

Induction of Apoptosis by *Coprinus disseminatus* Mycelial Culture Broth Extract in Human Cervical Carcinoma Cells

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ABSTRACT. Extract of *Coprinus disseminatus* (*pers. Fr.*) (*C. disseminatus*) culture broth (EDCB) inhibits proliferation and induces apoptosis in the human cervical carcinoma cells at 5 µg/ml. To determine whether the cell death induced by the EDCB recruits caspases or not, one of the exclusive pathways in cell death, we examined caspase-3 activity in this cell death process. The activity of caspase-3 was remarkably increased when the cell was treated with EDCB, and this activity was nullified by Z-VAD-FMK, a well known caspase-3 inhibitor. From these results, we would expect the EDCB to contain substances with the ability to induce apoptosis in the human cervical carcinoma cells. The extent of the EDCB induced apoptosis is cell line-dependent.

Key words: apoptosis/*Coprinus disseminatus*/mycelial culture broth/caspase-3

The non-edible mushroom *Coprinus*, a kind of Chinese medicine, has drawn considerable attention in China and Japan for its high antitumor activity. It is reported that the extract from the fruiting bodies of this mushroom is effective for treating cancers of sarcoma 180, cervical carcinoma, malignant melanomas, etc., by acting as bio- or immuno-potentiators (21). In the past couple of years, several papers have stated the potentiality of the extract as chemotherapeutic agents (21). However, the production of large amounts of these substances from the fruiting bodies of *Coprinus* is not easy (200–250 mg/kg fruiting body). Instead of this fruiting body, an extract from the type species of *C. disseminatus* mycelial culture broth (EDCB) was studied. EDCB showed the ability to inhibit the growth and induce the apoptosis of human cervical carcinoma cells.

Apoptosis, or programmed cell death, is characterized by a series of distinct morphological changes (19),

among them, cell shrinkage (4, 14), membrane blebbing (8), nuclear break down and DNA fragmentation (3, 12), and is essential for the development and maintenance of tissue homeostasis and the elimination of harmful cells in metazoan organisms. It is controlled by gene expression, which modulates a specific enzyme activity (5). Recently, a number of sophisticated papers have stated that the effectors of apoptosis are represented by a family of intracellular cysteine proteases known as caspases. Caspase-3 (CPP32), one of the key family proteins, is expressed in cells as an inactive 32-kDa precursor, and proteolytic processing is required to generate the 17 and 12 kDa subunits which dimerize to form an active enzyme that guide the process of apoptosis (6, 13, 15). In this study, we reported that caspase-3 was activated during the EDCB-induced apoptosis, and that EDCB reduced proliferation and DNA fragmentation in cultured cervical carcinoma cells. These results support the potential utility of this non-edible mushroom extract in cancer chemotherapy.

Materials and Methods

Materials

DMEM and F12 were purchased from Gibco Life Technologies Inc. (Grand Island, NY, USA). Fetal bovine serum (FBS) and horse serum (HS) were obtained from Flow Laboratory (North Ryde, N.S.W., Australia). MTT, proteinase K

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Abbreviations: *C. disseminatus*, *Coprinus disseminatus*; EDCB, extract of *C. disseminatus* mycelial culture broth; *C. pubescens*, *Coriolus pubescens*; EPCB, extract of *Coriolus pubescens* mycelial cultured broth; CDIII medium, modified Corn steep liquor-Dextrin medium; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; HS, horse serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; SDS, sodium dodecyl sulfate.

and RNase A were from Sigma (Tokyo, Japan). Penicillin G and streptomycin were from Wako Pure Chemicals (Tokyo). Ac-Asp-Glu-Val-Asp-MCA (Ac-DEVD-MCA) and Z-Val-Ala-Asp(OMe)-CH₂F (Z-VAD-FMK) were from Peptide Institute Inc. (Osaka, Japan). All other reagents were of analytical reagent grade.

Cell lines and culture conditions

Cervical carcinoma cell lines, HeLa, Caski and SiHa, were used in this study. The Caski and SiHa cells were a gift from Dr. T. Kanda, National Institute of Health, Japan, and were grown in DMEM supplemented with 10% FBS. HeLa cells (laboratory stock) were grown in DF medium (1:1 mixture of DMEM/F12) with 5% (v/v) each of heat-inactivated FBS and HS. Both culture media contained antibiotics (100 µg/ml each of penicillin G and streptomycin). All cultures were maintained at 37°C under a 90% humidified atmosphere and 5.0% CO₂.

Preparation of extracts from mushroom mycelial culture broth

The *C. disseminatus* (5475 strain) and *C. pubescens* (5225 strain) were obtained from the collection of The Mushroom Research Institute, Japan (Kiryu). Cultivation conditions of the both mushrooms were as follows. The mycelium was cultured at 24.5°C for 28 days in CDIII medium (1% glucose, 1% dextrin, 0.2% corn steep liquor and 0.1% yeast extract, pH 4.8) with shaking. The extract (soluble in ethyl acetate)

and water soluble substances from the cultured broth were prepared modified form according to von Wright *et al.* (17) (Fig. 1). Ethyl acetate extract of the CDIII in which the mycelium was not cultured was also prepared as above.

Cell growth assay and observation of cell morphology

Cell suspensions of 2.8–3.2 × 10⁴ cells/ml were prepared in DMEM or DF with 10% FBS or 5% (v/v) each of FBS and HS. The cells were pipetted into each well of a 96-well culture dish (0.1 ml cell suspension/well). After incubation for 24 hr at 37°C, the original culture medium was changed to medium containing EDCB or the extract from *Coriolus pubescens* mycelial cultured broth (EPCB). The cells were further incubated for the pre-determined optimal time. Cell growth was measured by the modified MTT method reported by Mosmann (10) and Alley *et al.* (1), measuring the absorbance at A_{570–620} using a plate reader (Toso, Type MPR-A4, Tokyo, Japan). The relationship between the inhibitory activity of those extracts and cell growth was calculated from the following equation:

$$SI = \frac{A_2 - A_0}{A_1 - A_0}$$

where A₀ is the absorption of the blank; A₁ is the absorption of the culture without mushroom culture broth extracts. A₂ is the absorption of the culture with EDCB (or EPCB). Each point is expressed as the average (within ±5%) of the values of 12 wells.

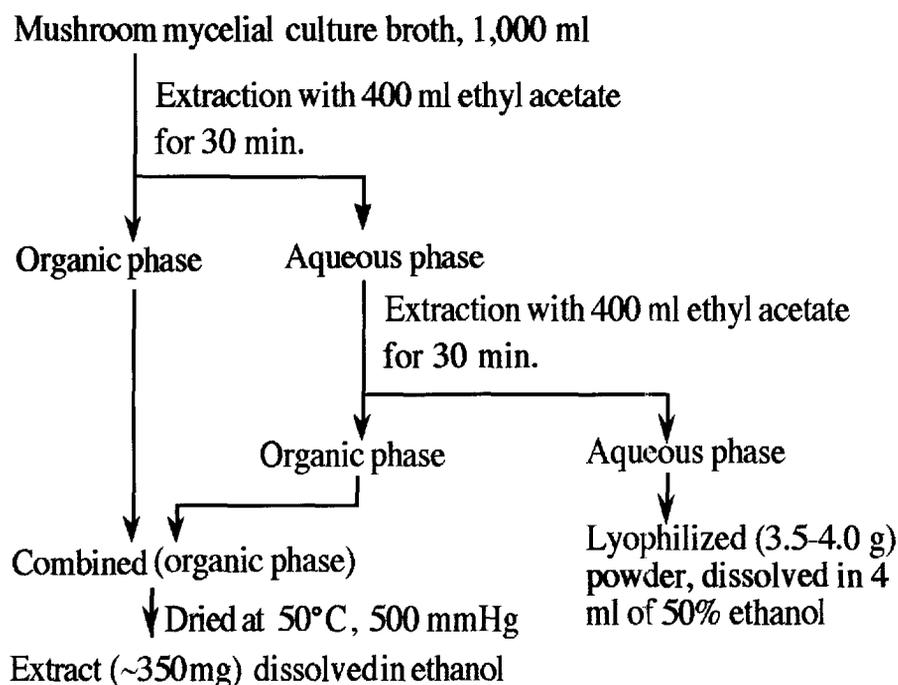


Fig. 1. Fractionation of *C. disseminatus* (or *C. pubescens*) mycelial culture broth (17).

The correlation between MTT absorbance and cell number was determined with a TATAI Eosinophil Counter (Kayagaki Irikakogyo Co., Ltd., Tokyo, Japan) using a microscope. Cell morphologies were observed by a light microscopy (Nikon, TMS-F).

Detection of DNA fragmentation

Extraction of chromatin DNA from the treated cells was carried out according to Ishizawa *et al.* (7) with the following modifications. The cultured cervical tumor cells, treated by EDCB/EPCB (conc. 5 $\mu\text{g}/\text{ml}$ at 37°C for 48 hr), were collected and resuspended in PBS to 2.5×10^6 cells/ml. The cell suspension (200 μl) was incubated with 0.5 mg/ml proteinase K, 0.05 mg/ml RNase A and 0.5% SDS and incubated at 37°C for 30 min. Three hundred μl of 6 M NaI solution (6 M NaI, 12.5 mM EDTA, 0.5% sodium lauroylsarcosine, 25 mM Tris-HCl and 10 μg glycogen, pH 8.0) was then added to the incubated cell-suspension as a carrier. The mixture was further incubated at 60°C for 15 min and was then supplemented with 500 μl of 100% isopropyl alcohol. After vigorous mixing, the mixture was left to stand at room temperature for 15 min. The precipitated DNA, obtained by centrifugation at $15,000 \times g$ for 15 min, was treated again with RNase A (0.1 mg/ml) at 37°C for 1 hr. To detect fragmented DNA, DNA obtained as above was subjected to electrophoresis in 1.5% agarose gels stained with ethidium bromide, and the electrophoresis patterns were observed.

Hoechst 33258 staining

The cultured Caski cells, control cells and treated by EDCB (conc. 5 $\mu\text{g}/\text{ml}$ at 37°C for 48 hr), were washed with PBS(-). They were then fixed with 1% glutaraldehyde at room temperature for 30 min. After washing with PBS(-), the fixed cells were stained with Hoechst 33258 for 15 min. The stained cells were rinsed with PBS(-) and observed under a fluorescence microscope (Nikon XF-EFD, Japan). Photographs were taken with Nikon AFX-A and Nikon FX35WA cameras, using Neopan-1600 film (Fuji Co., Japan).

Preparation of apoptotic extract and caspase-3 activation assay

The apoptotic extract was obtained by harvesting the cells (1×10^8 cells/ml) after treatment with the EDCB/EPCB (5 $\mu\text{g}/\text{ml}$) in the presence or absence of Z-VAD-FMK, a caspase inhibitor, at 37°C for 48 h. The cells were collected and lysed in ice-cold extraction buffer (50 mM HEPES-NaOH, pH 7.0, 5 mM EGTA, 2 mM MgCl_2 , 1 mM DTT, 1 mM PMSF, 1 $\mu\text{g}/\text{ml}$ leupeptin, 1 $\mu\text{g}/\text{ml}$ antipain). Lysis was completed by two cycles of freezing at -80°C and thawing at 4°C , to prevent nonspecific cleavage of proteins. Homogenates were clarified by centrifugation for 10 min at $15,000 \times g$. Supernatants were collected and diluted with dilution buffer (100 mM HEPES-KOH, pH 7.5, 10% sucrose, 0.1% CHAPS and 10 mM DTT)

for assays of enzymatic activity.

Prepared apoptotic extract (20 μg) were incubated in duplicate with Ac-DEVD-MCA (80 ng) (substrate for caspase-3) in a total volume of 100 μl of dilution buffer. This reaction was at 30°C for 30 min. The fluorescence of the cleaved substrates was measured at an excitation wavelength of 320 nm and an emission wavelength of 400 nm.

Results

Inhibition of the cervical carcinoma cell growth by EDCB

Effects of EDCB on the growth of various cancer cells were analyzed by MTT assay (Fig. 2). In the case of the extract prepared from media not cultured with mushroom, the inhibition of Caski cells growth was very slight (Fig. 2). Similar data were also obtained in the SiHa and HeLa cells (data not shown). The inhibitory profiles of EDCB in Caski, SiHa and HeLa cells were almost the same, and of them that of SiHa cells was the most sensitive. When the concentration of EDCB was higher than 2.5 $\mu\text{g}/\text{ml}$, the growth rate of various cancer cells was less than 50% (Fig. 2). In the case of aqueous phase extract prepared as shown in Figure 1, there was no inhibition of cell growth and the growth rate was greater than 70% of the rate of the cell cultured even at high concentrations (4 mg/ml) (data not shown). Although the effect of EPCB was similar to that of EDCB, the inhibition was less (Fig. 2). In the presence of Z-VAD-FMK (0.5 μM), however, the inhibitory profiles of EDCB on the growth of experimental cells showed they were significantly resistant observed

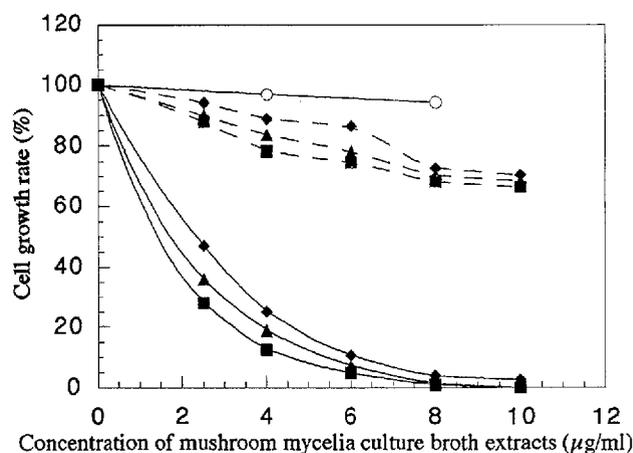


Fig. 2. Dose-dependency of the effect of EDCB (—) and EPCB (---) on the growth of various cervical tumor cells as measured by MTT assay. ○, control. Effect of ethyl acetate extract of CDIII media in which mushroom was not cultured on Caski cell growth. ▲, Caski; ■, SiHa; ◆, HeLa.

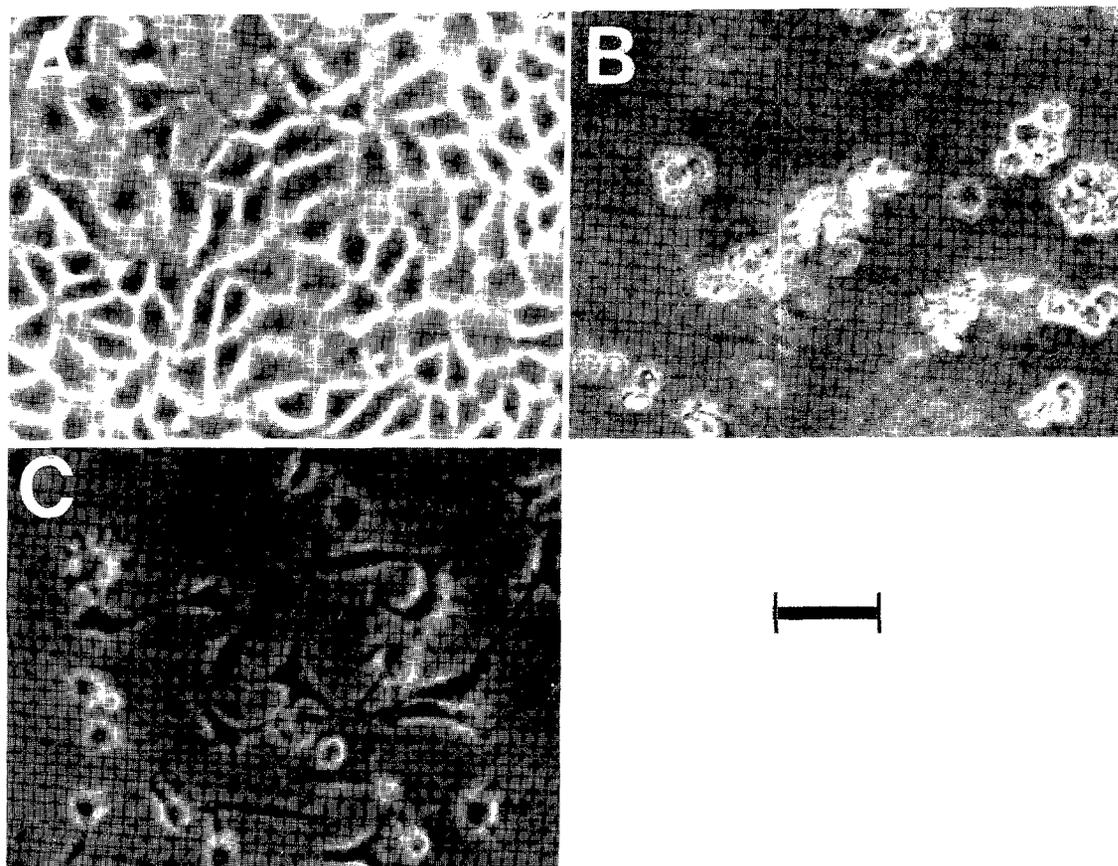


Fig. 3. Effect of EDCB on the morphology of Caski cells. Cells were cultured in normal medium (A) or with 5 $\mu\text{g}/\text{ml}$ of EDCB (B), or with 5 $\mu\text{g}/\text{ml}$ of EDCB in the presence of Z-VAD-FMK (1 μM) (C). Cell morphologies were observed after 2 days cultivation. Bar = 50 μm .

by MTT assay (data not shown).

Effects of the extract on cell-morphology

The effect of EDCB on Caski cells morphology was examined (Fig. 3). Caski cells, which attached themselves to the bottom of culture dish in normal medium, displayed some out growth processes (Fig. 3A). After 48 hr incubation with EDCB (5 $\mu\text{g}/\text{ml}$), the treated cells shrank and the cell number decreased (Fig. 3B). These morphological changes caused by the addition of EDCB was also observed in SiHa and HeLa cells (data not shown). At lower extract concentrations (less than 2 $\mu\text{g}/\text{ml}$), the treated cells displayed non-flattened shrinkage (data not shown). In the case of EPCB, the treated cells displayed non-flattened shrinkage even at higher concentrations (8 $\mu\text{g}/\text{ml}$) (data not shown). When the cells were treated with EDCB in the presence of Z-VAD-FMK (0.5 μM), the morphology of cells show only a small change (Fig. 3C), and most of the cells survived by staining with 0.4% trypan blue (data not shown).

Detection of DNA fragmentation

DNA fragmentation showing a ladder pattern is a marker for one kind of cell death, apoptosis (2, 3, 18). Therefore, the DNA fragmentation detected as ladder patterns in agarose gel electrophoresis caused by the addition of 5 $\mu\text{g}/\text{ml}$ extracts was analyzed (Fig. 4). In the case of Caski and SiHa cells, DNA fragmentation was detected (lanes 3 and 6). However, in case of HeLa cells, neither of the extracts induced complete fragmentation (lane 9). In addition, EPCB was unable to induce DNA fragmentation in any of those cell lines (lanes 2, 5 and 8).

Effects of EDCB on cell-nuclear morphology

The formation of nuclear fragments (apoptosis particles) is another known characteristic of apoptosis. The nuclear morphology of Caski cells treated with EDCB was observed by staining with a DNA-binding fluorescence reagent (Hoechst 33258) (Fig. 5). Nuclear fragments (apoptosis particles) were detected in cells

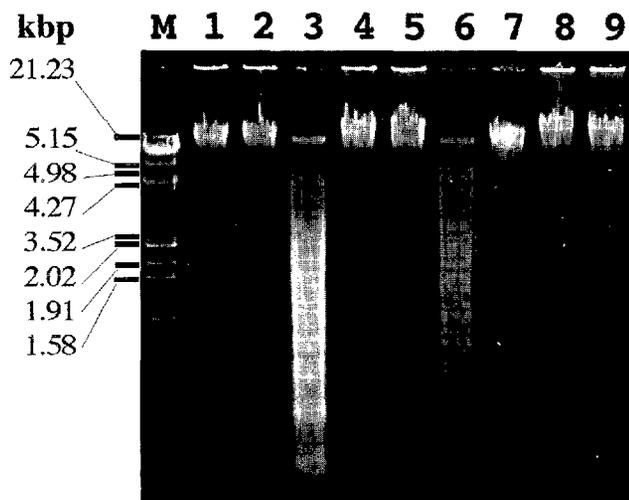


Fig. 4. Effect of the EDCB and EPCB on the DNA fragmentation of cultured tumor cells. Cells, Caski (lanes 1–3), SiHa (lanes 4–6) or HeLa (lanes 7–9) were treated with EDCB/EPCB and the soluble DNA fractions were electrophoresed in agarose gels. Lane M, marker DNAs prepared by the digestion of lambda-phage DNA with *EcoRI* and *HindIII*. Lanes 1, 4, 7, no addition; lanes 2, 5, 8, addition of EPCB; lanes 3, 6, 9, addition of EDCB. EDCB (or EPCB) were used at 5 $\mu\text{g}/\text{ml}$ for the various cell lines.

treated with EDCB (Fig. 5B), whereas no nuclear fragments were recognized in non-treated cells (Fig. 5A). Similar data were also obtained in the case of SiHa cells (data not shown). In the case of HeLa cells, however, no nuclear fragments were detected (data not shown).

Activation of caspase-3 protease by EDCB treatment

Caspases including caspase-3 have been reported to be involved in the apoptotic process (9, 20). Here, we investigated the effect of EDCB on alterations of enzymatic activity of caspase-3 and apoptotic process in human cervical carcinoma cells. The specific peptide was coupled to the fluorescent (4-methyl-coumaryl-7-amide) acetyl group (MCA). The pattern of increased activity of caspase-3 by EDCB correlated well with that of the extracts-induced DNA fragmentation. Figure 6 shows that caspase-3 activity was activated in the experimental cell lines after EDCB-induced apoptosis. In the case of Caski and SiHa cells, the activities of caspase-3 of cell extract were more than 1,500 units/mg protein. However, in the HeLa cells, the activities of caspase-3 of extract of cells were less than 200 units/mg protein. These results support the results in DNA fragmentation that EDCB is able to induce apoptosis in the Caski and SiHa cells, but not in the HeLa cells. The prevention of apoptosis by Z-VAD-FMA (inhibitor for caspases) has generally been attributed to its inhibition of an endonuclease acting in the late phase of apoptosis. Using the extracts (protein and DNA) of the Caski and SiHa cells that had been treated for 48 h with 5 $\mu\text{g}/\text{ml}$ EDCB in the presence of Z-VAD-FMA, we examined the effects of the inhibitor on caspase-3 activity and DNA fragmentation. The Z-VAD-FMA inhibited the ability of EDCB to increase caspase-3 activity (Fig. 6) and induced the DNA fragmentation (Fig. 7). The effect of Z-VAD-FMA in the cells was concentration-dependent.

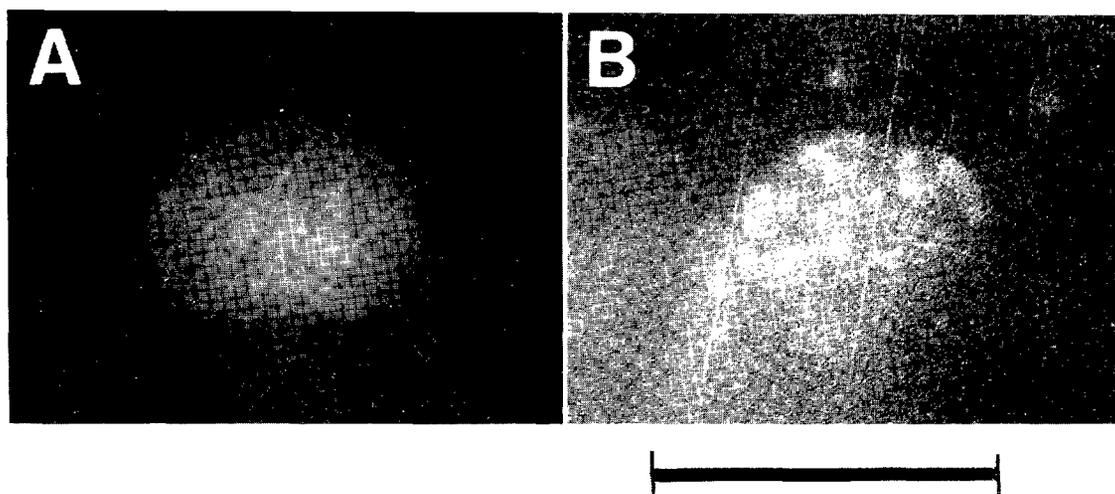


Fig. 5. Nuclear morphology of Caski cells treated with EDCB. The treated cells were stained with a DNA-binding fluorescence reagent (Hoechst 33258). (A), no addition; (B), addition of EDCB. The concentration of the extract was 5 $\mu\text{g}/\text{ml}$ for various cells. Cell morphology was observed under fluorescence microscope. Bar = 20 μm .

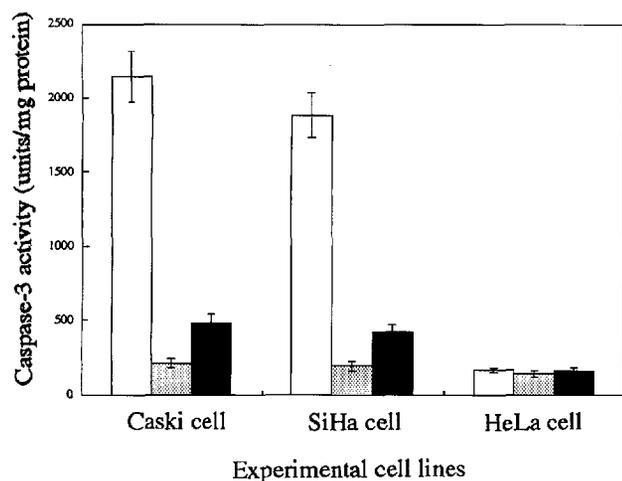


Fig. 6. Activity of caspase-3 in extracts from EDCB (□, ▨) or EPCB (■) treated culture cells. After treatment by EDCB for 48 h at 37°C in the presence (□) or absence (▨) of Z-VAD-FMK (1 μ M), the cells were lysed with cell lysis buffer. The apoptotic extracts (20 μ g) were incubated with Ac-DEVD-MCA (80 ng) for 30 min at 30°C in a total volume of 100 μ l of dilution buffer. The fluorescence of the cleaved substrates was then measured at an excitation wavelength of 320 nm and an emission wavelength of 400 nm.

Discussion

The experimental results described above suggested that EDCB induced apoptosis in human cervical carcinoma cells is cell-line dependent.

As shown in Figure 2, EDCB moderately inhibits the growth of cancer cells. For EDCB, no clear cell-line dependency on growth inhibition was detected. The inhibition of cell growth by EDCB was accompanied by shrinkage and shape changes of the cells (Fig. 3).

Since the discovery that caspase-3, a key effector protein in programmed cell death, exhibits homology with interleukin 1 β -converting enzyme, the involvement of proteolytic enzymes in apoptosis has been an issue of significant interest (16). We found that caspase-3 activity increased during EDCB-induced apoptosis. Along with DNA fragmentation (Fig. 4), cell-nuclear morphology (Fig. 5) and activation of caspase-3 (Fig. 6), these effects were cell line-dependent. Although EDCB clearly induced DNA fragmentation and increased caspase-3 activity in Caski and SiHa cells, this induction and activation were incomplete in HeLa cells. In most cell types, fragmentation of nuclear DNA into internucleosomal size fragments and activation of caspase-3 activity are the biochemical hallmarks of apoptosis (11). EDCB did not induce DNA fragmentation and activate caspase-3 activity when added directly to HeLa cells, suggesting that by itself it was incapable of inducing apoptosis. Whether Z-VAD-FMK, an inhibitor of caspase-3, inhibited cell death at nanomolar concentra-

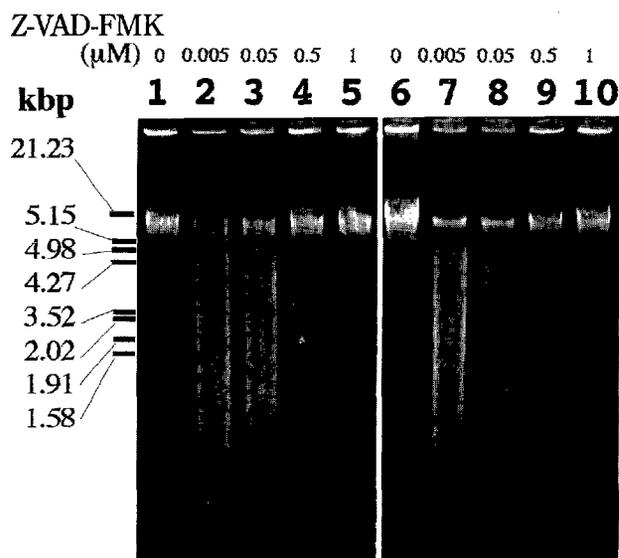


Fig. 7. Effects of the concentration of Z-VAD-FMK on the DNA fragmentation in Caski (lanes 1–5) and SiHa (lanes 6–10) cell lines when those cells were treated with EDCB. The cells were incubated with EDCB (5 μ g/ml) for 48 h at 37°C in the presence of Z-VAD-FMK (conc. 0, 0.005, 0.05, 0.5 and 1 μ M). The soluble DNA fractions were electrophoresed in agarose gels.

tion ranges, as shown in Figure 6B, it is clear that it could attenuate EDCB-induced apoptosis and patently inhibit Ac-DEVD-MCA cleavage in Caski and SiHa cell lines. Therefore, the Caski and SiHa cells might be induced to undergo to apoptosis by EDCB through the caspase-3 activation pathway. However, even though EDCB inhibited the growth of the HeLa cell, it could not activate caspase-3 activity or induce DNA fragmentation. This situation probably stems from the fact that EDCB is insufficient. To clarify this point, the separation and investigation of EDCB is now in progress. EDCB may thus provide a useful tool for the study of apoptosis as well as cell differentiation, and may also be useful in cancer chemotherapy.

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