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Antitumor Activity of *Paecilomyces japonica* is Mediated by Apoptotic Cell Death

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Abstract The aqueous extract from the cultural mycelium of *Paecilomyces japonica* showed cytotoxicity against several tumor cells including Jurkat, U937, HL-60, HepG2, BW5147.G.1.4, and NIH3T3. When the aqueous extract was fractionated by sequential organic solvent extractions using n-hexane and ethyl acetate, the ethyl acetate fraction appeared to contain the most cytotoxic activity, and the IC_{50} values for various tumor cells were in the range from 1.5 to 10.0 μg/ml. To elucidate the cellular mechanism underlying the induced cytotoxicity, the apoptotic DNA fragmentation along with the cell cycle progression was examined in Jurkat T cells following the ethyl acetate fraction treatment. In the presence of 2.5 μg/ml of the ethyl acetate fraction, apoptotic DNA fragmentation of the cells was detected within 1 h and increased upto 24 h in a time-dependent manner. Under the same conditions, a sub-G1 peak was detectable by flow cytometry. These results indicate that the cytotoxic effect of *P. japonica* on tumor cells is attributable to the induced apoptosis.

Key words: *Paecilomyces japonica*, silkworm pupa, cytotoxicity, antitumor effect, apoptosis

The genus *Cordyceps* including about 300 species is the largest single genus of insect parasites in the division Ascomycota and it belongs to the family Clavicipitaceae which is comprised in the order Clavicipitales [4, 12, 13]. Insects infectivity by *Cordyceps* are mainly in the order of Hemiptera, Diptera, Lepidoptera, Hymenoptera, and Coleoptera [12]. The infection in these insects occurs most commonly through the larvae.

Cordyceps possesses a sexual stage as well as nonsexual stage throughout its life cycle. It has been generally accepted that *Cordyceps* can produce ascospores for the sexual reproductive structure and conidia for the nonsexual reproductive structure [7, 10]. While *Cordyceps* has been classified based largely on the 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, Pseudogibellula, Sporothrix, Stilbella, Verticillium, and Beauveria* [10, 14].

Several *Cordyceps* species are known to be used as traditional medicine in China, Japan, and Korea [3]. Recently, mass production of these strains through artificial cultivation has been successfully established. This subsequently allows several *Cordyceps* species to be supplied for public demands, and employed as a target to search a new anticancer and immunomodulating drug. In Korea, *Paecilomyces japonica* is among the *Cordyceps* species artificially cultivated on a large scale using a silkworm pupa and has started to be consumed as a functional food believed to be effective in cancer prevention along with its therapy. However, there has been no research conducted to elucidate the mechanism underlying its inhibitory effect on tumor cells.

In the present study, the antitumor effect of an aqueous extract of *P. japonica* artificially cultivated on the silkworm pupas was examined, and using the active fraction obtained by ethyl acetate extraction from the aqueous extract, the cytotoxic effect against human acute T leukemia Jurkat cells was further investigated. The results show that the cytocidal effect of *P. japonica* on several tumor cells of human and murine origins is attributable to induced apoptosis.

Materials and Methods

Microorganism and Culture Conditions

P. japonica was initially obtained from the Korean Rural Development Administration. The strain was periodically subcultured on a Potato Dextrose Agar (PDA) medium (Difco Laboratories, Detroit, U.S.A.) and stored at 4°C. To prepare for a seed culture, the strain grown on a PDA

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medium was cultivated with shaking in a Potato Dextrose Broth (Difco Laboratories, Detroit, U.S.A.) for 7 days at 25°C. For a large-scale cultivation, 100 ml of the seed culture was inoculated into 2-l of polypropylene bag containing 500 g of sterilized silkworm pupas, and incubated at 25°C for 15 days under 70% relative humidity (RH). Sequentially, the culture was incubated at 20°C under 90–95% RH with an exposure to light for a period of 20 days to induce the fruiting body.

Mammalian Cell Culture

Human Jurkat T cells, human myeloid leukemia U937 cells, murine lymphoma BW5.1.4.7.G.1.4 cells, and human breast cancer MDA361 cells were kindly supplied by Dr. Albert A. Nordin (Gerontology Research Center, NIA/NIH, Baltimore, U.S.A.). Human promyelocytic leukemia HL-60 cells, human acute monocytic leukemia THP-1 cells, human hepatocellular carcinoma HepG2 cells, murine T cell leukemia EL-4, murine sarcoma 180 cells, and murine fibroblast NIH3T3 cells were obtained from the Korean Collection for Type Culture (KCTC, KRIBB, Taejon, Korea). Jurkat, U937, HL-60, and THP-1 were maintained in RPMI 1640 (Life Technologies, Gaithersburg, U.S.A.) containing 10% FBS (UBI, Lake Placid, U.S.A.), 20 mM HEPES (pH 7.0), 5×10^{-5} M β-mercaptoethanol, and 100 μg/ml gentamycin. The culture medium used for MDA361, BW5147.G.1.4, and NIH3T3 cells was Dulbecco's Modified Eagle's Medium (Life Technologies, Gaithersburg, U.S.A.) supplemented with 10% FBS, 20 mM HEPES (pH 7.0), 1 mM sodium pyruvate, 5×10^{-5} M β-mercaptoethanol, and 100 μg/ml gentamycin.

Cytotoxicity Assay

The cytotoxic effect of *P. japonica* on several tumor cells was analyzed by either MTT assay or [^{3}H]thymidine-incorporation as described previously [1]. For MTT assay, tumor cells were grown for 2 days with or without the compound to be tested. After the incubation, 50 μl of MTT solution (1.1 mg/ml) was added to each well and incubated for an additional 4 h. After centrifugation, the supernatant was removed from each well and then 150 μl of DMSO was added to dissolve the colored formazan crystal produced from MTT. OD values of the solutions were measured at 540 nm by using a plate reader.

The incorporation of [^{3}H]thymidine into DNA by Jurkat T cells treated with the aqueous extract of *P. japonica* was determined by adding of 5×10^4 cells to 1:48 dilution of the aqueous extract in a 96-well plate. After 44 h, 1 μCi/well of [^{3}H]thymidine was added for 4 h and assayed for the incorporation of [^{3}H]thymidine by the liquid scintillation.

DNA Fragmentation Analysis

In order to determine apoptotic DNA fragmentation induced in Jurkat T cells following the treatment by *P. japonica*, the isolation of apoptotic DNA fragments was performed as described by Bae *et al.* [2]. Briefly, the cells were harvested by centrifugation and then treated with a lysis buffer (1% NP-40, 20 mM EDTA, 50 mM Tris-HCl, pH 7.5). After centrifugation for 5 min at 1,600 ×g, the supernatant was collected and brought to 1% SDS, and treated for 2 h at 50°C with RNase A and subsequently with proteinase K for 2 h at 37°C. The DNA fragments were precipitated with 2.5 volume of ethanol in the presence of 5 M ammonium acetate. The DNA fragmentation was visualized by electrophoresis on a 2% agarose gel.

Flow Cytometry Analysis

Change of the cell cycle progression of Jurkat T cells following the treatment with the ethyl acetate fraction was analyzed by flow cytometry as described previously [9]. Approximately 1×10^6 cells were suspended in 100 μl of PBS, and then 200 μl of 95% cold ethanol was added while vortexing. The cells were incubated at 4°C for 1 h, washed twice with PBS containing 2% FCS, 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 the increased red fluorescence.

Results and Discussion

Morphology of *P. japonica*

After *P. japonica* was inoculated on a silkworm pupa, the growth of mycelium and formation of the fruiting body were kinetically investigated. The mycelium grew and covered as much as 40–50% of the surface of sterilized silkworm pupa within 5 days after inoculation, when incubated at 25°C under 70% RH (Figs. 1A and B). The entire surface as well as the internal region of the pupa appeared to be filled with the mycelium by 15 days after inoculation. At this time, the culture was transferred to 20°C under 90–95% RH and exposed to light for inducing the fruiting body (Fig. 1C). Under these conditions, the formation of the fruiting body was significantly enhanced in 20 days (Fig. 1D) and reached a maximum level in 30 days (Fig. 1E).

Inhibitory Effect of the Aqueous Extract of *P. japonica* on Tumor Cells

To obtain an aqueous extract of *P. japonica*, 30 g of the lyophilized culture was suspended in 300 ml of distilled water and incubated in a boiling water bath for 3 h. The aqueous solution was centrifuged at 12,000 rpm for 15 min to remove mycelium. To examine the inhibitory effect of

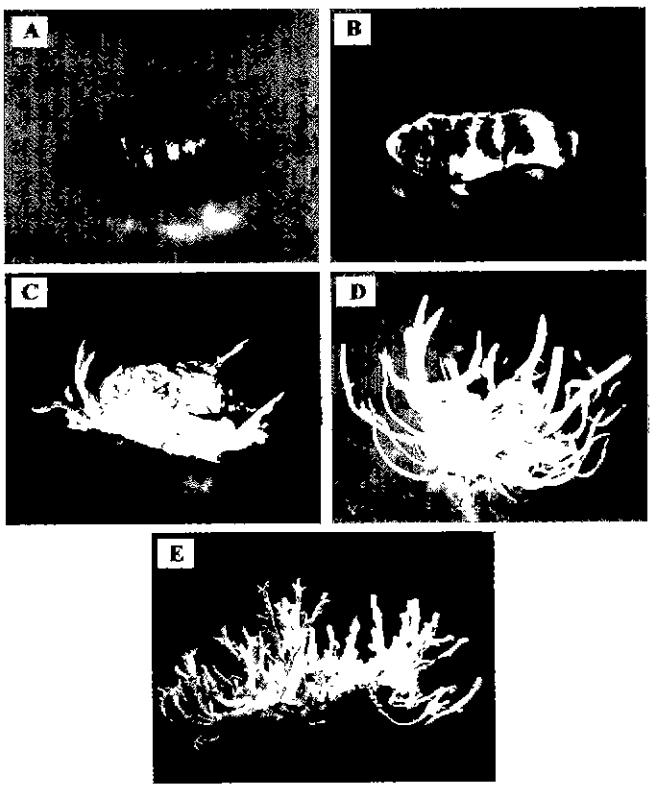

Fig. 1. Kinetic change of growth of *P. japonica* following the inoculation on a silkworm pupa.
After the sterilized silkworm pupa (A) was inoculated with *P. japonica* and incubated at 25°C under 70% RH, 40–50% of the surface of the silkworm pupa appeared to be covered with the mycelium in 5 days (B). The incubation was continued until the entire surface was covered with the mycelium which took 15 days (C), and then it was transferred to 20°C under 90–95% RH with exposure to light. The fruiting body was significantly produced in 20 days (D) and reached a maximum level within 30 days (E) after inoculation.

the aqueous extract on tumor cells, the aqueous extract was sequentially diluted with RPMI 1640 or DMEM, and added into a 96-well plate containing either 5×10^4 cells for Jurkat, U937, HL-60, and BW5147.G.1.4 or 5×10^3 cells for HepG2 and NIH3T3. After being incubated for 2 days, the cell proliferation was determined by MTT assay.

As shown in Fig. 2, the aqueous extract is cytotoxic for various tumor cells of human and murine origins at the concentration ranging from 1/24 to 1/384 dilutions. However, the proliferation of murine normal fibroblast NIH3T3 cells was not affected at the concentration from 1/192 to 1/384 dilutions, suggesting that normal fibroblast is less sensitive to cytotoxicity of *P. japonica* compared to the malignantly proliferating leukemia, lymphoma, and solid tumor cells.

It has previously been reported that *Cordyceps militaris* produced an antibiotic, cordycepin, which is known to inhibit polyadenylation of hnRNA in addition to the 45S RNA synthesis in Hela cells at concentration of 50 μg [5, 6]. Since the structure was identified as 3'-deoxyadenosine, the inhibitory effect of cordycepin on eukaryotic cells was

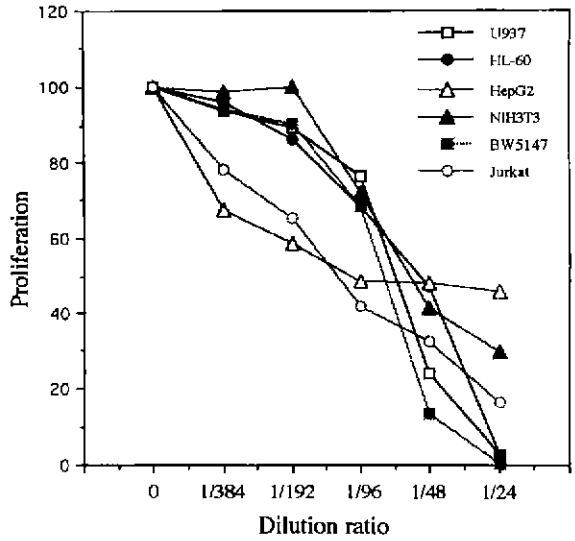

Fig. 2. Cytotoxic effect of the aqueous extract of *P. japonica* on various tumor cells.
The aqueous extract of *P. japonica* was serially diluted and added into the 96-well plate containing 5×10^4 cells for Jurkat, U937, HL-60, and BW5147.G.1.4 or 5×10^3 cells for HepG2 and NIH3T3. After 2 days, the cell proliferation was measured by MTT assay.

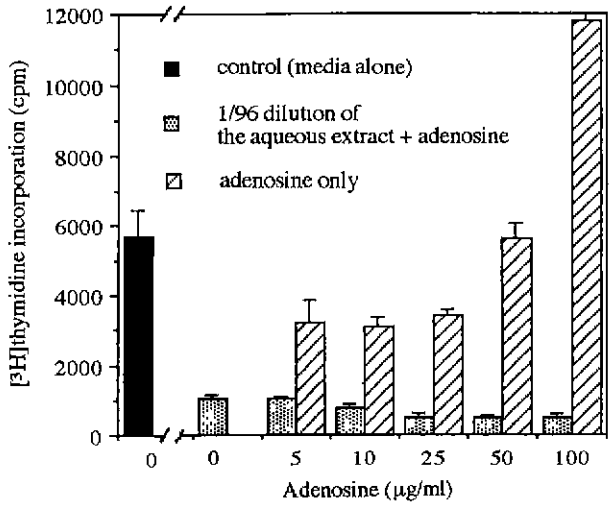

Fig. 3. Effect of adenosine on cytotoxicity of *P. japonica*.
To examine the effect of adenosine on the antitumor effect of the aqueous extract from *P. japonica*, the incorporation of [^{3}H]thymidine into DNA by Jurkat T cells treated with 1/96 dilution of the aqueous extract in the presence of serial dilutions of adenosine was determined by adding 5×10^4 cells to serial dilutions of the aqueous extract in a 96-well plate. After 2 days, 1 μCi/well of [^{3}H]thymidine was added for 4 h and its incorporation was assayed by liquid scintillation counter.

shown to be reversed by adenosine [5]. To determine whether the antitumor effect of the aqueous extract of *P. japonica* is mainly caused by cordycepin, the inhibitory effect of the aqueous extract on Jurkat cells was tested in the presence of various concentrations of adenosine. Although in the absence of the aqueous extract of *P. japonica* the proliferation of Jurkat T cells increased by

adenosine in a concenration-dependent manner upto 100 μg/ml, the inhibitory effect of the aqueous extract on Jurkat T cells was not reversed by adenosine. These results indicate that an antitumor activity of the aqueous extract from *P. japonica* is possibly due to other component(s) other than cordycepin (Fig. 3).

Fractionation of the Aqueous Extract by Organic Solvent Extraction

To purify the antitumor component from *P. japonica*, the aqueous extract was fractionated by an organic solvent extraction using n-hexane and ethyl acetate. When 200 ml of the aqueous extract was extracted with the same volume of n-hexane and sequentially with ethyl acetate (three times for each), 14.0 mg of the hexane fraction, 16.2 mg of the ethyl acetate fraction, and 638.8 mg of the remaining aqueous soluble fraction were recovered. The cytotoxicity of each fraction was examined by MTT assay using Jurkat T cells. The IC_{50} of the n-hexane fraction and aqueous fraction was 176 μg/ml and >200 μg/ml, respectively, whereas that of the ethyl acetate fraction was 1.5 μg/ml. Since cordycepin can be extracted by n-hexane from the aqueous extract of *C. militaris* [8, 11], these results confirm that an antitumor activity in the ethyl acetate fraction is not due to cordycepin. It is important to note that the Rf value of an authentic cordycepin appeared to be 0.19 on a silica gel TLC (developing solvent, n-hexane:ethyl acetate: methanol = 2:1:1), whereas the Rf values of the main components in the ethyl acetate extract appeared to be in a range from 0.46 to 0.73 under the same conditions (data not shown).

Cytotoxic Effect of the Ethyl Acetate Extract and Apoptosis Induction

The cytotoxic effect of the ethyl acetate extract from *P. japonica* on several tumor cells of human and murine origins was determined by MTT assay. The ethyl acetate extract is cytotoxic for various continuously proliferating tumor cells and the IC_{50} value appeared to be 1.5 μg/ml for Jurkat, 4.5 μg/ml for U937, 4.8 μg/ml for HL-60, 8.6 μg/ml for THP-1, 10.0 μg/ml for MDA361, 8.4 μg/ml for sarcoma 180, 2.0 μg/ml for BW5147.G.1.4, and 1.8 μg/ml EL-4 (Table 1). Most leukemia and lymphoma cells were found to be sensitive and cytotoxicity of *P. japonica* ranged at the concentrations from 1.5 to 10.0 μg/ml. However, human acute monocytic leukemia THP-1, human breast cancer MDA361, and murine sarcoma 180 were less sensitive to the ethyl acetate extract cytotoxicity. These results suggest that the cytotoxicity of *P. japonica* is more effective on leukemias and lymphomas than on solid tumors.

Table 1. Inhibitory concentration of the ethyl acetate extract from *P. japonica* against malignantly proliferating tumor cells.

Cell line	IC_{50} (μg/ml)
Jurkat	1.5
U937	4.5
HL-60	4.8
THP-1	8.6
BW5147.G.1.4	2.0
EL-4	1.8
Sarcoma 180	8.4

The IC_{50} indicates a concentration of the ethyl acetate extract which caused 50% decrease in the number of viable cells based on MTT assay. The cells were cultured in the presence of various concentrations of the ethyl acetate extract for 2 days

To determine the cellular mechanisms underlying the cytotoxicity, the effect of the ethyl acetate fraction on human acute Jurkat T-cell leukemia was investigated by focusing on the induction of apoptosis. When cells were treated with the ethyl acetate fraction at various concentrations of 0.3 to 5.0 μg/ml for 20 h, apoptotic DNA fragmentation was slightly detectable at the concentration of 1.3 μg/ml and reached a maximal level of between 2.5 and 5.0 μg/ml (Fig. 4A). This indicates that an apoptotic cell death of Jurkat by the ethyl acetate fraction of *P. japonica* is induced in a concentration-dependent manner. As shown in Fig. 4B, apoptosis of Jurkat cells was induced within 1 h in the presence of 2.5 μg/ml of the ethyl acetate extract and increased time-dependently. The maximal level of apoptosis was induced 24 h after treating the cells with the ethyl acetate fraction. In order to investigate the percentage of apoptotic change during the cell cycle, Jurkat cells were analyzed by flow cytometry after the ethyl acetate extract-treatment. As shown in Fig. 5, when cells were treated with either 2.5 or 5.0 μg/ml of the ethyl

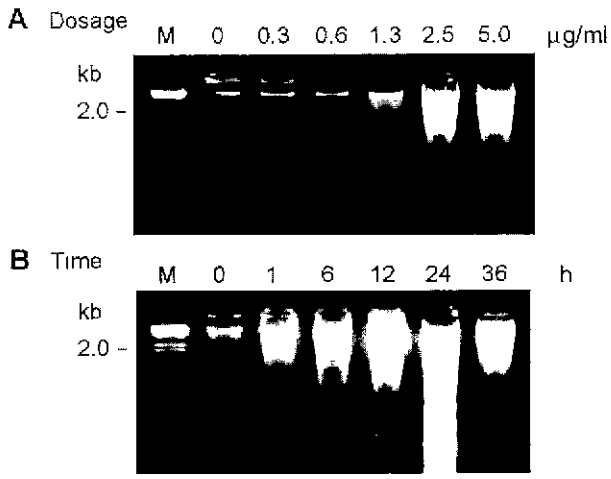

Fig. 4. Effects of the ethyl acetate fraction on apoptotic DNA fragmentation in Jurkat T cells.

Continuously proliferating Jurkat T cells (5×10^5) were collected after incubation for 20 h at the indicated concentration of the ethyl acetate extract from *P. japonica* (A), and collected at the indicated times while being incubated with 2.5 μg/ml of the ethyl acetate extract (B) to analyze apoptotic DNA fragmentation by the NP-40 lysis method using 2% agarose gel electrophoresis, as described in Materials and Methods.

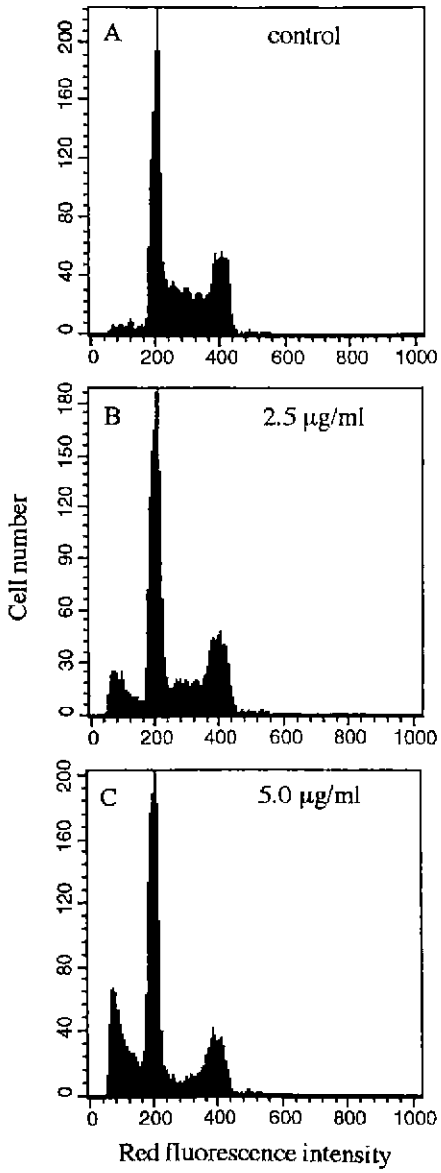

Fig. 5. Cell cycle analysis of Jurkat T cells by flow cytometry after treatment with the ethyl acetate extract from *P. japonica*.
An equal number of Jurkat T cells (10^4) were collected after incubation for 20 h either without (A) or with 2.5 μg/ml (B) and 5.0 μg/ml (C) of the ethyl acetate extract from *P japonica* The cells were analyzed by flow cytometry after staining of DNA with propidium iodide

acetate extract, it could easily be detected that the sub-G1 peak was undergoing apoptotic cell death. However, there was no detectable changes in the cell cycle distribution of treated cells under the same conditions.

Taken together, these results demonstrate that the cytotoxic effect of fungus *P. japonica* is attributable to the induced apoptosis, and suggest that the mycelium of *P. japonica* cultured on a silkworm pupa may contain a promising antitumor agent, other than cordycepin, that is more applicable to leukemia and lymphoma than to solid tumors.

Acknowledgments

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