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Cell and Nuclear Enlargement of SW480 Cells Induced by a Plant Lignan, Arctigenin: Evaluation of Cellular DNA Content Using Fluorescence Microscopy and Flow Cytometry

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Arctigenin is a natural plant lignan previously shown to induce G_2/M arrest in SW480 human colon cancer cells as well as AGS human gastric cancer cells, suggesting its use as a possible cancer chemopreventive agent. Changes in cell and nuclear size often correlate with the functionality of cancer-treating agents. Here, we report that arctigenin induces cell and nuclear enlargement of SW480 cells. Arctigenin clearly induced the formation of giant nuclear shapes in SW480, as demonstrated by fluorescence microscopic observation and quantitative determination of nuclear size. Cell and nuclear size were further assessed by flow cytometric analysis of light scattering and fluorescence pulse width after propidium iodide staining. FSC-H and FL2-W values (parameters referring to cell and nuclear size, respectively) significantly increased after arctigenin treatment; the mean values of FSC-H and FL2-W in arctigenin-treated SW480 cells were 572.6 and 275.1, respectively, whereas those of control cells were 482.0 and 220.7, respectively. Our approach may provide insights into the mechanism behind phytochemical-induced cell and nuclear enlargement as well as functional studies on cancer-treating agents.

Introduction

Biological particle sizing is often utilized in many biological studies. Nuclear size can be easily evaluated by analyzing the fluorescence pulse signal emitted from cells stained with a DNA labeling fluorescent dye such as propidium iodide (Kang *et al.*, 2010). The fluorescence pulse signal has three measurable parameters (height, width, and area), and pulse width highly correlates with particle size (Hoffman, 2009; Kang *et al.*, 2010).

Cell cycle arrest is one of the most important mechanistic targets of cancer treatment, used in both chemotherapy and chemoprevention (Surh, 2003; Shu *et al.*, 2010). Much research has been conducted to examine the effects of natural products and synthetic chemicals on the cell cycle of cancer cells (Surh, 2003; Kang *et al.*, 2007; Meeran and Katiyar, 2008; Jin *et al.*, 2010; Yasuda *et al.*, 2010). A cell cycle blocker, particularly one that targets the G_2/M phase, may enlarge nuclear size, possibly because the cell cannot enter mitosis even after sufficient DNA replication (Kang *et al.*, 2010). Thus, enlarged nuclei are one indication that cell cycle inhibitors function as cancer-suppressing agents. Flow cytometric analysis of the fluorescence pulse width can be a useful tool for measuring nuclear size.

Arctigenin, a natural dibenzyl butyrolactone lignan (Fig. 1), can be isolated from the Mongolian medicinal plant, *Saussurea salicifolia* (Kang *et al.*, 2007). Arctigenin has various known biological activities, including anti-cancer, anti-HIV, anti-inflammation, and neuroprotection (Eich *et al.*, 1996; Jang *et al.*, 2002; Awale *et al.*, 2006; Kang *et al.*, 2007; Zhao *et al.*, 2009; Yoo *et al.*, 2010). We recently showed that arctigenin inhibits cell proliferation by inducing cell cycle arrest at the G_2/M phase in both human colon cancer SW480 cells and human gastric cancer AGS cells (Kang *et al.*, 2007; Yoo *et al.*, 2010), though apoptosis was not pronounced in SW480 cells (Yoo *et al.*, 2010).

Based on our previous observations, we hypothesized that arctigenin may induce cell and nuclear enlargement because cancer cells are unable to enter mitosis even after sufficient DNA and protein synthesis. To verify this hypothesis, we used microscopy to observe morphological changes in SW480 cells after arctigenin treatment and flow cytometric analysis of the fluorescence pulse signal emitted from propidium iodide-stained cells to evaluate nuclear size. We also

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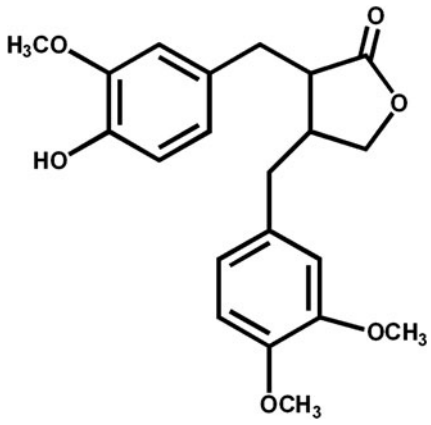

FIG. 1. Chemical structure of arctigenin.

investigated the effect of cell cycle phase on arctigenin-induced nuclear enlargement through fluorescence pulse width analysis.

Materials and Methods

Materials

Arctigenin, a natural plant lignan, was isolated from *S. salicifolia* (Kang *et al*., 2007) (chemical structure shown in Fig. 1) and dissolved in dimethyl sulfoxide for cellular treatment. The following antibodies were purchased: α-tubulin (Cell Signaling Technology, Danvers, MA), GAPDH (Cell Signaling), β-actin (Santa Cruz Biotechnology, Santa Cruz, CA), and lamin B1 (Abcam, Cambridge, United Kingdom).

Cell culture

SW480 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI-1640 supplemented with 25 mM HEPES, 10% (v/v) heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were cultured under humidified atmospheric conditions (95% air, 5% CO_2) at 37°C.

Western blot analysis

SW480 cells (4×10^5 cells per well) were seeded onto six-well plates, incubated for 24 h, and then treated with arctigenin. Total cell lysates were prepared and subjected to electrophoresis on 10% sodium dodecyl sulfate–polyacrylamide Tris-HCl gels. Additional procedures were performed as previously described (Kang *et al*., 2009; Yoo *et al*., 2010).

Microscopy and measurement of nuclear size

SW480 cell morphology was observed using an Olympus CK40 phase-contrast microscope (Tokyo, Japan). To measure nuclear size, detached cells were stained with propidium iodide as previously described (Kang *et al*., 2007, 2010). Fluorescence images were obtained with a Nikon TE2000U fluorescence microscope (Kanagawa, Japan) and the ProgRes CF^{cool} camera system (JENOPTIK, Jena, Germany). Nuclear size (diameter, μm) was determined using the circle measurement algorithm of the ProgRes Capture Pro 2.5 software (JENOPTIK) as previously described (Kang *et al*., 2010).

Flow cytometry

SW480 cells (3×10^5 cells per well) were seeded onto six-well plates, incubated for 24 h, and then treated with arctigenin. For flow cytometric analysis, cells were stained with propidium iodide. FSC-H, SSC-H, FL2-A, and FL2-W values were measured using a Becton Dickinson flow cytometer and the CellQuest Pro software (San Jose, CA) as previously described (Kang *et al*., 2010).

Statistical analysis

Data were expressed as the mean ± standard deviation. Statistical analysis was carried out by one-way analysis of variance followed by Dunnett's multiple comparison test using the GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA). $p<0.05$ was considered statistically significant.

Results

Arctigenin induces formation of giant cell shapes in SW480 cells

We examined morphological changes after arctigenin treatment in SW480 cells. Arctigenin triggered the formation of giant cell shapes in SW480 cells. Cells treated with arctigenin (50, 100 μM) for 48 h were much larger in size than those treated with the vehicle control (Fig. 2, Supplementary Video SV1, SV2). Phase-contrast microscopy also suggested that arctigenin treatment increased nuclear size.

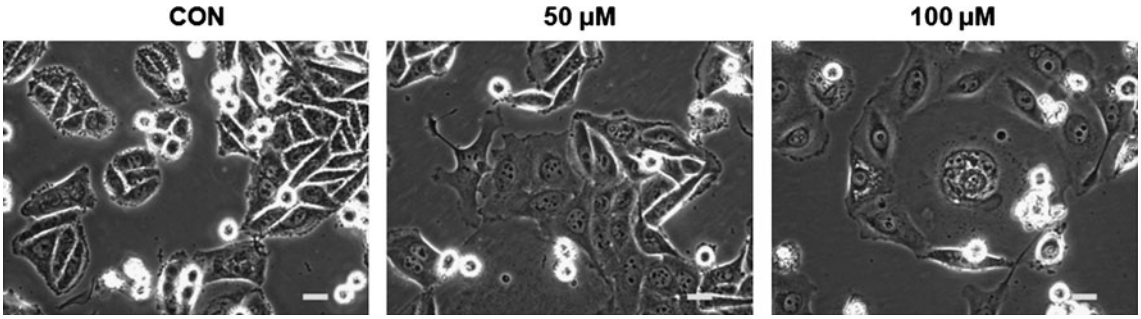

FIG. 2. Phase-contrast microscope images (bar = 20 μm) of changes in SW480 cell morphology induced by arctigenin treatment (50, 100 μM for 48 h).

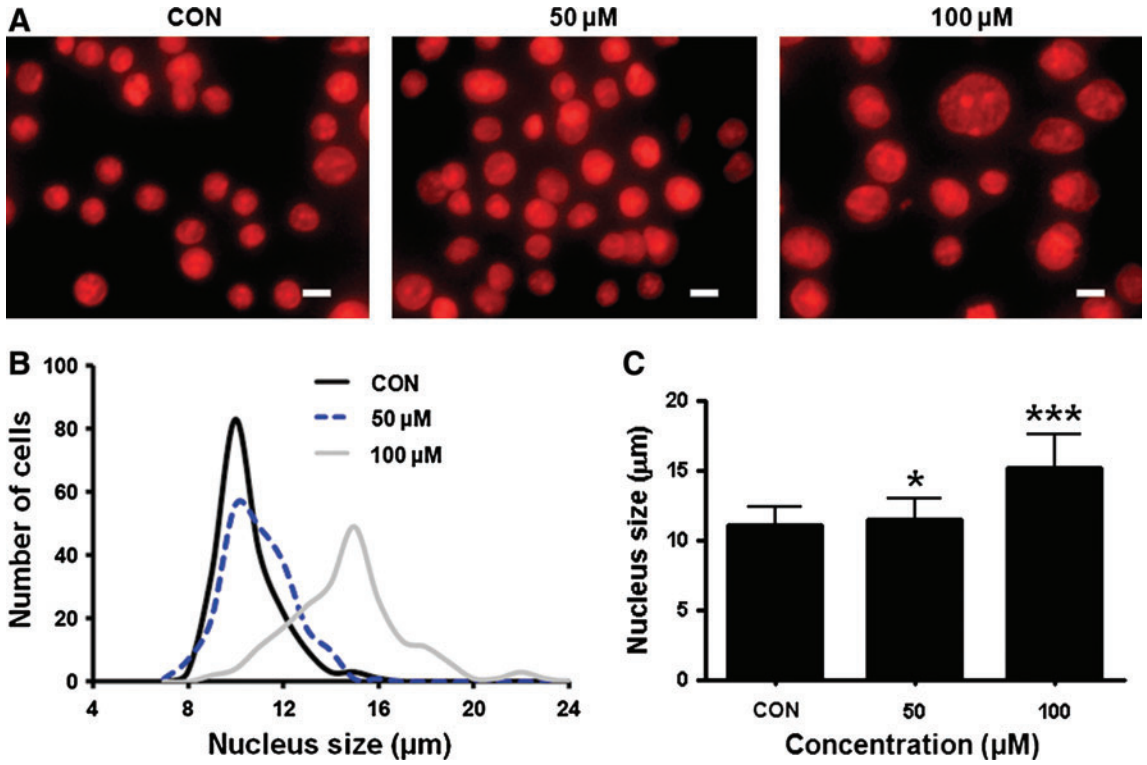

FIG. 3. Arctigenin-induced nuclear enlargement of SW480 cells by fluorescence microscopy. Cells were treated with arctigenin (50, 100 μM) for 48 h and stained with propidium iodide. **(A)** Fluorescence microscope images (bar = 10 μm). Nuclear size (diameter, μm) was determined using a circle measurement algorithm. **(B)** Distributions of nuclei size ($n = 200$). **(C)** Mean nuclear size. Bar represents the mean ± standard deviation ($n = 200$). *$p < 0.05$ and ***$p < 0.001$, compared with the vehicle-treated control (CON). Color images available online at www.liebertonline.com/dna

Fluorescence microscopy demonstrates that arctigenin induces nuclear enlargement in SW480 cells

For more precise observation of cell nuclei, we used fluorescence microscopy after propidium iodide staining. Arctigenin treatment (100 μM, 48 h) clearly induced formation of giant nuclei in SW480 cells (Fig. 3), which were quantified using the circle measurement algorithm of the microscope software. Arctigenin significantly increased nuclear size, as nuclear enlargement was more apparent in cells treated with 100 μM arctigenin than in cells receiving a lower dose (50 μM) (Fig. 3B, C). This result also correlates with the cell cycle results, as only 100 μM of arctigenin was able to induce G_2/M arrest (Fig. 4D).

Because arctigenin treatment induced nuclear and cell enlargement, we examined the expression levels of various cytoskeleton proteins by western blot analysis. Expression of cytosolic fibers α-tubulin (microtubule subunit) and β-actin (microfilament subunit) did not change with arctigenin treatment. In contrast, expression of lamin B1, a nuclear membrane structural component, remarkably decreased with arctigenin treatment (Fig. 5).

Flow cytometric analysis of fluorescence pulse signal confirms that arctigenin induces SW480 cell and nuclear enlargement

We had previously established a flow cytometric method of fluorescence pulse width analysis for the evaluation of nuclear size (Kang *et al.*, 2010). We used this tool here to analyze nuclear enlargement of SW480 cells after arctigenin treatment. We divided the region into four parts on the FL2-A versus FL2-W dot plot based on cell cycle phase (R1, G_0/G_1 phase; R2, S phase; R3, G_2/M phase; R4, whole singlet cell region, R1 + R2 + R3) (Fig. 4A). FSC-H values (which refer to cell size) of arctigenin-treated cells were distributed at higher positions than those of vehicle-treated control cells. Arctigenin induced a dose-dependent increase of FSC-H values; the mean values of FSC-H were 482.0 ± 17.8, 513.9 ± 12.9, and 572.6 ± 9.6 for 0, 50, and 100 μM of arctigenin treatment, respectively (Fig. 4B). These results were consistent with microscopic observations (Fig. 2). In addition, SSC-H values (which refer to cell granularity or internal complexity) significantly increased with 100 μM of arctigenin treatment (Fig. 4C). In the FL2-A histogram referring to the cell cycle, we were able to confirm arctigenin-induced G_2/M arrest, showing that only treatment with 100 μM of arctigenin could induce a G_2/M arrest (Fig. 4D). Finally, FL2-W values (which refer to nuclear size) of arctigenin-treated cells were also distributed at higher positions than those of vehicle-treated control cells. Only treatment with 100 μM arctigenin significantly increased FL2-W values; the mean values of FL2-W were 220.7 ± 2.3, 227.4 ± 3.4, and 275.1 ± 11.8 for 0, 50, and 100 μM of arctigenin treatment, respectively (Fig. 4E). These results were also consistent with fluorescence microscopic observations (Fig. 3).

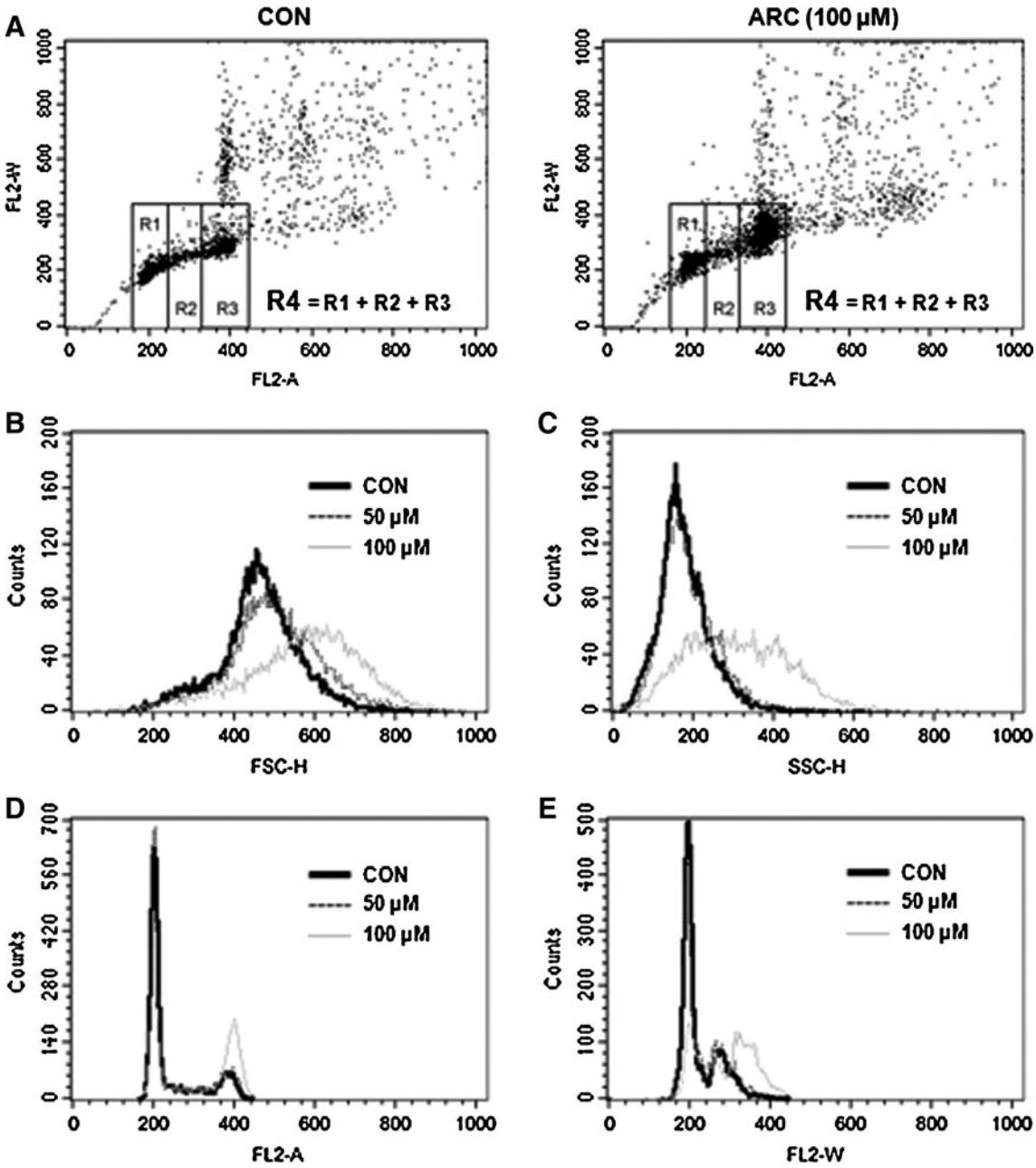

FIG. 4. Flow cytometric analysis to evaluate arctigenin-induced cell and nuclear enlargement in SW480 cells. Cells were treated with arctigenin (50 or 100 μM) for 48 h, harvested, fixed in 70% ethanol, and stained with propidium iodide. Representative data are shown from four independent experiments. FL2-A versus FL2-W dot plot **(A)** (R1, G_0/G_1 phase cells; R2, S phase cells; R3, G_2 + M phase cells; R4, whole singlet cells, R1 + R2 + R3). Histogram plots of FSC-H **(B,** cell size**)**, SSC-H **(C,** cell granularity**)**, FL2-A **(D,** cell cycle**)**, and FL2-W **(E,** nuclear size**)** when gated for R4. Overlay histograms are schematized using vehicle-treated control (CON), 50, and 100 μM arctigenin (ARC)-treated cell data.

Arctigenin-induced SW480 cell nuclear enlargement preferentially occurs during G_2/M phase

To examine the correlation between cell cycle phase and cell and nuclear enlargement in arctigenin-induced cells, we compared the FSC-H and FL2-W distributions of vehicle-treated control and arctigenin-treated cells gated for each cell cycle phase (R1, R2, and R3 as described in Fig. 4A) using a histogram plot (Fig. 6). FSC-H values of arctigenin-treated cells were distributed at higher positions than those of vehicle-treated control cells when gating for R1 or R3 (G_0/G_1 or G_2/M phase cells, respectively), indicating that the arctigenin-induced increase in cell size was not correlated with either early or late cell cycle phases (Fig. 6A, C). However, FL2-W values of arctigenin-treated cells were distributed at higher positions than those of vehicle-treated control cells when gating for R3 (G_2/M phase cells), whereas others were distributed similarly when gating for R1 or R2 (G_0/G_1 or S phase cells, respectively) (Fig. 6B). The difference in mean FL2-W values between vehicle-treated control and

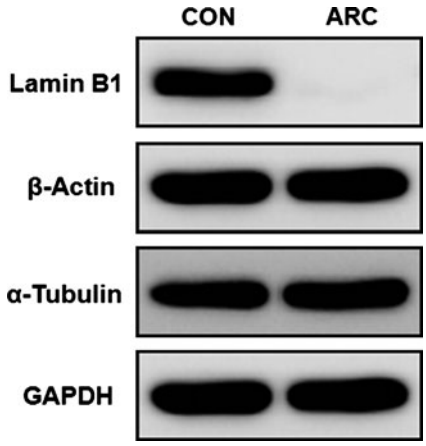

FIG. 5. Effect of arctigenin on cytoskeletal protein expression in SW480 cells. GAPDH was used as a loading control for western blot analysis. Cells were treated with arctigenin (ARC, 100 μM) for 48 h. CON, vehicle-treated control. Representative data are shown from three independent experiments.

arctigenin-treated cells (100 μM, 48 h) when gating for R3 (difference of 35.8) was significantly higher than those gated for R1 or R2 (difference of 13.7 and 15.1, respectively) (Fig. 6D). These data imply that arctigenin-induced nuclear enlargement preferentially occurs during the late G_2/M phase rather than the early G_0/G_1 or S phases of the cell cycle.

Discussion

Morphological changes are one of the most important indicators of cancer suppression activity which can be induced by anti-cancer agents and include differentiation, apoptosis, and formation of giant cell shapes (Munster *et al*., 2001; Cho *et al*., 2004; Rello-Varona *et al*., 2006). Cell and nuclear size analysis can be used to study the function of cell cycle inhibitors found in many cancer-treating agents. The formation of abnormal giant cell and nuclei may be an indirect indication of cell cycle arrest at late G_2/M phase. As cancer cells arrested at G_2/M are unable to enter mitosis even after synthesizing sufficient DNA and protein for cell division, they retain abnormal giant nuclei. Several previous studies support that treatment with suberoylanilide hydroxamic acid (histone deacetylase inhibitor), CGP 41251 (PKC inhibitor), AZD1152 (Aurora B kinase inhibitor), and ^{131}I-irridation (radiotherapy) induces G_2/M arrest in various cancer cells and they also trigger cell or nuclear enlargement (Begemann *et al*., 1996; Munster *et al*., 2001; Eriksson *et al*., 2008; Nair *et al*., 2009). External expression of AIE-75 PDZ-domain protein and Spindlin1, which are colon cancer-related antigen and the meiotic spindle-associated protein, respectively, can also induce G_2/M arrest and formation of multinucleated giant nuclei (Hirai *et al*., 2004; Zhang *et al*., 2008).

We had previously suggested that two properties are required for chemically induced nuclear enlargement. First, the chemical should be able to induce cell cycle arrest at later phases, and, second, the chemical should not induce apoptosis rapidly (Kang *et al*., 2010). Arctigenin induces G_2/M arrest and does not effectively induce apoptosis in human colon cancer SW480 cells, which contain a nonfunctional p53 (R273H/P309S) (Yoo *et al*., 2010). We predicted that arctigenin would induce the formation of giant nuclei, and our findings are in agreement with this prediction. Arctigenin (100 μM) induces a strong G_2/M arrest as well as apoptosis in human gastric cancer AGS cells (Kang *et al*., 2007), likely because these cells carry functional p53, the master controller of diverse cellular processes, including apoptosis. We thus speculated that the formation of giant nuclei in AGS cells (with functional p53) would be much weaker than that observed in SW480 cells. To test this idea, we will further investigate the arctigenin-induced nuclear enlargement of AGS cells in the presence or absence of various apoptosis inhibitors.

Along with our recent report (Kang *et al*., 2010), in the present study, we promote a further application of the flow cytometric method for evaluation of cell and nuclear size change after arctigenin treatment; these results were consistent with our microscopic observations. This is the first report of flow cytometric fluorescence pulse width analysis for phytochemical-induced nuclear enlargement. The fluorescence pulse width of cells stained with propidium iodide, FL2-W, correlates well with nuclear size. In addition to nuclear size described in this study, we suggest that other various biological particle sizes can also be evaluated by flow cytometric analysis of fluorescence pulse width using either organelle-specific or immunofluorescence staining.

Although arctigenin-treated cells synthesize sufficient DNA and protein for cell division, they are unable to enter mitosis due to arrest at G_2/M phase. Accordingly, both cells and nuclei are enlarged in shape, suggesting a relationship between cell and nuclear enlargement. We also found that arctigenin-induced nuclear enlargement preferentially occurs during the G_2/M phase by fluorescence pulse width analysis after propidium iodide staining. In contrast, arctigenin-induced cell enlargement was unaffected by cell cycle phase based on FSC-H analysis. However, more precise evaluation of cell size is needed to clarify this phenomenon using methods such as a fluorescence pulse width analysis after staining cells with fluorescein isothiocyanate (fluorescent dye for whole cellular protein) (Crissman *et al*., 1981), followed by quantification of cell size using another method (e.g., with a Coulter Counter).

We investigated whether the increased presence and reorganization of cytoskeletal proteins may contribute to cell and nuclear enlargement. Western blot analyses showed that arctigenin treatment did not change the expression of either α-tubulin or β-actin cytosolic fibers. However, as western blots represent overall protein expression in a cell population, the expression of α-tubulin and β-actin in single cells may actually increase by arctigenin treatment and lead to cell enlargement. Lamin B1 expression dramatically decreased, similar to that recently reported for etoposide-induced nuclear enlargement (Kang *et al*., 2010). Because lamins are known to be important for cell cycle control, DNA replication, and chromatin organization (Chi *et al*., 2009; Prokocimer *et al*., 2009), arctigenin may actually inhibit the formation of nuclear membrane via abnormal nuclear enlargement.

Conclusion

Here, we found by microscopic observation that a natural plant lignan, arctigenin, induces cell and nuclear enlargement in SW480 human colon cancer cells. We further performed flow cytometric analysis of light scattering and

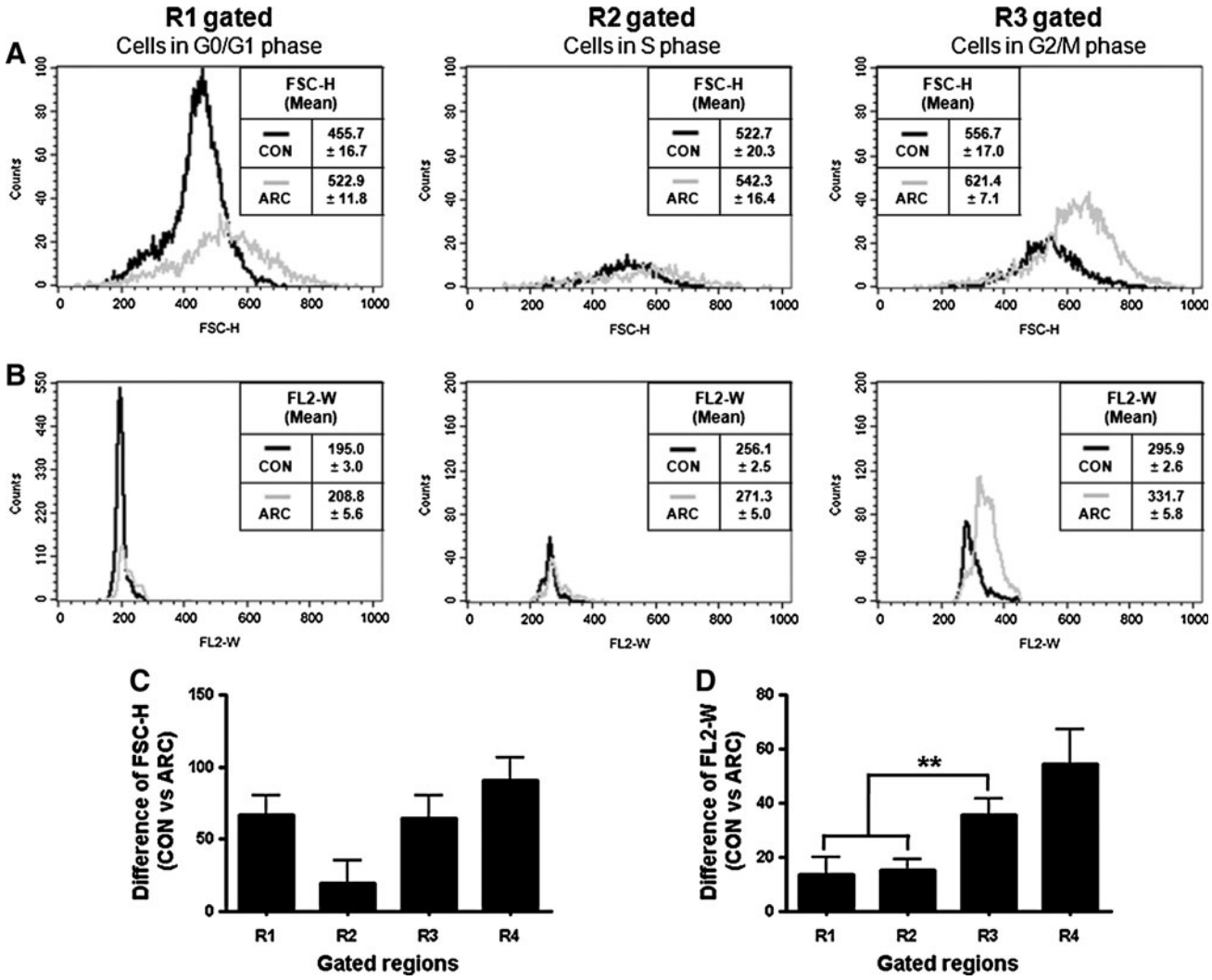

FIG. 6. Effects of cell cycle phase on arctigenin (ARC)-induced cell and nuclear enlargement in SW480 cells. FSC-H **(A,** cell size**)** and FL2-W **(B,** nuclear size**)** distributions were compared between vehicle-treated control and arctigenin-treated cells (100 μM, 48 h). Representative data sets are shown from four independent experiments. Histogram plots of FSC-H **(A)** and FL2-W **(B)** were schematized from cells in each cell cycle phase (left: R1 gated, G_0/G_1 phase cells; middle: R2 gated, S phase cells; right: R3 gated, G_2 + M phase cells; details explained in Fig. 5A). Overlay histograms schematized using vehicle-treated control (CON) and arctigenin (ARC)-treated cell data. The means ± standard deviation values of FSC-H and FL2-W are represented in each plot from four independent experiments. Differences of FSC-H **(C)** and FL2-W **(D)** mean values are shown between vehicle-treated control and arctigenin-treated cells depending on cell cycle phase (R1, R2, R3, and R4). $^{**}p < 0.01$.

fluorescence pulse signal analysis to evaluate cell and nuclear enlargement after arctigenin treatment. To our knowledge, this is the first report of flow cytometric fluorescence pulse width analysis for phytochemical-induced nuclear enlargement. Further, we also found that arctigenin-induced nuclear enlargement preferentially occurs during the G_2/M phase of the cell cycle. Thus, our approach may provide further mechanistic insight and be applied to various biological studies related to biological particle sizing, including functional studies on cancer chemotherapeutic and chemopreventive agents.

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Disclosure Statement

No competing financial interests exit.

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