Journal of Cellular Biochemistry 91:1159–1173 (2004)

Wheat Germ Lectin Induces G_2/M Arrest in Mouse L929 Fibroblasts

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Abstract Wheat germ lectin (WGA) is a cytotoxic lectin for many cell lines [Wang et al., 2000], but its underlying mechanism is not clear. In this report, we found that incubation of synchronized mouse L929 fibroblasts with WGA resulted in a dose-dependent reduction of intracellular incorporation of ^{3}H-thymidine and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide)-conversion activity ($IC_{50} \cong 0.4$ μM). Fluorescein-conjugated WGA was demonstrated to transport from the cell surface into the paranuclear region of cultured L929 cells within 30 min, and subsequently evoked lipid peroxidation of plasma membrane and vacuolation in the cytoplasm of these cells. Studies with tritiated thymidine incorporation, immunofluorescence microscopy, immunoblotting analysis and flow cytometry revealed that WGA inhibited cell cycle progression after one replication, resulting in G_2/M arrest and alteration of cell cycle regulatory proteins, particularly activation of $p21^{Cip1/WAF1}$ and suppression of cyclin B and cdc 2. Although there was an increase of cytosolic caspase 3 and bax protein expression, no apoptotic bodies were observed by both fluorescence and transmission electron microscopy. These results suggest that WGA arrested L929 proliferation after one cell cycle in the G_2/M phase through activation of the $p21^{Cip1/WAF1}$ and suppression of Cyclin B–Cdc2. J. Cell. Biochem. 91: 1159–1173, 2004.

Key words: wheat germ lectin; L929 fibroblast cell line; cyclin kinase inhibitor; growth arrest

Lectins belong to a class of proteins which bind to a specific group of carbohydrates and which also agglutinate erythrocytes. They are present in plants, animals and microorganisms, and certain lectins have wide biomedical applications. Wheat germ lectin (WGA) is one of the extensively studied plant lectins, partly because of its high stability and partly because of its binding specificity for two groups of carbohydrates, *N*-acetylglucosamine (GlcNAc) and *N*-acetylneuraminic acid (NeuNAc) [Monsigny et al., 1980]. Although fluorescence-coupled WGA is a commonly used tracer to study the distribution of cell-surface carbohydrates [Hooghe and Ooms, 1995; Jacobs and Lakes-Harlan, 1997], it also possesses remarkable anti-proliferative and insecticidal activities [Monsigny et al., 1980; Kim et al., 1993; Camby et al., 1996; Lorea et al., 1997; Gorelik et al., 2001; Ohba et al., 2003]. Its toxicity is not fully understood although its ability to dimerize and its sugar-specific binding capability may play important roles [Bryant et al., 1991; Karlsson, 1999; Gorelik et al., 2001; Muraki et al., 2002].

Numerous toxic plant lectins have been reported: some of them, such as mistletoe lectin from *Viscum album*, induced apoptosis [Bussing, 1996; Lyu et al., 2001], whereas others, including ricin from *Ricinus communis* [Endo et al., 1987; Keppler-Hafkemeyer et al., 1998] and Concanavalin A from Jack bean [Desrivieres et al., 1997] arrest cell cycle prior

Abbreviations used: WGA, wheat germ lectin; sWGA, succinylated wheat germ lectin; FITC-WGA, fluorescein-conjugated wheat germ agglutinin; LEL, lycopersicon esculentum lectin; RPMI 1640, Roswell Park Memorial Institute 1640; PI, propidium iodide; ^{3}H-Tdr, tritiated thymdine; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; MDA-TBA, malondialdehyde-thiobarbituric acid; PCNA, proliferating cell nuclear antigen.

Grant sponsor: University Grant Committee, Hong Kong.

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Received 18 August 2003; Accepted 10 September 2003
DOI 10.1002/jcb.10755

to induction of apoptosis. Cell cycle progression is a highly regulated process, which is mediated by a series of cell cycle control proteins: the cyclins, cyclin-dependent kinases and their inhibitors (CKIs). $P21^{Cip1/WAF1}$ is a broad acting CKI which integrates cytotoxic insults into growth arrest and apoptotic signaling pathways which ultimately determine cell fate [Smits and Medema, 2001]. The growth inhibitory activity of rice bran agglutinin [Miyoshi et al., 2001] and concanavalin A [Desrivieres et al., 1997] has been associated with activation of $p21^{Cip1/WAF1}$.

WGA is widely abundant in wheat germ, and a body of scientific evidence is available indicating its toxic effects on mammalian cells [Molkalewski and Thyberg, 1990; Kim et al., 1993; Pusztai et al., 1993], but the mechanism underlying its toxicity remains unclear. The present study was designed to investigate the action of WGA on cell proliferation and cell cycle progression in synchronized mouse L929 fibroblasts.

MATERIALS AND METHODS

Reagents and Cell Culture

Wheat germ agglutinin (WGA, L1020), Fluorescein-conjugated wheat germ agglutinin (FITC-WGA, FL1021), succinylated wheat germ agglutinin (WGA, L1020S) and *Lycopersicon esculentum* lectin (LEL, L1170) were purchased from Vector Laboratories (Burlingame, CA). They were dissolved in RPMI culture medium at appropriate concentrations for bioassays. Antibodies to bcl-2 (sc-492), bax (sc-526), caspase 3 (sc-7148), cyclin D (sc-8396), cyclin B (sc-752), p-cdc2 (sc-7989-R), cdc2 (sc-54), p21 (sc-6246), cdc25C (sc-327), and proliferating cell nuclear antigen (PCNA, sc56) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), while antibody to p53 (15801A) was obtained from BD Pharmingen (BD Biosciences, San Diego, CA).

Mouse connective tissue fibroblast L929 cell line (CCL1) was purchased from American Type Culture Collection and was maintained in RPMI medium supplemented with 10% fetal bovine serum and 100 μg/ml streptomycin and 100 IU/ml penicillin in a humidified incubator with 5% CO_2 in air at 37°C until use [Liu et al., 1996].

Cell Viability and Proliferation Assays

L929 cells at 1×10^5 cells/well/0.1 ml were cultured in 96-well culture plates (Nunc, Denmark) in the presence of serial concentrations of WGA, and sWGA for 24 or 48 h at 37°C in a 5% CO_2 incubator. Cells were either pulsed for the last 6 h with 0.5 μCi/10 μl/well of ^{3}H-thymidine (^{3}H-Tdr, specific activity 5 μCi/mmole, Amersham, Little Chalfont, UK) for analysis of anti-proliferative activity of lectins or reacted with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) for colorimetric MTT-based cytotoxicity assays [Liu et al., 2002]. All experiments were repeated at least three times.

Fluorescence Microscopy

L929 cells were cultured on Permanox 8-chamber cultured slides (Nalge-Nune, Naperville, IL) in the presence of serial concentrations of WGA for 24h before they were fixed with buffered formalin, stained with 0.01% acridine orange in 0.06 M phosphate buffer, pH 6.0, and differentiated with 0.1 M calcium chloride. The nuclear morphology was assessed using a fluorescence microscope (Axioskop, Zeiss, Germany) with a 450–490 nm excitation block filter and a 520-nm barrier filter [Liu et al., 2002]. Cells incubated with fluorescein-conjugated WGA (FITC-WGA) were observed under the confocal microscope (MRC1024 Bio-Rad, Hemelhempstead, UK) for demonstration of its intracellular localization.

Electron Microscopy

Treated cells were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 4 h, washed in the same buffer, and post-fixed with 1% aqueous OsO_4 for 30 min. After washing in distilled water, the preparations were dehydrated through graded ethanols (50, 70, 95, and 100%) and was then embedded in Spurr's medium, sectioned with an UltraCut R (Leica, Austria), double-stained with uranyl acetate and lead citrate, and examined with a Hitachi 7100 transmission electron microscope (Japan).

Lipid Peroxidation

The polyunsaturated fatty acyl chains in the cell membrane are highly susceptible to oxidative damage by free radicals, and the peroxidation process as a consequence of the lectin treatment was evaluated by a modified method of thiobarbituric acid reactivity test [Uchiyama and Mihara, 1978]. Briefly, lectin-treated L929 fibroblasts were incubated with thiobarbituric acid (TBA, T5500, Sigma, St. Louis, MO) at 100°C for 45 min before the absorbance of the colored

reaction product, malondialdehyde (MDA)-TBA, was read at 532 nm with a Millipore fluorometer.

Immunoblotting Analysis

Immunoblotting analysis was performed as described previously [Liu et al., 2002]. Briefly, cells were treated with WGA for 16 and 24 h, and the nuclear and cytosolic proteins were obtained using NE-PER Nuclear and Cytoplasmic Extraction Reagents (78833, Pierce Biotechnology, Rockford, IL). Equal amounts of denatured proteins were loaded and separated on a 10% SDS–polyacrylamide gel, and were then transferred to a polyvinylene difluoride (PVDF) membrane. After blocking with 2% gelatin, the membrane was stained with specific primary antibodies against proteins of two major groups: (1) apoptosis-related proteins: rabbit antiserum to bcl-2 (sc-492), rabbit antiserum to bax (sc-526), and rabbit antiserum to caspase 3 (sc-7148); and (2) cell cycle regulatory proteins: cyclin D (sc8396), cyclin B (sc-752), cdc2 (sc-54), p-cdc2 (sc7989-R), and cdc25C (sc-327); cyclin-kinase inhibitors, p21 (sc-6246) and p53 (15801A); and proliferating cell nuclear antigen (PCNA, sc-56), followed by secondary antibody IgG conjugated to horseradish peroxidase (Tago, Burlingame, CA), which was visualized using the Western Blot Chemiluminescence Reagent (DuPont NEN, Boston, MA).

Immunofluorescence Microscopy of $p21^{Cip1/WAF1}$ in L929 Cells

In order to demonstrate if the expression of $p21^{Cip1/WAF1}$ was in agreement with the actions in the nucleus, its subcellular localization in asynchronous L929 cells after treatment with WGA for 24 h was visualized by indirect immunofluorescence microscopy using monoclonal antibody against $p21^{Cip1/WAF1}$ [Alt et al., 2002]. Briefly, L929 were cultured with a serial concentration of WGA for 24 h on an 8-chamber slide (177402 Lab-Tek, Nalgene-Nunc, USA), and then fixed with cold acetone for 1 min at 4°C and cold methanol for 2 min, washed with PBS twice prior to incubation with primary monoclonal antibody against p21, followed by biotin-conjugated rat anti-mouse or anti-rabbit IgG antibodies and FITC-avidin D (A2001, Vector Laboratories, Inc.), and observed under the confocal microscope (MRC1024 Bio-Rad, Hemelhempstead, UK) for demonstration of its nuclear translocation.

Cell Cycle Analysis by Flow Cytometry

Since the cell cycle for L929 cells was about 16 h (9–10, 1–15, and 3–4 h for G_1, S, and G_2/M phases, respectively, unpublished data), the cells were synchronized at G_0/G_1 phase by serum starvation for two cell cycles (32 h) in RPMI culture medium supplemented with 0.5% FBS prior to WGA treatment. At selected different time-points of 4, 8, and 13 h after the release of serum starvation, corresponding to the G_1 and G_2/M phases of the cell cycle, WGA at a concentration of 0.5 μM along with culture medium supplemented with 2.5% FBS were added to the cells, and cultured at 37°C for a total of 24 h. The cells were then harvested, fixed in 70% cold ethanol, and subjected to DNA pattern analysis using flow cytometry. For biparametric analysis of immunofluorescence of $p21^{Cip1/WAF1}$ versus DNA content, at the end of the immunostaining with FITC-conjugated antibody against $p21^{Cip1/WAF1}$ (sc6246-FITC, Santa Cruz Biotechnology), cells were resuspended in PBS containing 1.0 μg/ml propidium iodide (PI, Boehringer Mannheim, Germany) and 200 μg/ml RNAse A (R4875, Sigma). The blue fluorescence of $p21^{Cip1/WAF1}$-bound FITC and the red fluorescence of DNA-bound PI in individual cells were measured at 488 and 575 nm by using a Beckman Coulter ALTRA flow cytometer and the results were analysed using Expo II and ModFit softwares (Beckman Coulter).

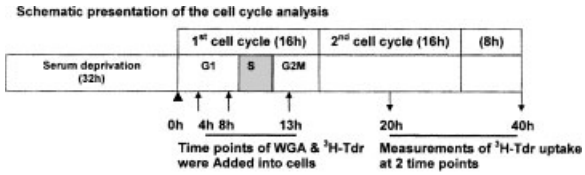
Thymidine Incorporation Assay

In order to obtain evidence of cell cycle progression after WGA treatment, synchronous L929 cells were co-incubated with 0.5 μM WGA and ^{3}H-Tdr at 4, 8, and 13 h, respectively, of the cell cycle (4 and 8 h were before, and 13 h was after the S phase). The rate of DNA synthesis in cells after WGA treatment over a 20 and 40 h period (corresponding to 1.5 and 2.5 cell cycles, respectively) was calculated by means of ^{3}H-Tdr uptake. Half of a cycle (8 more hours) was given at the end of 1st and 2nd cell cycles for detecting delayed cell cycle progression after WGA treatment, as depicted in the following scheme. [Scheme 1]

RESULTS

Cell Viability and Proliferation Assays

Figure 1a shows that ^{3}H-Tdr incorporation of L929 cells was significantly inhibited after 48 h



Scheme 1. Schematic presentation of the cell cycle analysis.

of treatment with WGA ($IC_{50} = 0.4$ μM). The proliferative ability of L929 cells declined from approximately 90% of the control level at 160 nM WGA to 30% at 800 nM WGA. Only about 20% of the control level was detected at a dose of 8 μM WGA. On the other hand, the proliferative activity of sWGA-treated L929 cells remained at a constant level even at the highest dose of 8 μM. MTT-assays also yielded results which paralleled findings from thymidine incorporation studies in that WGA at 800 nM strongly inhibited the activity of L929 cells (Fig. 1b). When WGA was succinylated to sWGA (with its binding activity for *N*-acetylneuraminic acid blocked), and then applied to culture cells, only negligible cytotoxicity was observed. However, the cytotoxicity of WGA cannot be solely attributed to its binding affinity for *N*-acetylneuraminic acid, because a dose-dependent decrease of viability of L929 cells was observed when they were exposed to equivalent doses of *Lycopersicon esculentum* lectin (LEL) which also possessed binding affinity for *N*-acetylglucosamine (Fig. 1c).

Morphological Observations

L929 cells appeared spindle-shaped with a centrally located nucleus, and adhered onto the surface of the cultured plate with cytoplasmic processes extending from the cell surfaces (Fig. 2a). The nucleus possessed more than one nucleolus with heterogeneous chromatin, and other organelles such as Golgi apparatus, rough endoplasmic reticulum (RER) and mitochondria were present in the cytoplasm. After treatment with WGA for 3 h, prominent vacuoles (Fig. 2b) and distended Golgi cisternae were observed, and aggregated chromatin increased gradually along with increased concentrations and length of WGA treatment (Fig. 2c–d). No apoptotic bodies were observed, however, in L929 cells after treatment with WGA at a range of 50–800 μM for 24 h (Fig. 2d). FITC-conjugated WGA appeared within minutes on the surface of L929 cells (Fig. 2f), were endocytosed into the cytoplasm, and was concentrated in the juxtanuclear and nuclear regions, as well as among cytoplasmic vacuoles within 4 h of treatment (Fig. 2g–h). Untreated L929 cells did not show any WGA signal (Fig. 2e).

Lipid Peroxidation

An increase of lipid peroxidation, up to two-folds, was observed in L929 cells treated with WGA up to 250 nM. Lipid peroxidation reached a plateau even when the dose of WGA was raised to 1 μM where all cells died (Fig. 3).

Immunoblotting Analysis

Figure 4a shows that the expression of the cell cycle related proteins, PCNA and cyclin D, remained at normal levels before declining with WGA treatment at 0.3–0.5 μM for 24 h. In contrast, the cyclin kinase inhibitors, $p21^{Cip1/WAF1}$ and p53, increased with WGA treatment in a dose-dependent manner, in accord with data on the accumulation of cells in G_2/M phase in the flow cytometric analysis. No protein was detected at the highest concentration treatment group as nearly all the cells were arrested or died after exposure to 0.6 μM of WGA. The mode of cell death may not be solely attributed to apoptosis as apoptotic bodies were not observed with fluorescence or electron microscopy, even although the expression of anti-apoptotic bcl-2 protein decreased and the apoptotic bax protein increased significantly at high doses of WGA treatment.

When the cells were exposed to WGA for one cell cycle (16 h), regulatory proteins typical for G_2/M phase, including cyclin B, p-cdc2, cdc2, and cdc25, expressed constitutively in untreated and cells treated with only 0.2 μM of WGA (Fig. 4b), but declined at both 0.4 μM and 0.6 μM of WGA (Fig. 4c), indicating a regulatory role of WGA on the cell cycle machinery at the G_2/M phase.

Immunofluorescence Microscopy of Cellular $p21^{Cip1/WAF1}$ and Caspase 3

Caspase 3 is a cysteine protease, a key effector molecule of apoptosis in mammalian cell, while $p21^{Cip1/WAF1}$ is a cyclin kinase inhibitor which plays an important role in cell progression of G_1–S and G_2–M phases. These two proteins were undetectable in exponentially growing L929 cells, but increased, in a dose-dependent manner, in the cytoplasm with increasing concentrations of WGA treatment (Figs. 5 and 6). It is noteworthy that $p21^{Cip1/WAF1}$ appeared primarily in the cytoplasm after treatment with

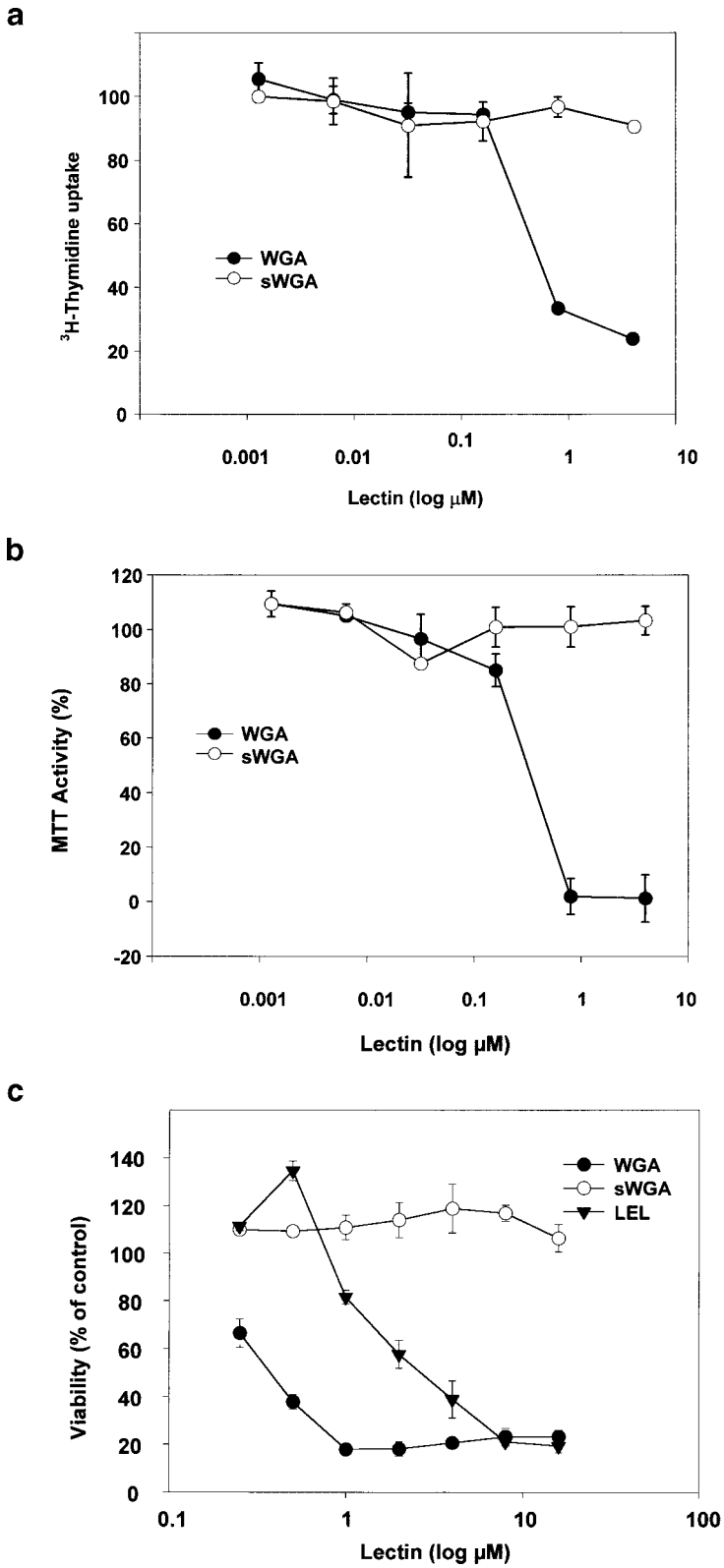


Fig. 1. Cytotoxicity of WGA and sWGA on exponentially growing L929 cells as measured by tritiated thymidine uptake (**a**) and MTT assay (**b**) (48 h, n = 3). A comparison of MTT assay of WGA, sWGA, and LEL on L929 cells for 24 h (**c**).

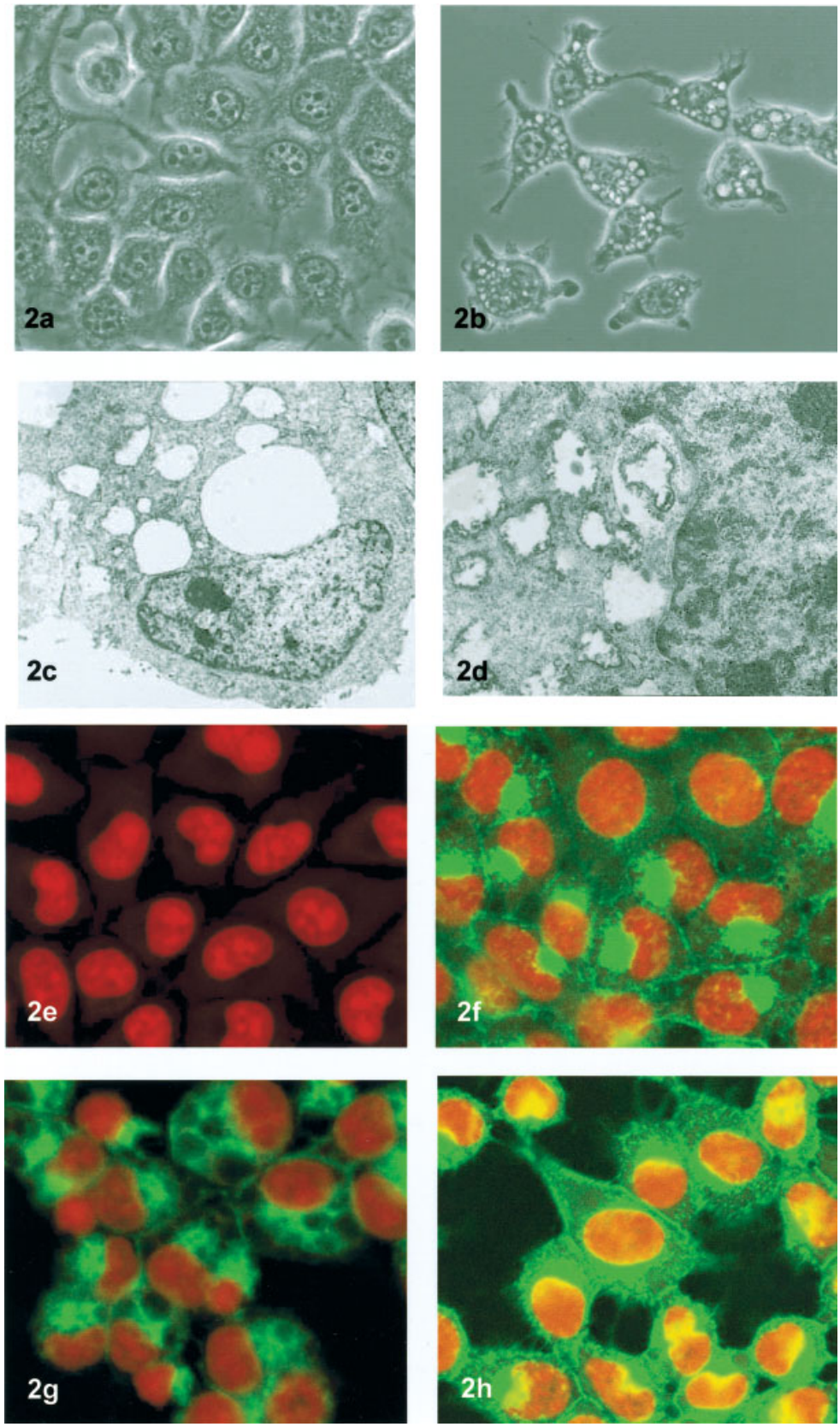


Fig. 2.

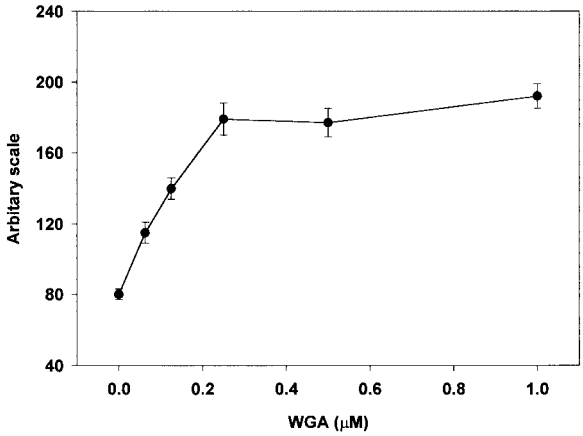


Fig. 3. Lipid peroxidation of L929 cells treated with WGA over a period of 24 h (n = 3).

0.3 μM WGA for 24 h (Fig. 6c–d), and it was further translocated into the nucleus at higher doses (0.4–0.5 μM) (Fig. 6e–h). $P21^{Cip1/WAF1}$ became predominant in the nuclei of the remaining cells when most cells died at the highest treatment dose (0.5 μM).

Cell Cycle Analysis by Flow Cytometry

The normal DNA pattern was observed in exponentially growing L929 cells incubated with doses of WGA up to 160 nM. However, prominent DNA fragmentation appeared in L929 cells exposed to WGA in doses higher than 320 nM. Nearly all DNA was degraded when WGA was applied at 800 nM concentration (data not shown). Since pronounced alteration of gene expression occurred in L929 cells at a concentration of 0.5 μM (Fig. 4), the cells were subjected to flow cytometric analysis. The results showed a time-dependent increase of annexin-V binding and propidium iodide incorporation, indicating possibly apoptotic changes in the cells (Fig. 7).

In order to demonstrate the mechanism by which WGA inhibited cell progression, L929 cells were synchronized by serum deprivation for a period of 32 h prior to treatment with 0.5 μM of WGA before (4 and 8 h) and after (13 h) the S-phase of the cell cycle. Serum deprivation accounted for about 75% of cells at G_0/G_1 phase and only 6% of cells at G_2/M phase (data not shown). Regardless of when L929 cells were exposed to WGA, G_2/M arrest (≥30% vs. 9.5% of control) without alteration of S phase (around 20%) was observed (Fig. 8).

Biparametric analysis of immunofluorescence of $p21^{Cip1/WAF1}$ versus DNA content revealed a relatively low increase of $p21^{Cip1/WAF1}$ immunofluorescence in G_1 phase but a sevenfold increase in G_2/M phase after WGA treatment for 12 h (Fig. 9), indicating that WGA did not interfere with cell progression through S phase but arrested cell proliferation at G_2/M phase.

Thymidine Incorporation Assay

In order to discriminate the action of WGA on the specific phase of the cell cycle (i.e., before or after S phase), synchronous L929 cells were coincubated with 0.5 μM WGA and ^{3}H-Tdr at H4, H8, and H13 after the release of serum deprivation. Cellular uptake of ^{3}H-Tdr over either a 20-h or a 40-h period (1.5 and 2.5 cell cycles) was measured. The results indicated that regardless of when L929 cells were exposed to 0.1–0.6 μM WGA during their G_1 phase (H4 and H8), the levels of ^{3}H-Tdr incorporation after one replication remained at the control level (Fig. 10a), and the untreated cells or cells treated with 0.2 μM WGA proceeded further through the second cell cycle, resulting in a twofold increase of ^{3}H-Tdr incorporation (2.25×10^5 vs. 1.1×10^5 for untreated cells after 24 and 40 h cultures, respectively). Cells treated with WGA ranging from 0.3 to 0.6 μM were arrested at the G_2/M phase of the first cell cycle. As these cells were unable to proceed to the second cell cycle, their ^{3}H-Tdr incorporation remained at a level similar to the first cell cycle (Fig. 10b).

A very low level (only 10–15%) of ^{3}H-Tdr incorporation was measured in untreated cells or cells treated with 0.2 μM WGA after 20 h if they were pulsed with ^{3}H-Tdr after their S phase (H13, Fig. 10a), but these cells had 1.0–1.1×10^5 counts after 40 h since they had passed through the S phase of the second cell cycle

Fig. 2. Phase contrast micrograph (330×) showing untreated L929 cells adhering onto the culture substratum (**a**). Vacuoles appeared in the cytoplasm of the cells after treatment with 0.4 μM WGA for 3 h (**b**). Transmission electron micrographs of L929 cells in the presence of 0.5 μM WGA for 24 h show numerous vacuoles and distended Golgi cisternae in the cytoplasm (**c–d**), but no apoptotic bodies in the nucleus (d) (1,200×). Confocal microscopy of L929 cells delineates the internalisation of FITC-conjugated WGA (0.4 μM) from the cell surface (**f**) into the cytoplasm among vacuoles, and then concentrated in both juxtanuclear and nuclear regions within 2 h of treatment (**g–h**). WGA signal was absent in untreated L929 cells (**e**) (400×).

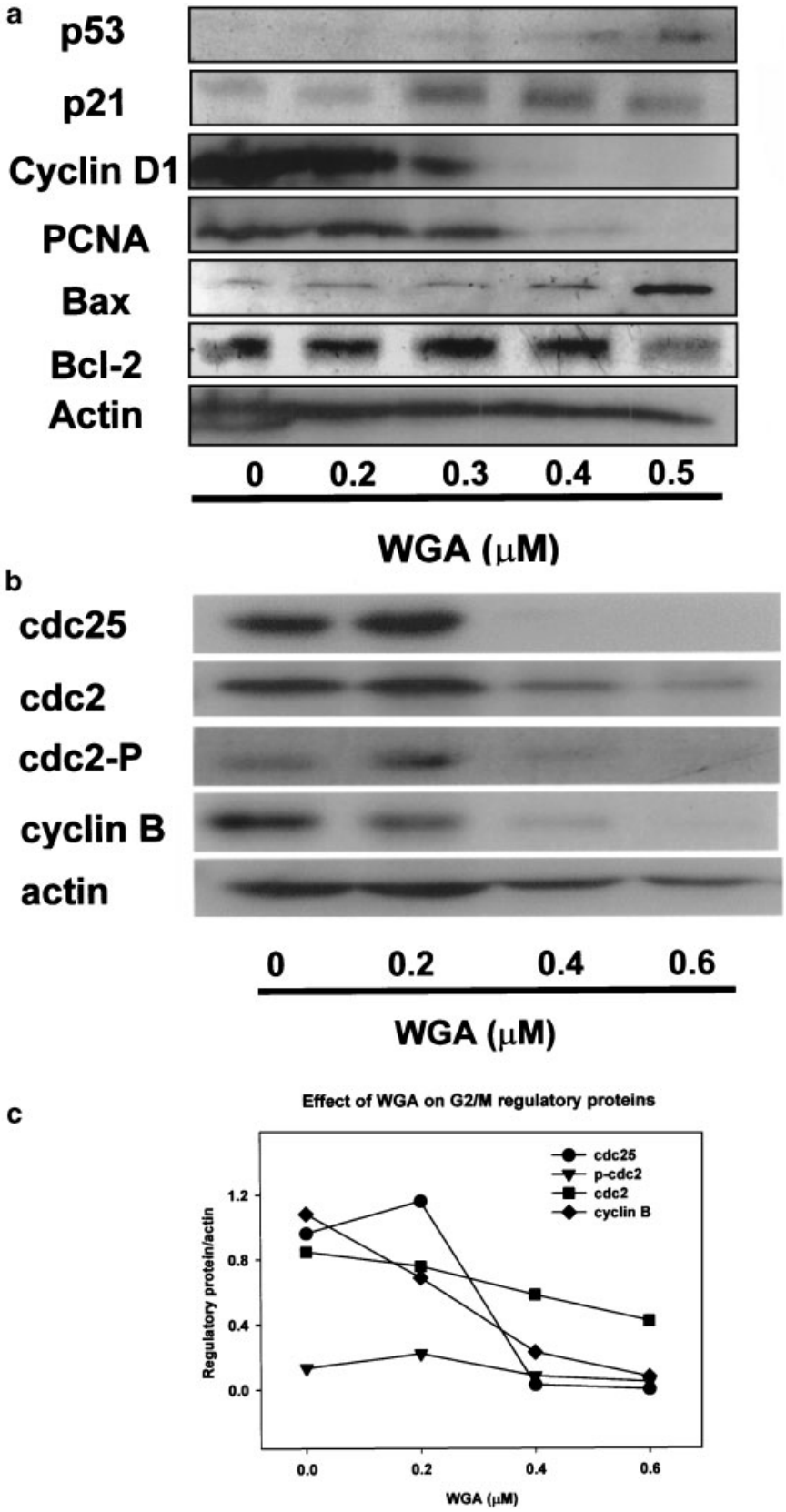


Fig. 4. Immunoblotting analysis of cell cycle regulatory proteins in L929 cells treated with WGA for 24 h (**a**) and 16 h (**b**). Only p21 and p53 were analyzed from nuclear protein lysates, while others were analyzed from total cell lysates. The expression of G_2/M regulatory proteins relative to actin was shown in panel (**c**).

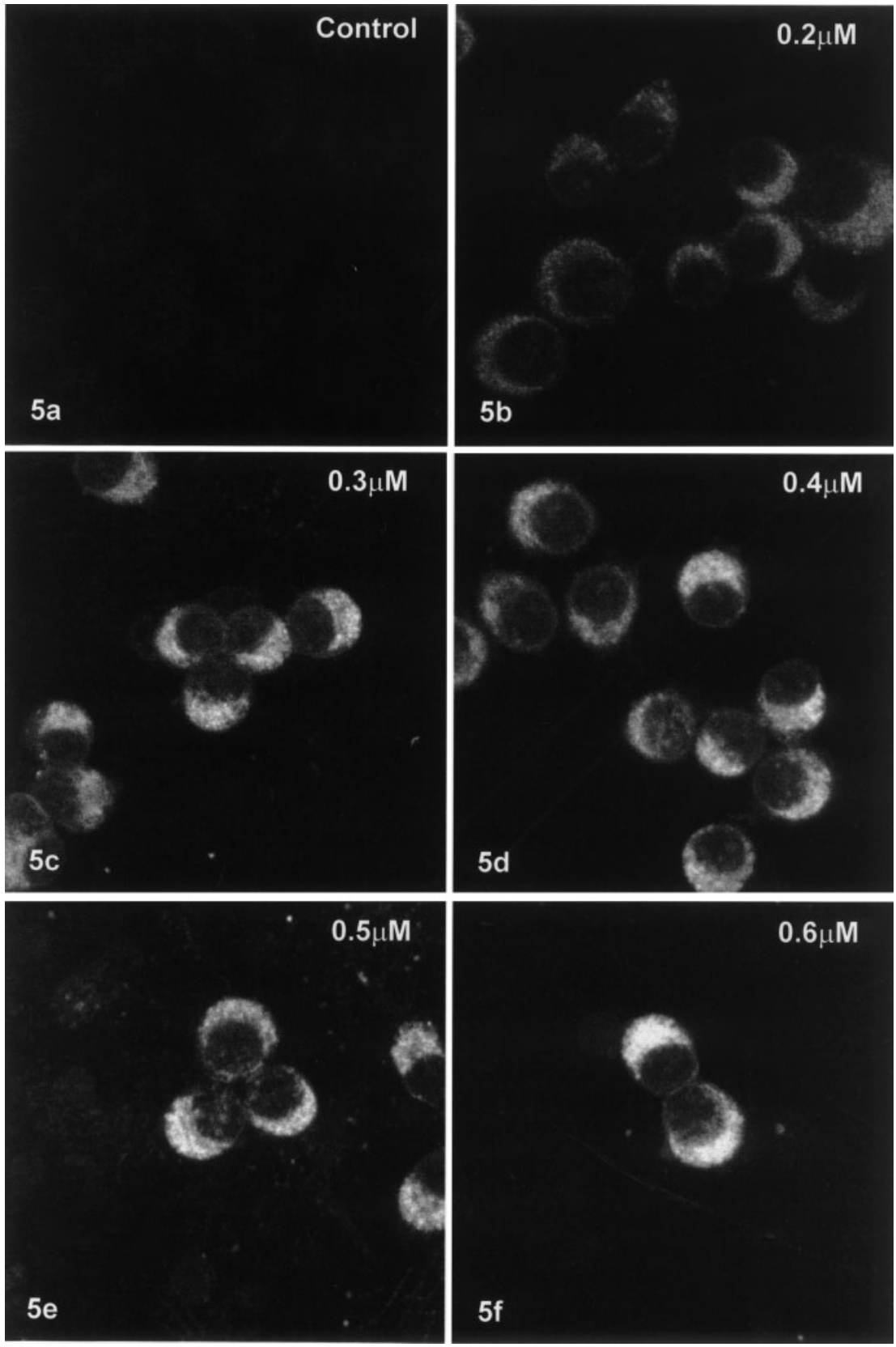


Fig. 5. Immunofluorescence microscopy shows the rise of caspase 3 in the cytoplasm of L929 cells treated with increasing doses of WGA (**a–f**) for 24 h (400×).

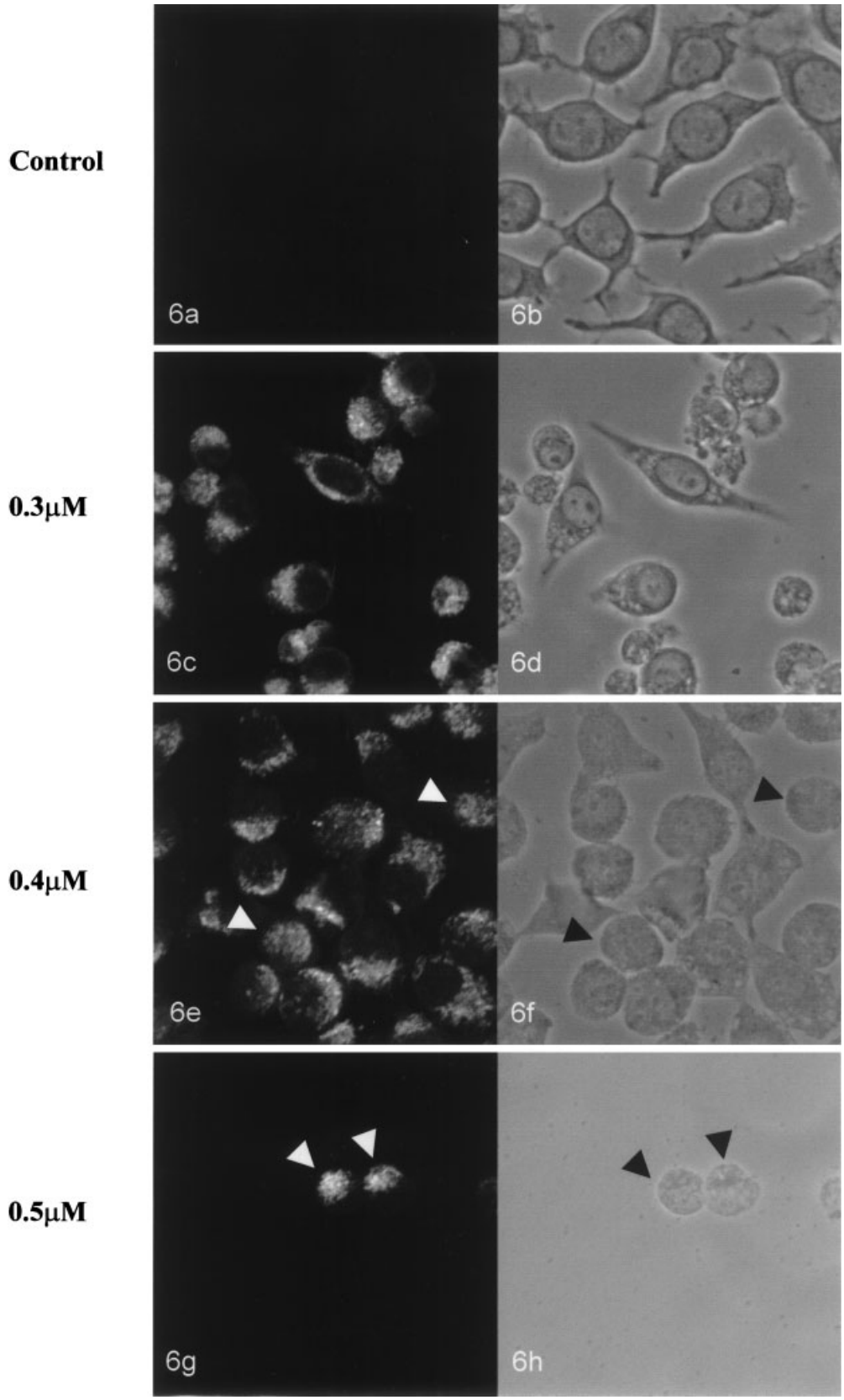


Fig. 6. Immunofluorescence microscopy shows that p21 was localized in the cytoplasm of L929 cells at low doses of WGA treatment but the protein was translocated into the nuclei (arrows) with increasing doses (0.4 and 0.5 μM) of WGA for 24 h (330×).

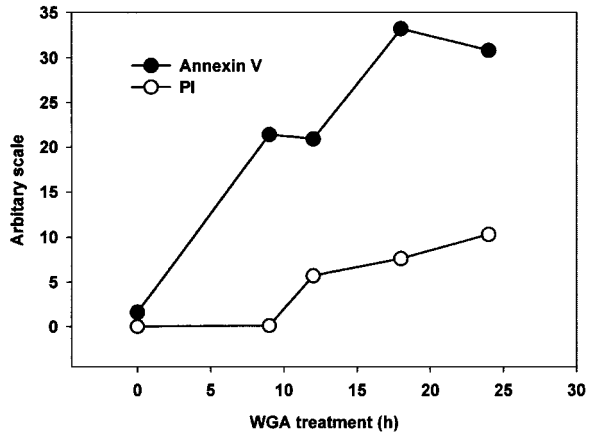


Fig. 7. A time-dependent increase of annexin-V binding and propidium iodide incorporation in L929 cells treated with 0.5 μM WGA for 24 h.

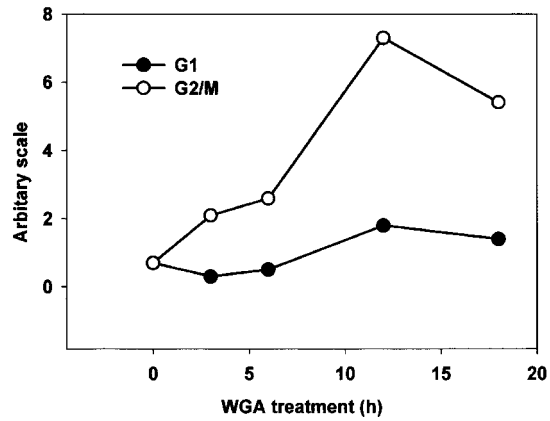


Fig. 9. Flow cytometric biparametric analysis of P21 immunofluorescence versus DNA content of synchronous L929 cells. After treatment in 0.5 μM WGA for 24 h, slight accumulation of p21 was observed in G_1 phase, which was significantly increased by about 10-fold in the G_2/M phase.

(Fig. 10b). For those cells treated with 0.3–0.6 μM WGA, they remained in the G_2/M phase of the first cell cycle for a total of 40 h, and thus their incorporated ^{3}H-Tdr remained at the basal level (H13, Fig. 10b). These results, taken together, indicated that WGA at doses of 0.3–0.6 μM did not interfere with G_1 nor S phases, but arrested cells at the G_2/M phase.

DISCUSSION

Lectins are carbohydrate-binding proteins of non-immune origin which agglutinate cells through their sugar-binding sites or precipitate

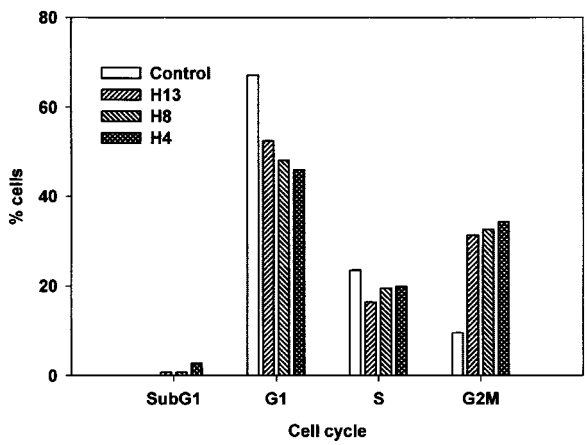


Fig. 8. Cell cycle analysis of synchronous L929 cells treated with 0.5 μM of WGA before (H4 and H8) and after (H13) S-phase of the cell cycle for 24 h. Cells arrested at G_2/M regardless of when WGA was administered.

polysaccharides or glycoconjugates [Goldstein et al., 1980]. They are divided into five groups according to their reactivity with monosaccharides which are the glucose/mannose, *N*-acetylglucosamine, fucose, galactose/*N*-acetylgalactosamine and sialic acid. It has been reported that WGA possesses binding specificity for two carbohydrate moieties, *N*-acetylglucosamine and *N*-acetyl-neuraminic acid [Monsigny et al., 1979]. The binding activity is changed if WGA is chemically modified by succinylation to form succinylated WGA (sWGA). There is some evidence to suggest that sWGA only binds to *N*-acetylglucosamine, but not *N*-acetyl-neuraminic acid [Monsigny et al., 1980]. It does not inhibit thrombin-induced rises in Ca^{2+} and PGI-2 production as compared to its native form [Grulich-Henn et al., 1988]. Our data show that the anti-proliferative activity of WGA toward L929 fibroblast cells was suppressed ($IC_{50} \cong 0.4$ μM and $IC_{50} > 10$ μM for WGA and sWGA, respectively) if it was succinylated, its *N*-acetylneuraminic acid-binding activity eliminated, and only *N*-acetylglucosamine binding activity remained the same as that of the native lectin. Moreover, *N*-acetylglucosamine binding activity alone was sufficient for inhibiting L929 cell proliferation ($IC_{50} \cong 2$ μM), indicating that lectin cytotoxicity was not solely mediated by sugar-binding activity.

WGA-binding has been detected in normal tissues [Chen et al., 1994; Maymon et al., 1994; Zschabitz et al., 1995] and in cancer cells [Kim et al., 1993]. It is often labeled with fluore-

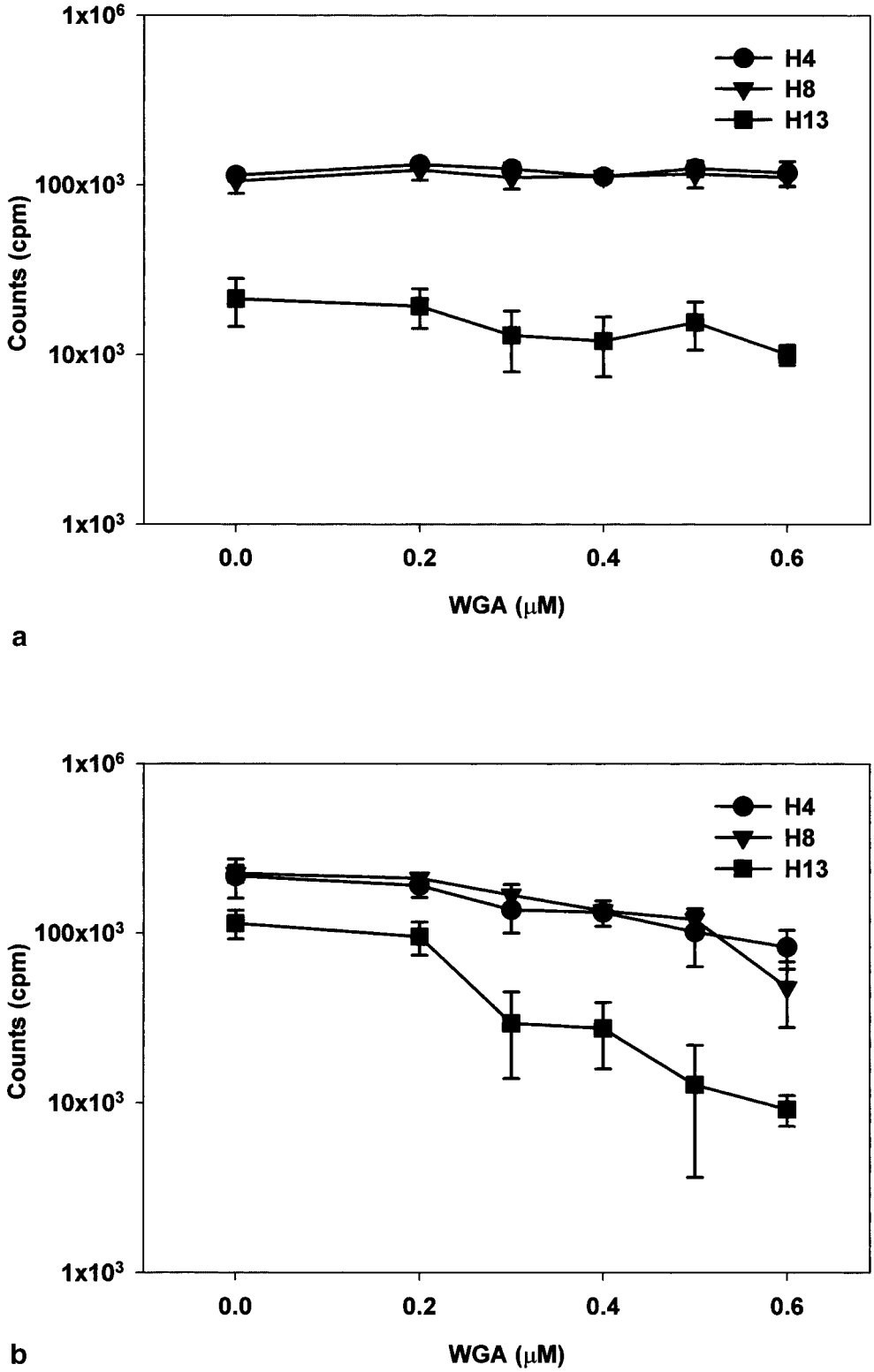


Fig. 10. Tritiated thymidine incorporation of synchronous L929 cells treated with 0.5 μM of WGA before (H4 and H8) and after (H13) S-phase of the cell cycle for a total of 20 h (1.5 cell cycle) (**a**), and 40 h (2.5 cell cycles) (**b**). Data are denoted as mean ± SD of four replicates.

scence, gold particles or horseradish peroxidase as tracers for bioassays [Alonso-Varona et al., 1995]. It is interesting to note that WGA, even at 0.4 μM, exerted a strong cytotoxic effect on tumor cells and L929 fibroblasts [Kim et al., 1993; Wang et al., 2000]. WGA induced apoptosis in tumor cells, particularly melanoma cells, but the mode of cytotoxic action on L929 cells has not been elucidated [Kim et al., 1993]. In the present study, we observed that WGA inhibited the ^{3}H-thymidine incorporation by L929 cells and it also reduced the tetrazolium reductase activity of these cells. L929 cells lost their cytoplasmic processes in the presence of WGA at a concentration of 0.3 μM, increased annexin-V binding and propidium iodide incorporation at 0.5 μM WGA in a time-dependent manner, and nearly all of them died at 0.8 μM concentration. Immunoblotting and immunofluorescence analysis further demonstrated constitutive expression of two apoptosis-related gene proteins, Bax and caspase 3, and down-regulation of the anti-apoptotic Bcl-2 protein at 0.5 μM WGA. These results indicated collectively that WGA induced apoptosis in L929 cells. However, we were unable to observe apoptotic bodies under both the fluorescence and transmission electron microscopes. L929 mouse fibrosarcoma cells are well documented to undergo apoptosis after treatment with tumor necrosis factor, but apoptotic phenotype (i.e., internucleosomal DNA fragmentation) was absent, and the situation was referred to as atypical apoptosis [Humphreys and Wilson, 1999]. It should be noted that apoptosis of L929 had also been induced by etoposide [Bonelli et al., 1996], ethanol [Mashimo et al., 1999], and cytochrome c [Takeyama et al., 2002], but prominent apoptotic bodies were only demonstrated in L929 cells by intracellular injection of cytochrome c [Takeyama et al., 2002]. Since WGA exhibited strong cytotoxicity on a battery of human cancer cell lines, and induced apoptotic bodies in human colon HT-29 carcinoma cells (unpublished data), experiments are being conducted to elucidate the mechanism(s) underlying the atypical apoptosis induced by WGA in L929 cells.

Studies by other laboratories have demonstrated that WGA was endocytosed after binding to the plasma membrane, and was then transported through an endosomal and/or a phagosomal network into the Golgi apparatus at the juxtanuclear position [Iida and Shibata, 1991; Vetterlein et al., 2002]. In the present study, we observed vacuoles of various sizes in the cytoplasm of cells after WGA treatment. We also noted that lipid peroxidation was increased twofold at 0.250 μM WGA when cell viability and protein expressions remained at normal levels. Lipid peroxidation and its product, malondialdehyde, had been demonstrated to induce oxidative damage and PCNA-dependent cell cycle arrest in human fibroblasts [Savio et al., 1998]. The generation of superoxide radicals, the products of lipid peroxidation, was found to interfere with bcl-2 protein expression in rat brain [Ekert et al., 1997]. It remains to be determined if the toxic effects of WGA is related to the production of oxygen free radicals by L929 cells.

The progress of a cell cycle is controlled by the joint action of the cyclins, cyclin kinases (CDKs), and their inhibitors (CKIs). CDKs are catalytic partners of cyclins, both of which promote cell proliferation, whereas CKIs exert an opposite regulation [Sherr, 1996]. Cyclin D-CDK4 and cyclin A/E-CDK2 complexes act sequentially in the G_1 phase of the cell cycle, with $p21^{Cip1/WAF1}$ and p27 proteins exerting inhibition at these complexes, thus preventing the cell from progressing into the S-phase. Cyclin B–cdc2 complex is present in the cytoplasm during interphase but is translocated rapidly into the nucleus for cell mitosis [Takizawa and Morgan, 2000]. The results of immunoblotting analysis in the present study showed that the expression of all tested regulatory proteins remained at normal levels at doses up to 0.3 μM, indicating that these regulatory proteins were not affected until the cells were treated with WGA at 0.4 μM or higher where the expression of cyclin D and PCNA were nearly undetectable. Since cyclin D is associated with PCNA and $p21^{Cip1/WAF1}$ in regulating cell progression from G_1–S phase, WGA is likely to interfere with cyclin and the inhibitors, particularly in the nuclear translocation of $p21^{Cip1/WAF1}$ as demonstrated by immunofluorescence microscopy, and subsequently affecting cell progression. However, biparametric analysis of immunofluorescence $p21^{Cip1/WAF1}$ versus DNA revealed an association of only onefold increase of $p21^{Cip1/WAF1}$ with G_1 phase whereas a sevenfold increase of $p21^{Cip1/WAF1}$ with G_2/M phase was observed (Fig. 9). No difference in ^{3}H-Tdr incorporation was noted among all WGA treatment groups after one replication, regardless of whether WGA was added at the 4 or 8 h in

the G_1-phase, indicating that $p21^{Cip1/WAF1}$ did not block L929 cells from progressing through G_1–S to G_2/M phases. Untreated cells and cells treated with WGA up to 0.3 μM proceeded to the G_1-phase of the second cell cycle where cyclin D and PCNA were expressed constitutively for DNA synthesis of the second cell cycle, resulting in high levels of both proteins in immunoblotting analysis (Fig. 4a). Cells treated with higher doses of WGA (0.4–0.6 μM) were arrested by $p21^{Cip1/WAF1}$ at G_2/M phase of the first cell cycle. These cells did not express cyclin D and PCNA, and thus both proteins were undetected by immunoblotting analysis after 24 h WGA treatment.

Apart from its inhibitory action on G1–S progression, $p21^{Cip1/WAF1}$ is also known to arrest cell progression by inhibiting cyclin B-cdc2 activities [Lu and Epner, 2000; Magrini et al., 2002], or by blocking the expression of Cdc25 [Ando et al., 2001], resulting in G_2/M growth arrest [Zhang et al., 2001]. Cyclin B–cdc2 was originally defined as M phase-promoting factor in meiotic frog eggs [Smits and Medema, 2001]; cdc2 is a catalytic unit while cyclin B is a regulatory subunit. The cyclin B–cdc2 complex is held in an inactive form by phosphorylation of cdc2 at threonine 14 and tyrosine 15, but confers activation by dephosphorylation activity of the protein tyrosine phosphatase cdc25 [Takizawa and Morgan, 2000]. The cytotoxicity of WGA has been associated with activation of $p21^{Cip1/WAF1}$ and cell cycle arrest at G_2/M, but the underlying mechanism was not clear [Miyoshi et al., 2001]. Based upon the present immunoblot findings with total cell lysate, perturbations in the interaction of cyclin B, p-cdc2, cdc2, and cdc25 are involved in the mechanism underlying the $p21^{Cip1/WAF1}$-mediated cytotoxic action of WGA. Experiments are being conducted to clarify whether WGA suppresses transcriptional and translational modification of cdc2, cyclin B, and cdc25 genes, or the nuclear translocation of cyclin B–cdc2 complex, and thus interfering with cell mitosis.

ACKNOWLEDGMENTS

The authors thank Ms. V.W. Chow for her excellent technical assistance.

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