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MISTLETOE (*Viscum album* var. *coloratum*) Growing on *Carpinus laxiflora* BL. Induces the Differentiation of Human Acute Promyeocytic Leukemia (HL-60) Cells

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ABSTRACT. The present study was undertaken to investigate the effects of mistletoe (*Viscum album* var. *coloratum*) growing on *Carpinus laxiflora* BL. on proliferation and differentiation of HL-60 acute promyelocytic leukemia cells. Aqueous extract and its $(NH_4)_2SO_4$ saturated fractions of the mistletoe exhibited potent anti-proliferation activity against HL-60 cells. Moreover, when HL-60 cells were treated with 0~30% and 30~70% $(NH_4)_2SO_4$ saturated fractions of the mistletoe, HL-60 expressed CD 66b or CD 14 cell surface antigens and showed activity to reduce nitroblue tetrazolium, indicating that mistletoe induces the differentiation of HL-60 into granulocytes or monocytes. To understand how mistletoe induces the differentiation, we investigated the expression of molecules for modulating the proliferation and differentiation of leukemia cells, such as c-Myc and myeloblastin. The 0~30% $(NH_4)_2SO_4$ saturated fraction of the mistletoe reduced the mRNA levels of c-Myc and myeloblastin in a time-dependent manner. The results indicate that the mistletoe induces the differentiation of HL-60 cells via the decrease of c-Myc and myeloblastin expressions. Thus, it is suggested that mistletoe has a therapeutic potential for the treatment of acute promyelocytic leukemia.

Keywords: HL-60 cells, Differentiation, Mistletoe, c-Myc, Myeloblastin.

INTRODUCTION

Mistletoe *(Viscum album),* a semiparasite of plant, has been used in adjuvant cancer therapy for decades, mainly in Europe (Jung *et al.*, 1990). Recent several studies have shown that mistletoe induces apoptosis of cultured tumor cells and lymphocytes, and stimulates the immune system (Bussing *et al.*, 1996; Hajto *et al.*, 1989, 1990). A number of studies on the components and anticancer activity of mistletoe have been made of European mistletoe (*V. album* L.). Lectins (Debray *et al.*, 1992; Franz *et al.*, 1981; Franz, 1986; Holtskog *et al.*, 1988; Lee *et al.*, 1992; Luther *et al.*, 1980; Olsnes *et al.*, 1982), alkaloids (Khwaja *et al.*, 1980, 1986, 1990; Khwaja and Manjikian, 1990) and viscotoxins (Schader and Apel, 1991) are the bioactive components in mistletoe. The most active compounds in cytotoxicity (Jung *et al.*, 1990; Wu *et al.*, 1995) and immunomodulatory effects (Gabius *et al.*, 1992; Hajto *et al.*, 1989) are considered to be linked to lectins. The European mistletoe lectins (*Viscum album* L. agglutinin, VAAs), composed of A- and B-chains which are D-galactose and/or N-acetyl-D-galactosamine-specific, have molecular weights between 55 and 63 kDa (Franz, 1986). The yellow berry Korean mistletoe (*Viscum album* L. *coloratum*), a subspecies of European mistletoe, also possesses anticancer activity (Kim *et al.*, 2000; Lyu *et al.*, 2001; Park *et al.*, 2001; Yoon *et al.*, 1999). A galactoside and N-acetyl-D-glucosamine-specific lectin (*Viscum album* L. *coloratum* agglutinin, VCA) with MW 60 kDa was isolated from Korean mistletoe (Park *et al.*, 1998; Lyu *et al.*, 2000).

On the other hand, the inhibitory effect of mistletoe on the growth of tumor cells indicates the possibility that

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mistletoe could induce the differentiation and/or apoptosis of tumor cells. Several reports have shown recently that leukemia cells are able to differentiate in response to various treatments. Moreover, these reports have suggested that terminally differentiated hematopoietic cells have a short life span and undergo apoptosis (Whyte *et al.*, 1993; Iwai *et al.*, 1994). Thus, it is of importance to examine whether mistletoe induces differentiation in addition to apoptosis.

In present study, we used the HL-60 cell line to examine the effect of Korean mistletoe (*Viscum album* var. *coloratum*) growing on *Carpinus laxiflora* BL on the induction of leukemia cell differentiation, because it has been reported that this promyelocytic leukemia cell line can be induced to terminally differentiate to morphologically mature granulocytes (Breitman *et al.*, 1980; Collins *et al.*, 1978).

MATERIALS AND METHODS

Preparation of extract from korean mistletoe and ammonium sulfate precipitation

Viscum album var. *coloratum* growing on *Carpinus laxiflora* BL was collected in winter in Jeju Island, South Korea and stored at 4°C until use. The leaves and twigs of mistletoes were cleaned and ground into a fine powder. The mistletoe powders (140 g) were extracted with 1 l of 25 mM 3-[morpholino]-propane-sulfonic acid (MOPS) buffer (pH 7.5) at 4°C. The extract was brought to 30% ammonium sulfate ($(NH_4)_2SO_4$) saturation by the addition of solid $(NH_4)_2SO_4$. After stirring at 4°C, precipitated proteins were collected by centrifugation (15,000 ×g, 30 min). The supernatant was brought to 70% saturation with solid $(NH_4)_2SO_4$. After an additional 10 min, the precipitated proteins were collected by centrifugation. The precipitated proteins were in 150 ml of 25 mM MOPS buffer and ultrafiltrated against 1 l of 25 mM MOPS buffer with YM 10 membrane to remove most of $(NH_4)_2SO_4$. Protein concentrations of the aqueous extract and its $(NH_4)_2SO_4$ saturated fractions were determined using Bradford method (Bradford, 1976), the aqueous extract and its $(NH_4)_2SO_4$ saturated fractions were stored at -70°C until use.

Cell culture

The human promyelocytic leukemia cell line, HL-60 was obtained from the Korean Cell Line Bank (KCLB). The cells were cultured at 37°C in a humidified atmosphere with 5% CO_2 in RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively). Exponentially growing cells were used throughout.

Effects of the aqueous extract and its $(NH_4)_2SO_4$ saturated fractions on the growth of HL-60 cells

Effect of the aqueous extract and its $(NH_4)_2SO_4$ saturated fractions of the mistletoe on the growth of HL-60 cells was determined by measurement of metabolic activity using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (Carmi chael *et al.*, 1987). HL-60 cells (3×10^5/ml) were treated for 4 days with the aqueous extract or its $(NH_4)_2SO_4$ saturated fractions of the mistletoe in 96-microwell plates. After incubation, 0.1 mg (50 μl of a 2 mg/ml solution) of MTT (Sigma-Aldrich) was added to each well and cells were then incubated at 37°C for 4 hours. Plates were centrifuged at 1000 rpm for 5 min at room temperature and the media was then carefully aspirated. 150 μl of dimethylsulfoxide was then added to each well to solubilize the formazan crystals. The plates were read immediately at 540 nm on a microplate reader (Amersham Pharmacia Biotech, UK). All experiments were performed three times and the mean absorbance values were calculated. Results are expressed as the percentage of inhibition that produced reduction of absorbance in the mistletoe-treated cells as compare with untreated controls.

Assays for cellular differentiation

HL-60 cells (3×10^5/ml) were treated with the $(NH_4)_2SO_4$ saturated fractions of the mistletoe at the protein concentrations varying from 1 to 10 μg/ml. After incubation for 4 days, cells were harvested and examined for signs of differentiation by analyzing cell surface antigen expression using fluorescence-activated cell sorter (FACS) and by nitroblue tetrazolium (NBT) reduction assay. To analyze cell surface antigens, cells were stained by direct immunofluorescent staining with fluorescein-isothiocyanate (FITC) conjugated mouse antihuman CD 66b or CD 14, and R-phycoerythrin (PE) conjugated mouse antihuman CD 33. Control studies were performed using non-binding mouse Ig G_1 or Ig M isotype antibodies. The antibodies were purchased from Pharmingen. Fluorescence was measured on a COULTER®EPICS ®XLTM Flow Cytometry (Beckman Coulter). For NBT reduction test, the treated cells (2.5×10^5/ml) were placed in 1 ml of RPMI-1640 media and mixed with 1 ml of NBT (1 mg/ml). After the addition of phorbol myristate acetate (PMA; 0.25 μg/ml), the cells were incubated at 37°C for 30 min. After the cells were harvested and resolved in 200 μl of DMSO, their absorbance at 580 nm was determined. The results are expressed as the mean percentage that produced increase of absorbance in the mistletoe-treated cells as compared with untreated controls.

RNA isolation and RT-PCR analysis

The HL-60 cells (3×10^5/ml) were treated with the $(NH_4)_2SO_4$ saturated fractions of the mistletoe and then harvested at 2, 4 and 6 days. The cells were washed with PBS at 4°C and homogenized. Total cellular RNA was extracted by Tri-reagent (MRC) according to the manufacturer's instructions. The RNA was quantified by reading the absorbance at 260 nm according to the methods described by Sambrook *et al.* (1989). The reverse transcription of 1 μg RNA was carried out with M-MLV reverse transcriptase (MBI). The generated cDNA was amplified by using primers. The primers for c-Myc (Watt *et al.*, 1983) were TCT GGA TCA CCT TCT GCT GG (S) and GCT CCT CTG CTT GGA CGG AC (A). For myeloblastin (Bories *et al.*, 1989), the primers were TTC TGC GGA GGC ACC TTG AT (S) and ATT GAG CTC CTG CAG GAC CT (A). For GAPDH (Weisinger *et al.*, 1999), the primers were CTT TGG TAT CGT GGA AGG ACT C (S) and GTC TAC ATG GCA ACT CTG AGG A (A). The (S) and (A) denote the forward and reverse primer. The polymerase chain reaction (PCR) was performed with a particular primer pair for 4 min at 94°C, followed by 35 cycles for 30 sec at 94°C (denaturing), for 30 sec at 55~60°C (annealing) and for 30 sec at 72°C (primer extension). The PCR products were detected on 1.2% agarose gel electrophoresis.

RESULTS

Effects of the aqueous extract and its $(NH_4)_2SO_4$ saturated fractions of the mistletoe on the growth of HL-60 cells

The effects of the aqueous extract and its $(NH_4)_2SO_4$ saturated fractions of the mistletoe on the growth of HL-60 cells were assessed by MTT assay. Briefly, HL-60 cells were cultured for 4 days in a medium containing the aqueous extract (100 μg/ml), 0~30% $(NH_4)_2SO_4$ saturated fraction (0.5, 1, 3 and 5 μg/ml) or 30~70% $(NH_4)_2SO_4$ saturated fraction (1, 3, 10 and 20 μg/ml). The MTT assay showed that the aqueous extract and its $(NH_4)_2SO_4$ saturated fractions markedly inhibited the proliferation of HL-60 promyelocytic leukemia cells (Table 1). Moreover, the effect of 0~30% $(NH_4)_2SO_4$ saturated fraction on the growth of HL-60 was significantly greater than 30~70% $(NH_4)_2SO_4$ saturated fraction.

Differentiation induction of HL-60 cells by the mistletoe

HL-60 cell differentiation by the $(NH_4)_2SO_4$ saturated fractions of the mistletoe was assessed by observing the expression of cell surface antigens. When HL-60 cells were exposed to the $(NH_4)_2SO_4$ saturated fractions of the mistletoe for 4 days, the HL-60 cells expressed CD 66b or CD 14 antigens (Fig. 1). At low concentrations (<5 μg/ml) of 30~70% $(NH_4)_2SO_4$ saturated fraction, the ability of HL-60 cells to express CD 66b or CD 14 antigens was unchanged compared to the control. However, the exposure of HL-60 cells to 10 μg/ml of 30~70% $(NH_4)_2SO_4$ or 3 μg/ml of 0~30% $(NH_4)_2SO_4$ saturated fraction markedly enhanced the expression of CD 66b or CD 14 antigens (Table 2). These results

Table 1. Inhibitory effects of the aqueous extract and the $(NH_4)_2SO_4$ saturated fractions of mistletoe on the growth of HL-60 cells

The fraction of mistletoe	Concentration (μg/ml)	Inhibition (%)
Aqueous extract	100	83.2 ± 1.14*
0~30% $(NH_4)_2SO_4$ saturated fraction	0.5	35.8 ± 3.24*
	1	67.9 ± 2.22*
	3	92.0 ± 0.47*
	5	92.7 ± 0.70*
30~70% $(NH_4)_2SO_4$ saturated fraction	1	21.6 ± 4.98*
	3	38.7 ± 3.75*
	10	78.2 ± 3.75*
	20	87.7 ± 1.94*

HL-60 cells (3×10^5/ml) were treated with aqueous extract or its $(NH_4)_2SO_4$ saturated fraction of mistletoe (*Viscum album* var. *coloratum*) for 4 days and measured for viability by MTT assay. All experiments were performed in triplicate. Data are represented as a mean ± S.D. *$p<0.05$ compared to the control.

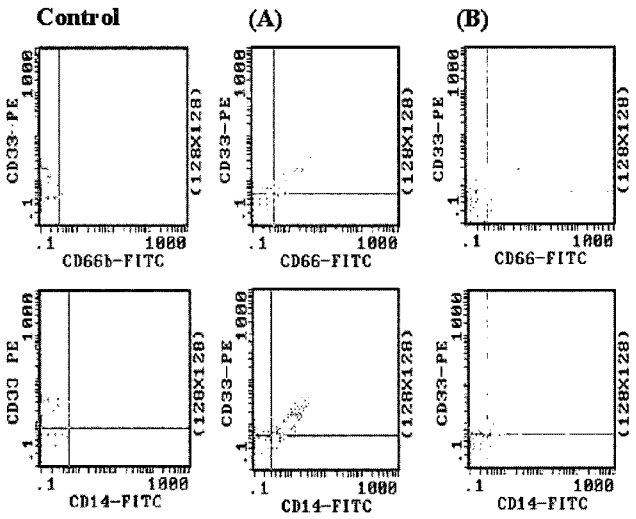

Fig. 1. Differentiation of HL-60 cells as induced by the $(NH_4)_2SO_4$ saturated fractions of mistletoe. HL-60 cells (3×10^5/ml) were exposed to (A) 0~30% $(NH_4)_2SO_4$ saturated fraction (3 μg/ml) or (B) 30~70% $(NH_4)_2SO_4$ saturated fraction (10 μg/ml) of mistletoe for 4 days, and then analyzed by flow cytometry.

Table 2. Effect of the $(NH_4)_2SO_4$ saturated fractions of mistletoe on the differentiation of HL-60 cells

The fraction of mistletoe	Concentration (μg/ml)	Expression of CD 66b (%)	Expression of CD 14 (%)
Control	0	0.42	1.29
0~30% $(NH_4)_2SO_4$ saturated fraction	1	3.88	3.91
	3	34.31	34.82
30~70% $(NH_4)_2SO_4$ saturated fraction	5	5.9	3.52
	10	27.45	20.23

HL-60 cells (3×10^5/ml) were treated with two concentrations of the $(NH_4)_2SO_4$ saturated fractions of mistletoe (*Viscum album* var. *coloratum*) for 4 days. Differentiation was determined by examining the expressions of CD 66b and CD 14 antigens.

Table 3. Differentiation of HL-60 cells induced by the $(NH_4)_2SO_4$ saturated fractions of mistletoe

The fraction of mistletoe	Concentration (μg/ml)	NBT reduction activity (%)
0~30% $(NH_4)_2SO_4$ saturated fraction	1	112 ± 2.7*
	3	149 ± 7.7*
30~70% $(NH_4)_2SO_4$ saturated fraction	5	108 ± 1.8*
	10	177 ± 8.0*

HL-60 cells (3×10^5/ml) were treated with two concentrations of the $(NH_4)_2SO_4$ saturated fractions of mistletoe (*Viscum album* var. *coloratum*) for 4 days. Differentiation was determined by NBT reduction. All experiments were performed in triplicate. Data are represented as a mean ± S.D. *$p<0.05$ compared to the control.

show that HL-60 cells are differentiated to mature granulocytes or monocytes by the mistletoe. In differentiated myeloid cells, PMA is capable of the induction of superoxide production. To evaluate the effect of the $(NH_4)_2SO_4$ saturated fractions of the mistletoe on the differentiation, the induction of superoxide production was measured using the NBT reduction assay. NBT, a water-soluble dye, is reduced to insoluble intracellular blue-black formazan by phagocytizing or membrane-stimulated granulocytes (Baehner *et al.*, 1976). After 4 days of incubation in various concentration of the $(NH_4)_2SO_4$ saturated fractions of the mistletoe, the activity of HL-60 cells reducing NBT increased (Table 3).

Expressions of c-Myc and myeloblastin mRNA in HL-60 cells treated with $(NH_4)_2SO_4$ saturated fraction of the mistletoe

In order to understand the induction mechanism of differentiation by the mistletoe, this study examined the expression levels of c-Myc and myeloblastin using RT-PCR analysis. The treatment of the HL-60 cells with the 0~30% $(NH_4)_2SO_4$ saturated fraction of the mistletoe resulted in a marked decrease of the c-Myc mRNA expression levels in a time-dependent manner (Fig. 2A). But, the 30~70% $(NH_4)_2SO_4$ saturated fraction of the mistletoe rarely decreased the expression of c-Myc mRNA (Fig. 2B). In addition, mRNA level of myeloblastin, a serine protease, was markedly reduced by the 0~

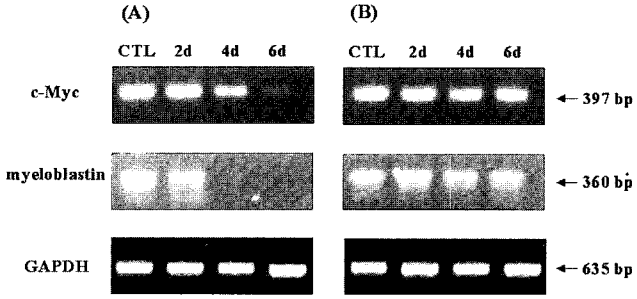

Fig. 2. Expressions of c-Myc and myeloblastin mRNA in HL-60 cells after the treatment with (A) 0~30% $(NH_4)_2SO_4$ saturated fraction (3 μg/ml) or (B) 30~70% $(NH_4)_2SO_4$ saturated fraction (10 μg/ml) of mistletoe at the indicated times. Reverse transcription polymerase chain reaction analysis of c-Myc and myeloblastin were performed as described in the method section.

30%, while the 30~70% $(NH_4)_2SO_4$ saturated fraction of the mistletoe slightly decreased the expression of myeloblastin mRNA in a time-dependent manner (Fig. 2).

DISCUSSION

This study investigated the effect of mistletoe (*Viscum album* var. *coloratum*) growing on *Carpinus laxiflora* BL. on the differentiation induction of HL-60 cells which can be induced to terminally differentiate to morphologically mature granulocytes or monocytes (Breitman *et al.*, 1980; Collins *et al.*, 1978; Gallagher *et al.*, 1979). To the best of our knowledge, this study shows for the first time that the mistletoe induces the differentiation of HL-60 acute myeloid leukemia cells through the down-regulation of c-Myc and myeloblastin.

We observed that the aqueous extract of mistletoe markedly inhibited the growth of HL-60 cells, while the 80% methanol extract of the mistletoe slightly decreased the growth of HL-60 cells (not shown), suggesting that the extract of the mistletoe induced the differentiation and/or apoptosis. In present study, we observed that the $(NH_4)_2SO_4$ saturated fractions of the aqueous extract of the mistletoe induced the differentiation of HL-60 cells into granulocytes or monocytes with the expression of

cell surface antigens and the NBT induction activity. Also, we observed the induction of apoptosis by flow cytometry. When HL-60 cells were stained with the DNA-specific fluorochrome, propidium iodide, the $(NH_4)_2SO_4$ saturated fractions of mistletoe were found to increase the proportion of sub-G_1 hypodiploid cells (not shown). Many studies have been performed on the induction of differentiation and apoptosis in HL-60 cells. HL-60 cells were induced to differentiate into granulocyte-like cells by dimethyl sulfoxide (Collins *et al.*, 1978) and retinoic acid (Breitman *et al.*, 1980; Collins *et al.*, 1977), or into monocyte (macrophage)-like cells by 1, 25-dihydroxyvitamin D_3 (McCarthy *et al.*, 1983) and phorbol diesters (Lotem and Sachs, 1979; Rovera *et al.*, 1979; Todd *et al.*, 1981). On the other hand, it was reported that ascorbate or parthenolide increases the 1, 25-dihydroxyvitamin D_3-induced monocytic differentiation of HL-60 cells (Quesada *et al.*, 1996; Kang *et al.*, 2002). It has also been reported that ascorbates, gallates or benzo (a) phenothiazines induce apoptosis (Sakagami and Satoh, 1997; Sakagami *et al.*, 1997; Satoh *et al.*, 1997). HL-60 leukemia cells are characterized by a high expression of c-Myc, a proto-oncogene (Iguchi-Ariga *et al.*, 1987; Kato and Dang, 1992). In order to understand some of the molecular events that occurred in HL-60 cells after the treatment of the $(NH_4)_2SO_4$ saturated fractions of the mistletoe, we investigated their effects on the c-Myc mRNA expression. The c-Myc expression level was dramatically reduced when the HL-60 cells underwent differentiation and/or apoptosis by the treatment of the 0~30% $(NH_4)_2SO_4$ saturated fraction of the mistletoe. c-Myc is widely known as a crucial regulator of cell proliferation in normal and neoplastic cells. Recently it has been shown that c-Myc activation can induce genomic instability and prevent the response of the $G_1 \rightarrow S$ checkpoint, allowing DNA synthesis despite the presence of eventual genetic damage (Felsher and Bischop, 1999; Sheen and Dickson, 2002). And, c-Myc deregulation is known to be able to enhance cell proliferation, arrest the differentiation, destabilize the genome equilibrium predisposing to pre-malignant phenotype (Felsher and Bischop, 1999; Felsher *et al.*, 2000). Myeloblastin is a protease that has been identified in the HL-60 leukemia cells. Myeloblastin is also known to be involved in the control of growth and differentiation of human leukemia cells (Bories *et al.*, 1989). It was reported that the protease is down-regulated during all-trans retinoic acid-induced differentiation in HL-60 and NB4 leukemia cells (Labbaye *et al.*, 1993). The 0~30% $(NH_4)_2SO_4$ saturated fraction of the mistletoe induced a marked decrease in the level of myeloblastin mRNA in a time-dependent manner. In addition, retinoic acid receptor-α (RAR-α) is known to mediate the granulocytic differentiation of HL-60 cells (Collins *et al.*, 1990). Therefore, further studies into the expression of RAR-α by the purified differentiation-inducing component of the mistletoe are planned.

In conclusion, the aqueous components of the mistletoe seem to induce the differentiation of HL-60 leukemia cells through the down-regulation of c-Myc and myeloblastin. These results support the possibility that the aqueous components of the mistletoe might have a therapeutic potential for treating human leukemia.

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