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# Induction of apoptosis in cultured cells by extracts from shiitake (*Lentinula edodes*) mycelial culture broth

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**Extracts from *shiitake* (*Lentinula edodes*) mycelial culture broth, by an organic solvent ethyl acetate, inhibited the proliferation of cultured cells. At lower concentrations (1.25–15 μg/ml), this inhibition, measured by the MTT assay, was dose- and cell line-dependent. Inhibition of tumor cells, such as Caski, SiHa, HeLa, HP-1 and A375, by *L. edodes*-436 extracts was stronger than inhibition of normal cells (3T3). At 20 μg/ml, the extracts induced changes in cell shape, DNA-fragmentation and the activation of caspase-3. The extracts also inhibited the binding of E2F protein to its promoter. The results suggest that extracts of *L. edodes* culture broth contain substances that have the ability to induce apoptosis in the cultured cells.**

Key Words——apoptosis; caspase-3; E2F factor; *Lentinula edodes*; mycelial culture broth.

Extracts of *L. edodes* (Berk.) Pegler fruiting bodies show prominent antitumor activity not only against allogeneic tumors such as Sarcoma 180, but also against various syngeneic and autochthonous tumors, by acting as bio- or immuno-potentiators (Mizuno et al., 1995; Mizuno, 1995; Chihara et al., 1969). There are several reports describing antitumour activity in *L. edodes* aqueous extracts (Sia and Candlish, 1999). However, there are no such reports about *L. edodes* organic solvent extracts.

Instead of fruiting bodies, the effects of organic solvent extracts of *L. edodes* mycelial culture broth on cultured cells (including cancer cells), effects on cell growth, DNA-fragmentation, activation of caspase-3 and binding of E2F protein to its promoter, were studied in this paper. DNA-fragmentation and activation of caspase-3 are characteristics that accompany with apoptosis (Yuan et al., 1993; Hale et al., 1996). The binding of E2F protein, regulated by the Rb gene-product, to its promoter allows the cell-cycle to proceed from G1 to S phase (Hinds and Weinberg, 1994; Weinberg, 1995). Therefore, the regulation of this binding is closely related to tumor suppression (Rieber and Strasberg-Rieber, 1998; Hinds and Weinberg, 1994).

Considering these reports, the obtained results suggest that extracts from *L. edodes* mycelial culture broth contain a factor that induces apoptosis in cultured cells.

## Materials and Methods

**Materials** Dulbecco's Modified Eagle's Medium (DMEM) and F12 were purchased from Gibco Life Technologies Inc. (N.Y., U.S.A..). Fetal bovine serum (FBS) and horse serum (HS) were obtained from Flow Laboratory (N.S.W., Australia). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), proteinase K and RNase A were from Sigma (Tokyo, Japan). 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), phenylmethanesulfonyl fluoride (PMSF), Hoechst 33258, penicillin G and streptomycin were from Wako Pure Chemicals (Tokyo, Japan). The substrate for caspase-3, Ac-Asp-Glu-Val-Asp-MCA (Ac-DEVD-MCA), was from Peptide Institute Inc. (Osaka, Japan). All other reagents were of analytical reagent grade.

**Cell lines and culture conditions** A normal cell line (3T3) and cancer cell lines (A375, Caski, SiHa, HP-1 and HeLa) were used as listed in Table 1. Mouse 3T3 cell-line, a well known normal cell line also described in the p53 and Rb genes (Wasylyk et al., 1994; L.-Frayssinet et al., 1995), was used as a normal cell line. Caski, SiHa and HP-1 cell lines were gifts from Dr. T. Kanda, National Institute of Health. Other cell lines were from our laboratory stock. Most cells (3T3, A375, Caski, SiHa and HP-1) were grown in DMEM supplemented with 10% FBS. HeLa cells were grown in DMEM-F12 medium (DF, 1 : 1 mixture of DMEM/F12) with 5% (v/v) each of 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 5.0% $CO_2$ and 90% humidity. After cultivation, cells were harvested using a trypsin-EDTA solution. This solution contained 0.25% trypsin and 0.02% EDTA in PBS(-) (phosphate-buffered saline without $Ca^{2+}$, pH 7.0).

**Preparation of extracts from the mycelial culture broth of *L. edodes*** The 436 and MM1 strains of *L. edodes* were

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Table 1. Cell lines used in this study.

| Cell line | Origin | | Characteristics | Situation in oncogenes | | Literature cited | Other |
|---|---|---|---|---|---|---|---|
| | Animals | Organs | | p53 gene | Rb gene | | |
| 3T3 | mouse | fibroblast | normal | normal | normal | Todaro, G. J. (1963) | |
| A375 | human | skin | malignant melanoma | mutant | mutant | Kroumpouzos, G. et al. (1994) | |
| Caski | human | cervil | carcinoma | low level | normal | Scheffner, M. et al. (1991) Smotkin, D. et al. (1986) | |
| SiHa | human | cervil | carcinoma | low level | normal | Scheffner, M. et al. (1991) Smotkin, D. et al. (1986) | |
| HP-1 | rat | fibroblast | carcinoma | low level | normal | Kanda, T. et al. (1987) | transformed cell of histerocarcinoma |
| HeLa | human | cervil | carcinoma | low level | normal | Scheffner, M. et al. (1991) | |

obtained from the collection of The Mushroom Research Institute, Japan (Kiryu, Japan). Cultivation conditions for *L. edodes* were as described by Raaska (1990) with the following modifications. The mycelia were cultured at 26°C for 21–35 days in CDIII medium (1% glucose, 1% dextrin, 0.2% corn steep liquor and 0.1% yeast extract, pH 4.8) or in starch medium (2.5% corn starch, 0.1% $KH_2PO_4$, 0.05% $CaCl_2 \cdot 2H_2O$, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.25% yeast extract, pH 4.8) with shaking. The broth was filtered through No. 2 filter paper (Toyo Roshi Co., Ltd., Tokyo, Japan). Ethyl acetate-soluble and water-soluble substances were prepared according to Wright et al. (1992) (Fig. 1). Ethyl acetate soluble-extracts of the CDIII or starch medium in which *L. edodes* mycelia had been not cultured were also prepared as described in the legend to Fig. 1.

For further purification, the ethyl acetate-soluble extract (as prepared in Fig. 1) from *L. edodes*-436 (CDIII) culture broth was resolved with 30% methanol. After the removal of the insoluble material (Fraction I) by centrifugation at 20,000 × g for 10 min, the supernatant was applied to an Excelpak SPE-UNI/154 (ES) mini-column (Yokogawa Analytical Systems, Japan) and the flow-through fraction was collected (Fraction II). The column was then eluted in a step wise fashion with 30% methanol (Fraction III), 35% acetonitrile (Fraction IV), 65% acetonitrile (Fraction V), 80% acetonitrile (Fraction VI), and finally 100% methanol (Fraction VII). The fractions were evaporated to dryness, redissolved in 50% ethanol, and used for the assay of E2F binding to its promoter (gel shift assay).

**Cell growth assay and observation of cell morphology**

Cell suspensions ($0.8 \sim 1.2 \times 10^4$ cells/ml) were prepared in DMEM or DF with 10% FBS or 5% (v/v) each of FBS and HS. Cell number was determined with a TATAI Eosinophil Counter using a microscope. The cells were seeded into a 96-well culture dish (1,000 cells/well) and cultured for 24 h at 37°C. The original culture medium was replaced with the medium containing *L. edodes* extracts. After culture for 72 h, the medium was replaced again with the same medium containing extracts, and inculation was continued for the pre-determined optimal time (72 h). Cell-growth was measured by the modified MTT method reported by Mosman (1983) and Alley et al. (1988), by measuring the absorbance at $A_{570-620}$ using a plate reader (Toso, Type MPR-A4, Tokyo, Japan). In-

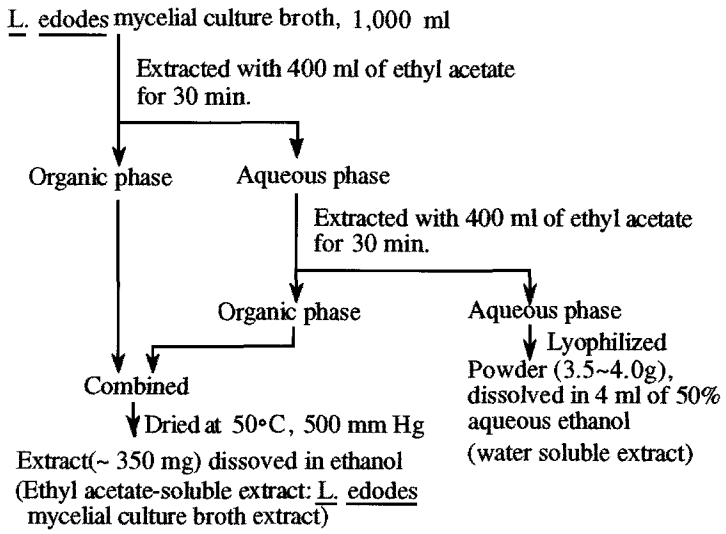


Fig. 1. Preparation of ethyl acetate- and water-soluble extracts from *L. edodes* mycelial culture broth.

hibitory activity (SI) of the *L. edodes* mycelial culture broth extracts on cell growth was calculated from the following equation:

$$\text{Cell growth rate } (\%) = \frac{A_2 - A_0}{A_1 - A_0}$$

where $A_0$ is the absorption of the blank, $A_1$ is the absorption of the culture without *L. edodes* mycelial culture broth extract, and $A_2$ is the absorption of the culture with *L. edodes* mycelial culture broth extract.

The MTT absorbance was proportional to cell number between $0.8 \times 10^4$ cells/ml and $1.0 \times 10^6$ cells/ml. Cell morphologies were observed by light microscopy (Nikon, TMS-F, Tokyo, Japan).

**Detection of DNA fragmentation** Cells (200 $\mu$l, $2.5 \times 10^5$ cells/ml) were seeded in a 60 mm culture dish with 4 ml of medium and cultured at 37°C for 48 h. Then the medium was replaced with medium containing *L. edodes* mycelial culture broth extracts (final conc. 20 $\mu$g/ml) without serum and incubated at 37°C for 48 h. The treated cells were collected by centrifugation at $150 \times g$ for 15 min and resuspended in PBS(-) to $2.5 \times 10^6$ cells/ml. Chromatin DNA was extracted from the treated cells according to Ishizawa et al. (1991) with the following modifications. The collected cell suspension (200 $\mu$l) was supplemented with 5 $\mu$l of 20 mg/ml proteinase K, 10 $\mu$l of 1 mg/ml RNase A, and 10 $\mu$l of 10% sodium dodecyl sulfate (SDS), and incubated at 37°C for 30 min. Then 300 $\mu$l of 6 M NaI solution (6 M NaI, 12.5 mM EDTA, 0.5% sodium lauroylsarcosine, 25 mM Tris-HCl and 10 $\mu$g/ml of glycogen, pH 8.0) was added to the incubated cell-suspension, and the mixture was further incubated at 60°C for 15 min. The precipitated DNA, obtained by the addition of 1 ml of isopropyl alcohol followed by centrifugation at $15{,}000 \times g$ for 15 min, was treated again with RNase A (1 mg/ml) at 37°C for 2 h. To detect fragmented DNA, DNA obtained as above was subjected to electrophoresis in 1.5% agarose gels containing 0.1 $\mu$g/ml of ethidium bromide as described by Smith et al. (1989). All operations were performed at room temperature (~20°C) unless otherwise stated.

**Measurement of caspase-3 activity** Cells ($2.5 \times 10^5$ cells/ml) treated with *L. edodes* mycelial culture broth extracts (20 $\mu$g/ml) at 37°C for 48 h were lysed in ice-cold

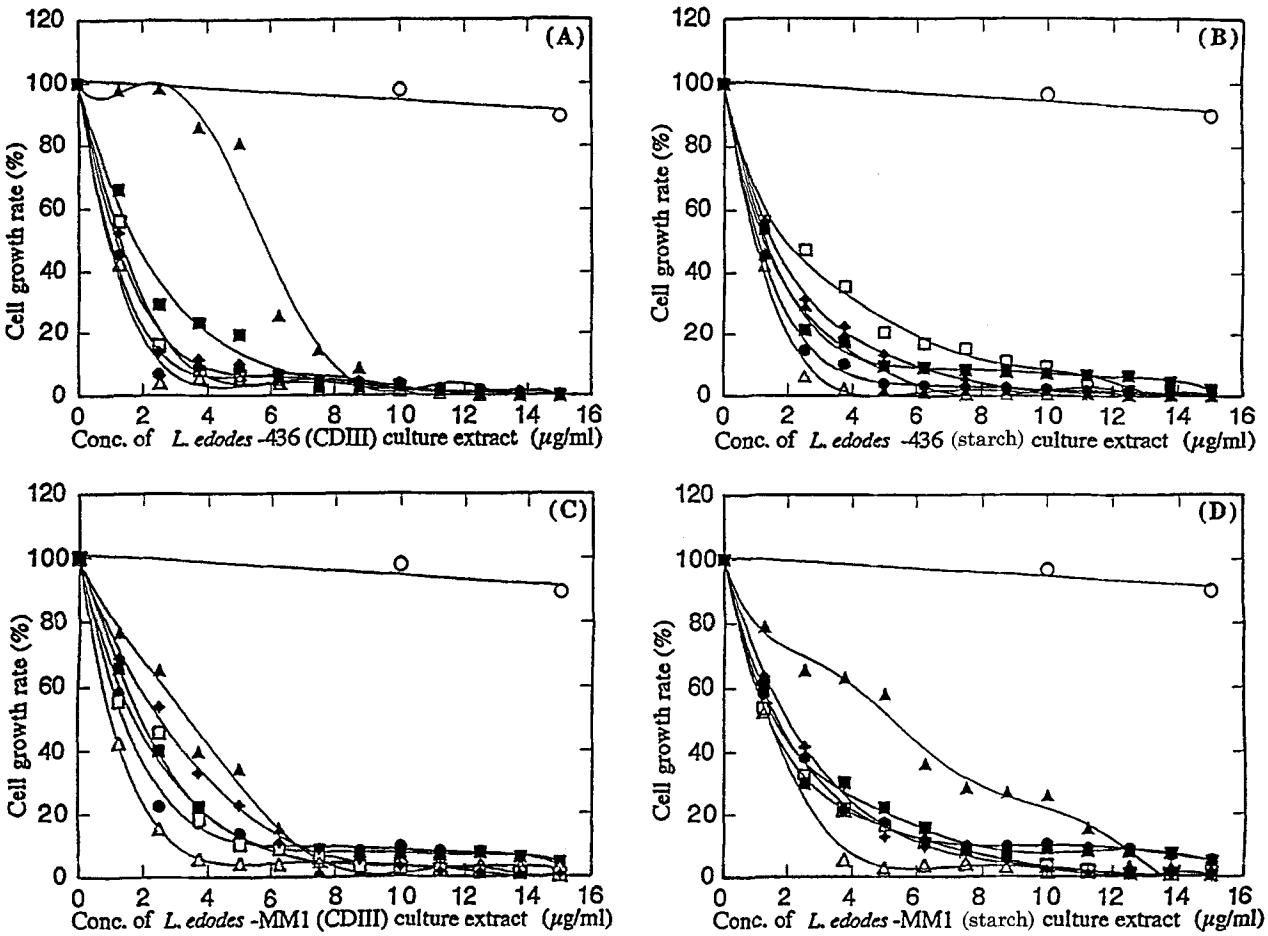


Fig. 2. Dose-dependency of the effect of *L. edodes* mycelial culture broth extracts on the growth of various cells as measured by the MTT assay: ○, control. Effect of ethyl acetate extracts of CDIII (A and C) or starch (B and D) media in which *L. edodes* had not been cultured on Caski cell growth. ▲, 3T3; ◆, A375; ●, Caski; △, SiHa; ■, HP-1; □, HeLa. The average values of MTT absorbance were calculated from the values (within ±5%) of 18 wells.

hypotonic buffer (20 mM Tris-HCl, pH 7.2, 1 mM EDTA and 0.5 mM PMSF). Lysis was completed by two cycles of freezing (at -80°C) and thawing (at 4°C). The supernatant of the homogenate, obtained by centrifugation for 10 min at 15,000×g, was diluted with dilution buffer (50 mM Tris-HCl, pH 7.5, 10 mM dithiothreitol and 0.1% CHAPS). The caspase-3 activity was measured as described by Aiuchi et al. (1998) using Ac-DEVD-MCA as the substrate. Assays were performed in duplicate.

**Analysis of E2F binding to its promoter (gel shift assay)** Preparations of a DNA fragment containing E2F protein-binding sites and the GST-E2F-1 fusion protein were carried out as described by Han et al. (1999). The DNA fragment (37.5 ng) and GST-E2F-1 (3.25 μg) were incubated at 37°C for 20 min in 15 μl of binding solution (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol and 50 μg/ml poly[d(I-C)]) with or without *L. edodes* mycelial culture broth extracts (150 ng or 300 ng, final conc. 10 μg/ml or 20 μg/ml) or ES mini-column-purified extract (60 ng, final conc. 4 μg/ml). After further incubation for 15 min at 30°C, the samples were electrophoresed in 0.5% Agarose-I gels, and the DNA was stained with ethidium bromide (1 μg/ml) in TAE buffer (40 mM Tris, 190 mM acetic acid and 1 mM EDTA, pH 8.0).

## Results

**Inhibition of the cell growth by extracts of *L. edodes* mycelial culture broth** The effect of ethyl acetate-soluble extracts from *L. edodes* mycelial culture broth on the growth of various cells was analyzed by the MTT assay (Fig. 2). In the case of extracts prepared from media not cultured with *L. edodes*, the inhibition of Caski cell growth was very slight (Fig. 2A–D). Inhibition of the growth of 3T3 cells by those exhacts was also slight (3.2% at 10 μg/ml and 8.4% at 15 μg/ml, data not shown). The inhibition profiles for *L. edodes*-436 (CDIII) culture broth extracts on 3T3 cells (Fig. 2A) differed extensively from those for other cells. In the case of A375, Caski, SiHa and HP-1 cells, the addition of 2 μg/ml of extract inhibited cell growth by 60–80%, and 4 μg/ml of extract inhibited it by 80–95%. In contrast, *L. edodes*-436 (CDIII) culture broth extract inhibited 3T3 cell growth by only 10%, even at 4 μg/ml (Fig. 2A). At 8 μg/ml, the inhibition reached 95%. Thus, compared with other cells, the same degree of inhibition of 3T3 cells was achieved by adding a two fold amount of extract. In cells other than 3T3 cells (Fig. 2B–D), the addition of 2 μg/ml of extract inhibited cell growth by 40–80%, while 4 μg/ml of extract inhibited it by 60–95%. Inhibition of 3T3 cells by *L. edodes*-MM1 (CDIII) (Fig. 2C) or *L. edodes*-MM1 (starch) (Fig. 2D) extract was also lower than those of other cells. In particular the inhibition by the *L. edodes*-MM1 (starch) mycelial culture broth extract was clearly lower than those of other cells (Fig. 2D). Among cell lines other than 3T3, HeLa cells showed lower sensitivity to *L. edodes*-436 (starch) extract than other cells (Fig. 2B), while SiHa cells showed higher sensitivity to all extracts than other cells (Fig. 2A–D). For all of the cells described above, the addition of 20 μg/ml of aqueous phase extract, prepared as shown in Fig. 1, inhibited cell growth by only 5%, and the growth rate was greater than 60% even at high concentrations (4 mg/ml).

**Effects of extracts on cell-morphology** The effect of

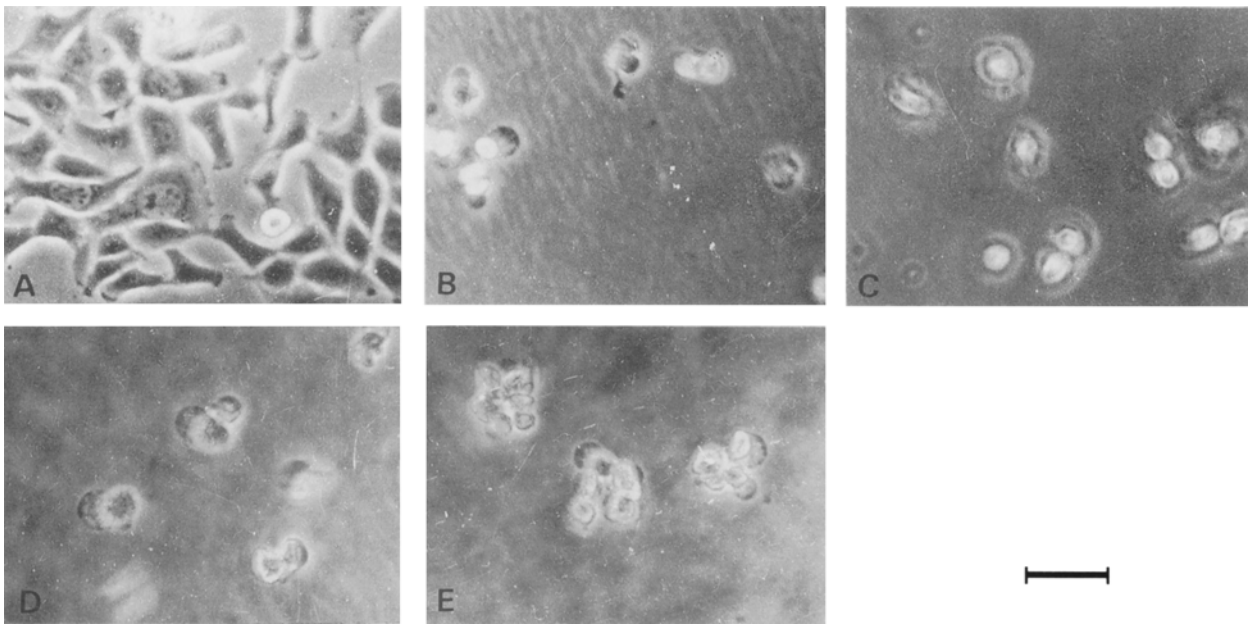


Fig. 3. Effect of *L. edodes* mycelial culture broth extracts on the morphology of Caski cells. Cells were cultured in normal medium (A). Cells were also cultured with 20 μg/ml extracts of *L. edodes*-436 (CDIII) (B), *L. edodes*-436 (starch) (C), *L. edodes*-MM1 (CDIII) (D) or *L. edodes*-MM1 (starch) (E). Cell morphologies were observed after 48 h of cultivation. Bar=50 μm.

various *L. edodes* mycelial culture broth extracts on the morphology of Caski cells was examined (Fig. 3). Caski cells, grown as attached cells on the bottom of a culture dish in normal medium, displayed some out-growth processes (Fig. 3A). After 48 h of cultivation with various extracts (20 $\mu$g/ml), the treated cells shrank and the cell number was decreased (Fig. 3B–E). After treatment with extracts of *L. edodes*-436 (starch) (C), *L. edodes*-MM1 (CDIII) (D) and *L. edodes*-MM1 (starch) (E), the cells became flattened in shape. These morphological changes, caused by the addition of extracts, were also observed in the 3T3, A375, SiHa, HP-1 and HeLa cells (data not shown). At lower extract concentrations (less than 5 $\mu$g/ml), the treated cells displayed non-flattened shrinkage (data not shown).

**DNA fragmentation in cultured cells treated with *L. edodes* mycelial culture broth extracts** DNA fragmentation showing a ladder pattern on agarose gel electrophoresis is another marker of apoptosis (Wyllie, 1980; Arends et al., 1990). The cultivation of 3T3, A375, Caski, SiHa, HP-1 or HeLa cells with 30 $\mu$g/ml of extract in the presence of serum or at low extract concentrations ($<$20 $\mu$g/ml) in the absence of serum did not induce DNA fragmentation (data not shown). Therefore, DNA frag-

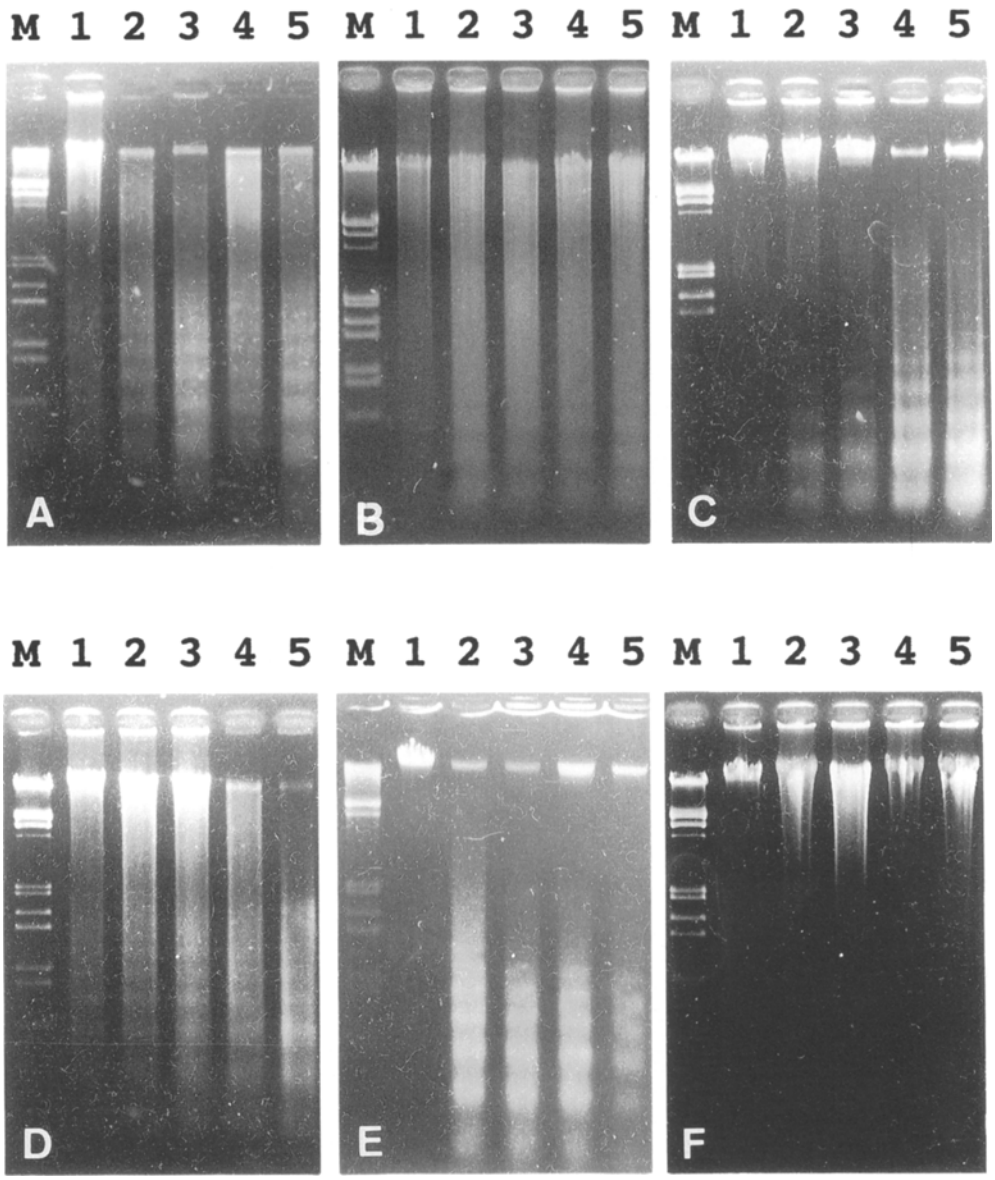


Fig. 4. Effect of *L. edodes* mycelial culture broth extracts on DNA fragmentation in cultured cells. 3T3 (A), A375 (B), Caski (C), SiHa (D), HP-1 (E), or HeLa (F) cells were cultured with extracts and the soluble DNA fractions were electrophoresed in agarose gels. Lane M, marker DNAs prepared by the digestion of lambda-phage DNA with *Eco* RI and *Hin* dIII. Lane 1, no addition; Lane 2, addition of *L. edodes*-436 (CDIII) extract; Lane 3, addition of *L. edodes*-436 (starch) extract; Lane 4, addition of *L. edodes*-MM1 (CDIII) extract; Lane 5, addition of *L. edodes*-MM1 (starch) extract. The concentration of each extract was 20 $\mu$g/ml in each cell culture.

mentation caused by the addition of 20 μg/ml of extracts in the absence of serum was analyzed (Fig. 4). DNA prepared from 3T3 cells not treated with extracts showed no fragmentation (Fig. 4A, lane 1). When the *L. edodes*-436 (CDIII) extract was added, slight DNA fragmentation was detected (lane 2). This fragmentation became more evident upon the addition of other extracts (lanes 3–5). DNA fragmentation in A375 cells (Fig. 4B) was clearly induced by the addition of any extract (lanes 2–5). In Caski cells (Fig. 4C), the addition of *L. edodes*-436 (CDIII) (lane 2) or -436 (starch) (lane 3) extract induced only slight DNA fragmentation, while fragmentation was clearly induced by *L. edodes*-MM1 (CDIII) (lane 4) and -MM1 (starch) (lane 5) extracts. The addition of *L. edodes*-436 (CDIII) extract to SiHa cells (Fig. 4D) induced weak fragmentation (lane 2), while other extracts induced clearer DNA fragmentation (lanes 3–5). In HP-1 cells, DNA fragmentation was induced by all extracts (Fig. 4E, lanes 2–5). The ability of *L. edodes*-436 (CDIII) extract to induce DNA fragmentation in HP-1 cells was the clearest (Fig. 4E, lane 2). In HeLa cells (Fig. 4F), none of the extracts induced fragmentation (Fig. 4F, lanes 2–5).

**Measurement of caspase-3 activity** The activation of caspases, including caspase-3, has been reported to be involved in the apoptotic process (Miura et al., 1993; Yuan et al., 1993). Caspase-3 activity in cultured cells was measured after the addition of *L. edodes* extracts (Fig. 5). When cells were treated with extracts obtained from broth (CDIII) in which *L. edodes* had not been cultured, the caspase-3 activities in the cell extracts were less than 300 units/mg protein. However, in the case of extracts of 3T3, A375 or Caski cells treated with *L. edodes* extracts, the activity was more than 1,500 units/mg protein. In SiHa and HP-1 cell extracts, the activities were more than 1,000 units/mg protein. In the case of HeLa cell extracts, the caspase-3 activities were less than 500 units/mg protein.

**Effect of extracts on E2F binding to its promoter** The effects of extracts on the binding of E2F protein to its promoter are shown in Fig. 6. A DNA fragment containing E2F binding sites was incubated with the GST-E2F-1 protein in the absence or presence of *L. edodes* mycelial culture broth extracts and subjected to electrophoresis in agarose gels. The DNA fragment electrophoresed at the front (Fig. 6A, lane 1) and the addition of GST-E2F retarded its movement (lane 2). The addition of 10 μg/ml of extracts caused a slight disturbance in the interaction between GST-E2F and the DNA fragment (Fig. 6A, lanes 3–6). The disturbances caused by *L. edodes*-MM1 (CDIII) (lane 5) and *L. edodes*-MM1 (starch) (lane 6) extracts appeared to be greater than those caused by other extracts (lanes 3, 4). At higher concentrations (20 μg/ml) (Fig. 6B), the disturbances were clearly observed (lanes 3–6). The extracts themselves did not interact with the DNA fragment (data not shown).

The effects of the extract and ES mini column-purified extract on the binding of E2F protein to its promoter are shown in Fig. 7. The DNA fragment (containing E2 promoters) electrophoresed at the front (lane 1), and the addition of the E2F protein factor (lane 2) retarded the movement of DNA. As already shown in Fig. 6, the extract inhibited the interaction of E2F with DNA (lane 3). For the ES mini-column-purified fractions, slight inhibition of E2F binding to the DNA fragment was observed with Fractions I, II, III, IV and VI (lanes 4–7 and 9). However, the addition of Fraction V caused a clear inhibition (lane 8).

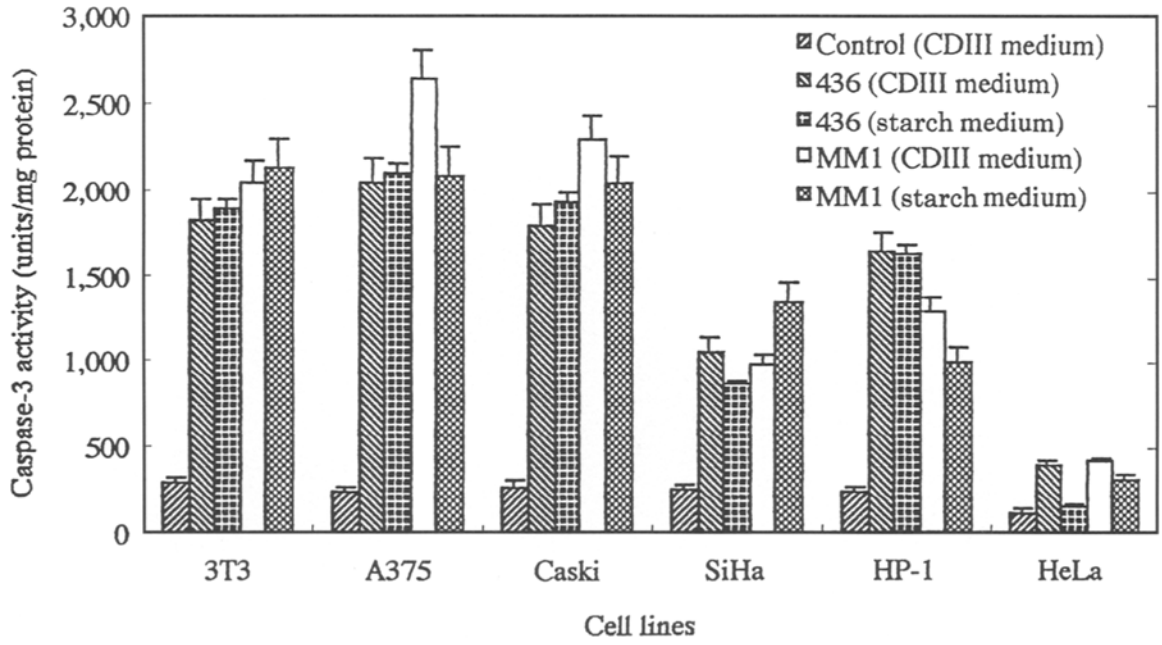


Fig. 5. Activity of caspase-3 in various cells treated with extracts (20 μg/ml) of *L. edodes* mycelial culture broth. As a control, cells were treated with the extract of CDIII medium in which *L. edodes* mycelium had not been cultured.

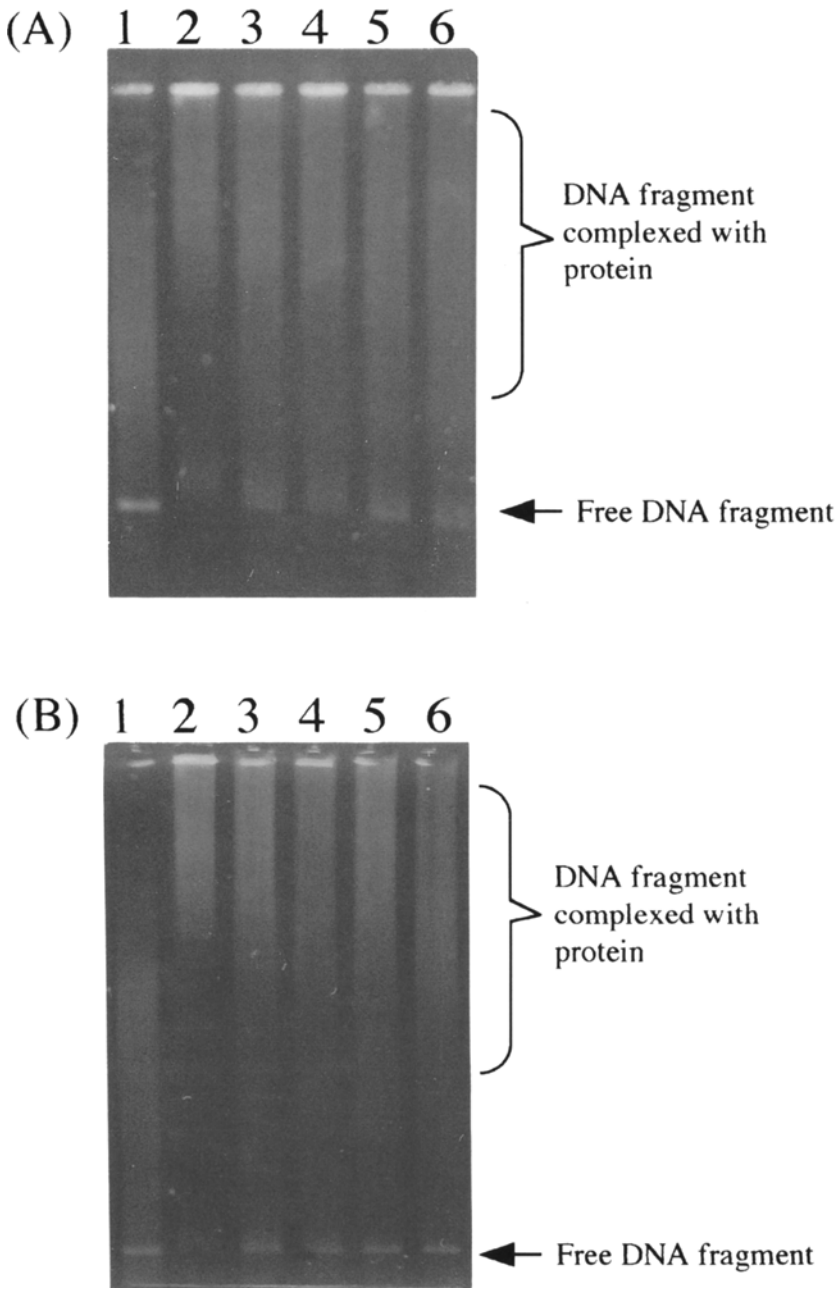


Fig. 6. Effect of *L. edodes* mycelial culture broth extracts on the binding of E2F to its promoter. (A) DNA fragment containing the E2F promoter alone (lane 1); a mixture of the DNA fragment plus GST-E2F-1 (3.25 $\mu$g) (lane 2), and a mixture of the DNA fragment plus GST-E2F-1 (3.25 $\mu$g) plus 150 ng (final conc. 10 $\mu$g/ml) of *L. edodes*-436 (CDIII) (lane 3), *L. edodes*-436 (starch) (lane 4), *L. edodes*-MM1 (CDIII) (lane 5) or *L. edodes*-MM1 (starch) (lane 6) extract were electrophoresed in a 1.5% agarose gel. The amount of DNA fragment was 37.5 ng/assay. (B) The same experiment as in (A) except the amount of each extract (300 ng/assay, final conc. 20 $\mu$g/ml) was different.

## Discussion

The effects of the various *L. edodes* mycelial culture broth extracts on cell growth, cell morphology, DNA-fragmentation, the activation of caspase-3, and E2F protein binding to its promoter were analyzed. As shown in Fig. 2A, *L. edodes*-436 (CDIII) extract inhibits the growth of cancer cells. The inhibition was moderate for normal cells (3T3 cells). This cell-line dependency was also observed for the *L. edodes*-MM1 (starch) extract. With other extracts, no clear cell-line dependency of growth inhibition was detected. Incubation of the cells with *L. edodes* extracts caused shrinkage and a change in cell shape (Fig. 3). *L. edodes* extracts induced DNA fragmentation (Fig. 4) and caspase-3 activation (Fig. 5). Although all of the extracts induced DNA fragmentation

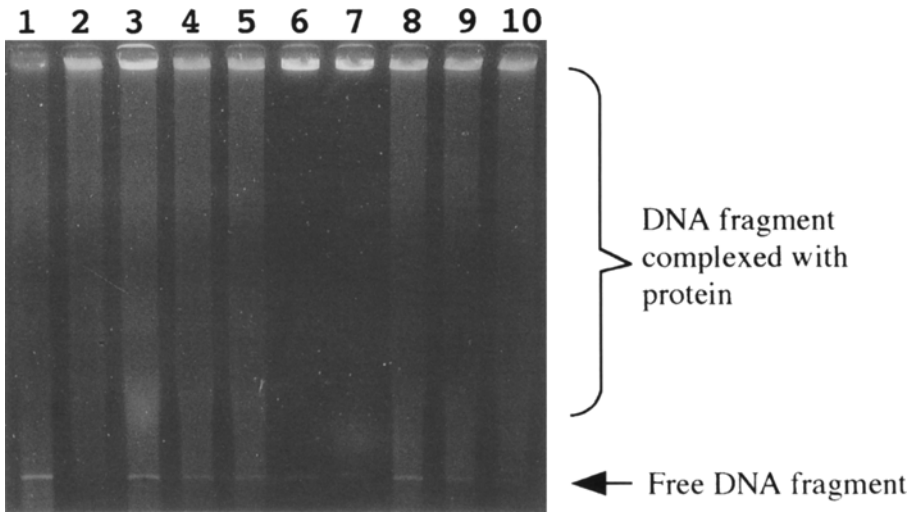


Fig. 7. Effect of the extract and the Excelpak SPE-UNI/154 (ES) mini-column-purified extract from *L. edodes*-436 (CDIII) culture broth on E2F binding to its promoter. The experiment was same as described for Fig. 6. Lane 1, DNA fragment alone; lane 2, DNA fragment plus GST-E2F-1 protein; lanes 3 to 10 were DNA fragment plus GST-E2F-1, plus extract (lane 3), plus ES mini column-purified extract Fraction I (lane 4), Fraction II (lane 5), Fraction III (lane 6), Fraction IV (lane 7), Fraction V (lane 8), Fraction VI (lane 9), and Fraction VII (lane 10). In each assay 300 ng of extract (final conc. 20 $\mu$g/ml) and 60 ng of each fraction (final conc. 4 $\mu$g/ml) was employed.

and increased caspase-3 activity in 3T3, A375, Caski, SiHa and HP-1 cells, these effects were not observed in HeLa cells. The data suggest that *L. edodes* extracts induce apoptosis through the caspase-3 activation pathway in 3T3, A375, Caski, SiHa and HP-1 cells. However, cell death caused by *L. edodes* extracts in HeLa cells is not related to caspase-3 activation.

A tumor suppressor gene product, the Rb protein, plays an important role as a negative regulator of cell-cycle progression (Scheffner et al., 1991; Weinberg, 1995; Levine, 1997; Hinds and Weinberg, 1994), and that role is reciprocal to that of E2F protein and cyclin D1 (Rieber and Strasberg-Rieber et al., 1998). RB protein works in part by binding to and inactivating the E2F protein, preventing the expression of the E2F-activated genes associated with G1/S cell-cycle progression (Hinds and Weinberg, 1994). All *L. edodes* extracts inhibited E2F binding to its promoter (Fig. 6). Activity was detected in the fraction eluted from the ES mini-column with 65% acetonitrile (Fig. 7). This suggests that the molecular target for cell death (including HeLa cells) caused by the extracts is the inhibition of E2F protein binding. The data also suggest the usefulness of the extracts as reagents for inhibiting the cell-cycle progression from G1 to S phase. The correlation between the cell-line dependencies of cell growth inhibition (or on DNA fragmentation) and the characteristics of the oncogene (Rb) in each cell line (summarized in Table 1) is not clear. The potency of various mushroom extracts to induce DNA fragmentation (Fig. 4) and activation of caspase-3 (Fig. 5) depends on the medium and the strain of mushroom. These uncertainties may originate from the fact that the extracts were crude, and also from the ability of oncogenes to induce apoptosis. Because the active substances obtained from the ethyl acetate phase were eluted from the ES mini-column with 65% acetonitrile, they are probably hydrophobic organic compounds, not proteins. This is the first report describing induction of apoptosis in cultured cells, suggesting antitumour effects, by an organic solvent extract from *L. edodes* mycelial culture broth. To clarify the physical characteristics of the active substances, purification of the extracts is now in progress.

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