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A novel apoptotic pathway as defined by lectin cellular initiation☆,☆☆

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Abstract

In this study of lectin-induced apoptosis we found that wheat germ agglutinin (WGA) initiated an accelerated type of programmed cell death developing after only 30 min of incubation with tumor cells. To analyze possible mechanisms, studies were focused using the WGA lectin whose carbohydrate specificity is well defined. We found that WGA could induce apoptosis by binding to either *N*-acetylneuraminic acid or *N*-acetylglucosamine (GlcNAc) on the cell surface of normal and malignant cells. We also showed that it is unlikely that WGA triggers apoptosis by binding to the carbohydrate portion of Fas. *CrmA* gene transfection did not inhibit WGA-mediated apoptosis of Jurkat cells. In addition, Jurkat-R cells selected for resistance to Fas signaled apoptosis manifested high sensitivity to WGA as did Fas-negative BL6 melanoma cells. WGA-induced apoptosis is also caspase-3-independent and was found to be triggered via a mitochondrial pathway. WGA induced a loss of transmembrane potential, disruption of the inner mitochondria membrane, and release of cytochrome *c* and caspase-9 activation after 30 min of cell interaction. Interestingly, *Bcl-2* gene transfection did not affect sensitivity of Jurkat cells to WGA. The Jurkat-R subline that has been shown to be Bax and Bak deficient and resistant to various apoptotic signals was highly sensitive to WGA-induced apoptosis. In summary, WGA triggers a unique pattern of apoptosis that is extremely fast, Fas- and caspase-3-independent, and is mediated via a mitochondrial pathway. However, its mitochondrial component is unrestrained by the loss of Bax and Bak or the upregulation of Bcl-2 expression.

Keywords: Apoptosis; Caspases; Mitochondrial changes; WGA; Lectin–carbohydrate interactions

Lectins are proteins or glycoproteins that are able to recognize and bind specific carbohydrates. This property of lectins has been widely used for analysis of the cell surface carbohydrates of normal and malignant cells [1–3]. A number of studies have shown that lectin binding to cell surface carbohydrates triggers various biological effects. Some lectins such as PHA, ConA are able to stimulate T lymphocyte proliferation. These lectins bind to the carbohydrate portion of the T cell receptor, leading to its crosslinking, activation, and stimulation of lymphokine production and T cell proliferation [4]. Some lectins, such as wheat germ agglutinin (WGA), ricin, abrin, mistletoe, LCA, $GS1B_4$ as well as ConA and PHA, are highly cytotoxic and are able to kill normal or malignant cells at relatively low concentrations [1,5–7]. Binding of these lectins to specific oligosaccharides on cell membranes is an important step in lectin-mediated cell killing. Cells that have lost these carbohydrates become resistant to the cytotoxic effect of these lectins. Selection of cells resistant to the cytotoxic effect of lectins was found to be a useful experimental approach for investigation of the oligosaccharide biosynthesis, intracellular transport, and stability of glycoproteins as well as their role in cell-to-cell interactions [2]. Lectin resistant tumor cell variants serve as a useful tool for investigation of the role of cell surface carbohydrates in tumor growth and metastasis formation [2,3,8,9]. It was shown through these models that the cytotoxicity of the lectin is mediated via induction of apoptosis [5–7,10].

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☆☆ *Abbreviations:* WGA, wheat germ agglutinin; sWGA, succinylated WGA; GlcNAc, *N*-acetylglucosamine.
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The mechanisms of apoptotic effects of lectins remain mostly unknown. It was found that abrin, ricin, and mistletoe lectin consist of A and B chains joined by disulfide bonds. The B chain that has high affinity to galactose or GlcNAc residues helps lectin binding to the cell surface and its internalization. The internalized A chain interacts with 60S ribosomal subunits and inhibits protein synthesis [9]. It is possible that chain A induces apoptosis. Cell resistance to the ricin and abrin was associated with a loss or masking of Gal/GlcNAc by sialic acid but showed no resistance to A chain mediated inhibition of protein synthesis [8,10,11]. In contrast to these lectins, WGA contains two identical chains, both of which have high affinity to GlcNAc with the dimer (GlcNAc)2 and trimer (GlcNAc)3 having 13 and 3700 times, respectively, higher affinity than the monomer. WGA has lower affinity to *N*-acetylneuraminic acid (sialic acid) [12].

In the present study we found that WGA lectins-induced apoptosis after short (30 min) incubation with tumor cells. The mechanisms of such accelerated apoptosis remain unknown. We previously found that WGA binding to cell surface carbohydrates is crucial for its apoptotic effect [5]. Tumor cells that were selected for resistance to WGA showed reduction in expression of sialic acid and/or Gal/GlcNAc [2]. It is possible that WGA could bind to the glycosylated portion of Fas or other death receptors, leading to their activation and transduction of the apoptotic signaling. The results of our studies indicate that WGA-mediated apoptosis is unique: it is extremely fast, independent of the Fas pathway and mediated via a mitochondria pathway but does not depend on the function of Bcl-2, Bax, and Bak.

Materials and methods

Tumor cell lines. Jurkat cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA). This Jurkat cell line is highly sensitive to Fas-mediated lysis and hereafter is referred to as Jurkat sensitive (Jurkat-S). The Jurkat cell subline resistant to Fas-mediated lysis (Jurkat-R) was previously selected for resistance to the agonistic anti-Fas mAb [13]. The Jurkat-S cell sublines transfected with the *CrmA* and *bcl-2* genes were provided by Dr. Daniel Johnson (University of Pittsburgh Cancer Institute) and have been previously described [14]. Caspase-3 deficient MCF-7 human breast tumor cell line and Fas negative B16F10BL6 (BL6) melanoma sublines were also used. All cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, streptamycin (50 μg/ml), and penicillin (50 U/ml) (Gibco-BRL, Grand Island, NY).

Reagents. WGA, succinyl WGA (sWGA), WGA-biotin, and sWGA-biotin were purchased from Vector Laboratories (Burlingame, CA). Recombinant caspases, anti-caspase-3 antibody, fluorogenic substrates Ac-YVAD-AMC, Ac-DEVD-AMC, Ac-VEID-AFC, and Ac-IETD-AFC, fluorescein isothiocyanate-annexin V, and propidium iodide (PI) were from BD Pharmingen (San Diego, CA). Vibrant Apoptosis Assay Kit #4 (YO-PRO-1 and PI), chloromethyl X-rosamine (CMXRos), nonylacridine orange (NAO), and hydroethidine (HE) have been purchased from Molecular Probes (Eugene, OR). FITC-VAD-FMK was from Promega (Madison, WI).

Lectin-induced apoptosis. Cells were incubated with WGA (10 μg/ml) for different time points, washed to remove unbound WGA, and then stained with FITC-Annexin V and PI or with YO-PRO-1 and PI according to the manufacturer's protocols. Briefly, after incubation with WGA, cells were washed and resuspended in a Binding Buffer, Annexin V-FITC (5 μl) and PI (10 μl) were added and cells were analyzed by flow cytometry. YO-PRO-1 apoptotic kit was used to exclude the possible binding of WGA to Annexin V and to confirm the results of WGA lectin-induced apoptosis. Cells were resuspended in PBS (0.5 ml), and 5 μl of the YO-PRO-1 diluted 1:10 and 5 μl of PI was added. Cells were incubated on ice for 30 min and analyzed by flow cytometry, measuring the fluorescence emission at 530 nm (FL1) and >575 nm for FL3.

DNA fragmentation assay. Tumor cells were incubated with WGA (10 μg/ml) for 16 h. After washing cells were lysed and DNA was phenol extracted and precipitated as described [5]. After incubation with ribonuclease A, DNA was extracted and precipitated again. The dissolved DNA (15 μg) of each sample was analyzed by electrophoresis on 1.2% agarose slab gel containing 0.2 μg/ml ethidium bromide. A preparation of *Hae*II-digested ϕX174 DNA was run as a marker.

Analysis of the mitochondrial changes in WGA-treated cells. Loss of the transmembrane potential ($\Delta\psi_m$) in WGA-treated cells was evaluated using the cationic lipophilic fluorochrome CMXRos [15]. Cells were incubated with WGA at 37 °C for 30 min–4 h, washed and incubated with the CMXRos (100 nM) for additional 30 min, and then analyzed by flow cytometry. To test the specificity of $\Delta\psi_m$ changes detected by CMXRos, WGA-treated cells were incubated with the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (50 M) for 10 min and then for 30 min in the presence of CMXRos (100 nM) [15]. Cell fluorescence was analyzed in FL-3 (wavelength 630 ± 22 nm). Nonylacridine orange (NAO) interacts with non-oxidized cardiolipin that exclusively localized in the inner mitochondrial membrane [16]. To test WGA-induced changes in the inner mitochondrial membrane, WGA-treated cells were incubated with 0.1 μM NAO for 15 min at 37 °C and level of fluorescence in FL-1 (wavelength 530 ± 30 nm) was analyzed by flow cytometry.

Analysis of WGA and sWGA binding to tumor cells. Jurkat cells were incubated with 10–30 μg of biotinylated WGA or sWGA for 30 min at 4 °C. After washing, cells were incubated with avidin-PE for additional 30 min at 4 °C. Cells were washed and fixed in 2% paraformaldehyde and analyzed by flow cytometry [17].

Caspase activity. Caspase activity in WGA-treated cells was analyzed using the fluorogenic substrates for caspase-1 (Ac-YVAD-AMC), caspase-3 (Ac-DEVD-AMC), caspase-6 (Ac-VEID-AFC), and caspase-8 (Ac-IETD-AFC). Jurkat cells were incubated with WGA (10 μg/ml) for different time periods at 37 °C. Cells were lysed and lysates were centrifuged at 10,000*g* for 10 min at 4 °C. Cytosolic fraction was mixed with the corresponding caspase substrate in the presence or absence of a caspase inhibitor z-VAD-FMK and incubated for 1 h at 37 °C. The level of liberated fluorogenic AMC was detected using fluorescent spectrophotometry (UV excitation of 380 nm and an emission wavelength of 430–460 nm (peak at 440 nm)). For AFC detection UV excitation was 400 nm and an emission wavelength was 480–520 nm (peak at 505 nm).

Western blot analysis. Jurkat cells were treated with WGA or VP-16 for 30 min, 2 h or 3 h. Cells were lysed in 0.5% NP-40, 10 mM Hepes, pH 7.4, 142 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml aprotinin. Proteins were resolved by SDS/PAGE and transferred to PVDF membranes, as previously described [14,18]. Following probing with a specific primary Ab and horseradish peroxidase-conjugated secondary Ab, the protein bands were detected by enhanced chemiluminescence (Pierce, Rockford, IL). The same membrane was reused after stripping to stain with anti-caspase 2, 3, 7, 8, and 9 antibodies. Rabbit anti-caspase-3 Ab, anti-caspase-2 mAb clone 35, anti-caspase-7 mAb clone B94-1 were from BD-PharMingen (San Diego, CA); Rabbit anti-caspase-9 specific for p35 (H-170) was from Santa Cruz.

Bak expression in Jurkat cells. Jurkat-S and Jurkat-R cells were incubated in 0.5% NP-40 lysis buffer for 30 min at 4 °C. The resultant lysates that contained both cytoplasm and mitochondria were resolved by SDS/PAGE and assessed by immunoblotting for the presence of Bak. Three different anti-human Bak Abs were used for blotting. The membranes were stripped and reprobed for β-actin to demonstrate equal loading. Anti-human Bak Abs were from Oncogene (Boston, MA, Ab-1, mouse clone AMO3, generated against recombinant BakΔC), Serotec (Oxford, UK, Ab-2, mouse mAb TC100, generated against recombinant BakΔC), and Santa Cruz (Santa Cruz, CA), Ab-3, goat Ab generated against synthetic peptide mapping within an internal region of 87 human Bak). Anti-Fas Ab CH-11 was purchased from Upstate Biotechnology (Lake Placid, NY); Anti-β-actin mAb clone AC-15 was from Sigma (St. Louis, MO).

Cytochrome c release assay. WGA-treated Jurkat cells were incubated in isotonic mitochondria buffer (20 mM sucrose, 20 mM Hepes, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 10 μg/ml leupeptin and aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Cells were disrupted by Dounce homogenization. Nuclei and debris were removed by 5 min centrifugation at 650*g*. The supernatant was centrifuged at 10,000*g* for 30 min at 4 °C to obtain the heavy membrane pellet (HM) and the resulted supernatant was further centrifugated at 100,000*g* for 1 h to yield a soluble cytosolic fraction S-100. Presence of cytochrome *c* in cytosolic fraction and HM was determined by Western blot analysis using anti-cytochrome *c* antibody (Pharmingen) as was described previously [19].

Results

WGA lectin cell surface binding and induction of apoptosis

To assess the apoptotic effects of WGA lectins, Jurkat-S cells were incubated with 10 μg/ml WGA for 30 min or 3 h, washed and stained with PI and annexin V-FITC. After 30 min of WGA treatments a substantial number (39%) of Jurkat cells were found to be annexin V positive cells and their proportion further increased up to 52% after 3 h of incubation with the lectin (Fig. 1A). In contrast, VP-16 (20 μM) showed no increase in apoptotic cells after 30 min and even after 3 h only slight increase in annexin positive apoptotic cells (up to 10%) was observed (Fig. 1A). VP-16 (20 μM) induced apoptosis in a high proportion (about 40%) of Jurkat-S cells only after 4 h of Jurkat-S cells treatment. Similarly, staurosporin and anti-Fas antibody triggered apoptosis in Jurkat cells only after 4 h of incubation (data not shown). These results indicate that WGA lectins have a unique ability of very fast triggering of

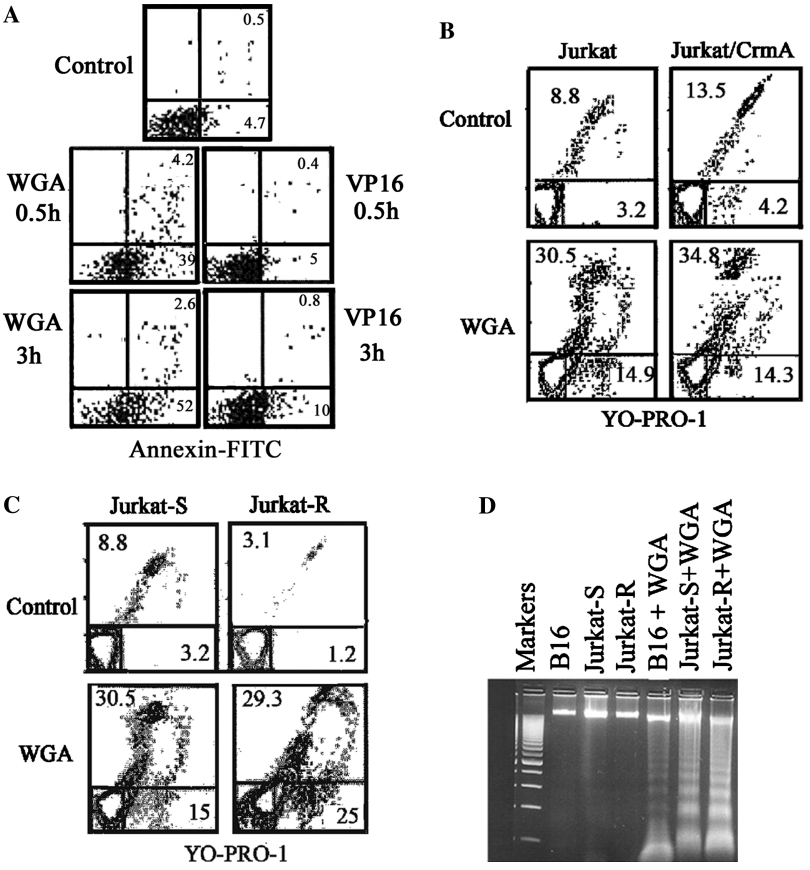

Fig. 1. Apoptotic effects of WGA lectins. (A) Jurkat-S cells (1×10^6) were incubated with WGA (10 μg/ml) for 30 min or 3 h. In parallel, Jurkat-S cells were treated with VP16 (20 μM). Cells were washed, stained with annexin V-FITC and PI, and analyzed by flow cytometry. The numbers indicate the percentage of Annexin V-FITC and PI positive cells. Jurkat-S and Jurkat-S cells transfected with the *CrmA* gene (B) or Jurkat cells that were selected for resistance to anti-Fas mAb (C) were incubated with WGA (10 μg/ml) for 30 min at 37 °C, washed and stained with YO-PRO-1 and PI. The percentage of YO-PRO-1 and YO-PRO-1 and PI positive cells is indicated. (D) B16 melanoma, Jurkat-S, and Jurkat-R cells were incubated with WGA (10 g/ml) for 16 h, DNA was isolated, and DNA fragmentation in tumor cells was analyzed on 1.25% agarose gel.

apoptotic changes. The rapid increase in annexin V positive cells after lectin treatment cannot be attributed to annexin V crossbinding to WGA. First, we did not detect binding of annexin V to WGA-conjugated beads (data not shown). Second, similar apoptotic effects of WGA were observed when another dye, YO-PRO-1, was used for testing their apoptotic effects. Annexin V binds to the exposed cell membrane phospholipid phosphatidylserine on apoptotic cells, whereas YO-PRO-1 is able to penetrate the cell membrane of apoptotic, but not normal, cells and binds to DNA, staining apoptotic cells green [20]. Using the YO-PRO-1 kit we found that apoptotic changes in tumor cells were also induced shortly (30 min) after cell incubation with WGA lectin (see Figs. 1B and C).

Lectins induce apoptosis by binding to the carbohydrate portion of cell surface glycoproteins or glycolipids. Prevention of their binding to cell surface by the haptenic sugar (GlNAc or chitin) abrogates their apoptotic effects [5]. It was shown that WGA binds with high affinity to *N*-acetylglycosamine (GlcNAc) residues and with low affinity to sialic acid [12]. Although WGA has low affinity to sialic acid, analysis of cell surface glycoproteins reacting with WGA revealed that the majority of WGA binding glycoproteins consist of sialylated plasma membrane proteins called high-molecular-weight acidic glycoproteins (HMWAG) [21,22]. To test the importance of sialylated glycoconjugates in triggering WGA-mediated apoptosis, we compared the apoptotic activities of WGA and succinylated WGA (sWGA) that are unable to bind sialic acid but bind to GlcNAc [23]. Jurkat cells were incubated with 10 μg WGA or sWGA for 30 min, washed and stained with YO-PRO-1 and PI. Control Jurkat had only 6.5% of apoptotic cells. After 30 min of incubation with sWGA the percentage of apoptotic cells only slight increased up to 9.9%, whereas 37.8% apoptotic cells were found after incubation with WGA. Even after 4 h of incubation of sWGA with Jurkat-S cells no apparent increase in apoptotic cells was found (data not shown).

These results suggest that WGA binding to the sialylated glycoproteins or glycolipids is important for the triggering of apoptosis. Binding of WGA to the cell surface is crucial for its apoptotic effect. It is possible that the differences in the apoptotic effects of WGA and sWGA may be due to the differences in their ability to bind Jurkat cells. Flow cytometric analysis showed that both WGA and sWGA bind to almost all Jurkat cells. However, WGA showed 10 times higher fluorescence than sWGA (data not shown). These differences are in accordance with the ability of WGA to bind both GlcNAc and sialic acid, whereas sWGA binds only GlcNAc containing glycoconjugates. It is possible that the lower binding of sWGA to Jurkat cells is insufficient to trigger apoptotic signaling. It is expected that increasing concentrations of sWGA will be able to induce higher levels of apoptosis. To test this, Jurkat cells were incubated with 30 μg/ml sWGA. Flow cytometric analysis showed that after 30 min of incubation with 30 μg/ml sWGA 53.4% of Jurkat cells were annexinV positive. A similar level of apoptosis (52.4%) was induced by WGA at 3 times lower concentration (10 μg/ml). These results suggest that sWGA (at high enough concentrations) binding to GlcNAc containing glyconjugates is also sufficient to induce apoptosis.

Analysis of the mechanisms of the apoptotic effect of WGA

It remains unclear what glycosylated molecules on cell surface are crucial for WGA-mediated apoptosis. It is possible that WGA triggers apoptosis by binding to the glycosylated portion of Fas or other cell death receptors. Binding of WGA to these receptors might cause their crosslinking and triggering of the apoptotic cascade. If it is the case, it is expected that the apoptotic effect of WGA would be lower in cells in which the Fas-mediated pathway was blocked by CrmA. It was shown that Jurkat cells transfected with the CrmA gene manifest increased resistance to Fas-induced apoptosis [13]. To test this, the parental Jurkat-S cell line and Jurkat-S cells transfected with the *CrmA* gene were incubated with WGA (10 μg/ml) for 30 min. The results of YO-PRO-1 staining indicate that the *CrmA* expression did not change sensitivity of Jurkat-S cells to the apoptotic effects of WGA (Fig. 1B).

When Jurkat-S cells were cultured in the presence of anti-Fas antibody it resulted in selection of Jurkat cells that showed complete resistance to anti-Fas antibody-mediated apoptosis. This subline has been termed Jurkat-R [13]. The selected Jurkat-R cells showed not only resistance to anti-Fas antibody but also became resistant to various apoptotic stimuli, such as UV light, staurosporin, VP-16, bleomycin, and cisplatin that induce apoptosis via mitochondria-mediated pathway [13]. We tested whether Jurkat-R cells will be also resistant to WGA-mediated apoptosis. When Jurkat-R cells were incubated with WGA for 30 min they showed high sensitivity to WGA-induced apoptosis (Fig. 1C). In the majority of experiments the sensitivity of Jurkat-R cells to WGA-mediated apoptosis was even higher than the parental Jurkat-S cells.

We found that B16F10BL6 (BL6) melanoma subline is resistant to anti-Fas mAb and FasL-mediated cytotoxicity. Flow cytometric analysis using anti-Fas mAb revealed that BL6 melanoma cells do not express Fas. These cells are also resistant to TRAIL and TNF-α (data not shown). However, when Fas negative BL6 melanoma cells were incubated with WGA for 1 h the percentage of annexin V positive cells increased from 7% (control) to 31.3%. To analyze the ability of WGA to induce DNA fragmentation, B16 melanoma, Jurkat-S,

and Jurkat-R cells were incubated with WGA (10 μg/ml) and 16 h later DNA was extracted and analyzed on 1.2% agarose gel. DNA from B16 melanoma as well as Jurkat-S and Jurkat-R treated with WGA showed an apparent ladder-like pattern of fragmented DNA (Fig. 1D).

To further investigate the mechanisms of WGA-mediated apoptosis, we tested the ability of WGA to trigger caspase activation. Jurkat cells were incubated with WGA or VP-16 for different time points, cells were lysed and analyzed using Western blot. WGA did not induce activation of caspase-2, -3, -7, and -8 during 0.5–3 h of treatment. WGA induced activation of caspase-9 and its activation was observed as early as 30 min of WGA treatment (Fig. 2A). Thus, activation of caspase-9 coincided with induction of apoptosis by WGA that occurred after 30 min of treatment. In contrast, treatment of Jurkat-S with VP-16 for 0.5-3 h did not induced activation of any tested caspases. Further, Jurkat-S cells that were incubated with WGA for 2–12 h, cells were lysed and cytosolic fractions were prepared. These cytosolic fractions were incubated with the caspase specific fluorogenic substrates. In agreement with our Western blot, no caspase-3 activity was found after 2 h of Jurkat cell incubation with WGA. The ability of WGA-treated Jurkat cells to cleave caspase-3 substrate DEVD-AMC was detected only after 5 h of incubation with WGA (Table 1). The caspase-3 activity in Jurkat cells was further increased following 10–12 h of incubation with WGA. WGA activates caspase-6 and -8 but this activation was found only following 5 h of WGA treatment (Table 1). Since apoptosis-associated membrane changes were found after 30 min of WGA treatment our Western blot and fluorescent substrate analysis suggest that caspases-2, -3, - 6, -7, and -8 are not primarily responsible for the initiation of WGA-induce apoptosis. Early induction of apoptosis coincided only with activation of caspase-9.

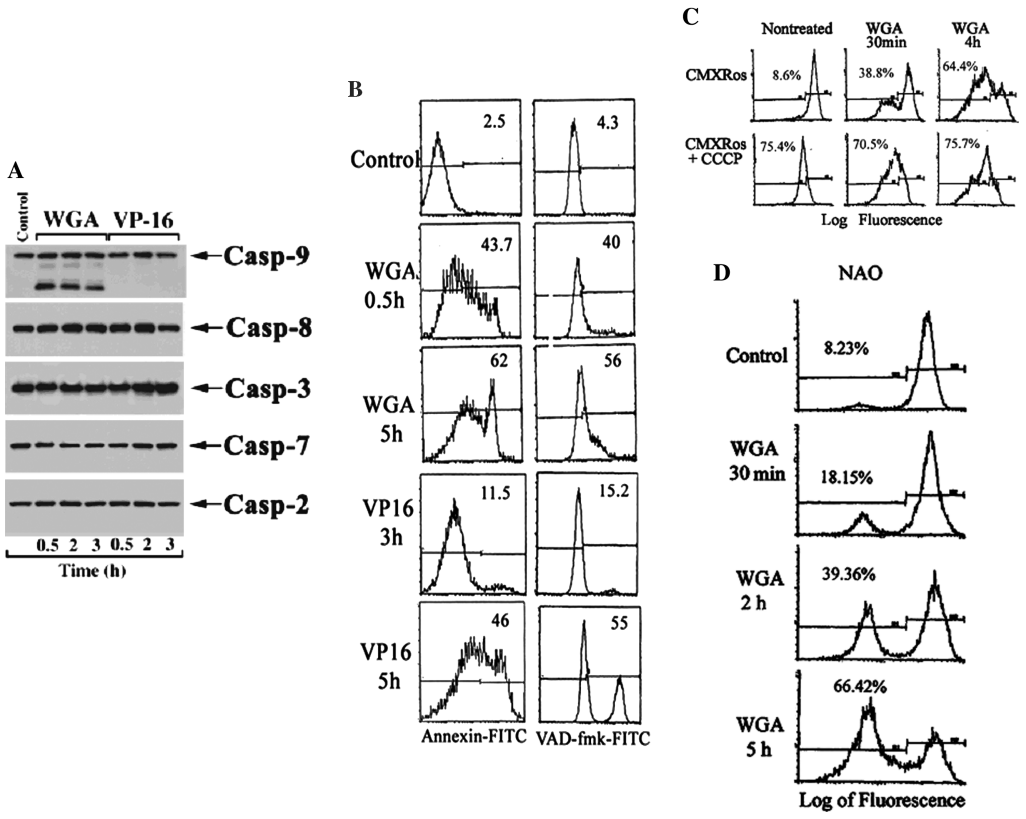

Fig. 2. Analysis of WGA-induced caspase activation and WGA-induced mitochondrial changes in Jurkat-S cells. (A) Jurkat cells were incubated with WGA (10 μg/ml) or VP-16 (20 μM) for 30 min, 2 h or 3 h. Cells were lysed and proteins were resolved by SDS–PAGE, transferred to polyvinylidene difluoride membrane, and stained with a specific primary antibody and horseradish peroxidase-conjugated secondary antibody. The protein bands were detected by enhanced chemiluminescence. (B) The comparative analysis of phosphatidylserine exposure and caspase activation in WGA-treated Jurkat cells. Jurkat-S cells were incubated with WGA (10 μg/ml) for 30 min or 5 h at 37 °C. In parallel, Jurkat-S cells were incubated with VP-16 (20 μM) for 3 or 5 h. Part of cells was stained with annexin V-FITC. Other part of cells was incubated with caspase substrate VAD-fmk-FITC and analyzed by flow cytometry. (C) WGA-induced loss of the mitochondria transmembrane potential ($\Delta\psi_m$). Jurkat-S cells were incubated with WGA for 30 min and 4 h, washed and incubated with CMXRos (100 nM) for additional 30 min. Some cells were preincubated for 10 min with CCCP (50 μM) and then with CMXRos. Cell fluorescence was analyzed in FL3 (wavelength 630 ± 22 nm). (D) WGA-induced changes in the inner mitochondria membrane. Jurkat cells were incubated with WGA for 30 min, 2 h, and 5 h. Cells were washed and incubated with 0.1 μM NAO for 15 min at 37 °C. The level of fluorescence in FL1 was analyzed by flow cytometry.

Table 1
Caspase activation in Jurkat-S cells treated with WGA lectin

Caspase	Substrate	Substrate OD				
		Time of WGA treatment (h)				
		0	2	5	10	12
Caspase 3	DEVD-AMC	175	193	611*	843*	857*
Caspase 6	VEID-AFC	181	155	271*	293*	281*
Caspase 8	IETD-AFC	96	104	159*	174*	174*

Jurkat-S cells were incubated with WGA (10 μg/ml) for 2–12 h. At different time points cells were lysed. The cytosolic fraction was isolated, mixed with the caspase substrate, and incubated for 1 h at 37 °C. The levels of liberated fluorogenic AMC were detected by spectrophotometry using UV excitation of 380 nm and an emission wavelength of 430–460 nm (peak at 440). For detection of liberated AFC UV excitation was 400 nm and an emission wavelength was 480–520 nm (peak at 505 nm).

* Significantly ($p < 0.05$) differs from the control group.

It is believed that apoptotic membrane changes are associated with the activation of caspase-8 and caspase-3 [24]. To further compare the timing of apoptosis and caspase activation, we compared the number of apoptotic cells and total activity of caspases by their ability to cleave VAD-fmk, a broad substrate for various caspases. Jurkat cells were incubated with VP-16 or WGA and after different time point cells were stained with FITC-labeled VAD-fmk substrate. Green cells with the activated caspases were detected by flow cytometric analysis. Jurkat-S cells incubated with VP-16 for 3 h had only small percentage of annexin V positive cells (11.5%) and caspase activation assessed by FITC-VAD was found in 15.2%. After 5 h of treatment with VP-16 the proportion of apoptotic and caspase activated cells substantially increased (46% and 55%, respectively) (Fig. 2B). In WGA-treated Jurkat cells 43.7% cells were annexin V positive after 30 min of treatment. In parallel, cleavage of the FITC-labeled VAD-fmk substrate in these cells was observed in about 40% cells. After 5 h of treatment with WGA, the proportion of annexin V positive and FITC-VAD stained Jurkat cells further increased up to 62% and 56%, respectively (Fig. 2B). It is of note that Jurkat-S cells treated with VP-16 had a very defined peak of cells with cleaved VAD-fmk-FITC, whereas in the WGA-treated cells a broad shift of FITC positive cells was found (Fig. 2B). This might be due to the differences in the profile of caspases activated by VP-16 and WGA in Jurkat-S cells. All data presented above indicate that WGA-mediated apoptosis is caspase-3- and caspase-8-independent. The ability of WGA to induce caspase-3-independent apoptosis was further confirmed in experiments in which WGA was able to induce apoptosis of caspase-3 deficient MCF-7 breast tumor cells (data not shown).

Apoptosis can be induced via mitochondria activation, leading to cytochrome *c* release, complexing of caspase-9 with Apaf-1, and activation of effector caspases, including caspase-3 [24]. Apoptotic changes in mitochondria include the generation of reactive oxygen species (ROS), calcium influx, opening of permeability transition (PT) pores, disruption of the transmembrane potential ($\Delta\psi_m$), and release of cytochrome *c* and other proapoptotic molecules [24]. Our data showed that WGA was able to activate caspase-9 in 30 min after treatment of Jurkat-S cells with WGA (Fig. 2A). To assess the ability of WGA lectin to induce changes in $\Delta\psi_m$, Jurkat cells were incubated with WGA and stained with chloromethyl-X-rosamine (CMXRos). Staining of cells with CMXRos gives bright red fluorescence that is reduced with loss of $\Delta\psi_m$ in the apoptotic cells. The protonophore CCCP causes a dissipation of the proton gradient buildup in the inner mitochondrial membrane and loss of $\Delta\psi_m$ even in the viable cells [15]. We therefore used CCCP to assess the specificity of changes in $\Delta\psi_m$ detected by CMXRos in WGA-treated cells. As shown in Fig. 2C, staining of control WGA untreated Jurkat cells with CMXRos resulted in a very bright red fluorescence that was lost when cells were pretreated with $\Delta\psi_m$ disrupting protonophore CCCP. Jurkat cells that were incubated with WGA for 30 min, washed and stained with CMXRos showed a loss of fluorescence in 38.8% of cells. After 4 h of WGA treatment the majority (64.4%) of cells lost $\Delta\psi_m$ (Fig. 2C).

Nonylacridine orange (NAO) interacts with non-oxidized cardiolipin, which is exclusively localized in the inner mitochondrial membrane [16]. Disruption of the inner mitochondria membrane results in loss of NAO staining. Viable Jurkat cells incubated with NAO showed very bright green fluorescence (Fig. 2D). However, after 30 min of incubation of Jurkat cells with WGA, 18.15% cells showed reduction in cell staining. With increase of incubation with WGA up to 2 and 5 h the percentage of Jurkat cells that lost NAO staining increased up to 39.36% and 66.42%, respectively (Fig. 2D). These results show that WGA induces disruption of the transmembrane potential and opening of permeability transition pores. These mitochondrial changes were similar in Jurkat-S and Jurkat-R cells and were observed shortly after cell incubation with WGA and coincided with the apoptosis-associated plasma membrane changes manifested in annexin V binding.

It was shown that loss of transmembrane potential $\Delta\psi_m$ and increase in permeability transition pores

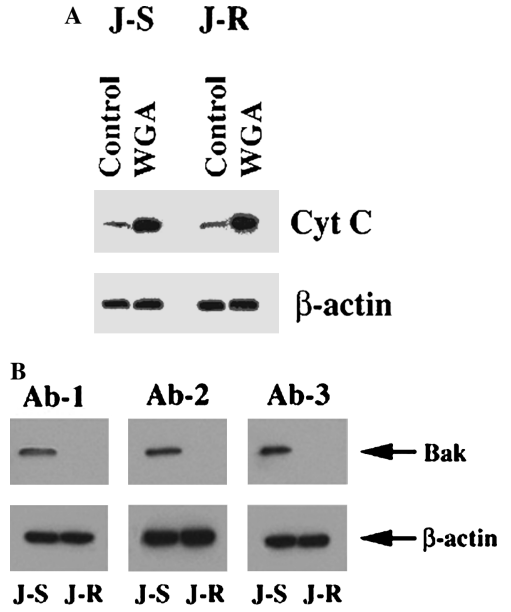

Fig. 3. (A) WGA-induced cytochrome *c* release. Jurkat-S and Jurkat-R cells were incubated with WGA for 30 min. The cytosolic fractions were isolated from WGA-treated and untreated cells and cytochrome *c* in these fractions was determined by Western blot analysis using anti-cytochrome *c* antibody. (B) Bak deficiency in Jurkat-R cells. Jurkat-S and Jurkat-R cells were incubated in 0.5% NP-40 lysis buffer for 30 min at 4 °C. The resultant lysates that contained both cytoplasm and mitochondria were resolved by SDS/PAGE and assessed by immunoblotting for the presence of Bak. Three different anti-human Bak Abs were used for blotting. The membranes were stripped and reprobed for β-actin to demonstrate equal loading.

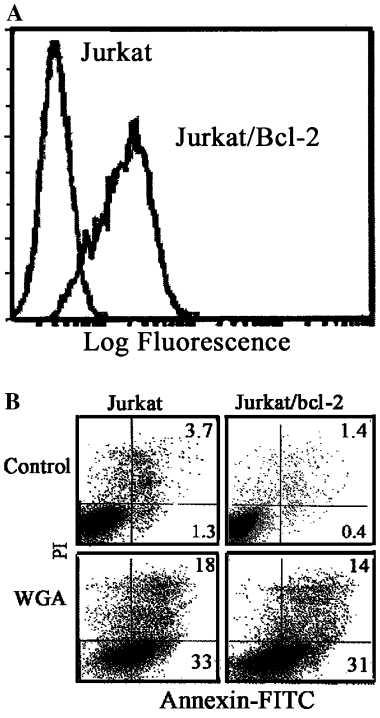

Fig. 4. (A) Bcl-2 expression in Jurkat-S cells transfected with murine *bcl-2* gene. Jurkat-S and Jurkat-S cells transfected with the murine *bcl-2* cDNA were permeabilized and stained with anti-murine bcl-2 monoclonal antibody conjugated with FITC. Cells were analyzed using flow cytometry. (B) Sensitivity of Jurkat-S cells transfected with the *bcl-2* gene to the apoptotic effect of WGA. Jurkat-S cells transfected with the *bcl-2* gene were incubated for 30 min with WGA (10 μg/ml), washed and stained with Annexin V-FITC and PI.

during apoptosis result in the release of cytochrome *c* [25]. We, therefore, tested whether WGA treatment induces cytochrome *c* release. Treatment of Jurkat-S and Jurkat-R cells with WGA for 30 min resulted in a rapid release of cytochrome *c* (Fig. 3A), confirming the involvement of the mitochondrial pathway in WGA-mediated apoptosis.

Jurkat-S cells cultured in the presence of anti-Fas antibody became resistant to anti-Fas antibody-mediated apoptosis. The selected Jurkat-R cells showed not only resistance to anti-Fas antibody but also became resistant to various apoptotic stimuli, such as UV light, staurosporin, VP-16, bleomycin, and cisplatin, that induce apoptosis via mitochondria-mediated pathway [13]. However, Jurkat-R cells were highly sensitive to the apoptotic effects of WGA (see Fig. 1C). The resistance of Jurkat-R cells to UV light, starosporin, VP-16, bleomycin, and cisplatin was found to be associated with Bax and Bak deficiency [26,27]. Wild type Jurkat cells have already been shown to be Bax deficient and this was confirmed in this Jurkat-R cell line [26,27]. Western blot analysis using three different anti-Bak antibodies showed lack of Bak in Jurkat-R cells (Fig. 3B). Since Jurkat cells are highly sensitive to WGA-mediated apoptosis this result suggests that the apoptotic effects of WGA are independent of the presence of Bax or Bak.

It was shown that overexpression of the antiapoptotic Bcl-2 molecules might increase resistance to the apoptotic effects of various stimuli. Indeed, transfection of Jurkat-S cells with the murine *Bcl-2* gene increased their resistance to various drugs inducing mitochondria-mediated apoptosis [14]. We analyzed the level of *Bcl-2* expression in the Bcl-2 transfected Jurkat-S cells. Using anti-murine bcl-2 antibody and flow cytometric analysis of the permebilized Jurkat cells we found that transfected cells express high level of bcl-2 (Fig. 4A). When *Bcl-2* transfected Jurkat-S cells were treated with WGA for 30 min a high level of apoptosis was induced. In this regard, no differences in the sensitivity between the parental and the *bcl*-2 transfected Jurkat cells were observed (Fig. 4B), indicating that Bcl-2 does not control WGA-mediated apoptosis.

Discussion

Our study has shown that WGA lectins are able to induce apoptosis of various tumor cells after a very short time (30 min) of incubation. WGA's ability to bind cell surface carbohydrates is essential for its cytotoxicity and haptenic sugar (GlNAc or chitin) could prevent

binding of WGA and its apoptotic effect. Succinylated WGA (sWGA) that binds GlcNAc but not sialic acid manifested lower apoptotic effects than WGA against Jurkat cells. Similarly, studies with human pancreatic cells showed that sWGA is much less cytotoxic than WGA [28]. The neuraminidase treatment of the pancreatic tumor cells reduced the apoptotic effect of WGA, further supporting the importance of sialic acid in WGA-mediated killing [28]. These results imply that sialylated glycoproteins are important in induction of WGA cytotoxicity. Our findings that sWGA is able to induce apoptosis indicate that GlcNAc containing glycoconjugates are also involved in WGA-mediated apoptotic effects. It remains unclear what particular cell membrane glycoconjugates are of prime importance for the apoptotic effect of WGA. Our studies imply that it is unlikely that WGA induces apoptosis via a Fas-mediated pathway. The expression of the transfected *CrmA* gene in Jurkat cells rendered them resistant to Fas-mediated apoptosis [13]. However, the same cells showed no resistance to WGA-induced apoptosis. In addition, Jurkat-R cell line selected for resistance to Fas-mediated signaling was highly sensitive to WGA-induced apoptosis. Similarly, B16 melanoma cells that do not express detectable levels of Fas and showed resistance to anti-Fas mAb, TRAIL, and TNF were sensitive to WGA-induced apoptosis.

Furthermore, Fas-mediated and WGA-mediated apoptoses have different kinetics. WGA is able to induce apoptosis after 30 min of incubation. In contrast, crosslinking of Fas with the anti-Fas antibody or treatment of Jurkat cells with VP-16, staurosporin, bleomycin, cisplatin or other apoptotic chemicals usually induced similar changes only after 4 h of treatment. Accelerated apoptosis is probably due to very fast binding to the cell membrane and very quick internalization of WGA. It was shown that WGA is internalized via a coated pit-small vesicle pathway, by 3 min WGA reaches vacuoles and endosomes. After 15 min WGA can be found in the *trans*-Golgi network [21,22]. This might indicate that WGA does not trigger apoptosis via cell surface molecules but probably via intracellular signaling following internalization. We found that agarose bead-bound WGA failed to induce apoptosis of Jurkat-S cells. To test whether inhibition of WGA trafficking might affect its apoptotic effect, Jurkat cells were treated with brefeldin A, a potent inhibitor of intracellular protein trafficking from the endoplasmic reticulum to the Golgi [29]. Although brefeldin A has been reported to induce apoptosis after 8 h of treatment [30], its apoptotic effect was not detectable after 1 h of incubation. However, in the several repeatable experiments preincubation of Jurkat-S cells with brefeldin A (10 μg/ml) for 30 min before treatment with WGA (10 μg/ml) enhanced the apoptotic effect of WGA (data not shown).

An increasing body of evidence indicates that accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER) might induce ER stress and trigger apoptosis. It was shown that glucose deprivation or treatment of cells with tunicamycin, the inhibitor of protein glycosylation, affects protein glycosylation and proper protein folding in the ER. This results in an induction of CHOP (C/EBP-homologous protein, also known as GADD153) and apoptosis [30]. However, our Western blot analysis using anti-CHOP mAb showed no induction of CHOP in Jurkat cells treated with WGA for 15–240 min (data not shown). Furthermore, RT-PCR showed no message for CHOP/GADD153 in WGA-treated cells (data not shown). These results argue against the possibility that WGA induces apoptosis via induction of ER stress associated CHOP.

Our studies indicate that WGA-mediated apoptosis is mediated via the mitochondrial pathway. Indeed, WGA treatment was associated with changes in inner membrane cardiolipin, increase in permeability transition, release of cytochrome *c*, and activation of caspase-9. The mitochondrial changes in Jurkat cells were induced quickly (30 min after WGA treatment) and coincided with the apoptosis-associated membrane changes. We found activation of caspase-3 and -8 after 5 h of WGA treatment that might be downstream of cytochrome *c* release and caspase-9 activation probably lead to augmentation of the apoptotic process. It is possible that internalized WGA comes into contact with the mitochondria and directly induces a cell death pathway. However, we have not seen this effect when looking at purified mitochondria treated with WGA. These purified mitochondria did not release cytochrome *c* nor SMAC in the presence of WGA (data not shown). We conclude that the mitochondria may be important for the process of cell death as mediated by WGA, however, the initiation mechanisms responsible for this activation need further investigation.

Although WGA-induced apoptosis is mitochondria mediated it shows several unique characteristics. The Jurkat-R cell line that was selected for resistance to Fas-mediated apoptosis manifests resistance to various apoptotic stimuli (UV light, staurosporin, VP-16) that act through disruption of mitochondrial function [13]. This resistance was found to be associated with lack of the proapoptotic Bax and Bak molecules in Jurkat-R cells [13]. However, Jurkat-R cells were highly sensitive to WGA-induced apoptosis, suggesting this apoptosis is Bax and Bak independent. The susceptibility of Jurkat-R cells to WGA suggests that the mitochondrial apoptotic cascade induced by WGA is differently regulated from those that are induced by VP-16, staurosporin or UV light. This conclusion is further supported by the finding that overexpression of the transfected *bcl-2* gene in Jurkat cells significantly blocked VP-16-induced mitochondrial pathway [13], but had no effect on WGA-mediated changes.

In summary, we have found that the apoptotic effects of the WGA lectin are very quick with cell death changes found after only 30 min of treatment. The unique kinetic profile of these agents signified the possibility of a novel pathway to apoptosis. WGA-mediated apoptosis was found to be Fas- and caspase-3-independent and involves mitochondrial apoptotic events. However, its mitochondrial component is Bax and Bak independent and unrestrained by Bcl-2 regulation. Discovering new mechanisms of apoptosis has significant scientific and clinical ramifications. It is clear from these results that WGA-induced programmed cell death offers a model to study such a unique and previously untested pathway to apoptosis.

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