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Astragalus membranaceus lectin (AML) induces caspase-dependent apoptosis in human leukemia cells

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Abstract

Objectives: Recently, plant lectins have attracted great interest due to their various biological activities such as anti-cancer, anti-fungal and anti-viral activities. We have reported earlier concerning anti-proliferation of human cancer cell lines by a galactose-binding lectin (AML), from a Chinese herb, *Astragalus membranaceus*. In the present study, detailed investigations into the mechanism of such anti-proliferation properties have been carried out.

Materials and methods: Mechanism of apoptosis initiation in K562 cells by AML was investigated by morphology, flow cytometry and western blot analysis.

Results: AML induced apoptosis in a caspase-dependent manner in the chronic myeloid leukemia cell line, K562. Furthermore, we observed that cytotoxicity and apoptosis of K562 cells induced by AML were completely abolished in presence of lactose or galactose.

Conclusions: Our results suggest that AML could act as a potential anti-cancer drug.

Introduction

Plant lectins are carbohydrate-binding proteins of non-immune origin with cell agglutinating ability, and are ubiquitous in nature (1,2). In recent years, plant lectins have attracted increased interest due to their various biological activities, such as agglutination, toxicity, anti-proliferation of cancer cells and immunomodulation, as well as having anti-fungal and anti-viral activities (3–10). It is now widely accepted that plant lectins have a great potential in treating, preventing and helping to diagnose various chronic diseases including cancer (6). Some plant lectins like mistletoe lectin have been used for many years as alternative therapy for breast cancer patients (11). Apoptosis of cancer cells by mistletoe lectins (MLs) is mainly *via* mitochondrial and/or death-receptor pathways (12–14). Most plant lectins induce apoptosis of cancer cells, mediated through caspase-dependent pathways (15–19).

So far, some plant lectins, such as ricin (20), WGA (21,22) have been reported (6,14) as possessing significant anti-cancer properties. Several studies have suggested a strong correlation between certain lectin-binding patterns and their biological effects in various tumours (6). MLs consist of a catalytic A-chain, which inhibits protein synthesis, and B-chain, which facilitates binding of appropriate carbohydrate residues on cell surfaces, thereby inducing receptor-mediated cell death (23,24).

Previously, we have isolated and characterized a novel lectin, AML, from the dried roots of *Astragalus membranaceus*, which has been used in China for many centuries as a traditional herbal medicine to treat various chronic diseases (25). AML is a monomeric protein with molecular mass of 31.5 kDa and a glycoprotein with 10.7% neutral sugar. AML is a galactose-binding lectin, which is best inhibited by D-galactose and its derivatives, with pronounced preference for *o*-nitrophenyl-β-D-galactopyranoside (8.3 mM). AML belongs to a legume lectin family, and has carbohydrate-binding specificities, and further similarities to other reported legume lectins, (6,9,25,26). Furthermore, AML has been found to inhibit proliferation of HeLa cells, the chronic myeloid leukemia (CML) cell line K562 and more human cancer cell lines (25). Some other legume lectins such as concanavalin A, AMML and French bean haemagglutinin have also been reported to have apoptosis induction in various types of cancer cells *via* different molecular mechanisms (9,26–28). In the

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present study, we have demonstrated that AML-induced apoptosis in CML is a caspase-dependent manner. Furthermore, we observed that cytotoxicity and apoptosis of CML cells induced by AML were completely abolished in the presence of lactose or galactose.

Materials and methods

Chemicals and reagents

Astragalus membranaceus lectin (AML) was extracted and purified as described previously by Yan *et al.* (2010). Annexin V/FITC (fluorescein isothiocyanate) was purchased from the Center for Human Disease Genomics, Peking University, Beijing, China. MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was obtained from Amresco, Solon, OH, USA. DAPI (4′, 6-diamidino-2-phenylindole, dihydrochloride) stain and RPMI 1640 medium were purchased from Invitrogen, Carlsbad, CA, USA. Bovine serum albumin (BSA), Dulbecco's modified Eagle's medium (DMEM), propidium iodide (PI) and fluorescein isothiocyanate (FITC) were purchased from Sigma, St. Louis, MO, USA. Mouse anti-α-tubulin (CB100999) and mouse anti-Bcl-2 (sc-7382) were obtained from California Bioscience, Inc., Coachella, CA, USA and Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA respectively. Rat anti-caspase-3 (M097-3), z-VAD-fmk and anti-mouse IgG coupled to horseradish peroxidase (HRP) antibodies were obtained from Medical and Biological Laboratories Company, Naka-ku, Nagoya, Japan. All other chemicals used were of analytical grade.

Tumour cell lines and culture conditions

Human leukemia cell line, CML K562, was obtained from the Chinese Academy of Medical Sciences, Beijing, China. Culturing and maintenance of K562 cells were performed as described previously (Yan *et al.*, 2009). For culturing K562 cells, RPMI 1640 medium was used, supplemented with 10% of FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2. Cell viability was checked by MTT assay as described previously (Yan *et al.*, 2010).

Cell morphology

Exponentially growing K562 cells (1×10^5 cells/ml) were treated with 20 μg/ml AML for 24 h. Apoptotic nuclear morphology was visualized after DAPI staining of AML-treated cells. Cells were first fixed in 3.7% paraformaldehyde for 10 min at room temperature and then washed three times in PBS and 0.1% Triton X-100 for 2 min. Fixed cells were then stained with DAPI (10 μg/ml), in the dark, for 10 min. After washing three times in PBS, cells were visualized using a fluorescence microscope (Olympus Corporation, Japan).

DNA fragmentation assay

Ladder patterns of DNA fragmentation were analysed using agarose gel electrophoresis. K562 cells were incubated with AML (20 μg/ml) for different time intervals, and their DNA (1×10^6) was isolated using the Apoptotic DNA Ladder Kit, Applygen Technologies Inc., (Beijing, China). After ethanol precipitation, DNA from each sample was subjected to electrophoresis with 1% agarose slab gel containing 0.2 μg/ml ethidium bromide (EB).

Flow cytometry

Apoptosis was analysed using annexin V labelling and PI staining followed by flow cytometry. After incubation with AML (10 μg/ml) for 0, 12, 24 and 48 h, K562 cells were harvested, washed in cold PBS and suspended in binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$). Cells (1×10^6 cells/ml) were then stained with 10 μl annexin V/FITC and 5 μl PI (50 μg/ml) and kept in the dark at room temperature for 15 min ahead of the flow cytometric studies (Becton-Dickinson, Franklin, NJ, USA).

Cell surface binding assay

Binding of AML to surfaces of K562 cells was detected by fluorescence microscopy and flow cytometry using FITC as fluorescence marker. First, AML and BSA (negative control) were labelled by FITC (1 mg/ml), which was dissolved in cold PBS and dialysed against PBS at 4 °C overnight; cell agglutination activity of the lectin remained unchanged after FITC-labelling (data not shown). The cells (4×10^5 cells/ml) were washed three times with PBS and 1 ml of cells were seeded into each well in a 24-well plate. AML and BSA were added to each well separately at final concentration of 10 μg/ml. Cells from 24-well plates, previously incubated for 30 min at 37 °C in CO_2 incubator, were then washed three times in cold PBS, then were observed under the fluorescence microscope and subjected to flow cytometry.

Western blot analysis

K562 cells treated with AML (10 μg/ml) for 0, 12, 24 and 48 h were washed in cold PBS and re-suspended in 200 μl of lysis buffer [50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 150 mM NaCl] containing protease

inhibitors [1 mg/ml aprotinin, 10 mg/ml leupeptin and 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride] and incubated for 30 min on ice. Protein extracts were collected by centrifugation (9720 g, 10 min) and mixed with equal volume of 5× sodium dodecyl sulphate (SDS) gel loading buffer (1 M pH 6.8 Tris-HCl, 10% SDS, and 0.01% bromophenol blue and 50% glycerol) and boiled for 5 min to completely denature protein. After SDS-PAGE, protein bands were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA), which were blocked in 0.5% fat-free dried milk in Tris-buffered saline (TBS) with 0.05% Tween 20 (TBST), for 1 h at room temperature, and washed in TBST three times. Subsequently, membranes were incubated overnight at 4 °C with appropriate primary antibodies at adequate dilutions, in blocking buffer (0.5% BSA in TBS). After washing in TBST, membranes were incubated with anti-rat or anti-mouse horseradish peroxidase-conjugated secondary antibodies (1:2000 dilutions) in the above-mentioned blocking buffer for 1 h at room temperature. Bands were detected using enhanced chemiluminescence kit (Applygen Technologies Inc., China).

Statistical analysis

Results are expressed as the means and standard deviations of triplicate measurements. Each experiment was performed at least three times. Statistical comparisons were made by student's *t*-test and $P < 0.05$ was considered statistically significant. Western blots are representative of three independent experiments.

Results

Cell morphological changes and apoptosis induced by AML

To demonstrate anti-proliferative action of AML during apoptosis, the lectin-treated tumour cells were stained with DAPI and observed under a fluorescence microscope, for any morphological changes in the cells. In the control cells, nuclei (with their DNA) were found to be round in shape and appeared to be homogeneously stained, whereas in AML-treated cells, chromatin condensation was at the periphery of nuclear membranes and fragmentation of nuclear bodies were observed (Fig. 1b,c). A hallmark observation of separated nuclear DNA in most cells undergoing apoptosis, is the appearance of DNA ladders due to its inter-nucleosomal degradation, resulting from activation of nuclear endonucleases. Such ladder-like patterns of DNA fragments were observed in 20 μg/ml AML-treated cells after 48 h incubation, however, during the period of

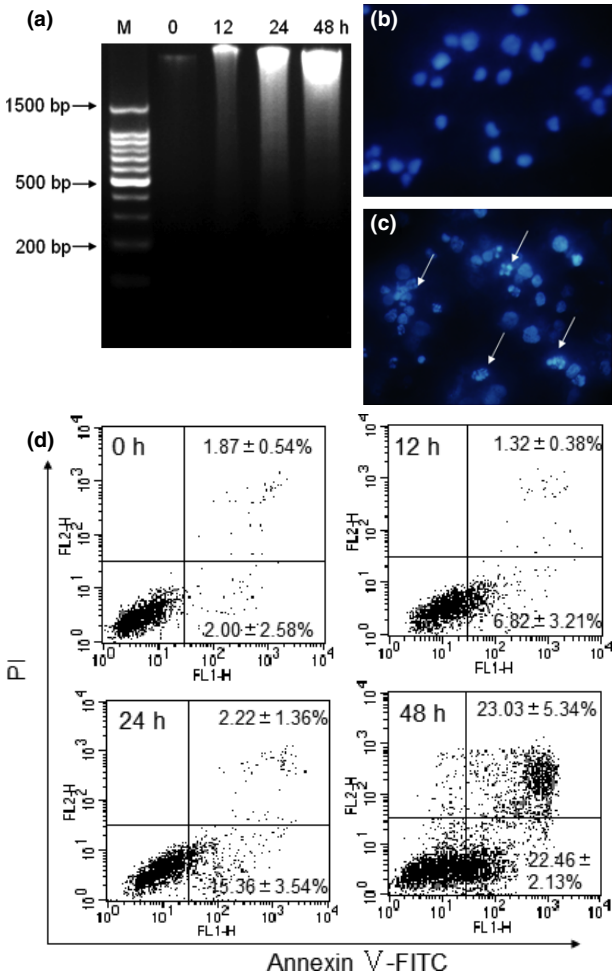

Figure 1. AML induced apoptosis in K562 cells. DNA fragmentation was checked by ethidium bromide staining (a). Cells were cultured with 20 μg/ml AML for 0, 12, 24 and 48 h respectively, 'M' represents marker. Morphological observation of nuclei was visualized using a fluorescence microscope and DAPI staining (400×). Cells were incubated in the absence (b) or presence (c) of 20 μg/ml AML for 24 h. Arrows indicate apoptotic features (condensed chromatin and nuclear fragmentation). Apoptosis of K562 cells was quantified using annexin V/PI staining and flow cytometry (d). Cells were treated with 10 μg/ml AML for 0, 12, 24 and 48 h respectively. Data expressed as mean ± SD, obtained from experiments performed in triplicate.

12–24 h, smear-like DNA degradation was observed in the AML-treated cells (Fig. 1a). Apoptosis of K562 cells by AML was occurred in a time-dependent manner.

Furthermore, to confirm this apoptosis-causing property of AML, the K562 cells were stained simultaneously with annexin V and PI. Early or late apoptosis was determined after a variety of time periods of AML treatment. As shown in Fig. 1d, in the region of 5% of cells which were not treated with AML were found either in early or late stage of apoptosis. Upon AML treatment, approximately 7% and 15% cells were found in early apoptosis

stages after 12 and 24 h respectively, and less than 5% total cells appeared to be in late apoptosis. It is interesting to observe that after 48 h, the proportion of early and late stage of apoptotic cells increased to 23.68% and 23.03% respectively and this ratio of apoptotic to non-apoptotic cells increased in a time-dependent manner. The above results clearly suggest that AML induces apoptosis in K562 cells.

Effects of caspase inhibitor in AML-induced cell death

A pan-caspase inhibitor, z-VAD-fmk, was used to ascertain involvement of caspases in AML-induced K562 cell death. Cells were treated with AML in the presence and absence of z-VAD-fmk. Anti-proliferative activity of AML was found to be significantly lower in the presence of z-VAD-fmk. Over 48 h AML treatment in the presence of the tested inhibitor, the anti-proliferative ratio was maintained in the region of 35%, which increased to 65% as concentration of AML increased. In particular, when AML was added at 5 μg/ml, the inhibitory ratio was less than 10% in the presence of inhibitor, whereas it was more than 30% in the absence of inhibitor (Fig. 2a). To confirm whether anti-proliferative activity of AML was through a caspase-mediated system of apoptosis, annexin V and PI staining was carried out with AML alone or with AML plus caspase inhibition. As shown in Fig. 2b, a significant decrease in early and late apoptosis was observed with AML and caspase inhibitor treated cells compared to only AML-treated cells. These results indicate that AML-induced apoptosis in our cells was mediated through caspase activation pathways.

Analysis of expression of Bcl-2 and caspase-3 by immunoblotting

Caspase-3 is known to play a vital role in caspase-dependent apoptotic pathways, which can be activated by relevant modulins, like Bcl-2, released from mitochondrial membranes. We investigated expression of caspase-3 and Bcl-2 in AML-treated cells by western blot analysis. Decreased levels of pro-caspase-3 expression and increased concentrations of the cleavage product of caspase-3, were observed in AML-treated K562 cells (Fig. 2c). Simultaneously, quantities of modulin, Bcl-2 were reduced with increase in AML treatment time. These results also clearly indicate involvement of mitochondria-mediated caspase-dependent apoptosis in AML-treated cells.

Cell surface binding by AML

Lectins recognize specific carbohydrate topology by conjugation with certain glycans on cell surfaces. Thus, cell

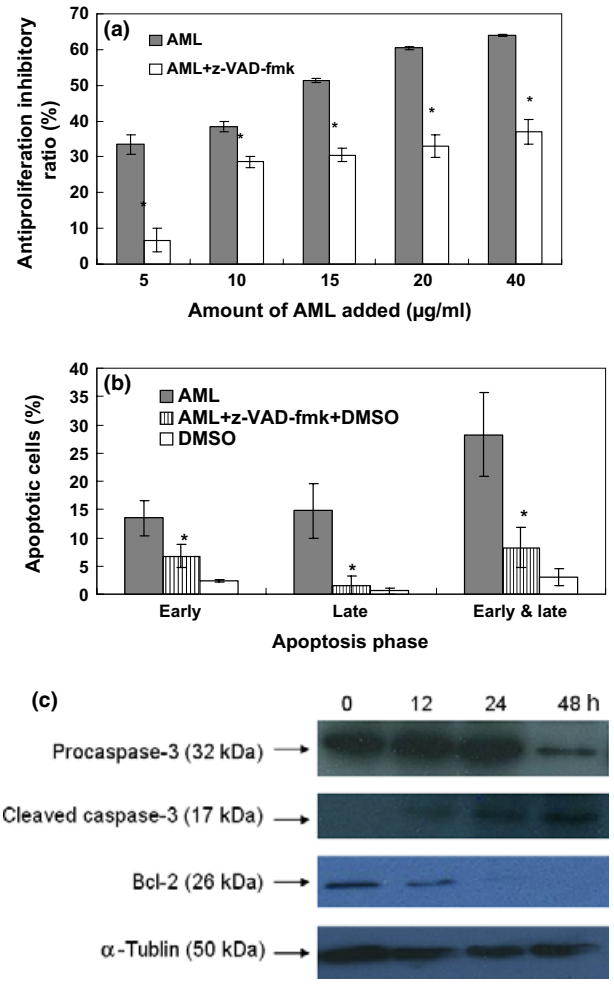

Figure 2. AML induced apoptosis through a mitochondria-mediated and caspase-dependent pathway. Cells were incubated in a variety of concentrations of AML and 40 mM z-VAD-fmk, for 48 h: cell viability was checked by MTT assay (a). Cells were incubated in 10 μg/ml AML and 40 mM z-VAD-fmk for 48 h; apoptosis was investigated using flow cytometry (b). Data expressed as mean ± SD, obtained from experiments performed in triplicate, $*P < 0.05$ versus AML group. The K562 cells were treated with 10 μg/ml AML for the variety of time periods, and levels of Bcl-2 and caspase-3 were detected by western blot analysis (c). α-tublin was used as equal control, and results are representative of experiments performed in triplicate.

surface binding of FITC-labelled AML to our cells was studied using fluorescence microscopy. AML was found to bind strongly to the cells compared to non-lectin treated controls, BSA treatment only (Fig. 3).

Effects of sugars on AML-induced cytotoxicity and apoptosis in K562 cells

We have previously reported the selective inhibition of AML haemagglutination activity by galactose or lactose (25). To test whether these sugars have any effect on the

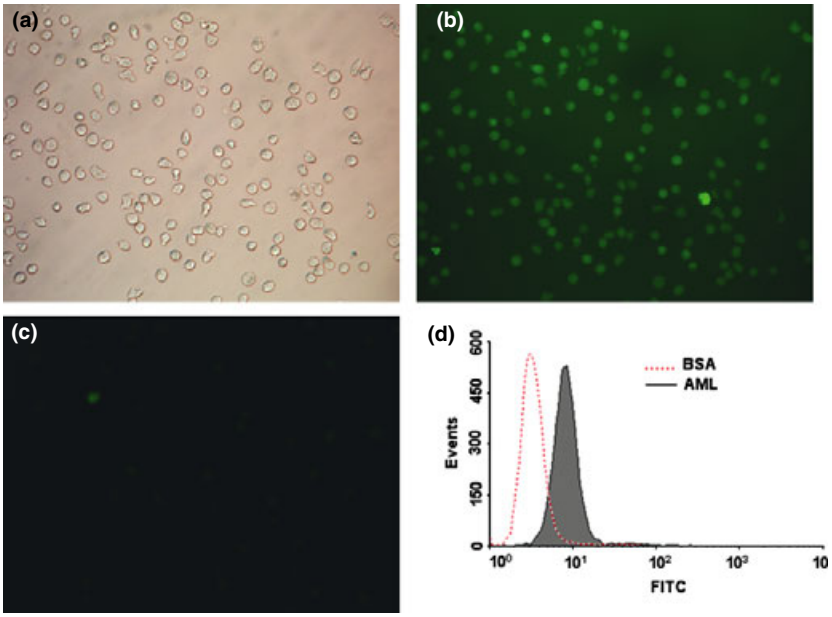

Figure 3. Observation of AML binding to K562 cell surface. K562 cells incubated with AML-labelled FITC, were observed using phase contrast microscopy (a, 200×) or fluorescence microscopy (b, 200×), and BSA-labelled FITC was used as negative control (c). Fluorescent intensity of cell surfaces was checked using flow cytometry (d); red dotted line represents cells bound with BSA as negative control. Results are representative of experiments performed in triplicate.

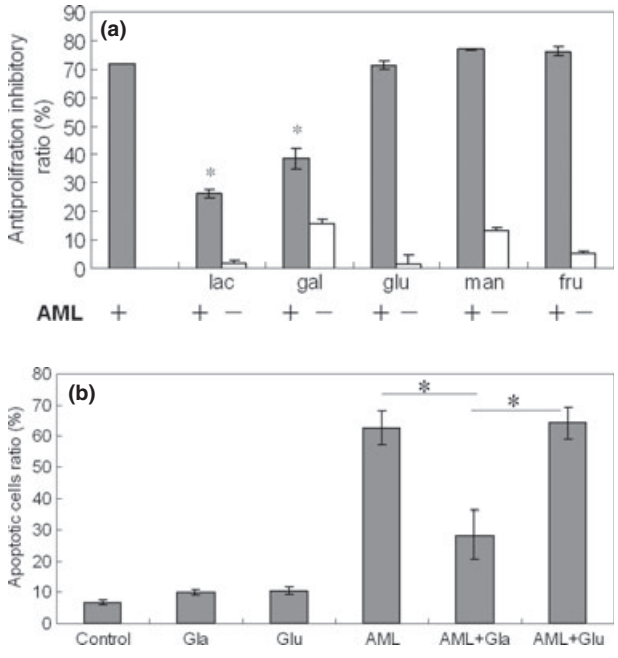

Figure 4. Effect of different sugars on anti-proliferative activity of AML on K562 cells. Cells were incubated with AML (20 μg/ml) and/or sugar (50 mM) (Lac for lactose, gal for galactose, glu for glucose, man for mannose, fru for fructose; ± cells incubated with or without AML). Data expressed as mean ± SD, obtained from experiments performed in triplicate, *$P < 0.05$ versus control.

anti-proliferative activity of AML, experiments were carried out in the presence of free carbohydrates such lactose and D-galactose. As negative control, cells were cultured with AML in the presence of other sugars such as glucose, mannose and fructose. Cells were cultured with sugars, without AML to discern effects of sugars on the cells. Anti-proliferative activity of AML was abrogated in the presence of galactose-containing sugars, lactose and D-galactose, while the other sugars did not cause any anti-proliferative effects of AML (Fig. 4a). Annexin V and PI staining indicated that apoptosis induced by AML was completely abrogated in the presence of galactose (Fig. 4b). These results suggest that cytotoxicity and apoptosis-causing AML to K562 cells were abolished, which could be due to binding with lactose or galactose, blocking biological activity of AML.

Discussion

Plant lectins are proteins or glycoproteins that are able to recognize and bind specific carbohydrates distributed on cell membranes. This property of lectins has been certified to trigger certain tumour cells to activate cell death (6). In our previous studies, we have found that AML was more inhibitory to K562 cells (IC_{50} = 15 μg/ml) compared to the HeLa cell line (25). In addition, compared to other lectins reported earlier, AML seemed to have more significant cell proliferation inhibition on CML cells (25,29,30). In our present investigation, results of DNA laddering and annexin V assays clearly indicated that AML can induce apoptosis in these CML cells, although apoptosis of HeLa cells by a further *Astragalus* lectin, AMML, has reported been (9).

Understanding intracellular mechanisms involved during human tumour cell apoptosis induced by lectins is of great importance for the development of lectin-based

anti-cancer drugs (14). Caspases, which belong to a family of cysteine proteases, have been well established as major players in apoptosis-causing mechanisms. Caspase-3, a key member of the caspase family, is known to initiate the cell death cascade after its activation by stimulation from mitochondria and/or cell death receptors (31,32). Our study using pan-caspase inhibitor, z-VAD-fmk, indicated that anti-proliferative activity of AML is mediated through a caspase-dependent mechanism. Furthermore, lower expression levels of anti-apoptotic protein, Bcl-2, further support our hypothesis that anti-proliferative activity of AML on CML cells is probably through mitochondrion-associated apoptotic signalling pathways (32,33). Induction of mitochondrial caspase-dependent apoptotic signalling by ingredients derived from plants or medicinal herbs has been well demonstrated in various cancer cells (13,14,17,34). Amongst reported plant lectins, some induce apoptosis in cancer cells through caspase-independent signalling way (15,30), but some lectins such as SNA-I/II, POL and PCL resemble AML in their molecular mechanisms of apoptosis in their corresponding cancer cells (17–19,35). As AML is capable of inducing apoptosis in CML cells by a traditional signalling pathway, it is reasonable to expect that AML can play an important role in treatment of cancers in the way of some other reported lectins (6,11,26).

Carbohydrate-binding specificity of lectins plays an important role in their biological activities. Investigations have been carried out to demonstrate similarities in signalling pathways of apoptosis induced by galactose-binding lectins like AML (6). Some plant lectins, such as those of mistletoe, which also bind specifically through lactose or galactose, bring about caspase-dependent apoptosis through mitochondria and/or death receptors of human tumour cells (12,13,36) and apoptosis of K562 cells caused by a D-galactose lectin (CvL) *via* a caspase-independent mechanism, has been reported (30). A Galβ1-3Gal-NAc-binding lectin (Jacalin) from jackfruit seeds inhibited CD45 tyrosine phosphatase activity of human B lymphocytes resulting in apoptosis (37). Thus, molecular mechanisms of lectin-induced apoptosis of tumour cells seems to be complex and cannot be judged simply by carbohydrate-binding property of lectins.

Lectin-mediated cytotoxicity is a complex process, and binding of lectin to appropriate carbohydrate moieties present on the cell membrane is presumed to be an initial step (38). In our study, cell surface binding of K562 cells was demonstrated using fluorescence-labelled AML (Fig. 3). Several other plant lectins showing cancer cell surface binding ability have been visualized with analogical lectin-labelling methods under fluorescence microscope (15,30,39). Furthermore, it was observed that lactose or galactose, added simultaneously with AML protected the K562 cells from death and apoptosis. Some previous reports have indicated that the anti-proliferative activity of plant lectins, including the well-known legume lectin concanavalin A, is inhibited by their special binding carbohydrates, but there are few reports that show that apoptosis-inducing ability can simultaneously be inhibited (7,27). Even though details of lectin-mediated cytotoxicity and apoptosis are not well known, our data clearly indicate that AML binds to appropriate galactose-containing receptors, which are responsible for triggering the apoptotic signals in K562 cells.

In conclusion, we have found that AML can induce apoptosis through caspase-dependent pathways in human K562 leukemia cells, and this lectin-mediated apoptotic mechanism is considered to have strong correlation with its sugar-binding specificity. To the best of our knowledge, this is the first time a detailed mechanism of apoptosis, induced by a plant lectin from *Astragalus* genus, has been reported. Our results may provide promising insights for pharmaceutical exploitation in the treatment of CML.

Acknowledgements

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