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Apple Phytochemical Extracts Inhibit Proliferation of Estrogen-Dependent and Estrogen-Independent Human Breast Cancer Cells through Cell Cycle Modulation

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Breast cancer is the most commonly diagnosed cancer in women in the United States. Dietary modification, particularly increased intake of fruits and vegetables, has been consistently associated with a reduced risk of various cancers, including breast cancer. Apples are a major source of dietary phytochemicals and flavonoids and possess potent antioxidant activity and antiproliferative activity in vitro. However, the molecular mechanisms of the anticancer properties of apple phytochemical extracts are not completely understood. In this study a possible mechanism by which apple extracts could inhibit cancer cell growth in vitro using estrogen-dependent MCF-7 and estrogen-independent MDA-MB-231 human breast cancer cell lines was analyzed. The data showed that apple phytochemical extracts significantly inhibited human breast cancer MCF-7 and MDA-MB-231 cell proliferation at concentrations of 10−80 mg/mL ($p < 0.05$). DNA flow cytometric analysis showed that apple extracts significantly induced G1 arrest in MCF-7 cells in a dose-dependent manner at concentrations >20 mg/mL ($p < 0.05$). At concentrations of 15, 30, and 50 mg/mL, apple extracts caused a greater increase in the G1/S ratio in MDA-MB-231 cells when compared with MCF-7 cells ($p < 0.05$). Cyclin D1 and Cdk4 proteins, the two major G1/S transit regulators, decreased in a dose-dependent manner after exposure to apple extracts. These results suggest that the antiproliferative activities of apple phytochemical extracts toward human breast cancer cells might be due to the modulation effects on cell cycle machinery.

KEYWORDS: Breast cancer; phytochemicals; fruits and vegetables; apples; cell cycle; antiproliferation; estrogen receptor

INTRODUCTION

Breast cancer is the most commonly diagnosed invasive cancer in women in the United States, with the number of cases diagnosed increasing worldwide (*1*). Among several distinct classes of tumors that exhibit different treatment responses, breast cancer is extremely difficult to treat (*2*). Patients with breast cancers that express estrogen receptors (ER) are generally associated with a better response to hormone therapy than those with cancers not expressing estrogen receptors (*3*). However, approximately one-third of breast cancer patients have been diagnosed with estrogen receptor-negative tumors, which are resistant to conventional hormonal or chemotherapeutic agents (*4*). New alternative breast cancer treatments and preventive strategies are in great need.

Dietary modification has been long proposed as an alternative strategy to lower the risk of cancer (*5, 6*). Among various dietary modification strategies, increased consumption of fruits and vegetables has been linked to a reduced risk of developing cancer by various epidemiological studies (*7, 8*) and, more specifically, to a reduced risk of breast cancer (*2, 9, 10*). More and more evidence has shown that fruit and vegetable phytochemicals play an important role in the prevention of cancers (*11*−*13*). We have proposed that the benefit of a diet rich in fruits and vegetables is attributed to the complex mixture of phytochemicals present in whole foods (*11*), because no proven health benefits of any single antioxidant compound have been observed in large-scale intervention studies (*14*−*17*). Such evidence suggests that whole foods, not individual compounds, should be thus characterized for the effects of reduced risk of cancers. In a review focusing on the survival of breast cancer patients and their intake of fruits and vegetables, five of the eight cohort studies showed an inverse relationship between vegetable and fruit intake and survival of breast cancer patients, with a 20−90% reduction in death risk (*43*). In contrast, recent

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research results on several cohort and case-control studies conducted in Europe did not support these protective effects of vegetables and fruits (*44*). Results from the European Prospective Investigation Into Cancer and Nutrition (EPIC) study showed that there was no significant association between vegetable or fruit intake and breast cancer risk. In the pooled analysis of cohort studies including 7377 cases of invasive breast cancer, results suggested that there was no significant association between fruit and vegetable consumption during adulthood and reduced risk of breast cancer (*45*). The different research results seen in these epidemiological studies may be due to the various study designs and the inherit system errors involved in the different types of studies.

Several epidemiological studies have linked apples, a very significant source of dietary flavonoids, to a reduced risk of cancers (*18, 19*). Apples have been shown to significantly lower lipid oxidation both in humans and in rats (*20, 21*). In previous studies, we demonstrated that apples contained abundant amounts of phenolic compounds and possessed potent antioxidant activity among commonly consumed fruits and vegetables tested in vitro (*22, 23*). We have shown that apples exhibited potent antiproliferative activity toward human liver cancer HepG2 cells and human colon cancer Caco-2 cells (*22, 24*). Recently, we showed that whole apple extracts prevented mammary cancer in a rat model in a dose-dependent manner (*25*). The doses of apple extracts used in this rat model could be extrapolated to doses comparable to human consumption of one, three, and six apples a day (*25*). Thus, it is of interest to further study the effects and mechanisms of apples toward the prevention of breast cancer.

Cell cycle machinery serves as a tight control on cell growth and cell proliferation. Thus, cell cycle modulation could serve as an effective method in the regulation of cell proliferation, and many chemotherapy agents exert their effects by interruption of cell cycle progression or by induction of apoptosis in cancer cells. Induction of apoptosis and inhibition of tumor cell proliferation have been used as markers to evaluate the anticancer activities of phytochemical compounds (*26*−*28*). To our knowledge, there are no studies that explain the mechanism of how apple extracts inhibit cancer cell proliferation. The objective of the present study was to (1) compare the antiproliferative effects of apple extracts on the ER-positive human breast cancer MCF-7 cell line and the ER-negative human breast cancer MDA-MB-231 cell line and (2) determine if the exhibited antiproliferative activities of apple extracts are associated with cell cycle regulation.

MATERIALS AND METHODS

Chemicals. Aluminum chloride, sodium hydroxide, methyl *tert*-butyl ether, methanol, and acetone were purchased from Fisher Scientific (Pittsburgh, PA). Gallic acid and metaphosphoric acid were obtained from ICN Biomedical Inc. (Costa Mesa, CA). Sodium nitrite, (+)-catechin, Folin−Ciocalteu reagent, hyrdrochloric acid, propidium iodide, Igepal, Triton X-100, and protease inhibitors [aprotinin; leupeptin; pepstain; phenylmethanesulfonyl fluoride (PMSF); sodium orthovanadate; sodium fluoride] were purchased from Sigma Chemical Co. (St. Louis, MO). All reagents used in the study were of analytical grade. Ultrapure Tris (base) and Tris (acid) were purchased from J. T. Baker (Phillipsburg, NJ). Phosphate-buffered saline (PBS), Minimum Essential Medium Alpha Medium (MEM), Hepes, insulin, penicillin, streptomycin, gentamicin, and fetal bovine serum were purchased from GIBCO (Life Technologies, Grand Island, NY).

Sample Extraction. Apples of the Red Delicious variety were obtained from the Cornell Orchard (Ithaca, NY). Fruits were cleaned and dried. The apple core was removed before extraction. The remaining parts were cut into small cubes for the homogenization process. Phenolics of fruits were extracted using a method previously reported by our laboratory (*22, 29*). Briefly, 100 g of fresh weight of apples were homogenized for 5 min with ice-cold 80% acetone (1:2 w/v) using a chilled Waring blender. The sample was then homogenized for an additional 3 min using a Polytron homogenizer. The homogenates were filtered under vacuum through a no. 2 Whatman filter paper on a Büchner funnel. The filtrate was evaporated at 45 °C until approximately 90% of the filtrate had been evaporated. The filtrate was then recovered with water to a final volume of 50 mL and stored at −40 °C until use.

Determination of Total Phenolic Content. The total phenolic content of samples was analyzed using the Folin−Ciocalteu colorimetric method described previously (*30*) and modified in our laboratory (*22*). Briefly, the appropriate dilutions of extracts were oxidized with Folin−Ciocalteu reagent, and the reaction was neutralized with sodium carbonate. After 90 min, the reaction mixture solutions were transferred to a 96-well plate, and the absorbance was measured against the blank at 760 nm using an MRX II DYNEX microplate reader (Dynex Technologies, Inc., Chantilly, VA) and was compared to a gallic acid standard curve. The results were expressed as mean (mg of gallic acid equiv/100 g of fresh apples) ± SD for three independent replications.

Determination of Total Flavonoid Content. Total flavonoid content was determined using a colorimetric method described previously (*22, 24*). Briefly, 0.25 mL of the apple extracts or (+)-catechin standard solution was mixed with 1.25 mL of distilled water in a test tube followed by addition of 75 μL of a 5% sodium nitrite solution. After 6 min, 150 μL of a 10% aluminum chloride solution was added and allowed to stand for another 5 min before 0.5 mL of 1 M NaOH was added. The mixture was adjusted to 2.5 mL with distilled water, and the absorbance was immediately measured at 510 nm using the MRX II Dynex microplate reader (Dynex Technologies, Inc.). (+)-Catechin was used as a standard, and the results were expressed as mean (mg of catechin equiv/100 g of fresh apples) ± SD for three independent replications.

Measurement of Antiproliferative Activity toward Human Breast Cancer Cells. The antiproliferative activities of apple extracts were measured by the MTS assay (MTS-based cell titer 96 non-radioactivity cell proliferation assay) (Promega, Madison, WI) described previously (*31*). Human breast cancer MCF-7 cells (American Type Culture Collection, ATCC, Rockville, MD) were maintained at 37 °C with 5% CO_2 in MEM containing 10 mM Hepes, 10 μg/mL insulin, 50 units/mL penicillin, 50 μg/mL streptomycin, 100 μg/mL gentamicin, and 10% fetal bovine serum (Gibco, Life Technologies). Human breast cancer MDA-MB-231 cells (ATCC) were maintained at 37 °C with 5% CO_2 in the same MEM but supplemented with 10% heat-inactivated fetal bovine serum. MCF-7 or MDA-MB-231 cells in growth media were placed in each well of a 96-well flat-bottom plate at a density of 2.5×10^4 cells/well. After 4 h of incubation at 37 °C with 5% CO_2, the growth medium was replaced by media containing different concentrations of apple extracts. Control cultures received the extraction solution minus the apple extracts, and blank wells contained 100 μL of growth medium with no cells. After 96 h of incubation, cell proliferation was determined by the colorimetric MTS assay. Cell proliferation (percent) was determined from the MTS absorbance at 490 nm reading for each concentration compared to the control. A minimum of three replications for each sample was used to determine the antiproliferative activity.

Analysis of Cell Cycle Distribution. Human breast cancer MCF-7 cells and MDA-MB-231 cells were seeded at a density of 1×10^6 cells/well in a 12-well plate. Six hours after seeding, MCF-7 cells were treated with different concentrations of apple extracts equivalent to 0, 10, 20, 40, and 60 mg/mL and were then harvested after a 15 h exposure. MDA-MB-231 cells were treated with 0, 15, 30, and 50 mg/mL apple extracts, and cells were harvested after a 24 h exposure. Trypsinized cells were centrifuged at 128*g* at 4 °C and were then resuspended to a density of 1×10^6 cells/mL in 0.1% sodium citrate solution containing 0.05 mg/mL propidium iodide and 0.1% (v/v) Triton X-100. After a 15 min exposure on ice in the dark, cells were filtered through a 41 μm nylon mesh (Laboratory Pak, Sefar America Inc., Kansas City, MO) and analyzed by flow cytometry (FASC system,

Becton Dickinson, San Jose, CA); at least 10000 cells per sample were analyzed. The percentage of cells in G1, S, and G2-M phases was analyzed by ModFit LT software (Verity Software House, Topsham, ME). All results were expressed as mean ± SD for three independent replications.

Protein Extraction and Western Blot Analyses. Protein extraction and Western blot analyses were conducted using the method described previously (*32*). Similar to the cell cycle analysis, cells were seeded at a density of 1×10^6 cells/well in 12-well plates. Six hours after seeding, MCF-7 cells were treated with different concentrations of apple extracts and were then harvested after a 15 h exposure. MDA-MB-231 cells were treated with apple extracts for 24 h before the cells were harvested. Each treatment was replicated in two wells, and the cells receiving the same level of treatment were combined together for protein extraction and further Western blot Analysis. Prior to analysis of cell cycle-related proteins, cell culture media were removed from the treatment plates and rinsed twice with ice-cold PBS. Cells were then scraped off in lysis buffer (50 mM Tris, pH 7.4; 1% Igepal; 150 mM sodium chloride; 1 mM EDTA) with protease inhibitors (1 μg/mL aprotinin; 1 μg/mL leupeptin; 1 μg/mL pepstain; 1 mM PMSF; 1 mM sodium orthovanadate; 1 mM sodium fluoride). Cell lysates were vortexed briefly and were placed on ice for 20 min to facilitate protein extraction. Lysates were then centrifuged at 12000*g* for 5 min at 4 °C, and protein concentrations of the lysates were determined using a Sigma Diagnostics Micro Protein Determination Kit and a Dynex Microplate Reader (Dynex Technologies). Electrophoresis was carried out on 15 μg of protein loaded onto 12% SDS−polyacrylamide gel, followed by transfer to Immobilon-P transfer membranes (*32*). The blots were blocked in 3% nonfat dry milk in PBS for 1 h at room temperature. After blocking, the membranes were incubated overnight at 4 °C with mouse monoclonal anticyclin D1 antibody, anti-cdk4 antibody (Sigma, St. Louis, MO), or rabbit polyclonal anti-Rb antibody (Phospho-specific, Thr826, Oncogene, San Diego, CA), respectively. Membranes were then incubated with the corresponding secondary antibody, anti-mouse IgG−HRP conjugate (anti-rabbit IgG−HRP conjugate for PhosphoRb detection) in PBS with 3% nonfat milk powder for 1 h under agitation at room temperature. The expression of human β-actin was analyzed as an internal standard for process control. Protein was visualized by the Enhanced Chemiluminescence kit (Cell Signaling Technology, Inc., Beverly, MA) (*32*). The densities of the specific protein bands were quantified by integrated optical densitometry using Labworks gel imaging and analysis software (UVP Laboratories, Upland, CA). All results were expressed as mean ± SD for three independent replications.

Statistical Analysis. Statistical analysis was conducted using SigmaStat version 8.0 (Jandel Corp., San Raphael, CA). Significance of the relationship was determined by analysis of variance using Minitab release 12 software (Minitab Inc., State College, PA).

RESULTS

The total phenolic content and the total flavonoid content of apple extracts were 296.3 ± 6.2 mg of gallic acid equiv/100 g of fresh apples and 82.7 ± 0.5 mg of catechin equiv/100 g of fresh apples, respectively.

As shown in **Figure 1**, proliferation of both MCF-7 and MDA-MB-231 cells was significantly inhibited in a dose-dependent manner by apple extracts above 10 mg/mL ($p < 0.05$). MCF-7 and MDA-MB-231 cell lines were inhibited by approximately 10% at a does of 10 mg/mL when compared to the control. However, the estrogen-independent MDA-MB-231 cells were more sensitive in the response to apple extracts when compared to the estrogen-dependent MCF-7 cells. At a dose of 30 mg/mL, MDA-MB-231 cell proliferation was inhibited by approximately 50%, whereas MCF-7 cell proliferation was reduced by 20%. The median effective concentration (EC_{50}) of apple extracts for inhibition of MCF-7 cell proliferation was 69.9 ± 5.1 mg/mL. Apple extracts demonstrated a more pronounced reduction against MDA-MB-231 cell proliferation,

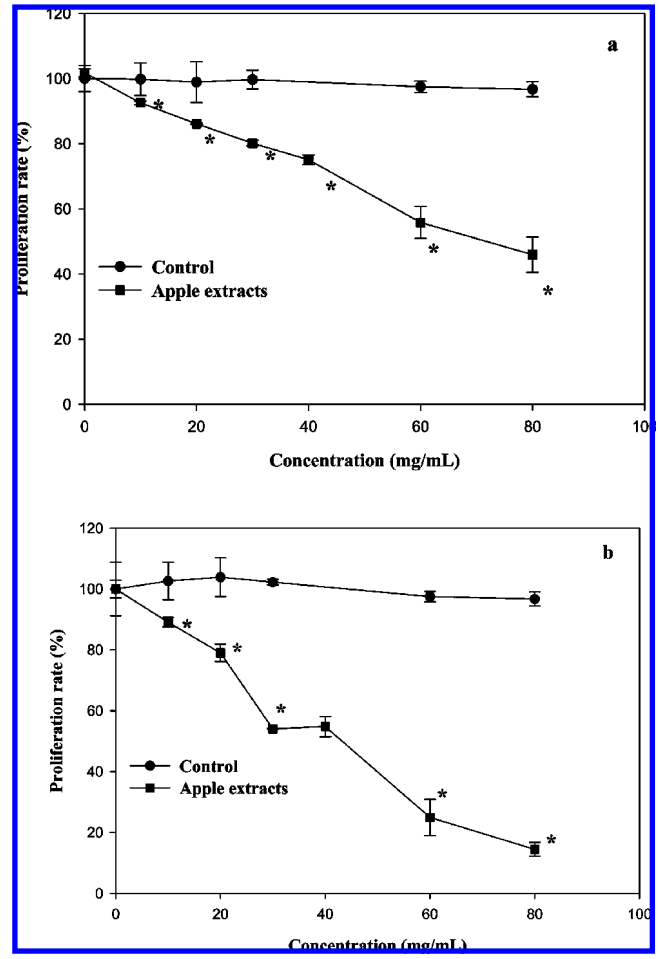

Figure 1. Inhibition of proliferation of human breast cancer MCF-7 cells (**a**) and MDA-MB-231 cells (**b**) by apple extracts (mean ± SD, $n = 3$). An asterisk (*) indicates a significant difference from the control at the same level ($p < 0.05$).

with an EC_{50} of 35.2 ± 2.4 mg/mL. There was no cytotoxicity toward either human breast cancer cell line at the doses tested, as measured by the methylene blue cytotoxicity assay (data not shown) (*13, 31*).

Time−kinetics experiments were carried out to determine the doubling time of each cell line and thus the best exposure time for the analysis. On the basis of our preliminary data, we found that the majority of proliferative MCF-7 cells entered the S phase 15 h after release from the G0 phase, whereas for MDA-MB-231 cells, 24 h after release from the G0 phase was the best test point. **Figure 2** shows the results of cell cycle distribution regulation by apple extracts for both MCF-7 (**Figure 2a**) and MDA-MB-231 cells (**Figure 2b**). The G1/S ratio was used as an index of G1 arrest. Apple extracts significantly induced G1 arrest in MCF-7 cells in a dose-dependent manner at concentrations >20 mg/mL ($p < 0.05$; **Figure 2a**). However, a more dramatic increase in the G1/S ratio was seen for the treated MDA-MB-231 cells at the same concentrations of apple extracts. At doses of 15, 30, and 50 mg/mL, apple extracts induced a significant increase in G1/S ratios of MDA-MB-231 cells in a dose-dependent manner ($p < 0.05$; **Figure 2b**). The G1/S ratio was 340% higher in MDA-MB-231 cells treated with 50 mg/mL apple extracts when compared to the control. At the dose of 60 mg/mL, the percentage of MCF-7 cells in G1 phase reached 73% of total cells compared to 69% in the control group ($p < 0.05$); the percentage of S phase cells declined from 24% in the control group to 17%, whereas a moderate increase from

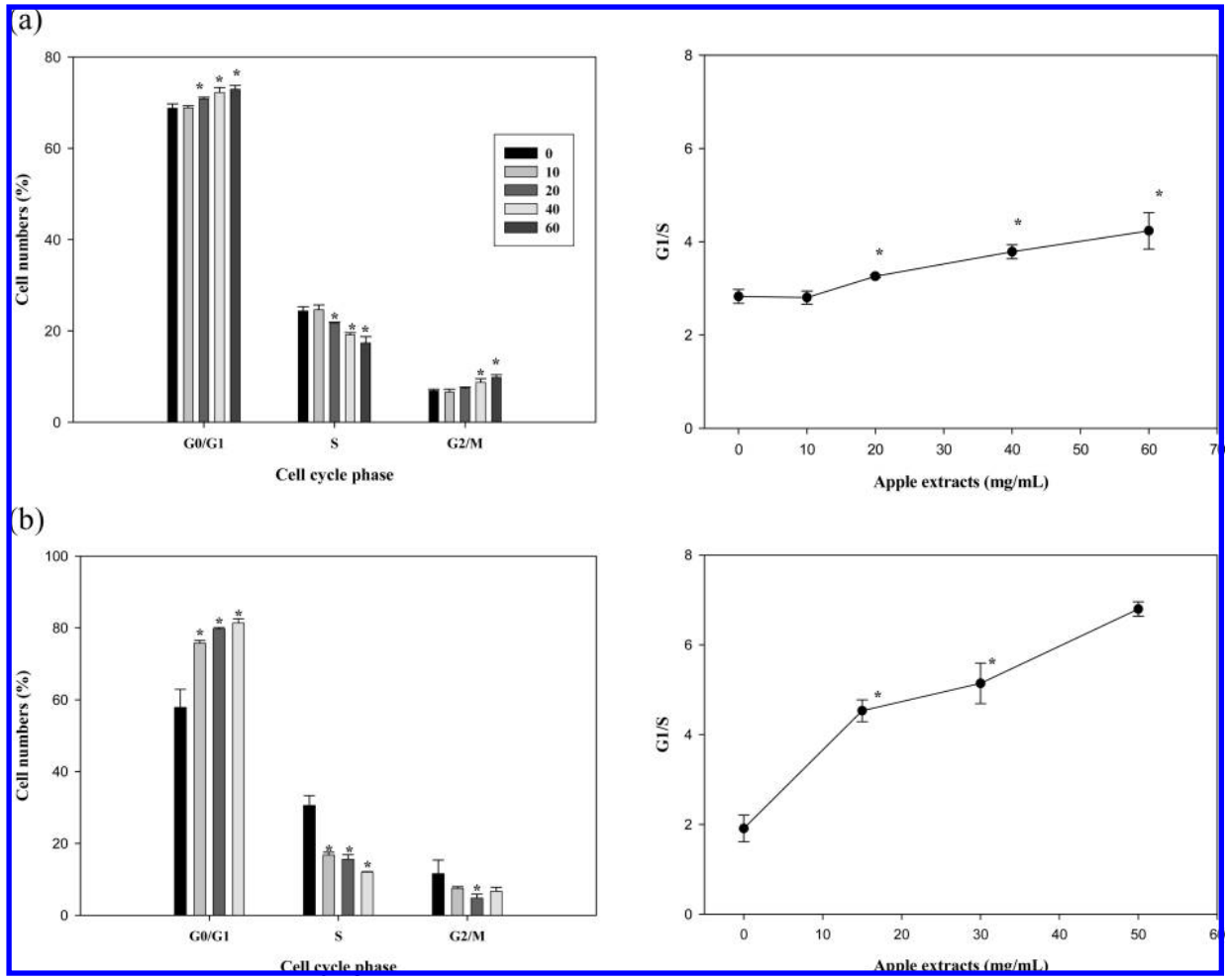

Figure 2. Induction of G1 arrest in the cell cycle progression of human breast cancer MCF-7 cells (**a**) and MDA-MB-231 cells (**b**) by apple extracts (mean ± SD, $n = 3$). An asterisk (∗) indicates a significant difference from the control ($p < 0.05$).

7 to 10% in G2-M phase cells was measured ($p < 0.05$). MDA-MB-231 cells exposed to 50 mg/mL apple extracts had a sharp increase in the percentage of G1 phase cells from 58 to 81% and a decrease from 30 to 12% in S phase cells ($p < 0.05$). A decrease in the percentage of G2-M phase MDA-MB-231 cells was observed at all of the doses tested.

The effects of apple extracts on cell cycle-related proteins in MCF-7 and MDA-MB-231 cells are shown in **Figures 3** and **4**. After exposure to apple extracts at doses of 10, 30, 50, and 100 mg/mL, cyclin D1 and Cdk4 protein expression in MCF-7 cells was down-regulated in a dose-dependent manner (**Figure 3**). At doses of 50 and 100 mg/mL, the cyclin D1 levels were significantly lower than that of the control group ($p < 0.05$). The Cdk4 levels were significantly lower than that of the control group with all of the doses tested ($p < 0.05$), suggesting stronger regulation effects of apple extracts toward Cdk4 than toward cyclin D1. The cyclin D1 and Cdk4 protein expression levels in MDA-MB-231 cells were down-regulated in a dose-dependent manner with the addition of apple extracts at doses of 10, 20, and 40 mg/mL (**Figure 4**). Both cyclin D1 and Cdk4 levels were significantly lower in MDA-MB-231 cells treated with 20 and 40 mg/mL apple extracts when compared to the control group. Rb is one of the key protein regulators that control cell phase transition from the G1 to S phase. At doses >40 mg/mL, the phosphorylated Rb expression was significantly lower than that of the control group in both cell lines ($p < 0.05$, **Figures 3** and **4**).

DISCUSSION

Epidemiological studies have consistently shown a significant inverse association between fruit and vegetable intake and breast cancer risk. Phytochemicals, such as phenolics and flavonoids, from fruits and vegetables may play a key role in reducing cancer risk (*11, 24, 33, 34*). Apples are the major contributors of dietary phytochemicals and flavonoids in the Western diet (*35*). Several studies have shown that higher consumption of apples is associated with lower cancer risk (*18, 19*). Previous studies showed that apple extracts exhibited potent antiproliferative activities toward human liver cancer HepG2 cells and human colon cancer cells, and these antiproliferative activities were not due to phenolic-induced hydrogen peroxide formation (*22, 36−38*). In this study, our data clearly showed that apple extracts inhibited the growth of both human breast cancer MCF-7 and MDA-MB-231 cells at doses ranging from 10 to 80 mg/mL. In our current study, we demonstrated that apple extracts inhibited breast cancer cell growth through estrogen-independent pathways because cell proliferation was significantly reduced in both human breast cancer cell types after exposure to the apple extracts. Because approximately one-third of breast cancer cases are estrogen-independent, and more resistant to antiestrogen therapy, it is worth noting that in our study the antiproliferative activity of the apple extracts was more pronounced against the estrogen receptor-negative MDA-MB-231 cells when compared to the estrogen receptor-positive MCF-7 cells.

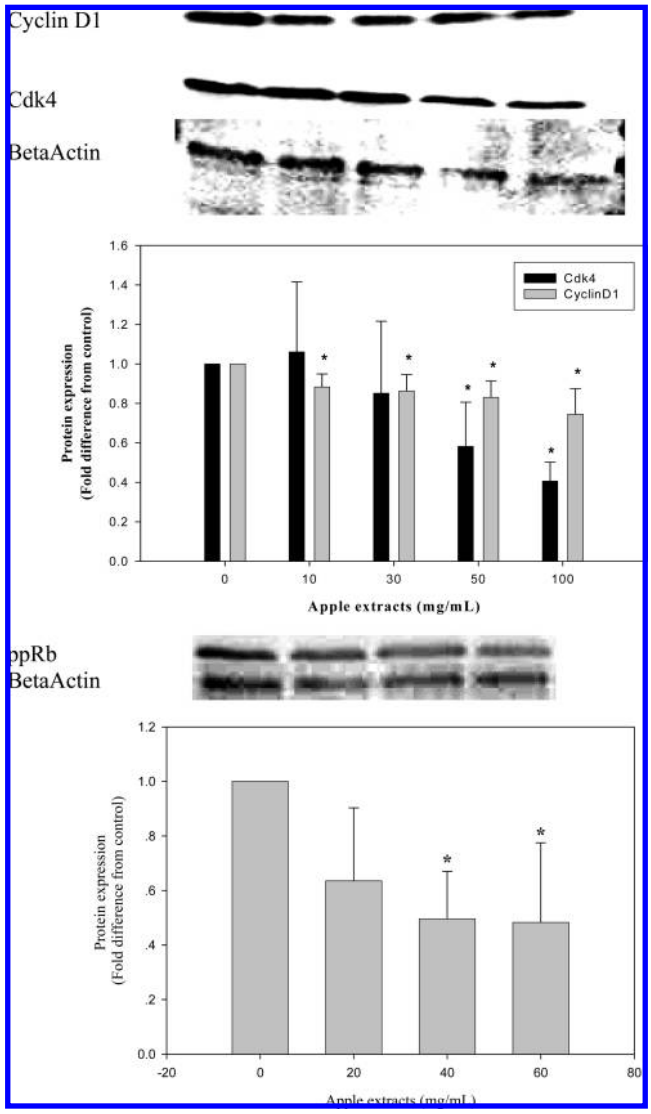

Figure 3. Regulation of cell cycle-related proteins in human breast cancer MCF-7 cells by apple extracts (mean ± SD, $n = 3$). An asterisk (*) indicates a significant difference from the control ($p < 0.05$).

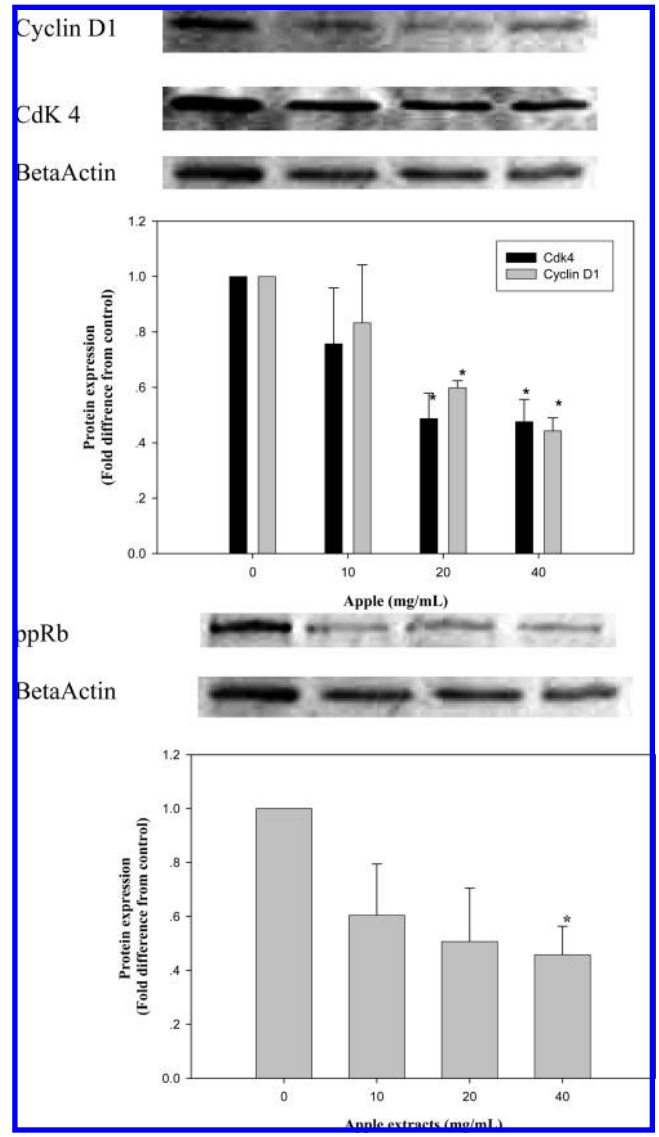

Figure 4. Regulation of cell cycle-related proteins in human breast cancer MDA-MB-231 cells by apple extracts (mean ± SD, $n = 3$). An asterisk (*) indicates a significant difference from the control ($p < 0.05$).

Cell cycle control has been proven to be a major event in ensuring accurate cell division, and the cell cycle regulators of cancer cells have been proposed as targets for chemoprevention treatment (*39*). Many studies have shown that phytochemicals derived from fruits and vegetables could interfere with cell cycle regulation machinery in vitro (*40*). Our data suggested that apple extracts, as a whole phytochemical cocktail, could cause a significant accumulation of cells in the G0/G1 phase in human breast cancer MCF-7 and MDA-MB-231 cells in a similar dose-dependent manner in vitro. It would be worth investigating the regulatory effects of apple extracts in vivo in the future. The blocking effect of apple extracts occurred at the G1/S transition, resulting from the significant increase of G0/G1 phase cells, a decrease of S phase cells, and a moderate increase of G2 phase cells. The cell cycle alteration effect of the apple extracts toward the modulation of the cell cycle machinery was different between MCF-7 and MDA-MB-231 cells. At doses of 10 mg/mL, apple extracts induced a 200% increase in the G1 arrest index in MDA-MB-231 cells, whereas no significant changes of the G1/S ratio were observed in MCF-7 cells under the same treatment. The estrogen receptor-negative MDA-MB-231 cell line was derived from a metastatic carcinoma. Patients with breast cancers that do not express estrogen receptors are generally associated with lower survival and poorer response to hormone therapy than those patients with tumors expressing estrogen receptors (*3*).

To further investigate in detail the effects of apple extracts on cell cycle alteration, the expression of several cell cycle-related proteins was determined by Western blot analysis (**Figures 3** and **4**). The expression and association of cyclin D1 and Cdk4 control the cell cycle events in early G1 phase by catalyzing the inactivation reaction of the tumor suppressor pRb through phosphorylation (*41, 42*). Through the G1 phase, Rb exists in a hypophosphorylated form and binds with the E2F family of transcriptional regulators. The cyclin D1/cyclin-dependent kinase complex catalyzes the phosphorylation of pRb into the hyperphosphorylated form and then releases the E2F transcriptional regulators subsequently. Thus, the phosphorylation of Rb is required for S phase entry. The antibody we used could specifically detect the phosphorylated Rb at the phosphorylation site at residue Thr826, which is the phosphorylation site catalyzed by the cyclinD1/Cdk4 complex. Our results showed that phosphorylated Rb expression was down-regulated in both cell lines after exposure to apple extracts at various doses (**Figures 3** and **4**). Overall,

our results showed that apple extracts could interfere with the cell cycle-related proteins. In particular, treatment with apple extracts significantly decreased the level of cyclin D1 and Cdk4 proteins. As a consequence, less phosphorylated Rb expression was observed due to lack of cyclin D1/Cdk4 complex, the catalytic factor. Theoretically, this regulation change will result in fewer E2F transcription regulators released from the ppRb/E2F complex, and thus fewer E2F targeted genes were expressed in response to the apple extract treatment.

In summary, our data showed that apple extracts markedly inhibited cell proliferation in both human breast cancer MCF-7 and MDA-MB-231 cells in a dose-dependent manner. The antiproliferative activity of apple extracts against MCF-7 and MDA-MB-231 cells could result from the induced G1 arrest with decreased expression of cyclin D1, Cdk4, and ppRb proteins. The modulation effects of apple extracts were more pronounced against estrogen receptor-negative MDA-MB-231 cells, compared to the estrogen receptor-positive MCF-7 cells.

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