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Suppressive effect of ethyl acetate extract of *Paecilomyces japonica* on cell cycle progression of human acute leukemia Jurkat T cell clone overexpressing Bcl-2

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Abstract

To understand antitumor activity of an edible mushroom *Paecilomyces japonica*, we have investigated its effect on the cell cycle. When Jurkat T cells transfected with vector (JT/Neo) or Bcl-2 gene (JT/Bcl-2) were treated with the ethyl acetate extract (4–6 μg/ml) of *P. japonica* for 40 h, JT/Neo cells underwent apoptosis with no detectable G_1-arrest, whereas JT/Bcl-2 cells that failed to induce apoptosis accumulated at the G_1 along with reduction of hyperphosphorylated Rb. The cdk4, cyclin E and A, required for the G_1-cdks that phosphorylate Rb, were markedly downregulated in JT/Bcl-2 cells, and the G_1-cdk inhibitor $p27^{Kip1}$ was significantly upregulated. GC–MS analysis identified a component structurally similar to diacetoxyscirpenol as the effective ingredient contributing to the G_1-arrest. These results demonstrate that *P. japonica* can arrest the cell cycle of JT/Bcl-2 cells at the G_1 by suppressing phosphorylation of Rb through downregulation of the activity of G_1-cdks, and thus potentiates apoptotic cell death.

Keywords: *Paecilomyces japonica*; Edible mushroom; Jurkat T cells; Rb phosphorylation; Bcl-2; G_1-arrest; GC–MS analysis

1. Introduction

Cordyceps, the largest single genus of insect parasites belonging to the family Clavicipitacea in the division of Ascomycota, has a sexual stage and also nonsexual stage throughout its life cycle (Breitenbach & Kranzlin, 1984; Moore-Landecker, 1996; Sung, Lee, & Yang, 1995). *Cordyceps* can produce ascospores for the sexual reproductive structure and conidia for the nonsexual reproductive structure (Evans, 1982; Kovayasi & Shimizu, 1983). While *Cordyceps* has been classified based largely on morphological characteristics, nonsexual stages of *Cordyceps* have also been assigned to several unique genuses, such as *Acremonium, Akanthomyces, Cephalosporium, Hirsutella, Hymenostilbe, Isaria, Nomuraea, Paecilomyces, Paraisaria, Pseudogilbellular, Sporothrix, Stilbella, Verticillium,* and *Beauveria* (Kovayasi & Shimizu, 1983; Sung et al., 1995).

Several *Cordyceps* species are known to be used as traditional medicine and healthful food in China, Japan, and Korea due to their pharmacological and physiological activities (Ban et al., 1998). Recently, in Korea *P. japonica*

Abbreviations: cdk, cyclin-dependent kinase; Rb, retinoblastoma protein; CKI, cyclin-dependent kinase inhibitor; FBS, fetal bovine serum; MOPS, 3-(*N*-morpholino)propanesulfonic acid; GC–MS, gas chromatography–mass spectrometry.

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among the *Cordyceps* species is artificially cultivated on a large scale using either the silkworm pupa or larvae, and has started to be consumed as a functional food believed to be effective in cancer prevention and therapy, and employed as a target to search for a new anticancer drug. However, the mechanism underlying the antitumor activity of *P. japonica* has not been completely elucidated. Previously, we demonstrated that when the aqueous extract from *P. japonica* artificially cultivated on the silkworm pupa was fractionated further by sequential organic solvent extractions using *n*-hexane and ethyl acetate, the ethyl acetate extract appeared to contain the most cytotoxic activity and the IC_{50} values for various tumor cells were in the range from 1.5 to 10 μg/ml (Park et al., 2000). It was also demonstrated that the cytotoxic activity of *P. japonica* toward human acute leukemia Jurkat T cells is attributable to induced apoptotic DNA fragmentation that was mediated by mitochondrial cytochrome *c* release with resultant activation of caspase-9 and -3, and cleavage of poly (ADP-ribose) polymerase (Park, Jun, & Kim, 2002). In the present study, we compared the ethyl acetate extract-mediated alteration in the cell cycle distribution of Jurkat T cells transfected with Bcl-2 gene (JT/Bcl-2) and Jurkat T cells transfected with vector (JT/Neo) to investigate whether the ethyl acetate extract of *P. japonica* can arrest the cell cycle progression of Jurkat cells under the conditions that apoptotic cell death is completely inhibited by ectopic overexpression of Bcl-2. In addition, the ethyl acetate extract was analyzed by the gas chromatography–mass spectrometry (GC–MS) to examine the active ingredient(s) affecting the cell cycle progression of Jurkat T cells overexpressing Bcl-2. The results show that the ethyl acetate extract of *P. japonica* suppresses the cell cycle progression of JT/Bcl-2 by G_1-arrest, and suggest that a component structurally similar to diacetoxyscirpenol may be responsible for the *P. japonica*-mediated arrest of the cell cycle.

2. Materials and methods

2.1. Microorganism and culture condition

P. japonica was obtained from the Korea Rural Development Administration. The strain was periodically subcultured on a Potato Dextrose Agar (PDA) Medium (Difco Laboratories, Detroit, USA) and stored at 4 °C. To prepare the seed culture, the strain grown on a PDA Medium was cultivated by shaking in a Potato Dextrose Broth (Difco Laboratories, Detroit, USA) for 7 days at 25 °C. For large-scale cultivation, 100 ml of the seed culture was inoculated into a 2 l polypropylene bag containing 500 g of sterilized silkworm pupas as described elsewhere (Park et al., 2000).

2.2. Ethyl acetate extraction of antitumor component from *P. japonica* by ethyl acetate extraction

To purify the active component for the antitumor activity in *P. japonica*, 30 g of the lyophilized culture of *P. japonica* on the silkworm pupas was suspended with 300 ml of distilled water and incubated in a boiling water bath for 3 h. The aqueous solution was centrifuged at 12,000 rpm to remove mycelium. This water soluble fraction was extracted with the same volume of *n*-hexane three times, and the remaining aqueous phase was then extracted with ethyl acetate three times. From the aqueous solution, approximately 16.2 mg of the ethyl acetate fraction was recovered as previously described (Park et al., 2000).

2.3. Human Jurkat T cell culture and cytotoxicity assay

Human acute leukemia T cell clones JT/Neo, JT/Bcl-2 were maintained in RPMI 1640 containing 10% FBS, 20 mM HEPES (pH 7.0), 5×10^{-5} M β-mercaptoethanol, and 100 μg/ml gentamycin. For the culture of both JT/Neo and JT/Bcl-2, G418 was added to the RPMI 1640 medium at a concentration of 400 μg/ml. The cytotoxic effect of the ethyl acetate extract on Jurkat T cells was analyzed by MTT assay reflecting cell viability as described elsewhere (Jang et al., 2002). Briefly, Jurkat T cells (5×10^4) were added to the serial dilution of the ethyl acetate extract in 96-well plates. After incubation for indicated time periods, 50 μl of MTT solution (1.1 mg/ml) was added to each well and incubated for an additional 4 h. The colored formazan crystal produced from MTT was dissolved in 150 μl of DMSO to measure the absorbance at 540 nm by a plate reader.

2.4. DNA fragmentation analysis

Apoptotic DNA fragmentation, induced in Jurkat T cells following the treatment by external stimulants, was determined as previously described (Jang et al., 2002). Briefly, the cells were harvested by centrifugation and then treated with a lysis buffer (0.5% Triton X-100, 5 mM EDTA, and 10 mM Tris–HCl, pH 7.4) for 20 min on ice. After centrifugation for 15 min at 14,000 rpm, the supernatant was collected and treated for 2 h at 50 °C with proteinase K and subsequently with RNase for 4 h at 37 °C. After extraction with an equal volume of buffer-saturated phenol, the DNA fragments were precipitated with 2.5 volumes of ethanol in the presence of 0.5 M NaCl and visualized following electrophoresis on a 1.2% agarose gel.

2.5. Preparation of cell lysate and Western blot analysis

Cellular lysates were prepared by suspending 5×10^6 Jurkat T cells in 200 μl of lysis buffer (137 mM NaCl, 15 mM EGTA, 1 mM sodium orthovanadate, 15 mM $MgCl_2$, 0.1% Triton X-100, 25 mM MOPS, and 2.5 μg/ml proteinase inhibitor E-64, pH 7.2). The cells were disrupted by sonication and extracted at 4 °C for 30 min. An equivalent amount of protein lysate (15–20 μg) was electrophoresed on 4–12% SDS gradient polyacrylamide gel with MOPS buffer. The proteins were electrotransferred to Immobilon-P membranes (Millipore Corporation, Bed-

ford, MA, USA), and then probed with individual primary antibodies. Detection of each protein was carried out with an ECL Western blotting kit (Amersham, Arlington, Heights, IL, USA) according to the manufacturer's instructions.

2.6. Flow cytometric analysis

The cell cycle progression of Jurkat T cell transfected with vector (JT/Neo) or Bcl-2 gene (JT/Bcl-2) following ethyl acetate extract treatment was analyzed by Flow cytometry as described elsewhere (Kim, Proust, Buchholz, Chrest, & Nordin, 1992). Approximately 1×10^6 cells were suspended in 100 μl of PBS, and 200 μl of 95% ethanol was added while vortexing. The cells were incubated at 4 °C for 1 h, washed with PBS and resuspended with 12.5 μg of RNase in 250 μl of 1.12% sodium citrate buffer (pH 8.45). Incubation was continued at 37 °C for 30 min before staining of the cellular DNA with 250 μl of propidium iodide (50 μg/ml) for 30 min at room temperature. The stained cells were analyzed on a FACScan Flow cytometer for relative DNA content, based on increased red fluorescence.

2.7. Analysis of ethyl acetate extract by gas chromatography–mass spectrometry

The GC–MS analysis was conducted with Hewlett–Packard (HP) 6890 gas chromatograph coupled to an HP5973N mass spectrometer. A HP-5MS capillary column filled with 5% Phenyl Methyl Siloxane was connected to the GC instrument. The GC analytical conditions were as follows: helium carrier gas flow rate, 0.7 ml/min; oven temperature program, 60 °C (hold for 15 min) rising to 280 °C; and split ratio, 30:1. The MS instrument was operated in the electron impact (EI) mode and scanned at 70 eV in an m/z range of 50–800 mass unit.

2.8. Replicates

Unless otherwise indicated, each result described in this paper is representative of at least three separate experiments.

3. Results

3.1. Effect of ethyl acetate extract of P. japonica on cell cycle progression of Jurkat T cells overexpressing Bcl-2

To confirm the protection effect of Bcl-2 on the cytotoxicity of the ethyl acetate extract of *P. japonica*, Jurkat T cells transfected with the vector (JT/Neo) or Bcl-2 gene (JT/Bcl-2) were treated with the ethyl acetate extract at concentrations ranging from 2 to 6 μg/ml for 40 h and the cell viability was assessed by MTT assay. As shown in Fig. 1(a), the transfectant of Bcl-2 gene was able to express significantly increased level of Bcl-2 protein. Although cytotoxic effect of the ethyl acetate extract on Jurkat T cells measured by MTT assay was reduced by ectopic overexpression of Bcl-2, it was not completely prevented (Fig. 1(b)). However, the apoptotic DNA fragmentation induced by the ethyl acetate extract appeared to be completely prevented by ectopic overexpression of Bcl-2 (Fig. 1(c)). It is noteworthy that the initial cell number (3×10^5/ml) for JT/Bcl-2 increased to 1.3×10^6/ml without the ethyl acetate extract, whereas it was similar to the initial

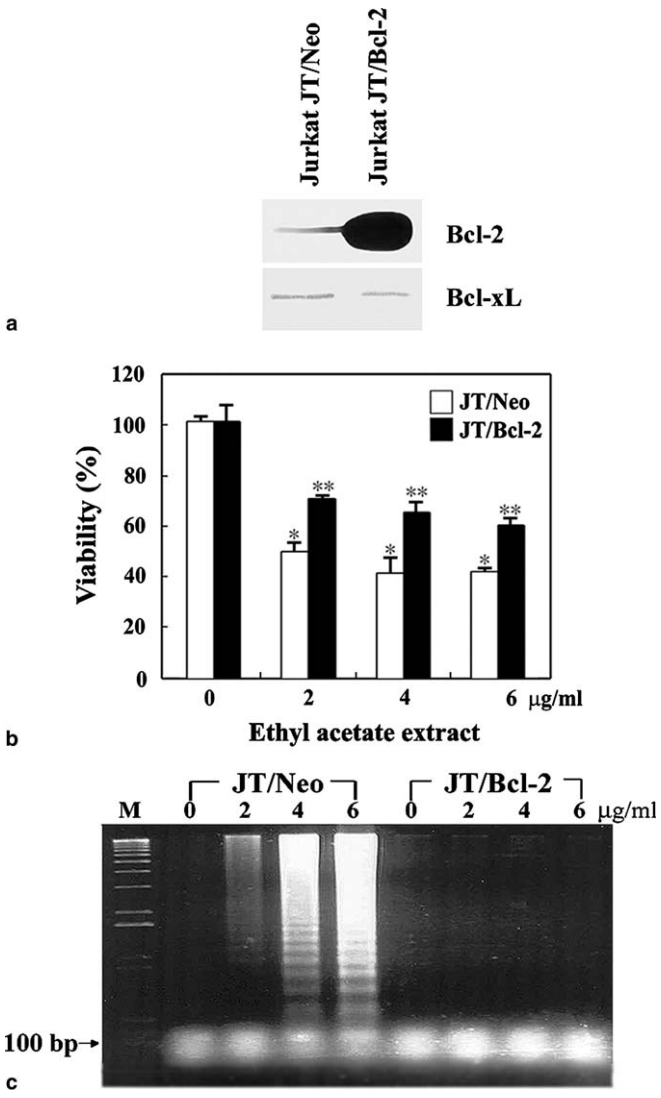

Fig. 1. (a) Effect of the ethyl acetate extract on cell viability of Jurkat T cells transfected with vector (JT/Neo) or Bcl-2 gene (JT/Bcl-2). Ectopic overexpression of Bcl-2 protein in Jurkat T cells transfected with Bcl-2 gene construct was confirmed by Western blot analysis. (b) Jurkat T cells overexpressing Bcl-2 (JT/Bcl-2) or control cells (JT/Neo) were incubated at a density of 4×10^4/well with various concentrations of the ethyl acetate extract in 96-well plates for 40 h and the final 4 h were incubated with MTT to assess the colored formazan crystals produced from MTT as an index of cell viability. (c) Equivalent cultures were processed to analyze apoptotic DNA fragmentation. $^*P < 0.005$ was obtained when the control and the ethyl acetate extract-treated group of JT/Neo were compared. $^{**}P < 0.0005$ was obtained when the control and the ethyl acetate extract-treated group of JT/Bcl-2 were compared.

cell number in the presence of 4–6 μg/ml of the ethyl acetate extract. Under these conditions, the effect of the ethyl acetate extract on the cell cycle progression of Jurkat T cells transfected with the vector (JT/Neo) or Bcl-2 gene (JT/Bcl-2) was analyzed by Flow cytometry. In JT/Neo cells, the sub-G_1 peak representing apoptotic cells was easily detectable and it increased dose-dependently (Fig. 2(a)). JT/Bcl-2 cells overexpressing Bcl-2, however, showed no detectable increase in the level of sub-G_1 peak, demonstrating the protective effect of Bcl-2 on ethyl acetate extract-induced apoptotic cell death (Fig. 2(b)). In addition, as compared to the continuously growing JT/Bcl-2 cells, the cells treated with 2–6 μg/ml of the ethyl acetate extract showed a dose-dependent increase in the level of G_1cells in proportion to the reduction of both the S and G_2/M cells. Kinetic analysis of the cell cycle progression of JT/Bcl-2 cells following treatment of 4 μg/ml of the ethyl acetate extract also showed that this accumulation in the G_1 phase began to be detectable at 15 h and enhanced by 40 h in accordance with the decrease of cells in the S and G_2/M phase (Fig. 3(b)). Although a significant level of apoptotic cells for JT/Neo was detectable at 15 h and appeared to increase in a time-dependent manner, there was no accumulation in the G_1 phase following treatment with the ethyl acetate extract (Fig. 3(a)). Comparison of the ethyl acetate-induced alterations in the cell cycle distribution between JT/Bcl-2 and JT/Neo cells suggested that the apoptotic cells were derived mainly from the cells being accumulated in the G_1 phase following treatment with the ethyl acetate. Consequently, these results demonstrate that the ethyl acetate extract of *P. japonica* can block the cell cycle progression of Jurkat T cells at the G_1 phase, and thus tumor cells with a high level expression of antiapoptotic proteins including Bcl-2 are also sensitive to the antitumor activity of *P. japonica*.

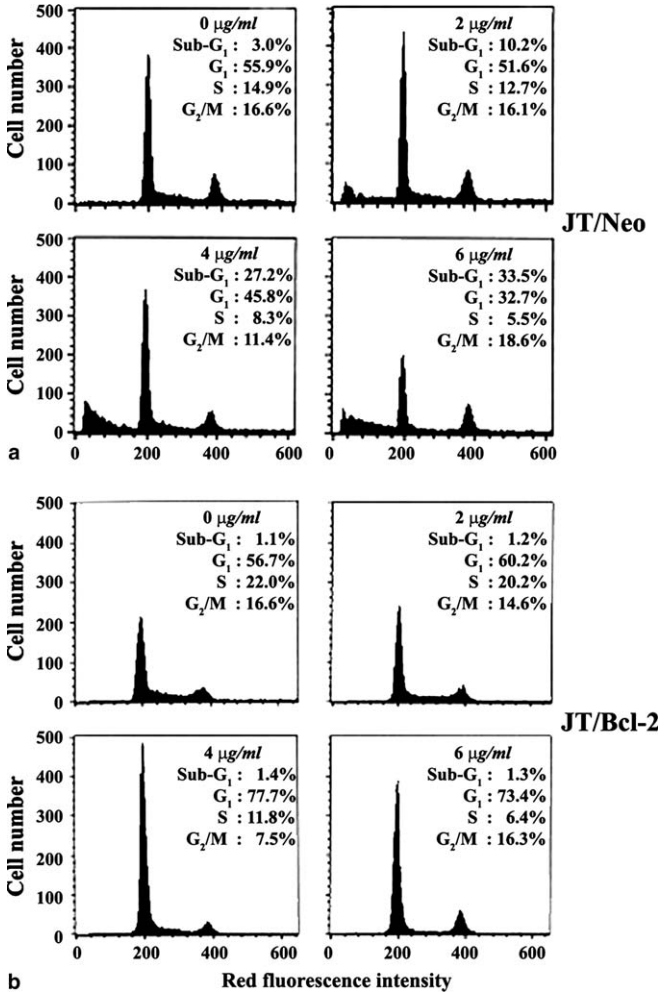

Fig. 2. Apoptotic change in the cell cycle distribution of Jurkat T cells transfected with vector (JT/Neo) (a) or Bcl-2 gene (JT/Bcl-2) (b) after treatment with various concentrations of the ethyl acetate extract for 40 h. After JT/Neo and JT/Bcl-2 were incubated in the presence of ethyl acetate extract under individual conditions, the cells were harvested. The analysis of cell cycle distribution was performed on an equal number of cells (5×10^5) by Flow cytometry after staining of DNA by propidium iodide.

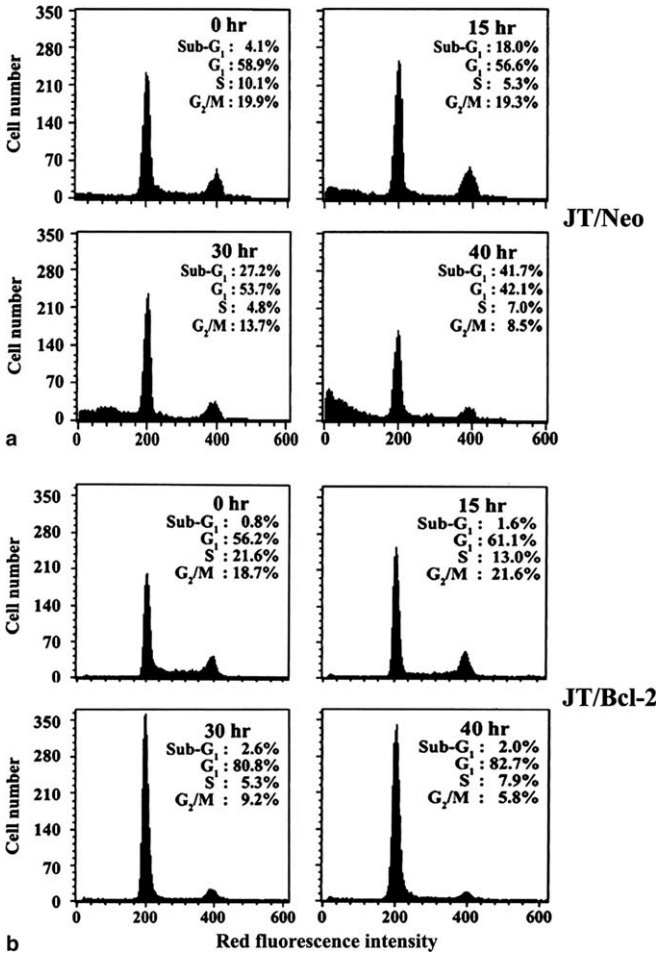

Fig. 3. Apoptotic change in the cell cycle distribution of Jurkat T cells transfected with vector (JT/Neo) (a) or Bcl-2 gene (JT/Bcl-2) (b) after treatment with 4 μg/ml of the ethyl acetate extract for various time periods. JT/Neo and JT/Bcl-2 were incubated at a density of 3×10^5/ml in the presence of ethyl acetate extract under individual conditions, the cells were harvested. The analysis of cell cycle distribution was performed on an equal number of cells (5×10^5) by Flow cytometry after staining of DNA by propidium iodide.

3.2. Decrease in the level of hyperphosphorylated form of retinoblastoma following treatment with ethyl acetate extract

Since the hyperphosphorylation of Rb protein in the G_1 phase allows the release of E_2F family from Rb and thus is essential for entering S phase (Helin et al., 1992; Krek et al., 1994; Weinberg, 1995), the failure of Rb phosphorylation during the cell cycle results in cell cycle arrest in the G_1 phase. To elucidate the mechanism underlying *P. japonica*-mediated G_1-arrest of the cell cycle progression, we have decided to investigate the effect of ethyl acetate extract on the phosphorylation level of Rb. When Western blot analysis was performed using a monoclonal anti-Rb, the hyperphosphorylated form of Rb was easily detectable in continuously growing JT/Neo and JT/Bcl-2 cells (Fig. 4). Although the level of hyperphosphorylated Rb did not decline significantly in JT/Neo cells after treatment with the ethyl acetate extract, it was significantly reduced in JT/Bcl-2 cells in a time-dependent manner. These results indicate that *P. japonica*-mediated G_1-arrest of the cell cycle progression of Jurkat T cells overexpressing Bcl-2 is associated with the failure of Rb phosphorylation required for the G_1–S transition, and suggest that the majority of JT/Neo cells being arrested in the G_1 phase, which is due to underphosphorylation of Rb after treatment with the ethyl acetate extract, might rapidly undergo apoptotic cell death.

3.3. Effect of ethyl acetate extract on the protein level of cdks and cyclins

Since the phosphorylation of Rb at the G_1 phase is mediated by G_1-cdks, it seems likely that perturbation of the kinase activity of G_1-cdks may have occurred in Jurkat T cells following treatment with the ethyl acetate extract. To test this prediction, the change in the protein level of cdks and cyclins was investigated in JT/Neo and JT/Bcl-2 cells after treatment with 4 μg/ml of the ethyl acetate extract. As shown in Fig. 5, the expression level of Bcl-2 protein in JT/Neo and JT/Bcl-2 cells, which was not affected by the treatment with the ethyl acetate extract, was confirmed by Western blot analysis. Under the same conditions, the proteins specific for cdk4, cdk6, cdk2, cdk7, and cdc2 were easily detectable in continuously growing JT/Neo and JT/Bcl-2 cells, and these protein levels remained relatively constant except for cdk4 and cdc2 whose levels appeared to be reduced in the presence of the ethyl acetate extract. While a remarkable decrease in the protein level of two cdk4 bands began to be detected in both JT/Neo and JT/Bcl-2 cells 15 h after treatment with the ethyl acetate extract, the reduction of the fast-moving cdk4, which was previously known as the active phosphorylated form of cdk4 (Solorzano, Rieber, & Rieber, 2000), was slightly more evident in JT/Neo cells. The decrease in the protein level of cdc2 was more significant in JT/Bcl-2 cells than in JT/Neo cells. Under these conditions, the protein levels of cyclin A, E, and B1 showed a marked decline in JT/Bcl-2 cells following treatment in a time-dependent manner (Fig. 6). The decline in the level of cyclins, however, was either undetectable or not significant in JT/Neo cells. Since cdk4/D-type cyclins, cdk2/cyclin A, and cdk2/cyclin E are among the critical G_1 cdks that phosphrylate Rb for the progression from G_1 to S phase (Adams, 2001), these results indicate that the ethyl acetate-mediated G_1-arrest of JT/Bcl-2 is associated with

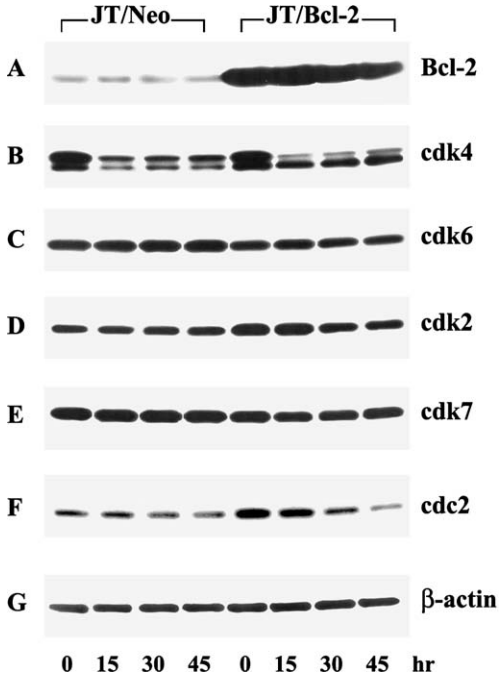

Fig. 5. Western blot analysis of the protein levels of Bcl-2 (A), cdk4 (B), cdk6 (C), cdk2 (D), cdk7 (E), cdc2 (F), and β-actin (G) in Jurkat T cells overexpressing JT/Bcl-2 gene (JT/Bcl-2) or control cells (JT/Neo) after treatment with the ethyl acetate extract for various time periods. Both Jurkat T cells overexpressing Bcl-2 (JT/Bcl-2) and control cells (JT/Neo) were incubated at a density of 3×10^5/ml with 4 μg/ml of the ethyl acetate extract and prepared for the cell lysates. Equivalent amounts of cell lysates were electrophoresed on 4–12% SDS gradient polyacrylamide gels and electrotransferred to Immobilon-P membrane. Western blot analysis was performed as described in Section 2.

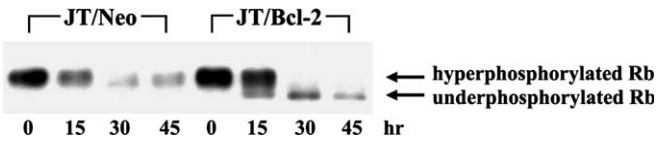

Fig. 4. Western blot analysis of the protein levels of Rb in Jurkat T cells overexpressing JT/Bcl-2 gene (JT/Bcl-2) or control cells (JT/Neo) after treatment with the ethyl acetate extract for various time periods. Both Jurkat T cells overexpressing Bcl-2 (JT/Bcl-2) and control cells (JT/Neo) were incubated at a density of 3×10^5/ml with 4 μg/ml of the ethyl acetate extract and prepared for the cell lysates. Equivalent amounts of cell lysates were electrophoresed on 6% SDS gradient polyacrylamide gels and electrotransferred to Immobilon-P membrane. The membrane was probed with anti-Rb and then with a horseradish peroxidase conjugated mouse monoclonal antibody. Detection of protein was performed using the ECL plus Western blotting detection system.

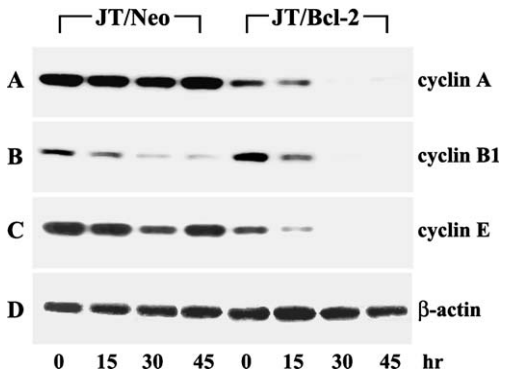

Fig. 6. Western blot analysis of the protein levels of cyclin A (A), cyclin B1 (B), cyclin E (C), and β-actin (D) in Jurkat T cells overexpressing JT/Bcl-2 gene (JT/Bcl-2) or control cells (JT/Neo) after treatment with the ethyl acetate extract for various time periods. Both Jurkat T cells overexpressing Bcl-2 (JT/Bcl-2) and control cells (JT/Neo) were incubated at a density of 3×10^5/ml with 4 μg/ml concentration of the ethyl acetate extract and prepared for the cell lysates. Equivalent amounts of cell lysates were electrophoresed on 4–12% SDS gradient polyacrylamide gels and electrotransferred to Immobilon-P membrane. Western blot analysis was performed as described in Section 2.

downregulation of cdk4, cyclin A and E, leading to dysregulation of G_1-cdks such as cdk4/D-type cyclins, cdk2/cycin A, and cdk2/cyclin E.

3.4. Upregulation of $p27^{Kip1}$ by ethyl acetate extract

Recent studies have shown that negative cell cycle regulators such as specific cdk inhibitors (CKIs) are essential for the control of cdk activities in response to intra or extracelllular signals (Morgan, 1995; Sherr & Roberts, 1995). $p27^{Kip1}$ downregulates the activity of G_1-cdks including cdk2 and is believed to be a main cdk inhibitor in T cells (Peter & Herskowltz, 1994). The change in the protein level of $p27^{Kip1}$following treatment with the ethyl acetate extract was also analyzed in order to understand the involvement of upregulation of CKIs in *P. japonica*-mediated G_1-arrest of Jurkat cells overexpressing Bcl-2. As shown in Fig. 7, the protein level of $p27^{Kip1}$ was significantly enhanced time-dependently, whereas the level of β-actin was not altered regardless of the presence of the ethyl acetate extract. The upregulation of $p27^{Kip1}$ like the remarkable reduction in the protein level of cyclin A and E, which can be directly associated with the ethyl acetate extract-induced G_1-arrest of the cell cycle, was not detectable in JT/Neo cells, possibly due to elimination of the majority of JT/Neo cells arrested at the G_1 phase by apoptotic cell death. Under these conditions, the upregulation of the other CKIs including $p16^{Ink4}$, $p21^{Waf1}$, and $p57^{Kip2}$ was not observed (data not shown). These results demonstrate that upregulation of $p27^{Kip1}$ is also associated with the *P. japonica*-induced G_1-arrest of JT/Bcl-2 cells.

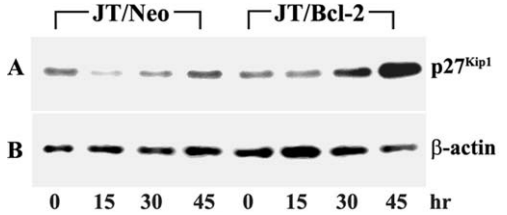

Fig. 7. Western blot analysis of the protein levels of $p27^{Kip1}$ (A) and β-actin (B) in Jurkat T cells overexpressing J/Bcl-2 gene (JT/Bcl-2) or control cells (JT/Neo) after treatment with the ethyl acetate extract for various time periods. Both Jurkat T cells overexpressing Bcl-2 (JT/Bcl-2) and control cells (JT/Neo) were incubated at a density of 3×10^5/ml with 4 μg/ml of the ethyl acetate extract and prepared for the cell lysates. Equivalent amounts of cell lysates were electrophoresed on 4–12% SDS gradient polyacrylamide gels and electrotransferred to Immobilon-P membrane. Western blot analysis was performed as described in Section 2.

3.5. GC–MS analysis of the ethyl acetate extract

To identify the active ingredient(s) in the ethyl acetate extract, which induces the G_1-arrest of Jurkat T cells overexpressing Bcl-2, the ethyl acetate extract dissolved in DMSO was analyzed by GC–MS. As shown in Table 1, the ethyl acetate extract did not contain nucleosides, glycosides, polysaccharides, amino acids and peptides, whereas it contained phthalic acid, stearic aicd, di(2-ethylhexyl)phthalate, cholesterol, and ergosterol as the main components, and their relative contents were 26.7, 6.9, 7.3, 13.0, and 17.2%, respectively. Since phthalic acid, stearic aicd, di(2-ethylhexyl)phthalate, cholesterol, and ergosterol were commercially available from Sigma–Aldrich, we decided to examine whether these individual compounds were capable of inducing growth-arrest of JT/Bcl-2 cells. When the cytotoxicity of phthalic acid, stearic aicd, di(2-ethylhexyl)phthalate, cholesterol, or ergosterol toward both JT/Neo and JT/Bcl-2 cells was examined at concentrations ranging from 0 to 10 μg/ml by MTT assay, neither cytotoxicity nor growth-retardation activity was detected (data not shown). These results exclude the possible involvement of any of these compounds in the ethyl acetate extract-mediated growth arrest of JT/Bcl-2 cells. It is noteworthy that another major component (relative contents (RC), 21.9%; retention time (RT), 27.6 min) of the ethyl acetate extract showed 46% homology with a toxic fungal metabolite diacetoxyscirpenol (anguidine) that belongs to the family of trichothecenes produced by various species of fungi imperfecti (Cole & Cox, 1981).

Table 1
Compounds identified by GC–MS analysis in the ethyl acetate extract of *P. japonica*

Compound	RT (min)[a]	Homology (%)[b]	RC (%)[c]
Diacetoxyscripenol	27.6	46	21.9
Phthalic acid	28.5	95	26.7
Stearic acid	32.7	94	6.9
Di-(2-ethylhexyl)phthalate	38.2	91	7.3
Cholesterol	46.7	99	13.0
Ergosterol	47.6	99	17.2

[a] Retention time; min.
[b] Homology of the peak to authentic compound in the data base; tentatively identified only by the mass spectrum.
[c] Relative contents; percentage of total.

Several studies reported the inhibitory effect of diacetoxyscirpenol (anguidine) on cell cycle progressoin in several tumor cells, without elucidating the underlying mechanisms (DeSimone, Greco, Lessner, & Bartolucci, 1986; Dosik, Barlogie, Johnston, Murphy, & Drewinko, 1978; Liao, Grollman, & Horwitz, 1976). Although the observed component in the ethyl acetate needs to be further purified and analyzed for understanding its molecular structure, these results suggest that a compound structurally similar to diacetoxyscirpenol is the active ingradient responsible for the G_1-arrest of JT/Bcl-2 cells.

4. Discussion

Recently, there have been some progresses in our understanding the pharmacological activities of the *Cordyceps*, which include immunomodulatory function (Shin et al., 2003; Weng, Chou, Lin, Tsai, & Kuo, 2002), improvement of diabetes-related metabolic diseases (Lo, Tu, Lin, & Lin, 2004), anti-inflammatory activity (Kim et al., 2003), and antitumor activity (Park et al., 2000, 2002; Zhang, Wu, Hu, & Li, 2004). In several in vitro and in vivo studies, it has been shown that anti-tumor activity of *Cordyceps* is mediated by inducing apoptosis of tumor cells or immuno-stimulating function toward macrophage (Park et al., 2000, 2002; Shin et al., 2003; Zhang et al., 2004). However, the regulatory effect of *Cordyceps* on the cell cycle progression in relation to antitumor activity has not been elucidated. In the present studies, we have first demonstrated that the ethyl acetate extract of *P. japonica*, which belongs to *Cordyceps* species and is artificially cultivated on silkworm pupas for mass production (Park et al., 2000), inhibits the cell cycle progression of human acute leukemia Jurkat T cells through the G_1-arrest resulting from the failure of phosphorylation of Rb protein. Since the ethyl acetate extract can induce a rapid apoptosis in Jurkat T cells via mitochondria-dependent apoptotic signaling pathway regulated by Bcl-2 and since this apoptotic cell death provides a difficulty in analyzing the ethyl acetate-mediated alteration of the cell cycle of Jurkat T cells, we have employed Jurkat T cell clone (JT/Bcl-2) overexpressing Bcl-2 to investigate the effect of *P. japonica* on the cell cycle under the conditions that the apoptosis induced by the ethyl acetate extract is completely inhibited. When the effect of the ethyl acetate extract on cell cycle distribution was compared between JT/Neo and JT/Bcl-2 by Flow cytometry, JT/Bcl-2 showed a dose- and time-dependent increase in the level of G_1 cells without an increase in the level of sub-G1 peak representing apoptotic cells. On the other hand, JT/Neo cells showed no accumulation in the G_1 phase, but a significant enhancement in sub-G_1 peak. These results demonstrate that the ethyl acetate extract of *P. japonica* can block the cell cycle progression of Jurkat T cells at the G_1 phase, and suggest that the cells being arrested in the G_1 phase may rapidly undergo apoptotic cell death in the absence of ectopic overexpression of Bcl-2.

Mammalian cell cycle progression is governed to a large extent by the sequential activation and inactivation of a series of cyclin-dependent kinases (cdks) (Grana & Reddy, 1995; Morgan, 1995). Activation of individual cdks at particular stages of the cell cycle requires first their physical association with appropriate subunits known as cyclins,and phosphorylation as well as dephosphorylation of the cdk at appropriate residues. During the cell cycle progression, the G_2-M transition requires the activation of cdc2/cyclin B, and progression from the G_1 to S phase involves activation of cdk4/cyclin D, cdk6/cyclin D, cdk2/cycin E, and cdk2/cyclin A. A negative cell cycle regulator, retinoblastoma (Rb) protein, which has been identified as a substrate of G_1-cdks, is known to play a critical role in determining G_1–S transition (Weinberg, 1995). In the G_0 and early G_1 phase, underphosphorylated form of Rb associates with transcription factors including E_2F family that are required for entering S phase (Helin et al., 1992; Krek et al., 1994). Rb becomes hyperphosphorylated by G_1-cdks in late G_1 phase resulting in the release of E_2F family. In order to understand the molecular mechanism underlying the ethyl acetate extract-mediated G_1-arrest in JT/Bcl-2, we have investigated whether the phosphorylation of Rb protein is affected by treatment with the ethyl acetate. Western blot analysis using a monoclonal anti-Rb revealed that the level of hyperphosphorylated Rb was significantly reduced in JT/Bcl-2 cells after treatment with the ethyl acetate extract, indicating that *P. japonica*-mediated G_1-arrest in JT/Bcl-2 is associated with the failure of Rb phosphorylation required for the G_1–S transition. Under these conditions, the level of hyperphosphorylated Rb protein did not decline significantly in JT/Neo cells, suggesting that the majority of JT/Neo cells arrested in the G_1 phase, due to the failure of Rb phosphorylation, might rapidly undergo apoptotic cell death. Western blot analysis further demonstrated that the protein levels of cdk4, cdc2, cyclin A, cyclin B1, and cyclin E were significantly reduced, whereas the protein level of $p27^{Kip1}$ was significantly enhanced in JT/Bcl-2 cells. Since the G_1-cdks catalyzing phosphorylation of Rb in the G_1 phase are cdk4 or cdk6 (associated with D-type cyclins) and cdk2 (associated with cyclin A or E), and since $p27^{Kip1}$ inhibits the activity of these G_1-cdks, these results indicate that the ethyl acetate-mediated G_1-arrest of JT/Bcl-2 accompanying the failure of Rb phosphorylation is correlated with downregulation of cdk4, cyclin A and E, and upregulation of $p27^{Kip1}$. Either the decline in the level of cyclins or upregulation in the level of $p27^{Kip1}$, however, was undetectable or not significant in JT/Neo cells, supporting our prediction that the majority of JT/Neo cells being arrested in the G_1 phase after treatment with the ethyl acetate extract were eliminated by apoptotic cell death. Previously, the advantage of ectopic expression of Bcl-2 was also reported in dissecting the relationship between p53-mediated G_1-arrest and p53-mediated apoptosis in murine M1 myeloid leukemia cells (Guillouf et al., 1995). Although p53-mediated G_1-arrest could not

be assessable in the M1 cells due to the simultaneous rapid induction of p53-mediated apoptosis, the G_1-arrest and underlying mechanism became unmasked when the apoptosis was suppressed by ectopic expression of Bcl-2.

GC–MS analysis revealed that the ethyl acetate extract contained phthalic acid, stearic aicd, di(2-ethylhexyl)phthalate, cholesterol, ergosterol, and a compound showing 46% homology to diacetoxyscirpenol (anguidine), as the main components. Except a component showing structural similarity to diacetoxyscirpenol, the components such as phthalic acid, stearic aicd, di(2-ethylhexyl)phthalate, cholesterol, and ergosterol were obtained from Sigma-Aldrich, and were individually tested. The results showed that none of these compounds possessed either cytotoxic or growth-retardation effect on JT/Bcl-2 cells at concentrations of up to 10 μg/ml. Recently, it has been reported that ergosterol, a fungal sterol, can be used like cholesterol by human cells to support membrane formation (Suarez et al., 2002), and stearic acid, a saturated fatty acid, at a concentration of ~85 μg/ml induces apoptotic cell death in PC12 cells via upregulation of Fas and Fas ligand expression (Ulloth, Casiano, & Leon, 2003). Our results and previous results suggest that phthalic acid, stearic aicd, di(2-ethylhexyl)phthalate, cholesterol, or ergosterol may not be the effective component responsible for the ethyl acetate-mediated G_1-arrest of JT/Bcl-2 cells. On the other hand, although the underlying mechanism remains to be elucidated, diacetoxyscirpenol (anguidine) has been reported to inhibit the cell cycle in tumor cells (DeSimone et al., 1986; Dosik et al., 1978; Liao et al., 1976). Recently, it has been shown that ergosterol peroxides and acetoxyscirpenediol, which possess cytotoxic activities against several solid tumor cells, were identified from methanol extract of *Paecilomyces tenuipes* that is a closely related fungus with *P. japonica* (Nam, Jo, Kim, Hyun, & Kim, 2001). The IC_{50} values of ergosterol peroxides and acetoxyscirpenediol against solid tumor cells were found to be 8.0–67.7 μg/ml and 0.4–1.3 μg/ml, respectively. Since the GC–MS data showed that the ethyl acetate extract of *P. japonica* also contains the component structurally similar to diacetoxyscirpenol, it seems likely that it might be the active ingredient that contributes to the G_1-arrest of JT/Bcl-2 cells following treatment of the ethyl acetate extract. Further purification of the effective component from the ethyl acetate extract and its molecular structure are under investigation.

In conclusion, these results demonstrate that the ethyl acetate extract from *P. japonica* is able to block the cell cycle progression of Jurkat T cells at the G_1 accompanying the failure in hyperphosphorylation of Rb protein when the ethyl acetate extract-induced apoptosis is completely prevented by ectopic overexpression of Bcl-2. The failure of Rb phosphorylation appears to be associated with down-regulation of cdk4, and cyclin E and A along with upregulation of $p27^{Kip1}$. Additional data suggest that a component of *P. japonica*, which is structurally similar to the family member of trichothecenes, may play a role in the G_1-arrest of JT/Bcl-2 cells following treatment of the ethyl acetate extract.

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