Apoptosis Induction Associated with Cell Cycle Dysregulation by Rice Bran Agglutinin[1]

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Effects of rice bran agglutinin (RBA) on human monoblastic leukemia U937 cells were examined in comparison with those of wheat germ agglutinin (WGA) and *Viscum album* agglutinin (VAA). These lectins inhibit cell growth, and several lines of evidence indicate that the growth inhibition is caused by the induction of apoptosis. We observed that RBA induces chromatin condensation, externalization of membrane phosphatidylserine, and DNA ladder formation, features of apoptosis. DNA ladder formation was inhibited by a general inhibitor against caspases, which are known to play essential roles in apoptosis. Flow cytometric analysis revealed that RBA and WGA cause G2/M phase cell cycle arrest with increased expression of Waf1/p21, while cell cycle arrest was not observed for VAA. These data indicate that RBA induces apoptosis associated with cell cycle arrest in U937 cells, and suggest that the induction mechanism for RBA is similar to that for WGA, but different from that for VAA.

Key words: apoptosis, cell cycle arrest, rice lectin, mistletoe, tumor cells, wheat germ.

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Lectins are proteins or glycoproteins of non-immune origin with specific binding affinities for the carbohydrate moieties of glycoconjugates (*1, 2*). Biological activities elicited by lectins include cell agglutination, mitosis, toxicity, and cell growth inhibition. Recently, some lectins have been shown to induce apoptosis, which would explain their cytotoxicity (*2–5*). Mistletoe lectins (*Viscum album* agglutinins, VAAs) are recognized as the main active constituents carrying the anti-cancer activities of mistletoe extracts that have been widely used for years in adjuvant cancer therapy in Europe. Recently, mistletoe lectins have been found to induce apoptosis in tumor cells, and their apoptosis-inducing mechanism has been clarified to some extent (*6–8*).

Rice bran agglutinin (RBA) was first isolated by Tsuda in 1979 (*9*), and some characteristics have been described (*9–11*). However, its biological activities on cancer cells have remained to be evaluated. RBA has features similar to those of wheat germ agglutinin (WGA) in terms of carbohydrate binding specificity and amino acid sequence (*11–14*), suggesting that as WGA has apoptosis-inducing activity (*2, 3*), RBA may as well.

In this study, we investigated the apoptosis-inducing activity of RBA and compared it with those of WGA and VAA.

MATERIALS AND METHODS

Materials—RBA and concanavalin A (Con A) were obtained from Wako Pure Chemical Industries, Osaka. RBA was coupled with CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech, Tokyo) according to the manufacturer's instructions to give RBA-agarose (5 mg/ml wet gel). Phytohaemagglutinin (PHA), WGA, and WGA-agarose (4–5 mg/ml wet gel) were from Seikagaku Kogyo, Tokyo and VAA was from Sigma Aldrich, Tokyo. Human monoblastic leukemia U937 cells and stomach cancer MKN-45 cells were obtained from Health Service Research Resources Bank, Osaka, Japan, and cultured in 10% fetal bovine serum in RPMI 1640 medium containing 50 U/ml penicillin, 50 μg/ml streptomycin, 2.5 μg/ml amphotericin B, and 50 μg/ml gentamycin at 37°C under 5% CO_2 as described previously (*15*). Alamar Blue was a product of Alamar BioSciences, Sacramento, CA, USA. A caspase inhibitor, carbobenzoxy-L-aspart-1-yl-(2,6-dichlorobenzoyl)oxy-methane (Z-Asp-CH_2-DCB), was obtained from Peptide Institute, Osaka. SYBR Green I was obtained from Molecular Probes, OR, USA. Hoechst 33342 (bisbenzimide H 33342 Fluorochrome) was obtained from Calbiochem-Novabiochem., CA, USA. Mouse monoclonal anti-Waf1/p21 antibody was from Santa Cruz Biotechnology, CA, USA.

Assay for Cell Proliferation—U937 cells (2×10^4) in 100 μl of culture medium were mixed with 100 μl of a filter-sterilized solution of three individual lectins in 48-well microculture plates. After culturing at 37°C for 42 h, the number of viable cells was determined by incubation with Alamar Blue as described previously (*16*), and the values

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Abbreviations: Con A, concanavalin A; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, 0.05 M phosphate-buffered saline (pH 7.4); PHA, phytohemagglutinin; RBA, rice bran agglutinin; RT, reverse transcription; WGA, wheat germ agglutinin; VAA, *Viscum album* agglutinin; Z-Asp-CH_2-DCB, carbobenzoxy-L-aspart-1-yl-(2,6-dichlorobenzoyl)oxy-methane.

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obtained were compared with those of controls incubated with vehicle only. Values for wells with the same number of cells as initially seeded represent 0% proliferation. In some cases, WGA-agarose and RBA-agarose (50 μl/well) were used in place of soluble lectins.

In the case of the adhesive cell line MKN45, trypsinized cells (2×10^4) were allowed to attach to the plate by incubation for 4 h before lectin samples were added.

Chromatin Condensation—U937 cells incubated in the culture medium in the presence or absence of three individual lectins at 37°C for 17 h were fixed with 1 % glutaraldehyde, washed with 0.05 M phosphate-buffered saline (PBS) at pH 7.4, and stained with Hoechst 33342 to examine chromatin condensation by fluorescence microscopy as described previously (*15*). MKN-45 cells treated similarly with lectins were trypsinized and the pelleted cells were used to examine chromatin condensation.

DNA Fragmentation—U937 cells were incubated in culture medium in the presence or absence of lectin. For DNA fragmentation analysis, 5×10^5 cells were pelleted by centrifugation, and DNA was isolated from the cell pellets as described by Sellins and Cohen (*17*). The DNA was then subjected to electrophoresis in 2% agarose gels, stained with SYBR Green I, and imaged and calculated with a FluorImager (Molecular Dynamics Japan, Tokyo) as described previously (*15*). To confirm apoptosis-associated DNA fragmentation, cells were incubated in the presence of lectin with a general caspase inhibitor, Z-Asp-CH_2-DCB (*18*), at 200 μM for 17 h, and DNA fragmentation was examined.

Cell Surface Phosphatidylserine—An Annexin V-FITC Apoptosis Detection kit (Medical and Biological Laboratories, Nagoya) was used for flow cytometric detection of apoptosis based on the localization of phosphatidylserine to the outer plasma membrane upon apoptosis (*19*). U937 cells treated with three individual lectins for various time periods were labeled with fluoresein isothiocyanate-labeled annexin V with a high affinity for phosphatidylserine according to the manufacturer's instructions. Flow cytometric analysis was performed using a flow cytometer EPICS XL System II (Coulter, Tokyo).

Cell Cycle Analysis—U937 cells (10^5–10^6 cells) incubated with individual lectins for various time periods were pelleted and treated with 1.2 ml of ethanol at –25°C for 4 h. After removal of ethanol, the fixed cells were suspended in 100 μl of 0.2 M Na_2HPO_4/0.1 M citrate buffer (96:4) containing 0.1% BSA, and 1 ml of propidium iodide solution (10 μg/ml) containing RNase A (10 μg/ml) in PBS was added. The mixture was incubated for 20 min, and cell cycle analysis was performed using a flow cytometer EPICS XL System II.

Western Blotting—U937 cells incubated with individual lectins for various time periods were lysed in PBS containing 1% Triton X-100. The lysates containing 100 μg protein each, as determined using Protein Assay (Nippon Bio-Rad Laboratories, Tokyo), were heated at 100°C for 2 min in the sample buffer for SDS-PAGE and examined by the Western blotting method. The polyvinylidene difluoride membrane with blotted proteins was probed with monoclonal anti-Waf1/p21 antibody and peroxidase-conjugated anti-IgG antibody by the chemiluminescence method using ECL Plus Western Blotting Detection Reagents (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Reverse Transcription (RT)–PCR—Total RNA was prepared from 5×10^5 cells treated with or without individual lectins using a Catrimox-14 RNA Isolation Kit ver. 2. 11 (Takara Shuzo, Tokyo) according to the manufacturer's instructions. RT-PCR was performed using extracted total RNA and Ready-To-Go RT-PCR Beads (Amersham Pharmacia Biotech). Amplified DNA was subjected to electrophoresis in 2% agarose gels, stained with SYBR Green I, and imaged and calculated using a FluorImager as described previously (*15*). Primers for Waf1/p21 designed according to the literature (*20*) were: 5′-GAC ACC ACT GGA GGG TGA CT-3′ and 5′-GGC GTT TGG AGT GGT AGA AA-3′, and those for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were: 5′-AAG GTC ATC CCT GAG CTG AA-3′ and 5′-CCC CTC TTC AAG GGG TCT AC-3′. The expected lengths of the PCR products for Waf1/p21 and GAPDH were 299 and 495 bp, respectively.

RESULTS

Effects on Cell Proliferation—When human monoblastic leukemia U937 cells were incubated with RBA or WGA, proliferation was inhibited in a concentration-dependent manner with IC_{50} values of about 13 and 5 μg/ml, respectively (Fig. 1). No cell growth inhibition was observed for

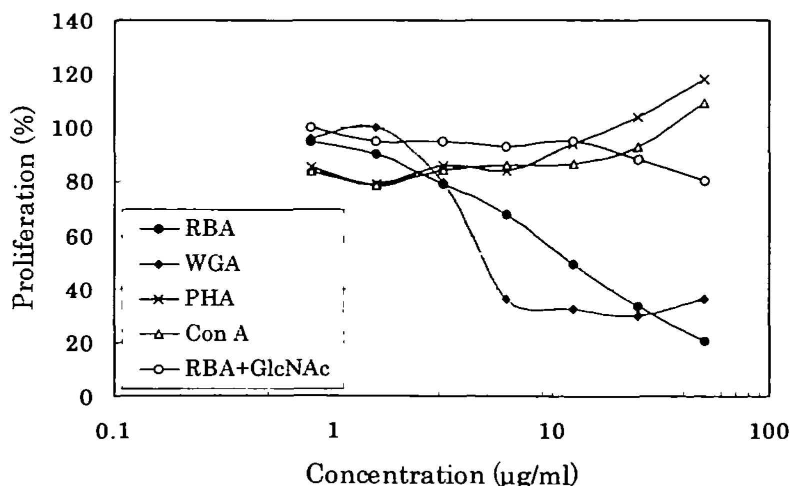

Fig. 1. **Effects of lectins on the growth of U937 cells.** RBA, WGA, or PHA dissolved in culture medium (100 μl) at various concentrations was added to 100 μl of a suspension of 2×10^4 cells in culture medium. After incubation at 37°C for 42 h, an aliquot of an Alamer Blue solution (20 μl) was added and fluorescence was determined after 2 h with excitation at 560 nm and emission at 590 nm. Each point represents the average of three determinations. The data include the results of experiments conducted with a hapten saccharide *N*-acetylglucosamine at 50 μM (○).

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Con A and PHA (Fig. 1).

The growth inhibition caused by RBA was prevented to a large extent in the presence of 50 μM *N*-acetylglucosamine (Fig. 1). Higher concentrations (100 and 200 μM) of *N*-acetylglucosamine caused cell growth inhibition themselves. When immobilized lectins were used, no inhibition of cell proliferation was observed (not shown).

Similar inhibition of the proliferation of human stomach cancer MKN-45 cells was observed with IC_{50} values of 42 and 30 μg/ml for RBA and WGA, respectively.

Chromatin Condensation—Hoechst 33342 staining of U937 cells treated with RBA, WGA, or VAA revealed chromatin condensation, one of the characteristic features of apoptosis (*21*) (Fig. 2). These lectins also induced chromatin condensation in MKN-45 cells (Fig. 3).

DNA Ladder Formation—When U937 cells were incubated with RBA for 17 h, DNA fragmentation in a nucleosome unit was induced (Figs. 4 and 5). Con A and PHA did not induce such DNA fragmentation (Fig. 4), while VAA and WGA induced DNA ladder formation (Fig. 5). The general caspase inhibitor Z-Asp-CH_2-DCB blocked the DNA fragmentation caused by VAA, WGA, or RBA (Fig. 5). These lectins were also found to induce DNA fragmentation in MKN-45 cells (not shown).

Externalization of Phosphatidylserine—Flow cytometric analysis revealed that the treatment with RBA, WGA, or VAA causes a time-dependent exposure of phosphatidylserine to the cell surfaces of U937 cells (Fig. 6). Externalization of this phospholipid is one of the characteristic

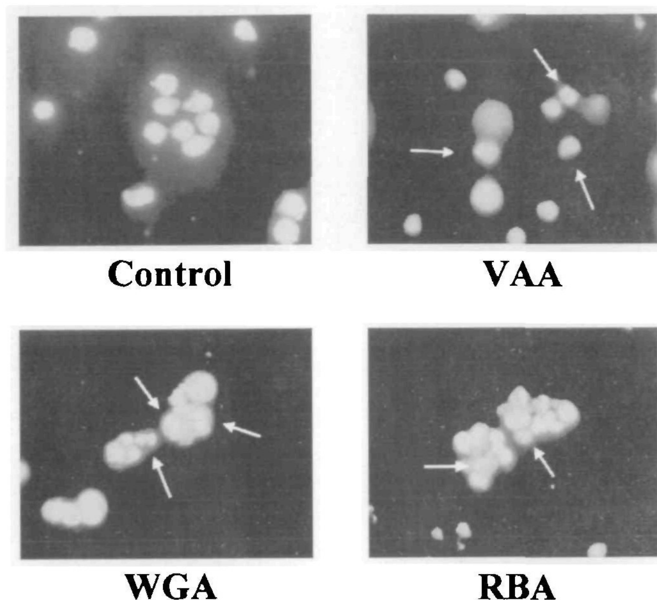

Fig. 2. **Chromatin condensation of lectin-treated U937 cells.** U937 cells untreated (control) or treated with VAA at 5 μg/ml, WGA at 25 μg/ml, or RBA at 25 μg/ml for 17 h were stained with Hoechst 33342 and examined by fluorescence microscopy. Cells with chromatin condensation are indicated by arrows.

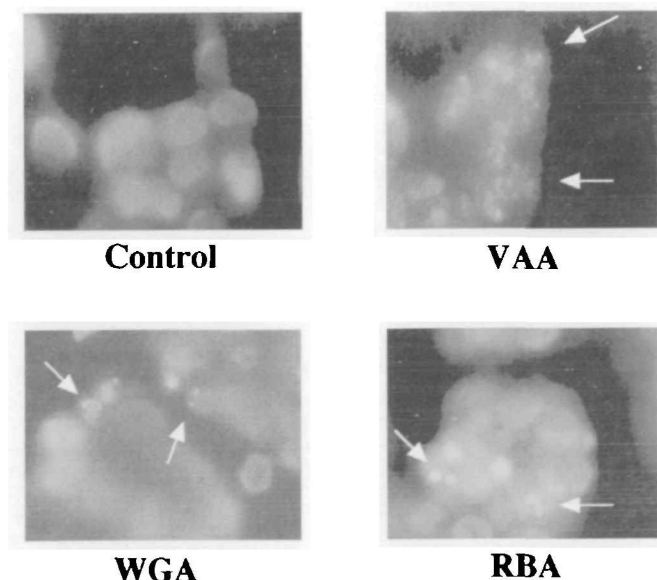

Fig. 3. **Chromatin condensation of lectin-treated MKN-45 cells.** MKN-45 cells untreated (control) or treated with VAA at 5 μg/ml, WGA at 25 μg/ml, or RBA at 25 μg/ml for 17 h were stained with Hoechst 33342 and examined by fluorescence microscopy. Cells with chromatin condensation are indicated by arrows.

Fig. 4. **DNA fragmentation in lectin-treated cells.** U937 cells were untreated (control, lane 1) or treated with PHA (lane 2), Con A (lane 3), or RBA (lane 4) each at 25 μg/ml for 17 h. Extracted DNA was subjected to 2% agarose gel electrophoresis, stained with CYBR Green I, and analyzed using a FluoroImager.

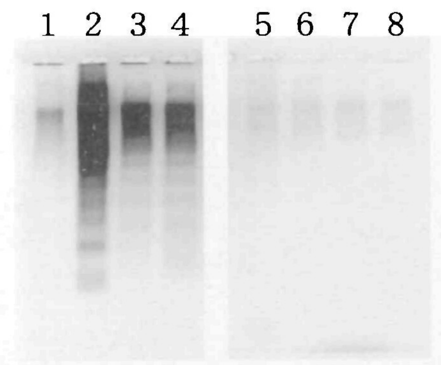

Fig. 5. **DNA fragmentation in lectin-treated cells and its inhibition by a caspase inhibitor.** U937 cells were untreated (control, lanes 1 and 5) or treated with VAA at 5 μg/ml (lanes 2 and 6), WGA at 25 μg/ml (lanes 3 and 7), or RBA at 50 μg/ml (lanes 4 and 8) for 17 h in the absence (lanes 1–4) or presence (lanes 5–8) of a caspase inhibitor, Z-Asp-CH_2-DCB. DNA was analyzed as described in the legend to Fig. 4.

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	0 h	2 h	4 h	6 h
VAA	Mean X: 0.552	Mean X: 0.741	Mean X: 8.88	Mean X: 43.8
WGA	Mean X: 0.87	Mean X: 4.63	Mean X: 5.33	Mean X: 5.37
RBA	Mean X: 0.853	Mean X: 2.71	Mean X: 3.38	Mean X: 4.28

Fig. 6. **Flow cytometric analysis of phosphatidylserine dislocation to the outer membrane.** U937 cells treated with lectins for various time periods were fixed in ethanol, stained with annexin V conjugated with fluorescein isothiocyanate, and analyzed using a flow cytometer.

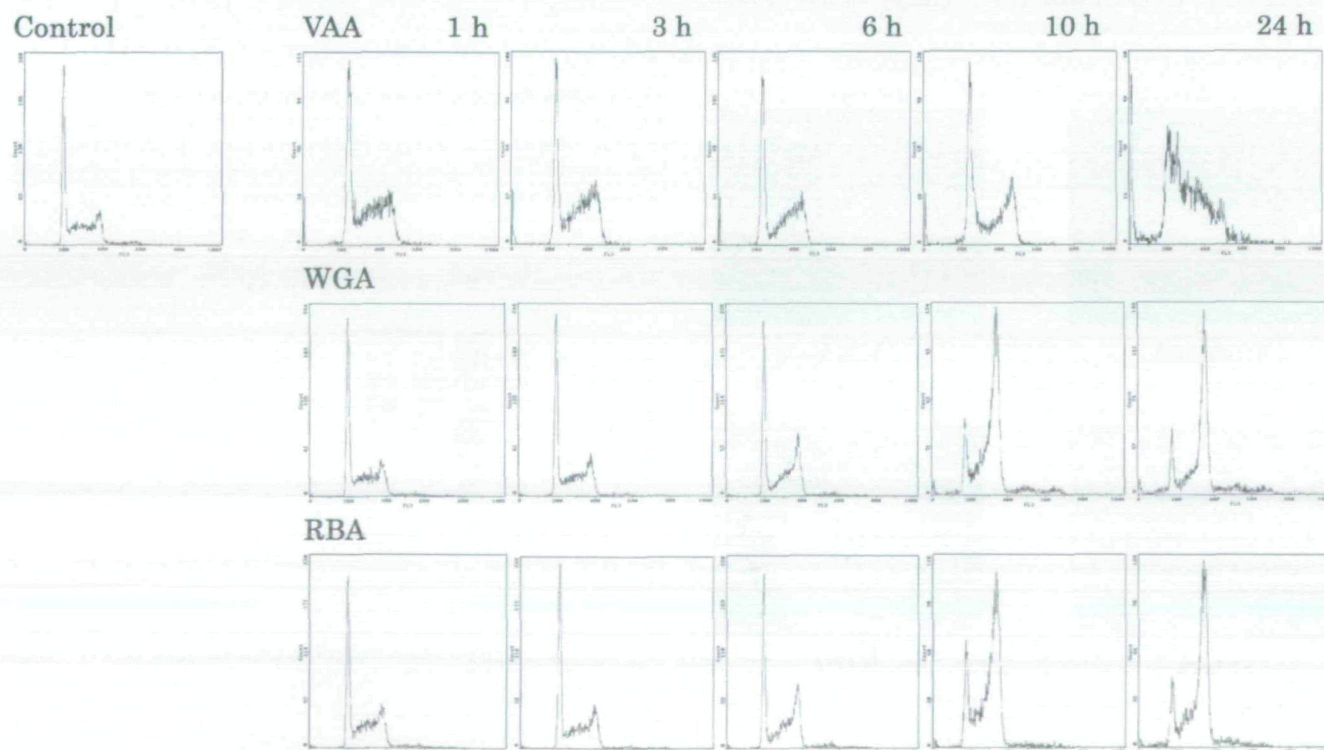

Fig. 7. **Effects of lectins on the cell cycle progression of U937 cells.** U937 cells untreated (control) or treated with VAA at 10 ng/ml, WGA at 25 μg/ml, or RBA at 50 μg/ml for various time periods were stained with propidium iodide and analyzed using a flow cytometer.

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TABLE I. **Cell cycle phase distribution (%) of U937 cells treated with individual lectins for various time periods.**[a]

Lectin	Phase	1 (h)	3 (h)	6 (h)	10 (h)	24 (h)
VAA	G0/G1	45.0	41.6	46.9	48.3	50.8
	S	30.1	32.4	29.6	19.1	31.7
	G2/M	24.9	26.0	23.5	32.6	17.5
WGA	G0/G1	50.7	49.8	43.3	16.6	15.0
	S	28.3	27.8	26.4	24.1	15.3
	G2/M	21.0	22.4	30.3	59.3	69.7
RBA	G0/G1	45.5	46.4	41.5	20.9	15.2
	S	31.4	26.8	29.2	27.3	20.6
	G2/M	23.1	26.8	29.3	51.8	64.2

[a]Untreated cells are 46.6% in G0/G1, 31.4% in S, and 22.0% in G2/M phases.

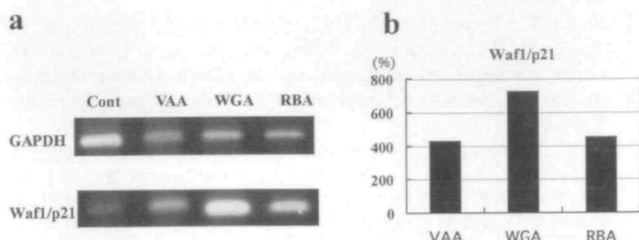

Fig. 8. **Effects of lectins on the expression of the mRNA for Wafl/p21.** RNA was extracted from U937 cells untreated (control) or treated with VAA at 5 μg/ml, WGA at 25 μg/ml, or RBA at 50 μg/ml for 17 h, and RT-PCR was carried out. DNA separated in 2% agarose gels was detected by staining with SYBR Green I and using a FluoroImager (a). The results are normalized using data for GAPDH and are expressed as the value relative to that for control (100%) in b.

features of apoptosis (*19*).

Cell Cycle Arrest—Flow cytometric analysis of U937 cells exposed to lectins indicated that RBA and WGA arrest cells in G2/M phase, unlike VAA, which shows no distinct arrest (Fig. 7 and Table I).

Expression of Waf1/p21—The results of RT-PCR suggest a marked elevation of the mRNA for Wafl/p21 upon exposure of U937 cells to lectins (Fig. 8). Western blotting analysis showed that these lectins induce the enhanced expression of WAF1/p21 at the protein level as well (Fig. 9).

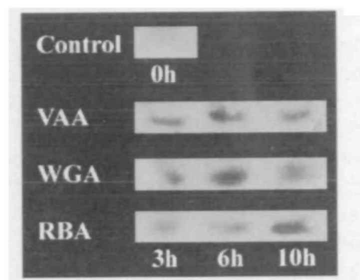

Fig. 9. **Effects of lectins on Wafl/p21 protein expression.** Cell lysates were prepared from U937 cells treated with VAA (5 μg/ml), WGA (25 μg/ml), or RBA (50 μg/ml) for various time periods. Samples containing 100 μg protein were subjected to Western blotting analysis using monoclonal antibody against Wafl/p21 in combination with peroxidase-conjugated anti-mouse IgG, and detected by chemiluminescence.

DISCUSSION

Several anti-tumor drugs are known to induce apoptosis in cancerous cells and apoptosis is considered to be a primary mechanism for their action (*22, 23*). Several lectins are now known to induce apoptosis (*2–8*). On the basis of the apoptosis-inducing activity of mistletoe lectins, Schumacher *et al.* (*24*) reported that recombinant mistletoe lectin is successful in treating human ovarian cancer cells transplanted into severe combined immunodeficient mice, and suggested its clinical application with post-operative instillation of the lectin in ovarian cancer patients. Thus, lectins with apoptosis-inducing activity may have potential as anti-cancer agents.

In the present study, we examined the anti-proliferation activity of RBA against human leukemia U937 cells and obtained several lines of evidence that this growth inhibition is caused by an apoptosis-inducing activity. We observed that RBA induces chromatin condensation, DNA ladder formation, and externalization of membrane phosphatidylserine. These are the characteristic features of apoptosis (*17, 19, 21*). We also found that the DNA ladder formation is inhibited by the general caspase inhibitor Z-Asp-CH_2-DCB (*18*). Caspases are known to play essential roles in apoptosis (*25, 26*). These data support the idea that RBA induces apoptosis in U937 cells. The present results for WGA and VAA are consistent with the previous findings that these lectins have apoptosis-inducing activity (*2, 3, 6–8*).

RBA is an *N*-acetylglucosamine-specific lectin. As expected, apoptosis induction by RBA is largely inhibited by 50 μM *N*-acetylglucosamine, suggesting the involvement of an interaction between RBA and cell surface glycoconjugates in the mechanism. Immobilized RBA as well as immobilized WGA fail to induce apoptosis in U937 cells, suggesting that lectin internalization is required to cause apoptosis, as has been proposed for *Gliffonia simplicifolia* 1-B_4 lectin (*2*).

Although RBA and WGA are both *N*-acetylglucosamine-specific lectins, RBA has a somewhat different carbohydrate specificity from WGA in that WGA exhibits specificity toward *N*-acetylneuraminic acid (*11*). Schwarz *et al.* (*3*) have reported that WGA-mediated cytotoxicity in pancreatic cancer cells involves high levels of WGA membrane binding, primarily to sialic acid residues. Thus, the trigger membrane glycoprotein bound by RBA to initiate apoptosis may be different from that for WGA.

An intimate association between apoptosis and cell cycle arrest has well been documented (*27–31*). For example, epigallocatechin gallate, a major constituent of green tea, has been shown to cause G0/G1 cell cycle arrest, which is considered to be responsible for the induction of apoptosis (*27*). We found here that RBA and WGA cause arrest at G2/M in the cell cycle progression of U937 cells. The present finding for WGA may be related to a report describing that the growth of the intestinal parasite *Giardia lamblia* is inhibited by WGA in association with G2/M phase arrest (*32*). In contrast to RBA and WGA, VAA does not induce cell cycle arrest at any phase. This finding is consistent with a recent report by Lyu *et al.* showing apoptosis induction in acute promyeloid leukemic HL-60 cells by Korean mistletoe lectins without cell cycle arrest (*8*).

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In the present study, we found that RBA- or WGA-induced G2/M arrest is associated with an up-regulation of Wafl/p21 expression. Wafl/p21 is an inhibitor of kinase activities of cyclin/ cyclin dependent kinase complexes, and is known to regulate at both the G1/S and the G2/M cell cycle transitions (*33*). Therefore, its enhanced expression would cause cell cycle arrest at G1, S, or G2/M phase.

There are several reports describing the association of G2/M arrest with Wafl/p21 induction. These include apoptosis induced by genistein (*28, 34*) and by anticancer quinones (*29*). In the case of HEK-293 cells, calcitonin receptor-mediated cell growth suppression has been shown to be accompanied by an induction of Wafl/p21 and G2/M arrest, but not due to apoptosis induction (*35*).

Con A has been shown to inhibit the growth of murine T-lymphoma EL4 cells due to cell cycle arrest at both the G1 and the G2/M phases (*36*). In this case, however, no increase in the level of Wafl/p21 was observed.

In addition to leukemic U937 cells, RBA induces apoptosis in human stomach cancer MKN-45 cells. Plant lectins are often highly resistant to degradation by gastric acid and intestinal enzymes, and can be recovered from faeces in an active form (*2*). RBA is highly resistant to heat denaturation and to proteolytic enzymes, suggesting that it is biologically active even after passage through the gastrointestinal tract (*10*). Future work is required to know whether or not ingested RBA can suppress animal and human cancers, especially gastrointestinal cancers, by inducing apoptosis.

Besides the apoptosis-inducing activities of plant lectins, it has recently been reported that a chicken lactose lectin-I induces apoptosis in lymphocytes, suggesting immunomodulatory properties of animal lectins (*37*). It would be interesting to examine whether the apoptosis-inducing activity of animal lectins plays a role in the regulation of cancer cells *in vivo*.

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