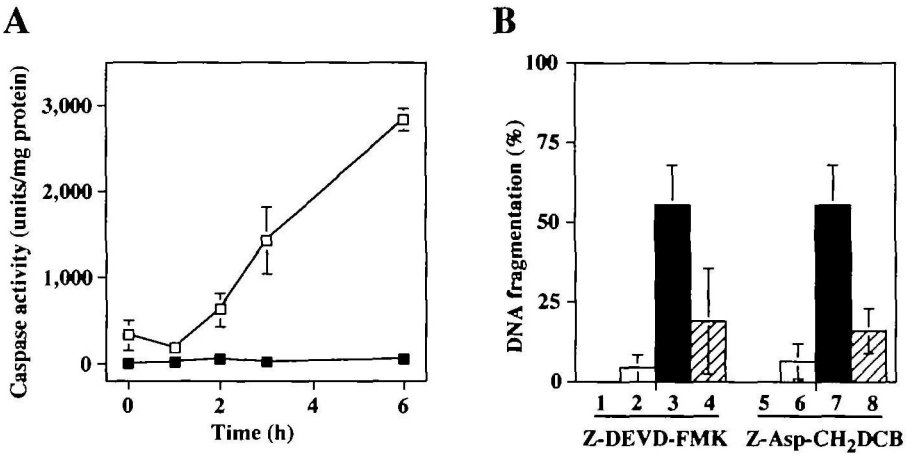

Fig. 4. **Analysis of the activities of caspase-1-like and caspase-3-like proteases during apoptosis induced by β-HIVS.** (A) Measurement of caspase-3-like (□) and caspase-1-like (■) activities. One unit is defined as the amount of enzyme required to cleave 1 pmol of the fluorescent substrate at 30°C in 30 min under the standard reaction conditions. The data are presented as the means ± SD of the results for three independent experiments. (B) Effects of inhibitors of caspases on DNA fragmentation induced by β-HIVS. HL-60 cells were incubated for 1 h with 50 μM inhibitor (columns 2, 4, 6, and 8), and then treated for an additional 3 h with 10^{-6} M β-HIVS (columns 4 and 8). Columns 1 and 5, untreated control cells; columns 3 and 7, β-HIVS-treated cells without pretreatment with an inhibitor. The data are presented as the means ± SD of the results for three independent experiments.

DEVD-FMK inhibits caspase-3-like activity specifically and Z-Asp-CH_2DCB is a broad spectrum inhibitor of the caspase family. Both inhibitors almost completely inhibited the DNA fragmentation that was otherwise induced by β-HIVS (Fig. 4B). These results suggest that a caspase-3-like protease but not a caspase-1-like one might be activated during apoptosis in HL-60 cells induced by β-HIVS.

Activation of MAP Kinases—The balance between the ERK and JNK-p38 pathways has been proposed to play an important role in the regulation of apoptosis (*22*): the activation of JNK and p38 promotes apoptosis, whereas the activation of ERK prevents it. As shown in Fig. 5A, ERK2 was activated by treatment with 10^{-6} M β-HIVS and the maximum activation of ERK2 of 42 kDa was detected 1 h after the start of treatment. Estimation of the activity of ERK2 from the extent of incorporation of ^{32}P into MBP with a Bio-imaging analyzer indicated that the extent of activation of ERK2 at 1 h after the start of treatment with β-HIVS was approximately 5-fold that in the case of untreated HL-60 cells (Fig. 5B). The activity of ERK2 then decreased. The activity of ERK1 of 44 kDa, which was much weaker than that of ERK2 in HL-60 cells, was not significantly changed by the β-HIVS treatment (results not shown). In contrast to that of ERK2, the activity of JNK increased gradually during treatment with 10^{-6} M β-HIVS and reached a maximum 3 h after the start of treatment (Fig. 5, C and D). The maximum activation of JNK corresponded to about 14 times the activity in control HL-60 cells. Both JNK1 of 45 kDa and JNK2 of 55 kDa were activated similarly by β-HIVS in a time-dependent manner, as judged on an in-gel kinase assay, although the activation of JNK2 was approximately half that of JNK1 (results not shown). JNK3 (48 kDa) was not detected under our experimental conditions (results not shown). As shown in Fig. 5E, p38 MAP-kinase was rapidly activated after the start of treatment with β-HIVS. The activity of p38 reached a maximum of 1 h after the start of treatment and the activity remained high for at least 6 h after the start of treatment (Fig. 5F). These features of the activation of JNK, p38, and ERK were quite different from those reported for Fas and various anticancer drugs.

To examine the contributions of ERK2 and p38 to the induction of apoptosis by β-HIVS, we incubated HL-60 cells with 10^{-6} M β-HIVS in the absence and presence of their specific inhibitors. As shown in Fig. 6A, the addition of either 1 or 10 μM PD98059, a specific inhibitor of MEK,

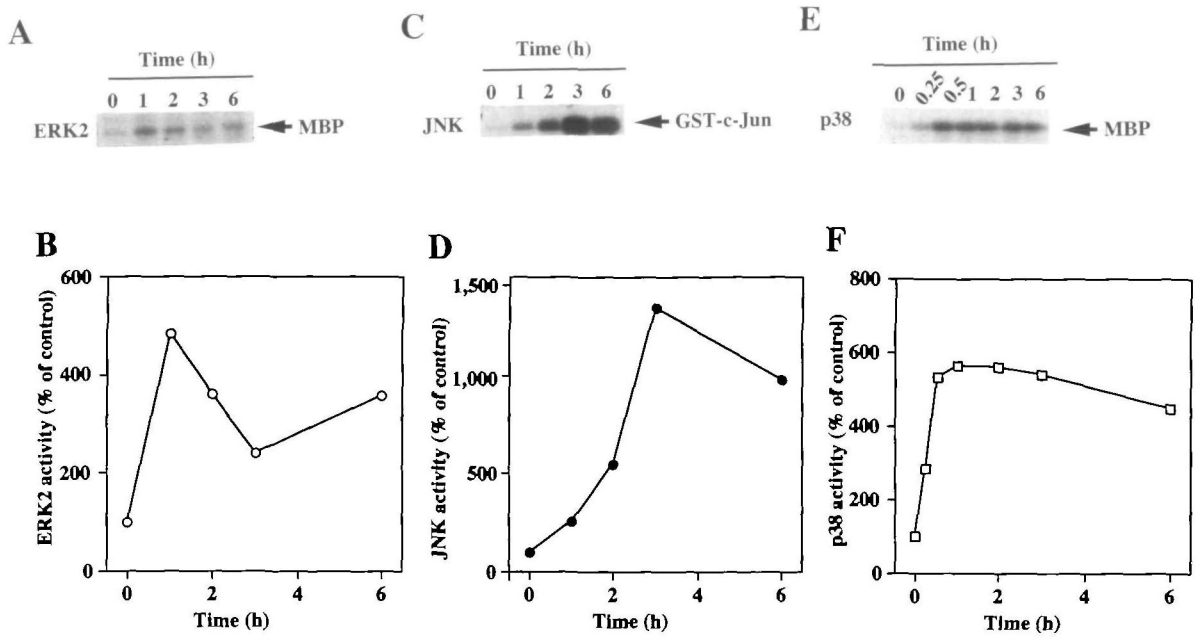

Fig. 5. **Effects of the treatment of HL-60 cells with β-HIVS on the activities of MAP kinases.** HL-60 cells were treated with 10^{-6} M β-HIVS for the indicated times. Cells were lysed with buffer as described under "MATERIALS AND METHODS." (A) ERK2 activity was detected by autoradiography as described under "MATERIALS AND METHODS." (B) ERK2 activity was quantified in terms of the incorporation of ^{32}P with a Bio-imaging Analyzer and ERK2 activity was determined relative to that in untreated HL-60 cells. (C) JNK activity was measured with glutathione S-transferase (GST)-c-Jun as the substrate as described in "MATERIALS AND METHODS." (D and F) The results in (C) and (E) were quantified with the Bio-imaging Analyzer. (E) After immunoprecipitation with p38-specific antibodies, p38 activity was measured by an *in vitro* kinase assay with MBP as the substrate.

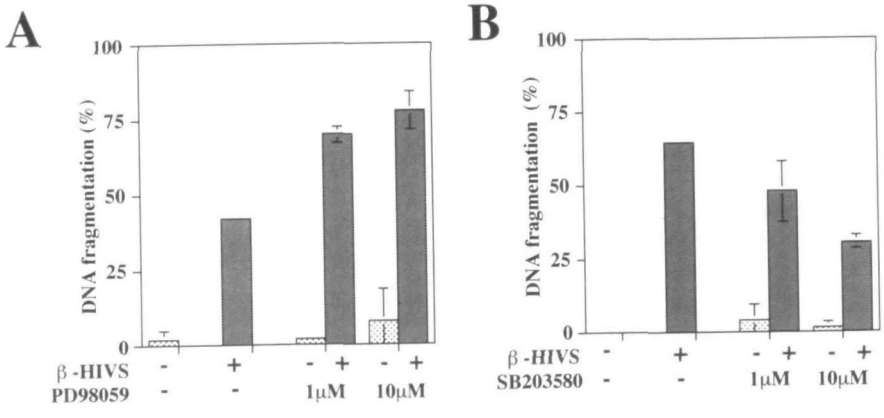

Fig. 6. **Effects of specific inhibitors of MEK and p38 on DNA fragmentation induced by β-HIVS.** HL-60 cells were pretreated with PD98059 or SB203580 for 1 h and then treated with 10^{-6} M β-HIVS for 3 h. After the cells had been collected, DNA fragmentation was measured as described in Fig. 2.

markedly promoted DNA fragmentation induced by β-HIVS. On the contrary, the specific inhibitor of p38, SB203580, significantly inhibited the DNA fragmentation in HL-60 cells induced by β-HIVS (Fig. 6B). These results support the proposal that p38 promotes apoptosis whereas ERK activation prevents it.

Figure 7 shows the effect of Z-Asp-CH_2DCB on the activation of JNK by treatment of HL-60 cells with β-HIVS. As is evident from this figure, the caspase inhibitor had no effect on the activation of JNK by β-HIVS. Z-DEVD-FMK also did not affect the activation of JNK by β-HIVS (results not shown), suggesting that JNK is located either upstream of caspases or is activated independently of the caspase signaling pathway.

DISCUSSION

In the present study, we found that β-HIVS was a potent inhibitor of the growth of cancer cells derived from various human solid tumors. Among the derivatives of shikonin, β-HIVS was the most potent inducer of cell death, as determined with human leukemia HL-60 cells. The death of HL-60 cells induced by β-HIVS was proved to be due to apoptosis by demonstration of the induction of caspase-3-like activity, and of the fragmentation of nuclei and DNA. Since the cell growth-inhibiting and apoptosis-inducing activities of β-HIVS were higher than those of shikonin, and since hydroquinone had no apoptosis-inducing activity (results not shown), both the basic structure of shikonin and

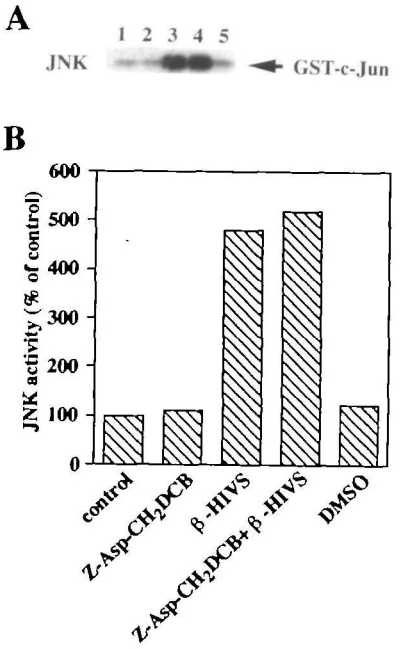

Fig. 7. **Effects of a caspase inhibitor on JNK activity.** (A) HL-60 cells were treated with Z-Asp-CH_2DCB or β-HIVS or both. After cells had been lysed with buffer, JNK activity was measured as described in the legend to Fig. 5. Lane 1, untreated control cells; lane 2, 50 μM Z-Asp-CH_2DCB for 4 h; lane 3, cells treated with 10^{-6} M β-HIVS for 3 h without pretreatment with Z-Asp-CH_2DCB; lane 4, cells incubated for 1 h with 50 μM Z-Asp-CH_2DCB, and then for an additional 3 h with 10^{-6} M β-HIVS; lane 5, cells treated with 2% DMSO for 4 h. (B) JNK activity was quantified with the Bio-imaging Analyzer.

the side chain structure seem likely to play a role in its function.

One feature of the effect of β-HIVS on HL-60 cells was that the concentrations needed to inhibit cell growth and to induce apoptosis were very low. The concentration for 50% inhibition of growth (IC_{50}) ranged from 10^{-8} to 10^{-6} M for various lines of human cancer cells, resembling the IC_{50} values of bufalin (*10*), VP16 (*16*), and 1α,25-dihydroxyvitamin D_3 (*10*). Another feature of the effect of β-HIVS was that it induced marked morphological changes in HL-60 cells. Moreover, staining of the actin filaments in HL-60 cells with phalloidin showed that β-HIVS changed both the profile and the distribution of actin filaments. We also found that caspase-3-like activity in HL-60 cells was increased by the treatment with β-HIVS. It should to be noted that neither a morphological change nor activation of the caspase-3-like protease of HL-60 cells was induced on treatment of the cells with anti-Fas antibodies (results not shown).

One other feature of the action of β-HIVS was that MAP kinases, such as JNK, ERK2 and p38, were activated within 1 h of the start of treatment of HL-60 cells with 10^{-6} M β-HIVS; JNK, composed of JNK1 and JNK2, was most strongly activated and the activity reached a maximum at 3 h. These features of the activation of MAP kinases were distinct from those reported for Fas and anticancer drugs. In addition, the relationship between JNK and caspases in the signaling pathway induced by β-HIVS differs from that in the pathway induced by Fas (*23, 24*). JNK activity started to increase 1 h after the start of treatment with β-HIVS and the activity was not inhibited by inhibitors of caspases such as Z-Asp-CH_2DCB or Z-DEVD-FMK. In Fas-induced apoptosis in Jurkat cells, in contrast, JNK activity is inhibited by inhibitors of caspases such as *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-FMK) (*23*). From these observations, we propose that JNK is located either upstream of caspases or is activated independently of the caspase signaling pathway in the β-HIVS-induced pathway to apoptosis. The mechanism of β-HIVS-induced apoptosis also seems to be different from that of etoposide-induced apoptosis for the following reason. Although Fujii *et al.* reported that shikonin at concentrations above 10^{-5} M inhibited topoisomerase II (*16*), the activity of topoisomerase II in HL-60 cells was barely affected by β-HIVS under our experimental conditions (results not shown). These results suggest the possibility that β-HIVS induces apoptosis through a novel pathway distinct from those reported thus far.

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