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Activation of c-Jun N-terminal Kinase 1 (JNK1) in Mistletoe Lectin II-Induced Apoptosis of Human Myeloleukemic U937 Cells

Raekil Park, Myung-Sunny Kim,* Hong-Seob So,* Byung-Hak Jung,† Sun-Rock Moon,‡ Sang-Young Chung,§ Chang-Bo Ko,* Bok-Ryang Kim‖ and Hun-Taeg Chung*¶*

Department of *Microbiology, Professional Graduate School of Oriental Medicine and †Internal Medicine, ‡Radiation Oncology, ‖Biochemistry, Wonkwang University School of Medicine, Iksan Chonbuk 570-749, and Department of §Surgery, Chonnam National University Medical School, Kwangju 501-757, Korea

ABSTRACT. Extracts of mistletoe (*Viscum album var. coloratum*) have been used for several decades as an anticancer immunomodulating agent in clinical fields. However, the mechanism by which the plant extracts kill tumor cells has remained elusive. We investigated the direct effects of beta-galactoside- and *N*-acetyl-d-galactosamine-specific mistletoe lectin II in inducing apoptotic death of U937 cells. Three distinct components of mistletoe, including beta-galactoside- and *N*-acetyl-D-galactosamine-specific lectin II (60 kDa), polysaccharides, and viscotoxin (5 kDa), induced apoptotic cell death, characterized by DNA ladder pattern fragmentation of U937 cells at 12 hr after treatment. Consistent with apoptosis of the cells, mistletoe extracts markedly increased the phosphotransferase activity of c-Jun N-terminal kinase 1 (JNK1)/stress-activated protein kinase (SAPK) in U937 cells. Among the three components, lectin II was the most potent in inducing apoptosis as well as JNK1 activation of U937 cells in a dose- and time-dependent manner. Catalytic activation of JNK1 induced by mistletoe lectin II was inhibited by the addition of peptide aC-DEVD-CHO, but not by aC-YVAD-CHO. In addition, mistletoe lectin II induced apoptosis in a variety of cell types including Jurkat T cells, RAW 264.7 cells, HL-60 cells, DLD-1 cells, and primary acute myelocytic leukemic cells. BIOCHEM PHARMACOL **60**;11:1685–1691, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. mistletoe lectin II; apoptosis; c-Jun N-terminal kinase; acute myelocytic leukemic cell

The anticancer properties of mistletoe have been known since ancient times [1]. Extracts from mistletoe are widely used in the treatment of cancers, but the mechanism of antitumor properties has not yet been clearly elucidated [2]. Mistletoe extracts are reported to stimulate or modulate non-specific immune functions, as they increase the number and activity of natural killer (NK) cells and large granular leukocytes [3, 4] and induce cytokines such as tumor necrosis factor-α (TNF-α), interferon gamma (IFN-γ), interleukin-1 (IL-1), IL-6, IL-10, and granulocyte–monocyte colony-stimulating factor (GM-CSF) [5]. They also increase the repair of UV-damaged DNA lesions and inhibit protein synthesis [6]. Mistletoe extracts have been found to be cytotoxic to animal tumor cells such as Dalton's lymphoma and Ehrlich ascites cells *in vitro* and to inhibit the growth of cell lines including lung fibroblast, Chinese hamster ovary, and human nasopharyngeal carcinoma cells [7].

¶ Corresponding author: Dr. Hun-Taeg Chung, Dept. of Microbiology, Wonkwang University School of Medicine, 344-2 Shinyong-dong, Iksan Chnonbuk 570-749, Korea. Tel. +82-653-850.6762; FAX +82-653-851.5066; E-mail: rkpark@wonnms.wonkwang.ac.kr
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Among the more than 1000 proteins in mistletoe extracts, the best characterized component, lectin, appears to share some resemblance to other plant toxins, including abrin, ricin, and modiccin, which have been extensively applied in clinical fields as anticancer adjuvants [8]. Mistletoe lectins, which belong to a group of heterodimeric plant toxins, are ribosome-inactivating proteins composed of two polypeptide chains: an A-chain, which inhibits protein synthesis, and a B-chain, which mediates entry into the cell via binding with appropriate carbohydrate receptors [9]. After translocation of A-chain into cytosol, the A-chain irreversibly modifies ribosomes to prevent interactions with elongation factor-2, resulting in impairment of the elongation process of polypeptide chains [10, 11]. In addition, treatment by mistletoe lectin decreases the proliferation of leukemic Molt-4 cells up to 50% [12]. Recently, several lines of evidence have suggested that mistletoe extracts decrease the number of cultured lymphocytes and mitogen-induced lymphoblasts via induction of apoptosis [2, 13]. However, little is known about the mechanism of apoptosis induced by mistletoe lectin, even though there is convincing evidence that lectin-mediated

anticancer properties are due to an activation of apoptotic processes.

Apoptosis is a tightly regulated process of programmed cell death characterized by clumping of chromatin, nuclear disruption, and formation of apoptotic bodies in cytosol [14]. The family of cysteine proteases, including caspase-1 and caspase-3, is necessary for programmed cell death [15, 16]. Recent studies have implicated JNK1,* known as SAPK, as an obligate component of the apoptosis cascade in PC12 cells [17]. JNK1, a member of the MAPK superfamily, is activated by a variety of stimuli including growth factors, cytokines, tumor promotors, protein synthesis inhibitors. UV irradiation, and oncogenes [18]. JNK1 in turn stimulates the transcriptional transactivation potential of activator protein-1 (AP-1) via phosphorylated c-Jun to induce apoptosis [19, 20].

This study was designed to investigate whether mistletoe lectin II directly induces apoptosis in target cells. Herein, we demonstrate that galactoside and *N*-acetyl-D-galactosamine-specific lectin II of Korean mistletoe (*Viscum album var. coloratum*) induce the internucleosomal DNA fragmentation of human monocytic U937 cells via the activation of JNK1. Mistletoe lectin II significantly stimulates the phosphotransferase activity of JNK1 in a dose- and time-dependent manner, and this activity is partially inhibited by caspase-3, 7 inhibitor, but not by caspase-1, 4 inhibitor. In addition, mistletoe lectin II induces apoptosis in a variety of cell types. The results suggest that the tumoricidal effects of mistletoe lectin II are mediated by activation of the apoptotic signaling cascade, caspase, and JNK that ultimately leads to DNA fragmentation of the target cells. Furthermore, there may be a cross-talk between caspase pathways and JNK1 in apoptotic processes induced by mistletoe lectin II.

MATERIALS AND METHODS

Extraction and Purification of Mistletoe Lectin II

Mistletoe lectin II was isolated from Korean mistletoe (*Viscum album var. coloratum*) by the methods detailed in Olsnes *et al.* [8]. Specifically, green parts of the plant were chopped into slices, transferred to a mortar, and frozen in liquid nitrogen. The frozen material was transferred to a Warring blender in a well-ventilated hood and ground into a fine power. Approximately 10 volumes of 10 mM Tris–HCl buffer (pH 8.3) containing 100 mM lactose were added and the suspension was stirred at 4° overnight. The suspension was filtered through cheesecloth and centrifuged at 10,000 × *g* for 20 min. After three extractions with ether, the supernatant was applied to a diethylaminoethyl (DEAE)-cellulose column equilibrated with 10 mM Tris–HCl buffer (pH 8.3). After washing with the same buffer, the bound toxin was eluted with 0.2 M NaCl. The eluted material was precipitated with $(NH_4)_2SO_4$ until 70% saturation and stirred at 4° for 12 hr. The precipitate was collected by centrifugation at 10,000 × *g* for 20 min and dissolved with 10 mM phosphate buffer (pH 6.5). The precipitate was applied to a carboxymethyl (CM)-Sepharose column (1.5 × 20 cm) equilibrated with 10 mM phosphate buffer (pH 6.5). After washing with the same buffer, a bound material was fractionated by sequential elution of 0.1 M NaCl into three components including 60,000 kDa peptide, 5000 kDa peptide, and non-peptide fraction of polysaccharide. Galactoside- and *N*-acetyl-d-galactosamine-specific lectin II (60,000 kDa) was further purified with a conconavalin A column (1.5 × 20 cm) equilibrated with 10 mM PBS (pH 7.4). The molecular weight of lectin II was about 60,000 by SDS–PAGE and SDS–capillary electrophoresis (CE) (data not shown). The polysaccharide fraction was purified with carboxymethyl (CM)-Sepharose and Sephadex G-100 columns, and its neutral sugar was confirmed by the phenol–sulfuric acid precipitation reaction. Viscotoxin was further purified with size-exclusion HPLC and its molecular weight was determined to be about 5000 by size-exclusion HPLC and SDS–PAGE [21].

Treatment of U937 Cells with Mistletoe Extracts

U937 cells were maintained in RPMI-1640 with 10% fetal bovine serum in a humidified chamber with 5% CO_2. The cells were incubated with mistletoe extracts including whole plant extract, galactoside- and *N*-acetyl-d-galactosamine-specific lectin II, polysaccharides, and viscotoxin for 12 hr.

DNA Extraction and Electrophoresis

DNA fragmentation was analyzed by 1.5% agarose gel electrophoresis. Genomic DNA was isolated with the Wizard Genomic DNA purification kit (Promega). After ethanol precipitation, 50 μg of DNA in each experimental group was subjected to electrophoresis on 1.5% of agarose at 50 V for 3 hr. DNA was visualized by staining with ethidium bromide.

Immunoprecipitation and In Vitro *Kinase Assay of JNK1*

To measure JNK1 activity, U937 cells treated with or without mistletoe extracts were lysed at 4° with 1 mL of ice-cold lysis buffer containing 1% Triton X-100, 10 mM Tris–HCl, pH 7.6, 50 mM NaCl, 0.1% BSA, 1% aprotinin, 5 mM EDTA, 50 mM NaF, 0.1% 2-mercaptoethanol, 5 μM phenylarsine oxide, and 100 μM sodium vanadate. U937 cell lysates were immunoprecipitated with 1 μg of anti-JNK1 antibody (Santa Cruz Co.) on ice for 1 hr. Immune complex was precipitated with 10% formalin-fixed *Staphylococcus aureus* on ice for 1 hr, and then washed twice with PAN buffer containing 10 mM piperazine-*N*-*N*'-bis[2-

* *Abbreviations:* aC-DEVD-CHO, acetyl-Ase-Glu-Val-Asp-aldehyde; aC-YVAD-CHO, Z-Tyr-Val-Ala-Asp-aldehyde; JNK1, c-Jun N-terminal kinase 1); MAPk, mitogen-activated protein kinase; SAPK, stress-activated protein kinase; PI, propidium iodide; and GST, glutathione *S*-transferase.

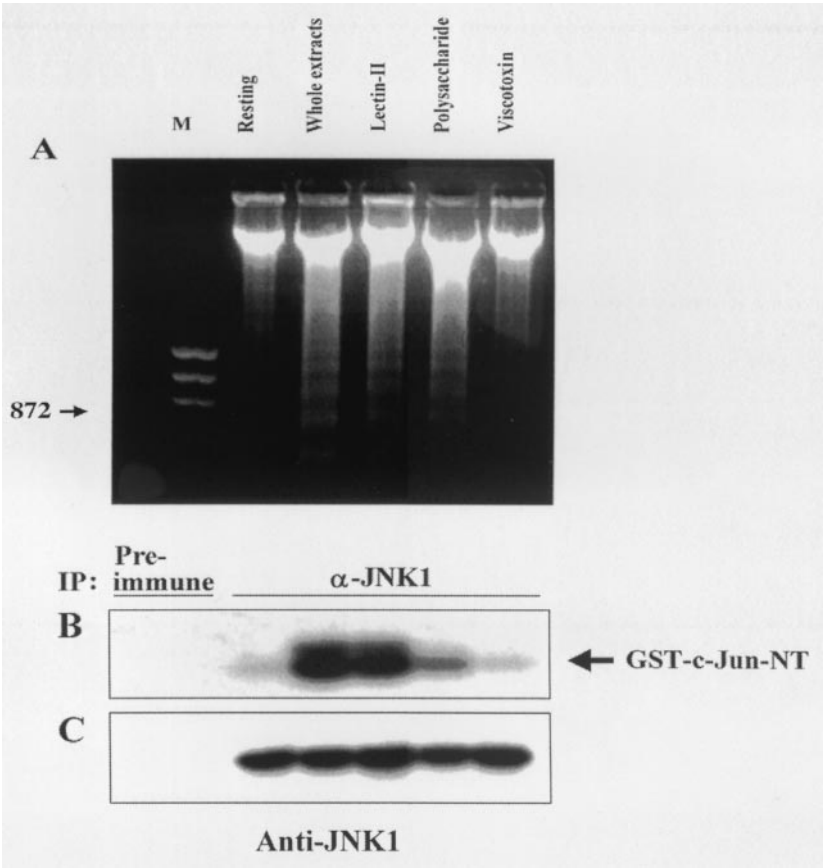

FIG. 1. Mistletoe extract induced the DNA fragmentation and JNK activation of U937 cells. (A) Cells (2×10^7 cells) were treated with three different components of mistletoe extract including whole plant extract (100 ng/mL), galacoside- and N-acetyl-D-galactosamine-specific lectin II (100 ng/mL, 60,000 kDa), polysaccharide (1 μg/mL), and viscotoxin (100 ng/mL, 5000 kDa) for 12 hr at 37° in a humidified incubator with 5% CO_2. DNA (50 μg/each lane) from U937 cells was extracted by the phenol/chloroform method and separated on 1.5% agarose gel. (B) JNK activity was measured by the immune complex kinase assay using GST-c-Jun-NT as detailed in Materials and Methods. (C) Immunoprecipitation of JNK was confirmed by immunoblot for JNK1.

ethanesulfonic acid] (PIPES) pH 7.0, 1% aprotinin, and 100 mM NaCl. JNK1 activity was detected by phosphorylation of GST-c-Jun N-terminus$_{1-79}$ fusion proteins in an *in vitro* kinase assay and was assessed by incorporation of [γ-^{32}P]ATP (1 μCi/sample) with 1 μg of GST-c-Jun N-terminus protein in kinase reaction buffer containing 20 mM Tris, pH 7.5, 20 mM $MgCl_2$, 2 mM dithiothreitol (DTT), and 20 μM cold ATP at 30° for 15 min [20]. A phosphorylation reaction was terminated by the equal amount of 2X Laemmli sample buffer and boiled for 5 min. The proteins were separated on 10% SDS–PAGE and transferred onto nitrocellulose membrane. The membranes were probed for JNK1. ^{32}P-labeled GST-c-Jun N-terminus was detected by autoradiography and quantified by Phosphorimager analyzer (Fuji Co.).

Western Blotting

After autoradiographic exposure to x-ray film, the nitrocellulose membrane was incubated with blocking buffer (5% skim milk in TBS-T containing 25 mM Tris–HCl, pH 7.4, 0.14 N NaCl, and 0.05% Tween 20) for 1 hr, and the membrane was incubated with rabbit polyclonal anti-JNK1 (Santa Cruz Co.) for 2 hr at room temperature. Horse radish peroxidase-labeled goat anti-rabbit immunoglobulin G antibody (1:5000 diluted) was added to the membrane, which was further incubated for 1 hr. The immunoreactive bands were visualized with enhanced chemiluminescence reagents (ECL, Amersham Life Science).

Flow Cytometric Analysis of Apoptosis

Flow cytometric analysis of apoptotic cell death was performed as described [22]. U937 cells (10^7 cells/group) treated with mistletoe extracts for 12 hr were harvested, washed with Hanks' balanced salt solution (HBSS), and then stained with 50 μg/mL of propidium iodide in 0.1% Nonidet P40. After washing unbound dye out, the cells were subjected to flow cytometric analysis to estimate DNA fragmentation. Data were collected and analyzed by the Consort 3.0 program (Becton-Dickinson).

Statistical Analysis

All values are represented as means ± standard deviation (SD) of triplicates. Results with $P < 0.05$ were considered statistically significant.

RESULTS

Effects of Mistletoe Extracts on DNA Fragmentation and JNK1 Activation of U937 Cells

To investigate whether mistletoe extracts directly induce apoptotic cell death of U937 cells, DNA from cells treated with mistletoe extracts including lectin II, viscotoxin, or polysaccharide was isolated and separated on 1.5% agarose gel (Fig. 1A). Apoptosis of the cells was known to be identified by the appearance of DNA fragmentation equiv-

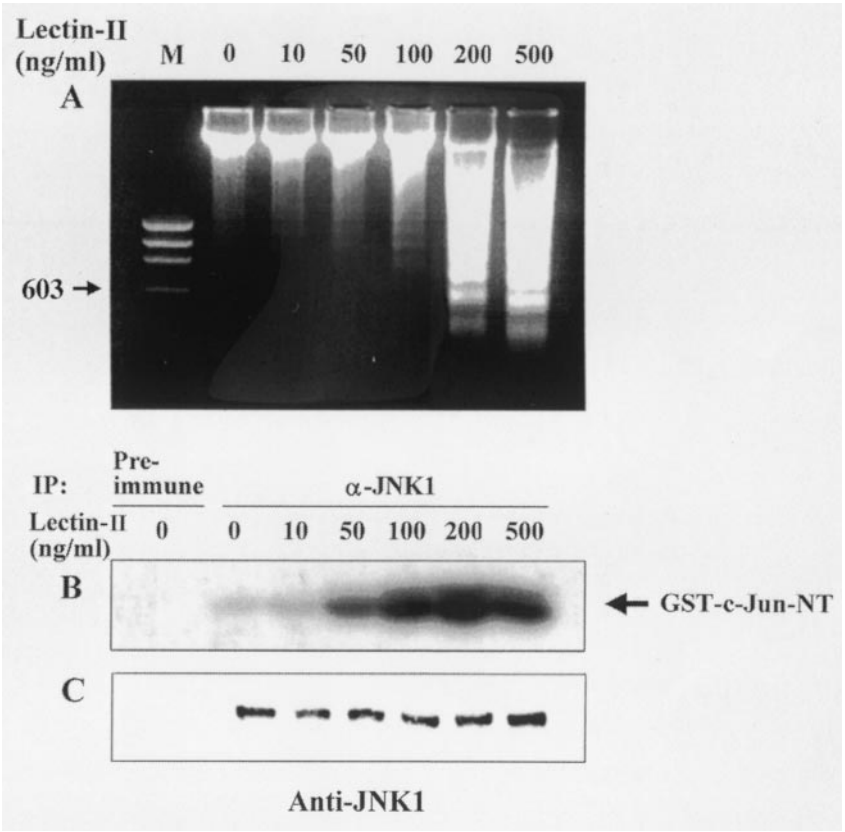

FIG. 2. Lectin II of mistletoe induced the apoptosis and JNK activation of U937 cells in a dose-dependent manner. The cells were treated with various amounts of lectin II for 12 hr. (A) DNA of the cells was extracted and separated on 1.5% agarose gel. (B) U937 lysates were immunoprecipitated with anti-JNK antibodies, and the immune complex was then reacted with GST-c-Jun-NT and [γ-^{32}P]ATP at 37° for 30 min. The phosphotransferase activity was visualized by autoradiogram. (C) Immunoprecipitation of JNK was confirmed by immunoblot for JNK1.

alent to approximately 200 base pairs and multiples thereof [8]. Whole extract (100 ng/mL) of mistletoe induced a prominent DNA ladder characteristic of apoptosis in U937 cells after 12 hr, whereas genomic DNA of resting cells was not cleaved. Galactoside- and *N*-acetyl-D-galactosamine-specific mistletoe lectin II also induced the DNA fragmentation of the cells at 100 ng/mL after 12 hr. U937 cell treated with polysaccharide fraction (1 μg/mL) of mistletoe showed a typical DNA ladder pattern of apoptosis, but the degree of DNA cleavage was less extensive than with whole plant toxin or lectin II. U937 cells treated with viscotoxin (100 ng/mL) showed a faint DNA ladder.

We next examined whether apoptosis of U937 cells induced by mistletoe extracts was mediated by activation of JNK1. JNK1 activity was determined by an *in vitro* kinase assay using GST-c-Jun N-terminal$_{1-79}$ protein as a substrate in anti-JNK1 immunoprecipitates of U937 cells. Both whole plant extract and lectin II significantly increased the phosphorylation of the JNK1 substrate, GST-c-Jun N-terminal protein, which represented the activation of JNK1 (Fig. 1B). The polysaccharide fraction of mistletoe also induced JNK1 activation. However, viscotoxin could not induce the activation of JNK1 in U937 cells. Anti-JNK1 immunoblot confirmed that equivalent amounts of JNK1 were immunoprecipitated (Fig. 1C). These data suggested that mistletoe extracts, including lectin II and polysaccharide, might induce the apoptosis of U937 cells via activation of JNK1.

DNA Fragmentation and JNK1 Activation of U937 Cells by Galactoside-Specific Lectin II in a Dose- and Time-Dependent Manner

We next examined the time- and dose-dependent patterns of DNA fragmentation and JNK1 activation in U937 cells with mistletoe lectin II because lectin II was the most potent component to induce apoptosis and JNK1 activation of U937 cells among the mistletoe extracts. U937 cells (10^7 cells/group) were treated with various concentrations of lectin II for 12 hr, after which the DNA fragmentation and JNK1 activity were evaluated (Fig. 2). A DNA ladder of U937 cells was observed at 50 ng/mL and increased dramatically above 100 ng/mL of mistletoe lectin II. However, it was not observed under resting conditions of U937 cells (Fig. 2A). Consistent with DNA fragmentation of the cells, mistletoe lectin II significantly increased JNK1 activity at 50 ng/mL, this activity reaching a maximum level at 100 to 200 ng/mL of lectin II (Fig. 3B). A concentration of lectin II below 10 ng/mL could not induce the DNA cleavage or JNK1 activation of U937 cells.

DNA fragmentation of U937 cells treated with 100 ng of lectin II appeared as early as 6 hr after treatment and showed a definitive ladder pattern after 12 hr (Fig. 3A). Treatment with mistletoe lectin II resulted in JNK1 activation after 6 hr, which reached a peak after 12 hr in a time-dependent manner (Fig. 3B). The intensity of phosphotransferase activity of JNK1 in U937 cells treated with lectin II for 12 hr was about 10-fold greater than in the

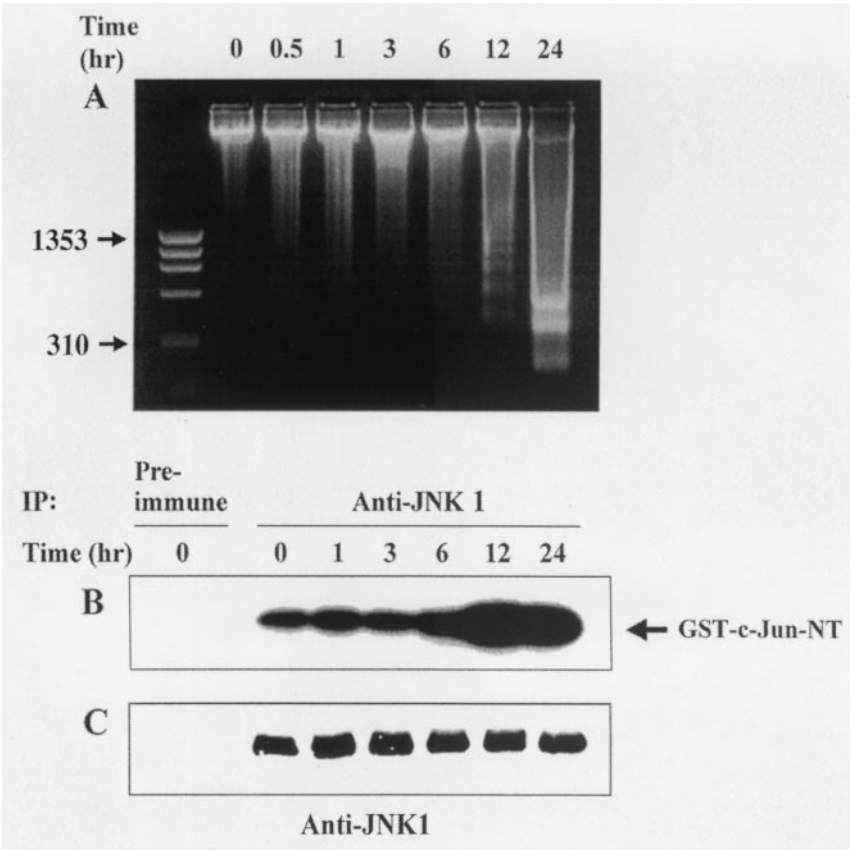

FIG. 3. Time-dependent DNA fragmentation and JNK activation of mistletoe lectin II on U937 cells. (A) The cells were treated with mistletoe lectin II (100 ng/mL) for various periods and their DNA extracts separated on 1.5% agarose gel. (B) U937 lysates were immunoprecipitated with anti-JNK antibodies, and the immune complex was then reacted with GST-c-Jun-NT and [γ-^{32}P]ATP at 37° for 30 min. The activity of JNK was visualized by autoradiogram. (C) Immunoprecipitation of JNK was confirmed by immunoblot for JNK.

control group. To verify the efficiency of immunoprecipitation, the membranes were immunoblotted for JNK1. The results demonstrated that the same amounts of JNK1 were precipitated in each experimental group with the exceptions of pre-immune immunoprecipitates (Figs. 2C and 3C).

Flow Cytometric Analysis of Mistletoe Lectin II-Induced Apoptosis in Various Types of Cells

To verify the apoptotic property of mistletoe lectin II in various types of cells including U937, HL-60, RAW 264.7, Jurkat, DLD-1, and acute myelocytic leukemic (AML) cells, flow cytometric analysis of DNA was performed to estimate the percentage of apoptotic cells after 12 hr (Table 1). When U937, HL-60, RAW 264.7, and Jurkat cells had not been treated with lectin II, the percentage of apoptotic cells was less than 5–7%. However, it was increased more than ten times up to 52–65% in cells treated with 100 ng/mL of mistletoe lectin II. DLD-1 cells were more resistant to lectin II and remained as normal up to 75% of diploid DNAs. AML cells were apoptotic up to 10% in the resting state and were susceptible to be killed by adding lectin II (65%, 6-fold increase). Interestingly, normal peripheral neutrophils and lymphocytes were relatively resistant to lectin II, and there was no significant difference in apoptotic cell death upon lectin II treatment.

Partial Dependence of JNK1 Activation on Caspase-3 during Mistletoe Lectin II Treatment

Since the caspase cascade has been reported to be a critical component of apoptosis, we tested the role of DEVD motif-specific caspases including caspase-3 and caspase-7 in the process of JNK1 activation by mistletoe lectin II. U937 cells were treated with lectin II (100 ng/mL) combined with either aC-DEVD-CHO (400 μM), a caspase-3, 7 inhibitor, or aC-YVAD-CHO (400 μM), a caspase-1, 4 inhibitor, for 12 hr. The cell lysates were immunoprecipitated with anti-JNK1 antibodies and the immune complex

TABLE 1. Hypodiploid DNA contents of various cell types treated with mistletoe lectin II

	Apoptosis (%)	
Cell types	Medium	Lectin II
U937 cells	3 ± 1.4	65 ± 3.7
HL-60 cells	5 ± 2.3	60 ± 2.6
RAW 264.7 cells	4 ± 1.8	52 ± 3.9
Jurkat cells	3 ± 1.3	55 ± 2.5
DLD-1 cells	4 ± 2.3	25 ± 2.6
AML cells	10 ± 2.8	65 ± 5.1
Normal PMNL	3 ± 1.6	3 ± 1.2

Cells were treated with mistletoe lectin II (100 ng/mL) for 12 hr. AML; acute myelocytic leukemic cells. PMNL; polymorphonuclear leukocytes. Data represented as means ± SD of quadraplicates.

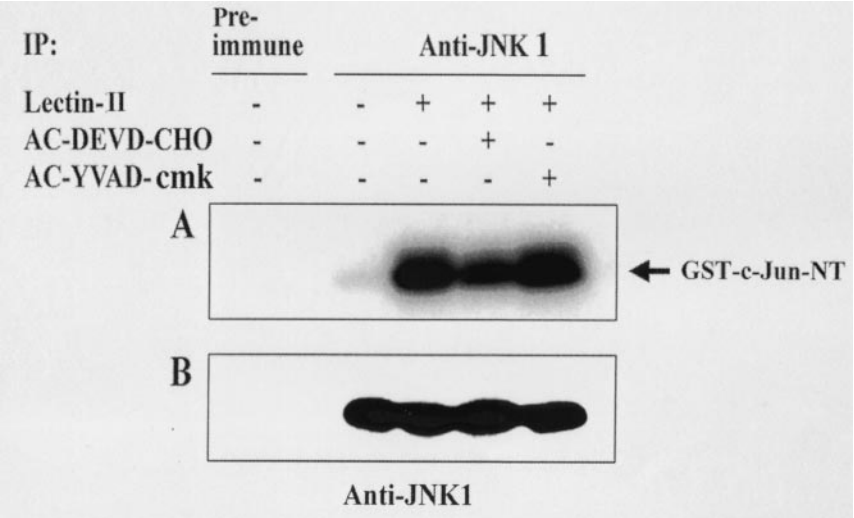

FIG. 4. DEVD motif-specific caspases were connected the JNK signaling pathway in the process of apoptosis. To mistletoe lectin II (100 ng/mL)-treated U937 cells were added either DEVD (400 μM) or YVAD (400 μM) peptides for 12 hr. (A) U937 cell lysate was immunoprecipitated with anti-JNK antibodies, and the phosphotransferase activity of the JNK immune complex was then measured as described previously. (B) Immunoprecipitation of JNK was confirmed by immunoblot for JNK.

in vitro kinase assay for JNK was then performed (Fig. 4A). Mistletoe lectin II-induced JNK activation was significantly inhibited by adding aC-DEVD-CHO, but not by the addition of aC-YVAD-CHO. Immunoprecipitation was confirmed by immunoblot for JNK1 (Fig. 4B). The results suggested that JNK1 activation was connected with DEVD motif-specific caspases including caspase-3 and caspase-7 in an apoptotic process mediated by mistletoe lectin II.

DISCUSSION

We have demonstrated that galactoside- and *N*-acetyl-d-galactosamine-specific mistletoe lectin II induces apoptosis in U937 cells and that this is mediated by activation of JNK1/stress-activated protein kinase. The mechanism of direct destructive effects by mistletoe extracts on cancer cells has been poorly understood. Previously, it was suggested that interaction of the mistletoe lectin B-chain with appropriate receptors on cancer cells could activate distinct signaling pathways that lead either to cytotoxicity or apoptosis of the cell. One possibility for direct cytotoxicity is that mistletoe extracts increase the number and activity of natural killer cells that mediate direct cell death without prior sensitization or major histocompatibility complex (MHC) restriction [3]. Another is apoptotic death of cancer cells. The evidence of mistletoe-induced apoptosis was that mistletoe extracts increased the hypodiploid DNA contents of the target cells and induced DNA fragmentation of the target cancer cells [2].

Recently, Bussing *et al.* reported that whole plant extracts from mistletoe decreased the number of cultured lymphocytes and the blastogenesis of T cells via induction of apoptosis [13]. They also suggested that cytotoxic effects of mistletoe extracts were mediated by lectin II, with apparently no contribution of other components, such as viscotoxin and polysaccharide, to the cell killing [2, 13]. However, contrary with other reports, the polysaccharide fraction of Korean mistletoe (*mistletoe Viscum var. coloratum*) also induced DNA fragmentation and JNK1 activation of the target cells [2]. This result might be due to differences in the effectiveness of the drug extracts. It is well known that the composition and effectiveness of mistletoe extracts are highly dependent on the host tree and time of harvest [23]. Apoptosis induced by mistletoe lectin II was not only specific for the U937 cell type, but also for other cell types including Jurkat T, RAW 264.7, HL-60, DLD-1, and primary acute myelocytic leukemic cells (Table 1). DLD-1 cells were less sensitive than the other cell types to mistletoe lectin II. Interestingly, mistletoe lectin II could not effectively induce the apoptosis of normal neutrophils and lymphocytes. These data indicate that lectin II or polysaccharide may mediate cytotoxicity in cancer cells. In other words, transformed cancer cells were more sensitive to apoptotic death mediated by mistletoe lectin II than normal cells.

One major pathway in programmed cell death requires the activation of the caspase cascade, including the cysteine protease of the interleukin-1β-converting enzyme (ICE), caspase-1, and cysteine protease protein (CPP) 32 family proteins, caspase-3 [24, 25]. JNK is also known to be an obligatory element of the apoptotic response [18, 19]. The possibility of the communication between these two pathways has been suggested [9]. Frisch *et al.* reported that caspases must be activated for JNK activation in anoikis. Similary, stimulation of the Fas receptor induced JNK activation in a caspase-dependent manner [26]. These results suggest that the JNK pathway may be connected to proteolytically active components in the caspase cascade. Our results demonstrated that inhibitory peptide of caspase-3 and caspase-7 suppressed the JNK activity mediated by mistletoe lectin II (Fig. 4). Consistent with our result, DEVD motif-specific caspases were recently reported to cleave and activate MAPK/extracellular signal-regulated kinase (ERK) kinase kinase 1 (MEKK1), the upstream effector of JNK, in anoikis [27]. These results indicate that the proteolytic activity of caspase-3, 7 may also be connected with the JNK pathway of apoptosis.

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