**Musca domestica** Larva Lectin Induces Apoptosis in BEL-7402 Cells through a Mitochondria-Mediated Reactive Oxygen Species Way

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A lectin from *Musca domestica* larva was purified by affinity chromatography on a glucose-Sepharose 4B column. *Musca domestica* larva lectin (MLL) inhibited the growth of BEL-7402 cells in a time and concentration-dependent way. The results of Hoechst 33258 staining indicated that MLL induce BEL-7402 cells apoptosis based on the typical apoptotic morphological changes. Subsequently, we found that MLL treatment abrogated glutathione antioxidant system and induced mitochondria to generate reactive oxygen species (ROS) accumulation, resulting in reduction of mitochondrial transmembrane potential. The induction of cell apoptosis was caused by the upregulation of Bax, the downregulation of Bcl-2, the cytochrome c release and the activation of the caspase pathways.

**Key words** lectin; *Musca domestica* larva; antitumor; apoptosis; mitochondrial path

Cancer was the disease with proliferation disorder and apoptosis obstacle. Apoptosis, a type of programmed cell death, is an active process playing an essential role in embryonic development, homeostasis, remodeling, surveillance, and host defense mechanisms. Redundant and potentially deleterious cells were eliminated in the apoptosis process, and apoptosis was arguably the most potent defense against cancer. Apoptosis is regulated by a network of signaling pathways and transcription factors, which is possible targetting for a rational tumor therapy. In several apoptotic pathways, mitochondria played a critical role in apoptotic death induced by various stresses agents. Cellular stresses trigger caspase activation by promoting release of mitochondrial components, including cytochrome c, into the cytoplasm from the mitochondrial intermembrane space. Reactive oxygen species (ROS) generation is a cell death initiation signal that contributes to mitochondrial dysfunction and the subsequent drop in cellular ATP levels.

*Musca domestica* is a common insect of the family Muscidae. About 90% of all flies occurring in human habitations are houseflies. *Musca domestica* can transport disease-causing organisms, but they could keep their normal physical conditions even living in the environment full of pathogenic microorganisms. According to “Compendium of Materia Medica,” *Musca domestica* larvae powder is an important component of Chinese herbal medicine, people use it to cure eczema, osteomyelitis, decubital necrosis and malnourishment stagnation. Some Indian indigenous people cure trauma with fresh larvaees because the larvaees eat necrotic tissue as their food. There are several activated substances in *Musca domestica*, such as prophenoloxidase, antibacterial protein/peptide, lysozyme and lectin.

Lectins, a class of carbohydrate-binding proteins, were known to be able to agglutinate cells. They were ubiquitous in nature, such as in microorganisms, animals and plants. And they played important roles in cellular events like proliferation, antibacterial, signal transduction, and apoptosis.

Several lectins have been shown to have antitumor activities. It was reported that lectins isolated from mistletoe induced apoptosis in tumor cells U937 cells via the caspase dependent way. And it also proved that mistletoe lectin showed more powerful antitumor effect on mice by oral route. Sophora flavescens lectin induced apoptosis of HeLa cells in a time and dose-dependent manner.

In 2007 Hou reported that the total extract of *Musca domestica* larvae showed its antimicrobial activity and *in vitro* anti-tumor activity. However, what is specific the antitumor products in *Musca domestica* larvae and what is the function of the products are still not clear. So the objective of this study was to investigate the anti-tumor activity of the lectin from *Musca domestica* larva and explore its potential apoptotic induction mechanism.

**MATERIALS AND METHODS**

*Musca domestica* Larva Lectin *Musca domestica* was purchased from Tianjin Sanitation and Epidemic Prevention Station (Tianjin, P.R. China). *Musca domestica* larva lectin (MLL) was purified as previously described.

**Cell Culture** Human liver cancer cells BEL-7402 and human normal liver cells L-02 were purchased from Tianjin Medical University (Tianjin, P. R. China). All cells were routinely cultured in RPMI-1640 medium containing 10% fetal bovine serum, 100 U/ml streptomycin, 100 U/ml penicillin, and 2 mm l-glutamine in a humidified cell incubator with an atmosphere of 5% CO₂ at 37°C.

**3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay** The growth inhibitory effect of human liver cancer cells BEL-7402 and human normal liver cells L-02 was measured by MTT assay. The cell suspension (1×10⁵/ml) was partitioned into the wells of 96-well plates (Costar, U.S.A.) with 0.1 ml/well and cultured. After 24 h, different concentrations of MLL were injected to the wells (0.1 ml/well). The control group was given the same volume of dilute solution without any drugs. After 24 h, 48 h and 72 h culture, 5 mm MTT solution (50 μl/well) was added to the wells. After another 4 h culture, the supernatant was discarded and dimethyl sulfoxide (DMSO) was added (100 μl/well). The absorbance (A) was read at a wavelength of 570 nm.
As shown in Fig. 2, MLL inhibited the proliferation of BEL-7402 and L-02 cells was investigated by MTT assay.

RESULTS

Western Blotting In order to discuss the potential apoptotic induction mechanism, the expression of characteristic apoptosis protein was investigated by Western blotting. The BEL-7402 cells were treated with MLL for 0, 6, 12, 24 and 48 h or co-incubated with the inhibitors (NAC and z-V AD-FMK) for 48 h. Both adherent and floating cells were collected and incubated with 100 μM DCF-DA for 30 min at 37°C. Then cells were washed with PBS and the relative levels of fluorescence were quantified in a spectrophotofluorimeter. The measured fluorescence values were expressed as a percentage of the fluorescence in control cells. Glutathione peroxidase (GSH-PX) was measured according to the kits.

Flow Cytometric Analysis of Mitochondrial Transmembrane Potential (∆ψm) Cells were treated with MLL for the indicated time and harvested by centrifugation at 1000 g for 5 min. Then the cells were stained with 1 μg/ml rhodamine-123 and incubated for 30 min at 37°C. Mitochondrial membrane potential (∆ψm) was analyzed by flow cytometer (Olympus, Tokyo, Japan).

Fluorescence Morphological Examination After cultured with MLL at the concentration of 5, 20, 35, 50, 65, 80 μg/ml for 24, 48 and 72 h, respectively. The viability of cells was determined by the MTT assay. Results represent means ± S.D.

ROS and Mitochondrial Transmembrane Potential

Hoechst 33258 Staining Apoptotic nuclear morphology was assessed with Hoechst 33258 purchased from Sigma (St. Louis, MO, U.S.A.). After 0 h and 48 h cultured in the RPMI-1640 medium, the cells were harvested, and then stained with Hoechst 33258 at 37°C for 20 min. The cells were washed and suspended again respectively in phosphate buffered saline (PBS) for morphologic observation under a fluorescent microscope (Olympus, Tokyo, Japan).

Measurement of Intracellular ROS The level of intracellular ROS was quantified by fluorescence with 2',7'-dichlorofluorescin dictate (DCF-DA), a nonfluorescent compound, is deacetylated in viable cells to 2',7'-dichlorofluorescein (DCF) by ROS. Cells were collected and incubated with 100 μM DCF-DA for 30 min at 37°C. Then cells were washed with PBS and the relative levels of fluorescence were quantified in a spectrophotofluorimeter. The measured fluorescence values were expressed as a percentage of the fluorescence in control cells.

MTT Assay In order to discuss the toxicity of MLL between the cancer cell and the normal cell, the effect of MLL on BEL-7402 and L-02 cells was investigated by MTT assay. As shown in Fig. 2, MLL inhibited the proliferation of BEL-7402 and L-02 cells in a dose and time dependent manner.

MLL possesses the abilities of remarkable inhibition especially on BEL7402 cells (IC50 35 μg/ml, 24 h), desirably, its toxicity to the normal liver cell L-02 cells is not obvious in the same dose and time, all these results suggested that MLL bears a greater susceptibility of the malignant cells.

Fluorescence Morphological Examination After cultured with MLL at the concentration of 5, 20, 35, 50, 65, 80 μg/ml for 24, 48 and 72 h, respectively. The viability of cells was determined by the MTT assay. Results represent means ± S.D.

Cell Cycle and Ratio of Apoptotic Cells The collected cells were fixed with 500 ml PBS and 10 ml 70% ethanol at 4°C overnight; then after washing twice with PBS, the cells were incubated with 1 ml propidium iodide (PI) staining solution (PI 50 mg/l and RNase A 1 g/l) for 30 min at 4°C in the dark condition. Samples were analyzed by a flow cytometer (Becton Dickinson, U.S.A.).

Lectin MLL was purified by affinity chromatography using glucose-Sepharose 4B. The electrophoretic patterns of MLL were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the absence and presence of 2-mercaptoethanol, the peak with molecular weight of 59 kDa was observed (Fig. 1).

Cell Cycle and Ratio of Apoptotic Cells The results of the cell cycle distribution are presented in Table 1. After BEL-7402 cells treated with 35 μg/ml of MLL at various time points, an arrest of cell cycle at G1 phase (from 25.2 to 48.6%) and the significantly increased apoptotic rates (from 1.38 to 12.5%) were observed (Table 1).
The increasing ROS production induced a variety of apoptotic signals and declined in cellular GSH content. GSH played a vital role in maintaining cellular ROS balance. In Fig. 4, the reduction of the activity of GPX enzyme and the increase in the content of ROS, suggested that MLL abrogated the GSH antioxidant system and led to ROS accumulated in large amount. Furthermore, after pretreated with ROS inhibitor N-acetyl-cysteine (NAC), the level of ROS was significantly inhibited. And the inhibition effort of MLL was blocked by NAC to BEL-7402 cells (Fig. 5). Treatment with NAC, which inhibited the production of intracellular ROS, significantly reversed the cell death induced by MLL treatment.

BEL-7402 cells were treated with MLL (35 μg/ml) for 0, 24 and 48 h, respectively. ∗p<0.05 and ∗∗p<0.01 vs. 0 h.

The increasing ROS production induced a variety of apoptotic signals and declined in cellular GSH content. GSH played a vital role in maintaining cellular ROS balance. In Fig. 4, the reduction of the activity of GPX enzyme and the increase in the content of ROS, suggested that MLL abrogated the GSH antioxidant system and led to ROS accumulated in large amount. Furthermore, after pretreated with ROS inhibitor N-acetyl-cysteine (NAC), the level of ROS was significantly inhibited. And the inhibition effort of MLL was blocked by NAC to BEL-7402 cells (Fig. 5). Treatment with NAC, which inhibited the production of intracellular ROS, significantly reversed the cell death induced by MLL treatment.

MLL treatment to BEL-7402 cells resulted in a rapid loss in ΔΨm in a time-dependent manner (Fig. 6). Flow cytometric results revealed that high level of Rhodamine123 binding to the mitochondrial of untreated BEL-7402 cells. There is a decrease in the fluorescence after 12 h exposure to MLL. However, a significant decrease in the fluorescence (M2) was observed at 24 h (22.17%) after MLL treatment. Furthermore, loss in ΔΨm was blocked by ROS inhibitor (NAC).

Western Blotting By Western blotting analysis, p53 started increasing from as early as 6 h, reached a maximum level at 24 h and persisted up to 48 h. At 48 h, the level of p53 was found to decrease. The level of Bcl-2 was highly expressed in BEL-7402 cells and then decreased to low level after MLL treatment. On the other hand, the level of Bax increased significantly after 6 h and attained a peak at 24 h (Fig. 7). After treatment with MLL, the level of Bax protein was increased while the level of Bcl-2 was decreased, sug-
suggesting that MLL induced apoptosis through up-regulation of Bax and down-regulation of Bcl-2 level.

Cytosolic cytochrome \(c\) is a factor necessary for activation of apoptosis. It also showed that cyt \(c\) was translocated from the mitochondrial membrane to the cytosol. The expression of cyt \(c\) in cytosol was increased while that in mitochondria was decreased in the time depend way. In further research the process could be inhibited by NAC.

It has been demonstrated that induction of cell death is associated with initial activation of several procaspases, which lead to sequential activation of downstream caspase family protease. According to expression of caspase-3 and caspase-9, BEL-7402 cells showed their activation of caspases after being treated with MLL. We observed a strong increase in caspases protein levels, and activation of the caspases was a time-dependent manner. To further define the role of caspase in MLL-induced apoptosis, cells were pre-treated with caspase inhibitor Z-V AD-FMK (100 \(\mu\)mol/l) 2 h before MLL. MLL-induced apoptosis was blocked by the broad caspase inhibitor Z-VAD-FMK.

**DISCUSSION**

MTT showed that MLL inhibited BEL-7402 cells proliferation in a dose and time-dependent manner, even MLL showed little inhibition to normal human cells L-02 cells. In my view the carbohydrate polymer in lectin has identified function, the carbohydrate polymer itself maybe has identified activity on cells, especially the malignant liver cells. Many articles have reported that lectins from plant bearing a greater susceptibility of the malignant cells, such as the polygonatum cyrtonema lectin.21) And research on what is the accurate structure and how the carbohydrate polymer identifies the different kind cells are being conducted. The
apoptosis induced by MLL was also confirmed by the observed changes in nuclear morphology. Compared to control cells, MLL treated BEL-7402 cells revealed nuclear fragmentation. And the nucleus and cytoplasm fragment would form apoptotic bodies that could be engulfed by phagocytes.

Many antioxidant agents and DNA-damaging agents induce apoptotic cell death by arresting the cell cycle at the G1, S, or G2/M phase.\textsuperscript{23} Our data demonstrated that MLL induced cell apoptosis by arresting BEL-7402 cells at the G1 phase. Components of the DNA damage checkpoint were essential for surviving exposure to DNA damaging agents which led to cell cycle arrest, DNA repair, and apoptosis in eukaryotes.\textsuperscript{23} Tumor suppressor p53 protein mediated checkpoint control and the apoptotic program, was critical for maintaining genomic integrity and preventing tumorigenesis.\textsuperscript{24} It has been reported that p53 may induce two sets of genes upon stress signals. One set mainly functions in cell growth control and the other set act on apoptosis, such as Bcl-2.\textsuperscript{25} Our data demonstrated that in BEL-7402 cells possessing p53, MLL-induced cells arrest and apoptosis were accompanied by the up-regulation of p53 protein level in a dose-dependent manner. These results indicated that the activation of the p53 pathway may be involved in MLL-induced cell cycle arrest and apoptosis in BEL-7402 cells.

Oxidative stress, characterized by overwhelming ROS, resulted in membrane lipid peroxidation, nitration of proteins, and degradation of DNA, all of which are associated with the course of apoptosis.\textsuperscript{26} Cells were normally able to defend themselves against ROS damage through the use of enzymes such as NAC, superoxide dismutases, lactoperoxidases, glutathione peroxidases and peroxidorexins. In our research MLL did not show the antitumor activity to cells defend themselves against ROS damage through the use of enzymes that control apoptosis.\textsuperscript{30} Our studies revealed that MLL treated BEL-7402 cells revealed nuclear fragmentation. And the nucleus and cytoplasm fragment would form apoptotic bodies that could be engulfed by phagocytes.

Tumor suppressor p53 protein mediated checkpoint control and the apoptotic program, was critical for maintaining genomic integrity and preventing tumorigenesis.\textsuperscript{24} It has been reported that p53 may induce two sets of genes upon stress signals. One set mainly functions in cell growth control and the other set act on apoptosis, such as Bcl-2.\textsuperscript{25} Our data demonstrated that in BEL-7402 cells possessing p53, MLL-induced cells arrest and apoptosis were accompanied by the up-regulation of p53 protein level in a dose-dependent manner. These results indicated that the activation of the p53 pathway may be involved in MLL-induced cell cycle arrest and apoptosis in BEL-7402 cells.

Acknowledgements We gratefully acknowledge the financial support by the National High Technology Research and Development Program of China (863 Program, No. 2007AA10Z319), the Chinese National Natural Science Foundation (20676103) and Tianjin Natural Science Foundation (07JCZDJC02900).

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