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Indole-3-carbinol Inhibits the Expression of Cyclin-dependent Kinase-6 and Induces a G_1 Cell Cycle Arrest of Human Breast Cancer Cells Independent of Estrogen Receptor Signaling*

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Indole-3-carbinol (I3C), a naturally occurring component of *Brassica* vegetables such as cabbage, broccoli, and Brussels sprouts, has been shown to reduce the incidence of spontaneous and carcinogen-induced mammary tumors. Treatment of cultured human MCF7 breast cancer cells with I3C reversibly suppresses the incorporation of [^{3}H]thymidine without affecting cell viability or estrogen receptor (ER) responsiveness. Flow cytometry of propidium iodide-stained cells revealed that I3C induces a G_1 cell cycle arrest. Concurrent with the I3C-induced growth inhibition, Northern blot and Western blot analyses demonstrated that I3C selectively abolished the expression of cyclin-dependent kinase 6 (CDK6) in a dose- and time-dependent manner. Furthermore, I3C inhibited the endogenous retinoblastoma protein phosphorylation and CDK6 phosphorylation of retinoblastoma *in vitro* to the same extent. After the MCF7 cells reached their maximal growth arrest, the levels of the p21 and p27 CDK inhibitors increased by 50%. The antiestrogen tamoxifen also suppressed MCF7 cell DNA synthesis but had no effect on CDK6 expression, while a combination of I3C and tamoxifen inhibited MCF7 cell growth more stringently than either agent alone. The I3C-mediated cell cycle arrest and repression of CDK6 production were also observed in estrogen receptor-deficient MDA-MB-231 human breast cancer cells, which demonstrates that this indole can suppress the growth of mammary tumor cells independent of estrogen receptor signaling. Thus, our observations have uncovered a previously undefined antiproliferative pathway for I3C that implicates CDK6 as a target for cell cycle control in human breast cancer cells. Moreover, our results establish for the first time that CDK6 gene expression can be inhibited in response to an extracellular antiproliferative signal.

Considerable epidemiological evidence suggests that high vegetable diets correlate with low breast cancer risk (1, 2). This phenomenon is likely due to the diverse spectrum of dietary and environmental compounds that can regulate the function and proliferation of mammalian cells by influencing hormone receptor signal transduction pathways (3, 4). Several classes of these naturally occurring hormone-like chemicals have been implicated in the control of tumor cell growth and as chemopreventative agents. One such substance is the dietary compound indole-3-carbinol (I3C),[1] an autolysis product of a glucosinolate, glucobrassicin, which occurs in *Brassica* vegetables such as cabbage, broccoli, and Brussels sprouts (5, 6). A recent screen of 90 potential chemopreventative agents in a series of six short term bioassays relevant to carcinogen-induced DNA damage, oxidative stress, and tumor initiation and promotion, revealed I3C to be one of only eight compounds effective in all assays (7). Several studies have shown that exposure to dietary I3C markedly reduces the incidence of spontaneous and carcinogen-induced mammary tumors in rodents (8, 9). For example, I3C administered in the diet or by oral intubation prior to treatment with carcinogen reduced the incidence of 7,12-dimethyl-benz(a)anthracene-induced mammary tumors in rodents by 70–90% (6, 10). Consistent with these results, dietary supplementation with cabbage or broccoli, vegetables that are good sources of I3C, also resulted in decreased mammary tumor formation in 7,12-dimethyl-benz(a)anthracene-treated rats (11). Also, in a long term feeding experiment, in which female mice consumed synthetic diets containing I3C, spontaneous mammary tumor incidence and multiplicity were reduced by 50% and tumor latency was prolonged compared with untreated control animals (9). I3C also has anticarcinogenic effects on other cancer types, such as hepatic tumors (12) and can reduce benzo(a)pyrene-induced neoplasia of the forestomach (6).

I3C has been shown to have an antiestrogenic activity *in vivo*, which has been proposed to account for some of its protective and antiproliferative effects on mammary tumor formation. Part of this effect may be due to alterations in estrogen metabolism, since oral administration of I3C to humans increased estradiol 2-hydroxylation by approximately 50% in both men and women (13) and also increased the levels of estradiol hydroxylation activity in female rats (14). In addition, I3C was shown to block the estradiol-induced proliferation of long term confluent cultures of human breast cancer cells (15).

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[1] The abbreviations used are: I3C, indole-3-carbinol; Ah, aromatic hydrocarbon; BrdUrd, 5-bromo-2′-deoxyuridine; CAT, chloramphenicol acetyltransferase; CDK, cyclin-dependent kinase; CS, calf serum; DIM, 3,3′-diindolylmethane; ER, estrogen receptor; ERE, estrogen response element; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ICZ, indolo(3,2-*b*)carbazole; NFDM, nonfat dry milk; PBS, phosphate-buffered saline; Rb, retinoblastoma; HPLC, high pressure liquid chromatography; IP, immunoprecipitation.

A major complication in interpreting the physiological results is that I3C is extremely unstable in acidic solution and does not withstand exposure to the low pH environment of the stomach (16). A relatively large fraction of I3C is converted into several acid-catalyzed derivatives with distinct biological activities that appear to mediate the antiestrogenic effects of I3C. Two of the most active acid products of I3C that have been identified are 3,3′-diindolylmethane (DIM) and indolo(3,2-*b*)carbazole (ICZ) (17).

A general picture has emerged indicating that many of the long term antiestrogenic biological activities of I3C probably result from the actions of one or more of its acid-catalyzed derivatives (17, 18). ICZ appears to mediate its antiestrogenic effects by the direct binding to the Ah receptor (aromatic hydrocarbon or dioxin receptor), inducing cytochrome P450 *CYP1A1* gene expression (19), which can alter estrogen metabolism, thereby effectively decreasing the amount of circulating estrogen. This reduction in circulating estrogen leads to the decreased growth of estrogen-responsive mammary tissue and, presumably, a protective effect against breast cancer. ICZ exhibits only a very weak affinity for the ER but is the most potent Ah receptor agonist among the characterized I3C acid-catalyzed derivative compounds. In contrast, I3C has approximately 100,000-fold lower affinity for the Ah receptor compared with ICZ (K_d of 190 pM) and 3000-fold lower affinity compared with DIM (K_d of 90 nM) (17). Thus, it is unlikely that I3C mediates its activities directly through the Ah receptor, and conceivably this dietary indole may exert many of its direct growth-inhibitory effects through a signal transduction pathway distinct from the antiestrogenic effects of its acid-catalyzed products. However, virtually nothing is known about the mechanism by which I3C mediates its antiproliferative effects on human breast cancer cells. The I3C-regulated cellular processes are likely to be complex, however, because both estrogen-independent and estrogen-dependent pathways may potentially be under indole control.

Analogous to most other antiproliferative signaling molecules in mammalian cells, the I3C growth suppression pathway probably targets specific components and stages within the cell cycle. The eukaryotic cell cycle is composed of four phases (G_1 (gap 1), DNA synthesis (S), G_2 (gap 2), and mitosis (M)) as well as an out of cycle quiescent phase designated G_0. In normal mammary epithelial cells, an intricate network of growth-inhibitory and -stimulatory signals are transduced from the extracellular environment and converge on G_1-acting components, which, through their concerted actions, stringently regulate cell cycle progression (20, 21). The final targets of these growth signaling pathways are specific sets of cyclin-cyclin-dependent kinase (CDK) protein complexes, which function at specific, but overlapping, stages of the cell cycle (22–24). An active CDK complex phosphorylates retinoblastoma (Rb) protein family members. Rb and its related proteins sequester the E2F transcription factor, and the phosphorylation of Rb proteins by the active CDKs releases E2F, which then induces the expression of genes involved in the initiation of DNA synthesis (25). In the G_1 phase of the cell cycle, certain cyclins (C, D1, D2, D3, E) are necessary for activation the G_1 CDKs (CDK2, CDK4, and CDK6), while, in a complementary manner, several of the small proteins associated with cyclin-CDK complexes (p15, p16/Ink4a, p21/Waf1/Cip1, p27/Kip1, p57/Kip2) have been shown to act as specific inhibitors of cyclin-dependent kinase activity (26–29). The loss of normal cell cycle control in G_1 has been implicated in mammary tumor development and proliferation. Up to 45% of human breast cancers show an aberrant expression and/or amplification of cyclin D1 or cyclin E (30, 31). In other studies, inappropriate expression and/or mutation of certain G_1-acting proto-oncogenes, growth factors, and their cognate receptors has been observed in both human and rodent mammary tumor cells (31–33). Furthermore, many tumors exhibit a loss in expression or function of certain tumor suppressor genes (such as p53), which modulate cell cycle events late in the G_1 phase (34, 35).

Regulated changes in the expression and/or activity of cell cycle components that act within G_1 have been closely associated with alterations in the proliferation rate of normal and transformed mammary epithelial cells (4). For example, the estrogen-induced activation of CDK4 and CDK2 during progression of human breast cancer cells between the G_1 and S phases is accompanied by the increased expression of cyclin D1 and decreased association of the CDK inhibitors with the cyclin E-CDK2 complex (36). In rat tumor cells derived from 7,12-dimethyl-benz(a)anthracene-induced mammary adenocarcinomas, glucocorticoids induce a G_1 cell cycle arrest and alter expression of cell cycle-regulated genes (37). Thus, it seemed likely that dietary indoles control the emergence and proliferation of breast cancer cells by regulating G_1-acting cell cycle components in mammary epithelial cells. In this study, we demonstrate that the dietary indole I3C, but not the acid-catalyzed derivative DIM or ICZ, inhibits the growth of human MCF7 breast cancer cells and induces a G_1 cell cycle arrest in an ER-independent manner. Strikingly, this growth arrest is accompanied by the selective inhibition in expression of CDK6 transcripts, CDK6 protein, and the activity of CDK6, which correlates with a decrease in endogenous Rb phosphorylation.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium, fetal bovine serum (FBS), calf serum (CS), calcium-free and magnesium-free phosphate buffered saline (PBS), L-glutamine, and trypsin-EDTA were supplied by BioWhittaker (Walkersville, MD). Insulin (Bovine), 17β-estradiol, and tamoxifen ((*Z*)-1-(*p*-dimethylaminoethoxyphenyl)-1,2-diphenyl-1-butene) citrate salt were obtained from Sigma. [^{3}H]Thymidine (84 Ci/mmol), [γ-^{32}P]ATP (6,000 Ci/mmol), and [α-^{32}P]dCTP (3,000 Ci/mmol) were obtained from NEN Life Science Products. Indole-3-carbinol (I3C) was purchased from Aldrich. I3C was recrystallized in hot toluene prior to use, while ICZ and DIM were prepared and purified as described (17). The sources of other reagents used in the study are either listed in the following methods or were of the highest purity available.

Cell Culture—The human breast adenocarcinoma cell lines, MCF7 and MDA-MB-231, were obtained from the American Type Culture Collection (Rockville, MD). MCF7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 10 μg/ml insulin, 50 units/ml penicillin, 50 units/ml streptomycin, and 2 mM L-glutamine. MDA-MB-231 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 50 units/ml penicillin, 50 units/ml streptomycin, and 2 mM L-glutamine. Both cell lines were maintained at 37 °C in humidified air containing 5% CO_2 at subconfluency. I3C, tamoxifen, and estradiol were dissolved in Me_2SO (99.9% HPLC grade, Aldrich) at concentrations 1000-fold higher than the final medium concentration. In all experiments, 1 μl of the concentrated agent was added per ml of medium, and for the vehicle control, 1 μl of Me_2SO was added per ml of medium.

[^{3}H]Thymidine Incorporation—Breast cancer cells were plated onto 24-well Corning tissue culture dishes. Triplicate samples of asynchronously growing mammary cells were treated for the indicated times with either vehicle control (1 μl of Me_2SO/ml of medium) or varying concentrations of I3C, estradiol, and/or tamoxifen. The cells were pulsed for 3 h with 3 μCi of [^{3}H]thymidine (84 Ci/mmol), washed three times with ice-cold 10% trichloroacetic acid, and lysed with 300 μl of 0.3 N NaOH. Lysates (150 μl) were transferred into vials containing liquid scintillation mixture, and radioactivity was quantitated by scintillation counting. Triplicates were averaged and expressed as counts/min/well.

Flow Cytometric Analyses of DNA Content—Breast cancer cells (4×10^4) were plated onto Corning six-well tissue culture dishes. A final concentration of 100 μM I3C was added to three of the wells, and vehicle control (1 μl of Me_2SO/ml of medium) was added to the other three. The medium was changed every 24 h. Cells were incubated for 96 h and hypotonically lysed in 1 ml of DNA staining solution (0.5 mg/ml propidium iodide, 0.1% sodium citrate, 0.05% Triton X-100). Nuclear emit-

ted fluorescence with wavelength greater than 585 nm was measured with a Coulter Elite instrument with laser output adjusted to deliver 15 milliwatts at 488 nm. Nuclei (10,000) were analyzed from each sample at a rate of 300–500 cells/s. The percentages of cells within the G_1, S, and G_2/M phases of the cell cycle were determined by analysis with the Multicycle computer program provided by Phoenix Flow Systems in the Cancer Research Laboratory Microchemical Facility of the University of California, Berkeley.

Western Blot Analysis—After the indicated treatments, cells were harvested in radioimmune precipitation buffer (150 mM NaCl, 0.5% deoxycholate, 0.1% Nonidet P-40, 0.1% SDS, 50 mM Tris) containing protease and phosphatase inhibitors (50 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 0.1 μg/ml NaF, 10 μg/ml β-glycerophosphate). Equal amounts of total cellular protein were mixed with loading buffer (25% glycerol, 0.075% SDS, 1.25% 2-mercaptoethanol, 10% bromphenol blue, 3.13% stacking gel buffer) and fractionated by electrophoresis on 10% (7.5% for Rb) polyacrylamide, 0.1% SDS resolving gels. Rainbow marker (Amersham Life Sciences) was used as the molecular weight standard. Proteins were electrically transferred to nitrocellulose membranes (Micron Separations, Inc., Westboro, MA) and blocked overnight at 4 °C with Western wash buffer and 5% NFDM (10 mM Tris HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20, 5% nonfat dry milk) (PBS, 5% nonfat dry milk for Rb). Blots were subsequently incubated for 1 h at room temperature for rabbit anti-CDK2, -CDK4, -CDK6, and -ER antibodies (Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), catalog numbers sc-163, sc-260, sc-177, and sc-543, respectively), 2 h at room temperature for goat anti-p21, rabbit anti-cyclin E, and rabbit anti-p27 antibodies (Santa Cruz Biotechnology, catalog numbers sc-397, sc-198, and sc-528, respectively), and overnight at 4 °C for mouse anti-cyclin D1 and mouse anti-Rb antibodies (Santa Cruz Biotechnology, catalog number sc-246; Pharmingen (San Diego, CA), catalog number 14001A, respectively). Working concentration for all antibodies was 1 μg/ml Western wash buffer (PBS for Rb). Immunoreactive proteins were detected after incubation with horseradish peroxidase-conjugated secondary antibody diluted to 3×10^{-4} in Western wash buffer, 1% NFDM (goat anti-rabbit IgG (Bio-Rad); rabbit anti-mouse IgG (Zymed, San Francisco, CA); donkey anti-goat IgG (Santa Cruz Biotechnology)). Blots were treated with ECL reagents (NEN Life Science Products), and all proteins were detected by autoradiography. Equal protein loading was ascertained by Ponceau S staining of blotted membranes.

Immunoprecipitation and CDK6 Kinase Assay—Breast cancer cells were cultured for 0, 15, 24, 48, or 96 h in growth medium with or without 100 μM I3C and then rinsed twice with PBS, harvested, and stored as dry pellets at −70 °C. For the immunoprecipitation, cells were lysed for 15 min in immunoprecipitation (IP) buffer (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 0.1% Triton X-100) containing protease and phosphatase inhibitors (50 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 0.1 μg/ml NaF, 10 μg/ml β-glycerophosphate, 0.1 mM sodium orthovanadate). Samples were diluted to 500 μg of protein in 1 ml of IP buffer, and samples were precleared for 2 h at 4 °C with 40 μl of a 1:1 slurry of protein A-Sepharose beads (Pharmacia Biotech, Uppasala, Sweden) in IP buffer and 1 μg of rabbit IgG. After a brief centrifugation to remove precleared beads, 0.5 μg of anti-CDK6 antibody was added to each sample and incubated on a rocking platform at 4 °C for 2 h. Then 10 μl of protein A-Sepharose beads were added to each sample, and the slurries were incubated on the rocking platform at 4 °C for 30 min. The beads were then washed five times with IP buffer and twice with kinase buffer (50 mM HEPES, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 0.1 μg/ml NaF, 10 μg/ml β-glycerophosphate, 0.1 mM sodium orthovanadate). Half of the immunoprecipitated sample was checked by Western blot analysis to confirm the immunoprecipitation.

For the kinase assay, the other half of the immunoprecipitated sample was resuspended in 25 μl of kinase buffer containing 20 mM ATP, 5 mM dithiothreitol, 0.21 μg of Rb carboxyl-terminal domain protein substrate (Santa Cruz Biotechnology, catalog number sc-4112), and 10 μCi of [γ-^{32}P]ATP (6000 Ci/mmol). Reactions were incubated for 15 min at 30 °C and stopped by adding an equal volume of 2 × loading buffer (10% glycerol, 5% β-mercaptoethanol, 3% SDS, 6.25 mM Tris-HCl, pH 6.8, and bromphenol blue). Reaction products were boiled for 10 min and then electrophoretically fractionated in SDS-10% polyacrylamide gels. Gels were stained with Coomassie Blue to monitor loading and destained overnight with 3% glycerol. Subsequently, gels were dried and visualized by autoradiography.

Isolation of Poly(A)$^+$ RNA and Northern Blot Analysis—Poly(A)$^+$ RNA was isolated from MCF7 cells treated with either 100 μM I3C or vehicle control (1 μl of Me_2SO/ml of medium) for 5 or 15 h as described previously (38). For Northern blot analysis, 10 μg of poly(A)$^+$ RNA was electrophoretically fractionated in 6% formaldehyde, 1% agarose gels, transferred onto Nytran nylon membranes (Schleicher and Schuell), and UV-cross-linked in a UV Stratalinker (Stratagene, La Jolla, CA). Membranes were preannealed with 100 μg/ml denatured salmon sperm DNA in hybridization buffer (5 × Denhardt's reagent, 5 × SSC, 50% formamide) and subsequently hybridized for 16–20 h with cDNA probes [α-^{32}P]dCTP-labeled by random primer extension (Boehringer Mannheim). For detection of CDK6 transcripts, the cDNA probe was added to the hybridization buffer at a concentration of 6 million cpm/ml. CDK6 membranes were washed twice for 10 min with 2 × SSC at room temperature followed by two 1-h-long washes with 0.2 × SSC, 0.1% SDS at 60 °C. For detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), CDK2, and CDK4 transcripts, the cDNA probe was added to the hybridization buffer at a concentration of 2 million cpm/ml. These membranes were subsequently washed with 2 × SSC twice at room temperature for 10 min followed by two 30-min washes at 60 °C in 0.2 × SSC, 0.1% SDS. The membranes were placed under film and analyzed after 4-h to 6-day exposures at −80 °C. CDK2, -4, and -6 transcripts were detected using purified *Bam*HI fragments of the pCMVcdk2, pCMVcdk4, or pCMVcdk6 plasmids containing the respective cDNA for each of the CDKs. The CDK plasmids were a generous gift from Ed Harlow's laboratory as described (39). GAPDH transcripts were detected with a 560-base pair *Xba*I/*Hin*D III cDNA fragment of the corresponding cDNA.

Quantitation of Autoradiography—Autoradiographic exposures were scanned with a UMAX UC630 scanner and band intensities were quantified using the NIH Image program. Autoradiographs from a minimum of three independent experiments were scanned per time point.

Transfection Procedure—Subconfluent breast cancer cells were propagated for at least 1 week in either low (5% CS) or high serum (10% FBS) containing medium prior to plating onto 60-mm Corning dishes 24 h before transfection in the appropriate medium. DNA-lipofectamine (Life Technologies, Inc.) mixtures were prepared by mixing 1 μg of ERE-vit-CAT reporter (−596 to +21 of the *Xenopus laevis* vitellogenin B1 genomic clone plus a consensus estrogen response element (ERE) subcloned into the *Hin*dIII site upstream of the chloramphenicol acetyltransferase (CAT) reporter gene in the SVOCAT vector, a generous gift from D. J. Shapiro (University of Illinois)) with 5 μl of lipofectamine in a total volume of 100 μl for 15 min at room temperature. The cells were washed twice with serum-free medium, and 100 μl of the liposome complex was added to each plate. After a 5-h incubation at 37 °C, the transfection was terminated by adding an equal volume of 2 × media containing either 10% CS or 20% FBS. Complete medium was replaced 18–24 h post-transfection, at which time appropriate agents (*e.g.* estrogen, tamoxifen, and I3C) were added. pCAT-basic vector (Promega, Madison, WI), which contains the promoterless CAT cDNA, was used as a negative control to determine background CAT activity.

CAT Assay—Cells were harvested by washing in PBS, resuspended in 100 mM Tris-HCl, pH 7.8, and lysed by three freeze/thaw cycles, 5 min/cycle. Cell lysates were heated at 68 °C for 15 min and centrifuged at $1.4 \times 10^4 \times g$ for 10 min, and the supernatant fractions were recovered. CAT activity in the cell extracts containing 20–50 μg of lysate protein was measured by a quantitative nonchromatographic assay (40). The enzyme assay was carried out in 100 mM Tris-HCl, pH 7.8, 1 mM aqueous chloramphenicol, and 1 μCi of [^{3}H]acetyl coenzyme A (final reaction volume of 250 μl). The reaction mixture was overlaid with 4 ml of Econofluor scintillation fluorochrome (NEN Life Science Products). CAT activity was monitored by direct measurement of radioactivity by liquid scintillation counting. Measurements of CAT activity were in the linear range of the assay as determined by a standard curve using bacterial CAT enzyme (0.01 units; Pharmacia), the positive control for CAT enzymatic activity. Reaction mixtures were incubated at 37 °C for 2–6 h. Mock-transfected cells were used to establish basal level activity for both assays. The enzyme activity was expressed as CAT activity produced per μg of protein present in corresponding cell lysates as measured by a Bradford assay, and the results show averages of triplicate samples.

5-Bromo-2′-deoxyuridine (BrdUrd) Incorporation and Indirect Immunofluorescence—MCF7 cells grown on eight-well Lab-Tek Permanox slides (Nalge Nunc International, Naperville, IL) were treated for 96 h with either vehicle control (1 μl of Me_2SO/ml of medium), I3C, tamoxifen, or both I3C and tamoxifen at the indicated concentrations followed by incubation with fresh medium containing a final concentration of 100 μM BrdUrd (Sigma) at 37 °C for 2 h. Cells were washed with PBS, fixed for 30 min in 4% paraformaldehyde, and rinsed with PBS, and DNA was denatured by incubation in 0.12 N HCl at 37 °C for 1 h. After neutralization in two changes of 0.1 M borate buffer over 10 min, cells

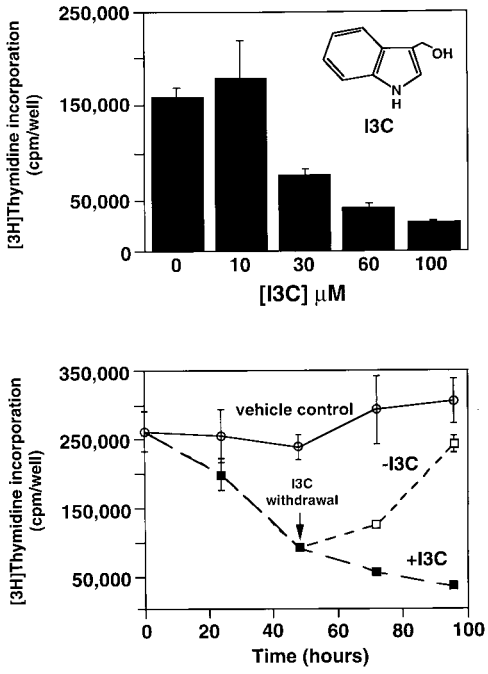

FIG. 1. **Effects of I3C on DNA synthesis in MCF7 breast cancer cells.** *Top panel*, MCF7 cells were plated at 20,000 cells/well on 24-well tissue culture dishes (forming a subconfluent monolayer) and treated with the indicated concentrations of I3C (see structure) for 48 h. Cells were labeled with [^{3}H]thymidine for 3 h, and the incorporation into DNA was determined by acid precipitation as described under "Experimental Procedures." The reported values are an average of triplicate samples. *Lower panel*, MCF7 cells were treated with 100 μM I3C (*+I3C*, ■) or with the Me_2SO vehicle control (*vehicle control*, ○) for a 96-h time course. After 48 h of I3C treatment, the I3C-containing medium in a subset of the cell cultures was replaced with medium containing only the vehicle control (*−I3C*, □), and the time course continued for an additional 48 h. At the indicated time points for each condition, the cells were labeled with [^{3}H]thymidine for 3 h, and the incorporation into DNA was determined by acid precipitation. The reported values are an average of triplicate samples.

were washed in PBS and blocked for 5 min in PBS containing 4% normal goat serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Cells were then incubated with mouse monoclonal anti-BrdUrd antibody (DAKO Corp. (Carpinteria, CA); diluted 1:80 in PBS) for 60 min at 25 °C. After five washes with PBS, cells were blocked for 5 min in PBS containing 4% normal goat serum. Cells were then incubated in anti-mouse rhodamine-conjugated secondary antibody (Jackson ImmunoResearch Laboratories; diluted 1:300 in PBS) at 4 °C for 30 min. Finally, cells were washed five times with PBS, mounted with 50% glycerol, 50 mM Tris (pH 8.0), and examined on a Nikon Optiphot fluorescence microscope. Images were captured using Adobe Photoshop 3.0.5 and a Sony DKC-5000 digital camera. Nonspecific fluorescence, as determined by incubation with secondary antibody alone, was negligible.

RESULTS

I3C Reversibly Inhibits the Growth and Induces a G_1 Cell Cycle Arrest of Human MCF7 Breast Cancer Cells—As an initial test to determine whether dietary indoles can directly regulate the growth of human breast cancer cells, MCF7 cells were cultured at subconfluency in medium supplemented with 10% FBS and 10 μg/ml insulin and treated with several concentrations of I3C for 48 h. Cells were then pulse-labeled with [^{3}H]thymidine for 3 h to provide a measure of their proliferation. Analysis of [^{3}H]thymidine incorporation revealed a strong dose-dependent inhibition of DNA synthesis with half-maximal response at approximately 30 μM I3C (Fig. 1, *upper panel*). Treatment with I3C above 200 μM was toxic to the cells. The lowest concentration of I3C that maximally inhibited the growth of MCF7 cells without affecting viability was 100 μM, and this level of the indole was therefore routinely used in subsequent experiments. HPLC analysis of the culture medium from I3C-treated cells revealed that this indole remained stable during the entire course of the experiment.[2] In the low pH environment of the stomach, I3C is converted into several acid-catalyzed products including DIM and ICZ, which bind to and activate the Ah receptor. Neither of these acid-catalyzed products had any significant effect on the incorporation of [^{3}H]thymidine into MCF7 breast cancer cells after 48-h treatments with concentrations that were above the K_d for the Ah receptor (data not shown). In multiple experiments, ICZ and DIM caused only an average of 10% inhibition of DNA synthesis under these conditions, which was not statistically significant.

Time course studies of I3C addition and withdrawal demonstrated that the I3C growth suppression of MCF7 breast cancer cells is completely reversible; therefore, this compound did not affect cell viability. For example, analysis of DNA synthesis over a 96-h time course revealed that 100 μM I3C inhibited [^{3}H]thymidine incorporation by 80% after 72 h and by greater than 90% after 96 h of treatment (Fig. 1, *lower panel*). The rate of [^{3}H]thymidine incorporation in untreated cells was approximately equal to the rate observed 48 h after indole withdrawal (Fig. 1, *lower panel*). Even after prolonged exposure (50 days), the I3C-induced growth arrest was readily reversible (data not shown).

To assess the cell cycle effects of I3C, MCF7 cells were treated with or without 100 μM I3C for 96 h and were then hypotonically lysed in the presence of propidium iodide to stain the nuclear DNA. Flow cytometry profiles of nuclear DNA content revealed that I3C induced a cell cycle arrest of these breast cancer cells. As shown in Fig. 2, I3C treatment altered the DNA content of the MCF7 cell population from an asynchronous population of growing cells (Fig. 2, *upper panel*) in all phases of the cell cycle (26% in G_1, 52% in S phase, and 22% in G_2/M phase) to one in which most (73%) of the I3C-treated breast cancer cells exhibited a 2n DNA content (Fig. 2, *lower panel*), which is indicative of a G_1 block in cell cycle progression. These results suggest that I3C suppresses cell growth by inducing a specific block in cell cycle progression.

I3C Rapidly Abolishes the Production and Activity of the CDK6 Cell Cycle Component and Reduces the Endogenous Phosphorylation of Rb—To determine potential downstream targets of the I3C-activated pathway that induces the cell cycle arrest, the expression of components that function within the G_1 phase of the cell cycle was examined during a time course of I3C treatment. MCF7 cells were treated with or without 100 μM I3C for the indicated time periods (Fig. 3), and the production of G_1 CDKs, cyclins, and CDK inhibitors was examined by Western blot analysis. Among the array of cell cycle components tested, only CDK6 protein levels were rapidly and significantly reduced in response to I3C treatment. The level of CDK6 is reduced within 24 h of indole treatment, and by 96 h, CDK6 production is essentially abolished (Fig. 3, *upper panel*). Importantly, no effect was observed on the expression of the two other G_1-acting cyclin-dependent kinases, CDK2 and CDK4. This result demonstrates the specificity of the I3C response. In addition, I3C did not alter the level of either cyclin D1 or cyclin E, both of which have been shown to be regulated in other studies. Estrogen and progesterone stimulate and antiestrogens inhibit cell cycle progression of the T47D human breast cancer cell line at a point in the early G_1 phase of the cell cycle with corresponding changes in cyclin D1 and p21 expression (41, 42). Quantitative analysis of autoradiographs from

[2] L. Bjeldanes, unpublished data.

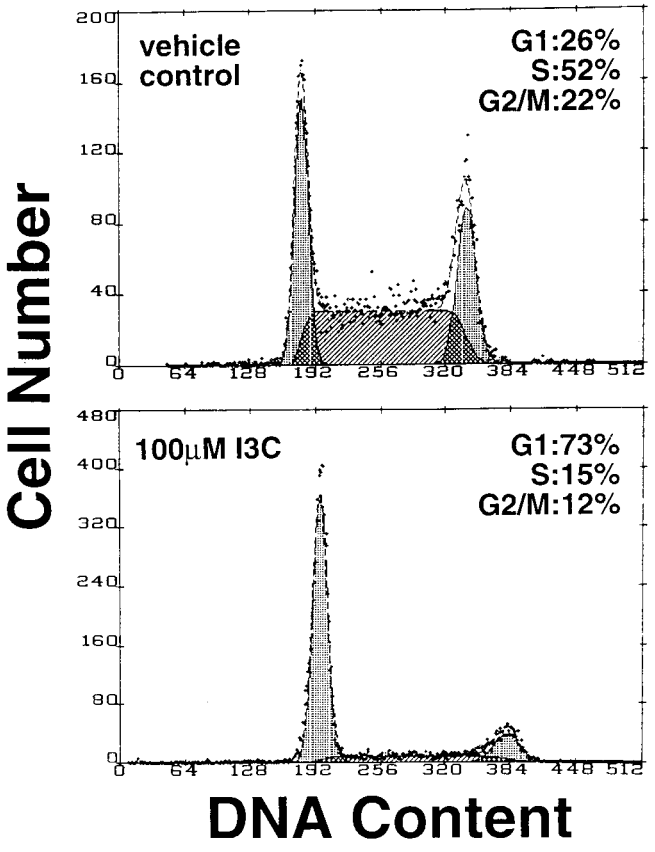

FIG. 2. **Effects of I3C on cell cycle phase distribution of MCF7 breast cancer cells.** MCF7 cells were treated with 100 μM I3C or with the vehicle control (Me_2SO) for 96 h. Cells were then stained with propidium iodide, and nuclei were analyzed for DNA content by flow cytometry with a Coulter Elite laser. A total of 10,000 nuclei were analyzed from each sample. The percentages of cells within the G_1, S, and G_2/M phases of the cell cycle were determined as described under "Experimental Procedures."

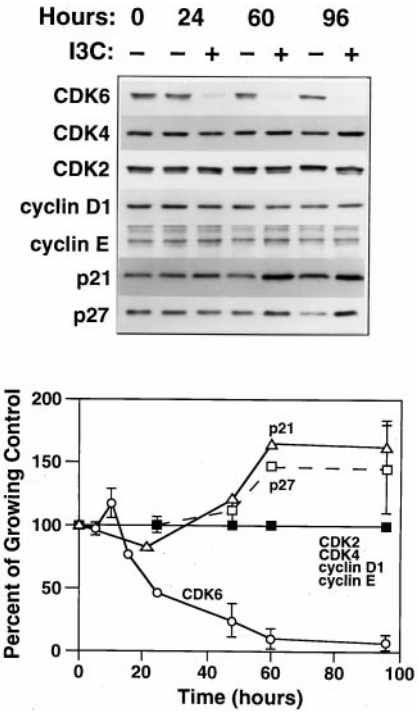

FIG. 3. **Effects of I3C on expression of G_1 cell cycle proteins in MCF7 breast cancer cells.** *Upper panel*, MCF7 cells were treated with 100 μM I3C or with the vehicle control (Me_2SO) for the indicated times, and the protein production of the G_1 cell cycle components was determined by Western blot analysis using specific antibodies. The same cell extracts were utilized for the analysis of each cell cycle protein, and equal sample loading was confirmed by Ponceau S staining of the Western blot membrane. *Lower panel*, the relative level of each cell cycle component shown in the representative Western blot in the *upper panel*, as well as from other Western blots with additional time points, was quantitated as described under "Experimental Procedures." The percentage of growing control was calculated by dividing the densitometry measurements of I3C-treated cells by the densitometry measurements of vehicle control-treated growing cells for each assay (CDK6 (○), p21 (△), p27 (□), CDK2, CDK4, cyclin D1, and cyclin E (■)).

more detailed time courses (Fig. 3, *lower panel*) revealed that of all the cell cycle components, only CDK6 protein levels changed early enough to coincide with the inhibition of DNA synthesis (see Fig. 1), suggesting a relationship between these two effects of I3C. Western blot analysis also demonstrated that I3C gradually stimulated the levels of the p21 and p27 cell cycle inhibitors by 50% only after the cells begin to display their maximal cell cycle arrest.

The phosphorylation of the Rb protein by the CDKs is critical for progression through the cell cycle, while in G_1 cell cycle-arrested cells, the Rb protein remains hypophosphorylated (43). To determine potential functional connections between the decreased expression of CDK6 and the cell cycle arrest, the effects of I3C on the ability of CDK6 to phosphorylate Rb *in vitro* and the level of total phosphorylated endogenous Rb protein were compared through a time course of I3C treatment. MCF7 cells were treated with and without I3C for a 96-h time course, and at specific time points, the immunoprecipitated CDK6 was assayed for Rb phosphorylation activity. As shown in Fig. 4 (*top panel*), the level of CDK6-mediated Rb phosphorylation activity was strongly inhibited by I3C in a manner consistent with the decreased expression of CDK6 protein. No change was observed in CDK6 activity at 15 h. However, by 24 h of treatment, there was a significant reduction in the ability of immunoprecipitated CDK6 to phosphorylate the GST-Rb fusion protein *in vitro*. The CDK6 activity in the I3C-treated cells remained low throughout the remainder of the time course. In a parallel experiment using I3C-treated and untreated MCF7 cells, the level of endogenous phosphorylated Rb protein was assessed in Western blots. The hyperphosphorylated and hypophosphorylated forms of Rb migrate at characteristic sizes in SDS-polyacrylamide gel electrophoresis (44, 45). As shown in Fig. 4 (*middle panel*), by 48 h of treatment, I3C causes a significant decrease in the amount of hyperphosphorylated Rb (*ppRb*) compared with untreated cells cultured for the same time period. The levels of *in vitro* CDK6 activity and endogenous phosphorylation of Rb were compared by quantitating the phosphorylation patterns at the 48-h time points. As shown in the *lower panel* of Fig. 4, I3C caused a similar inhibition of Rb phosphorylation in both assays. This result suggests a connection between the I3C-mediated decrease in CDK6 activity, the lack of endogenous Rb hyperphosphorylation, and the observed cell cycle arrest.

I3C Inhibition of CDK6 Transcript Expression—Because the level of CDK6 protein is rapidly reduced by indole treatment, it seemed likely that I3C regulates the level of CDK6 transcripts. Poly(A)$^+$ RNA was isolated from MCF7 breast cancer cells treated with and without 100 μM I3C for the indicated times, and electrophoretically fractionated samples were examined by Northern blot analysis. As shown in Fig. 5 (*upper left panel*), 15 h of treatment with I3C caused a specific reduction in the level of all three of the CDK6 transcripts. By 24 h of I3C treatment, the expression of CDK6 transcripts were undetectable (data not shown). Consistent with the unaltered protein production observed for CDK2 and CDK4, the transcript levels for these CDKs remained unchanged in either the presence or

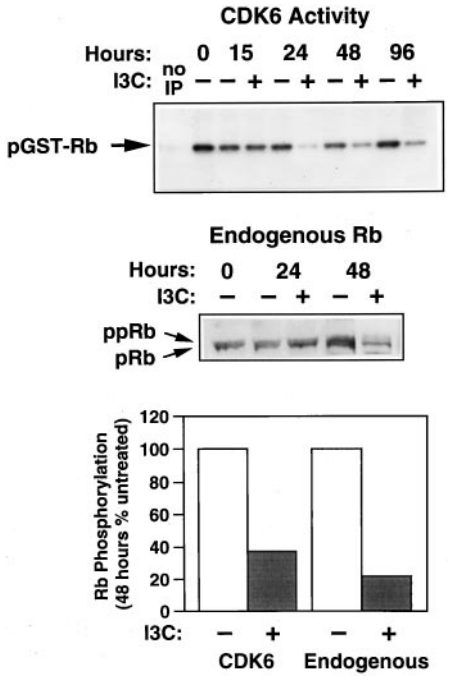

FIG. 4. **Effects of I3C on CDK6 kinase activity and endogenous Rb phosphorylation in MCF7 cells.** *Upper panel*, MCF7 cells were treated with 100 μM I3C or with the vehicle control (Me_2SO) for the indicated times. CDK6 was immunoprecipitated from cell lysates and assayed for *in vitro* kinase activity using the C terminus of the Rb protein as a substrate (GST-Rb). One control immunoprecipitation (*no IP*) contained rabbit anti-IgG with no added anti-CDK6 antibodies in untreated MCF7 cell lysates. The kinase reaction mixtures were electrophoretically fractionated, and the level of [^{32}P]Rb (*pGST-Rb*) was analyzed by autoradiography. *Middle panel*, MCF7 cells were treated with 100 μM I3C or with the vehicle control (Me_2SO) for the indicated times, the cell extracts were electrophoretically fractionated, and Western blots were probed with anti-Rb antibodies. The level of endogenous Rb phosphorylation was determined by the characteristic migration of the hyperphosphorylated (*ppRb*) and hypophosphorylated (*pRb*) forms of Rb. *Lower panel*, the levels of observed Rb phosphorylation at the 48-h time points from the CDK6 kinase assay and the Western blot were quantitated as described under "Experimental Procedures." The level of Rb phosphorylation in the untreated control was set at 100%, and the inhibition by I3C was determined by dividing the densitometry measurements of untreated cells by the densitometry measurements of I3C-treated cells for each assay.

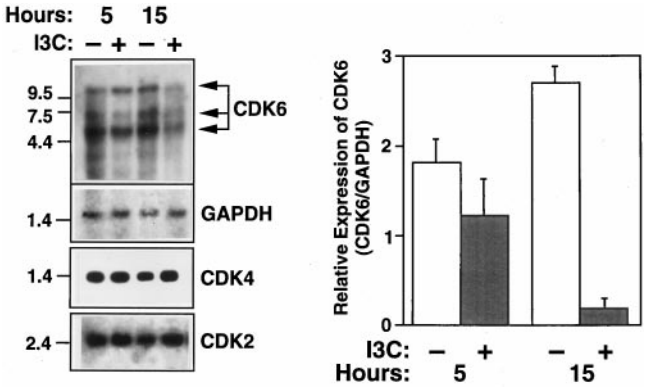

FIG. 5. **Effects of I3C on the expression of G_1 CDK transcripts in MCF7 breast cancer cells.** Poly(A)$^+$ RNA isolated from MCF7 cells treated with or without 100 μM I3C for 5 or 15 h. The RNAs were electrophoretically fractionated, and Northern blots were probed for CDK6, CDK4, and CDK2 transcripts (*left panels*) as described under "Experimental Procedures." As a loading control, the CDK6 Northern blots were reprobed for GAPDH, which is a constitutively expressed transcript. The x-ray film exposure times were 6 days for CDK6 and 2 h for CDK4, CDK2, and GAPDH. Molecular size standards in kilobases are indicated in the *left panel*. The band intensities of CDK6 and GAPDH transcripts for each condition were quantitated (*right panel*) as described under "Experimental Procedures." CDK6 mRNA levels were normalized to GAPDH mRNA levels by dividing the band intensities of CDK6 by the band intensity for GAPDH. The reported values represent an average of three independent experiments.

absence of I3C (Fig. 5, *lower left panels*). It is worth mentioning that MCF7 cells produce a significantly lower amount of CDK6 mRNA compared with the level of either CDK2 or CDK4 transcripts. A 6-day x-ray film exposure is required to detect the CDK6 mRNA bands with 10 μg of poly(A)$^+$ sample, whereas the same blot reprobed for CDK2 or CDK4 can be developed within 2 h. Autoradiographs from multiple experiments were quantitated, and CDK6 mRNA concentrations at each time point were normalized to the level of GAPDH transcripts, a constitutively expressed gene. No significant effect of I3C on CDK6 transcript levels was observed after 5 h of treatment. By 15 h, I3C caused a 5-fold reduction in CDK6 mRNA levels (Fig. 5, *right panel*). These changes in CDK6 transcript levels appear to account for the reduction in CDK6 protein because CDK6 protein levels begin to decrease in response to I3C between 15 and 21 h of treatment (Fig. 3, *lower panel*). Taken together, our results demonstrate that under conditions in which I3C induces a cell cycle arrest of MCF7 breast cancer cells, the gene expression and protein expression of CDK6, a G_1-acting cell cycle component, is selectively reduced.

The Regulation of CDK6 Production Is Specific for the Antiproliferative Effects of I3C—An important question is whether the I3C-induced reduction in CDK6 expression is a specific I3C response or whether it is a general consequence of the inhibition of cell growth. To distinguish these possibilities, the effects of I3C were compared with those of the antiestrogen tamoxifen, a well known inhibitor of the proliferation of estrogen-treated MCF7 cells (46). The I3C treatments were performed using MCF7 cells cultured in medium supplemented with 10% FBS, which contains enough estrogen and growth factors to maintain the cells in a proliferative state. To first demonstrate that tamoxifen can selectively inhibit an estrogen-responsive reporter plasmid in the presence of 10% FBS, ER expressing MCF7 breast cancer cells were transiently transfected with the ERE-vit-CAT reporter plasmid, which contains the vitellogenin promoter with four estrogen response elements linked upstream and driving the bacterial CAT gene. The cells were treated for 48 h with the indicated combinations of estrogen, tamoxifen, and/or I3C (Fig. 6), and the reporter gene activity was assayed by monitoring the conversion of [^{3}H]acetyl-CoA and unlabeled chloramphenicol into [^{3}H]acetylchloramphenicol. As shown in Fig. 6, the medium used for I3C-induced growth suppression (10% FBS) has endogenous estrogen and growth factors at a sufficient concentration to cause a high basal level of reporter gene activity in MCF7 cells transiently transfected with the ERE-vit-CAT reporter plasmid compared with the low serum (5% CS) condition. Low serum medium, as opposed to serum-free medium, was chosen because MCF7 cells cultured in serum-free conditions were not transfection-competent. Treatment with 100 nM estrogen further stimulated ERE-vit-CAT activity above each of the basal serum levels, whereas tamoxifen inhibited the serum-induced ERE-vit-CAT reporter plasmid activity by 70%. Treatment with I3C had no effect on the ER responsiveness of the ERE-vit-CAT activity; nor did this dietary indole modulate the antagonistic effects of tamoxifen (Fig. 6). Thus, under the FBS-containing conditions utilized to culture MCF7 cells, tamoxifen acts as a potent antagonist of ER responsiveness, while I3C has no apparent effects on ER function.

To test whether I3C and tamoxifen affected the absolute number of S phase cells, MCF7 cells were treated for 48 h with 100 μM I3C and/or 1 μM tamoxifen, and the cells were exposed

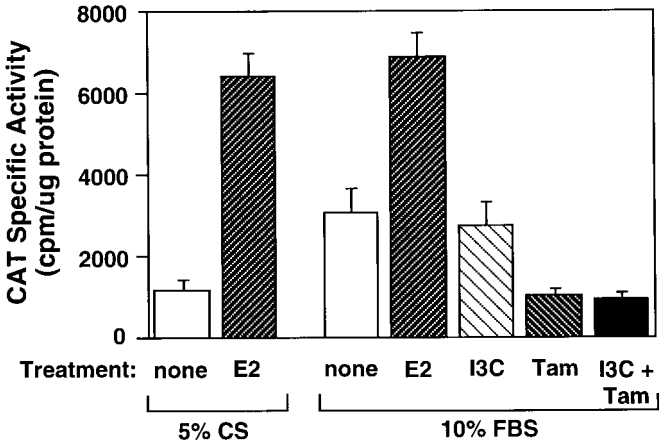

FIG. 6. **Effects of I3C and tamoxifen on the ERE-vit-CAT reporter plasmid activity in MCF7 breast cancer cells.** MCF7 cells were first cultured for 1 week in either 5% CS or 10% FBS. Cells were then transfected with the ERE-vit-CAT reporter plasmid, which encodes four EREs within the vitellogenin promoter linked upstream to and driving the bacterial CAT gene. Transfected cells were treated with the indicated combinations of 100 nM 17β-estradiol (*E2*), 100 μM I3C, and/or 10 μM tamoxifen (*Tam*) or with a vehicle control (*none*) for 48 h and assayed for CAT activity by a quantitative method that measures the conversion of [^{3}H]acetyl coenzyme A into [^{3}H]acetylchloramphenicol. CAT specific activity is the CAT activity produced per μg of protein present in the corresponding cell lysates. The reported values are an average of four independent experiments of triplicate samples.

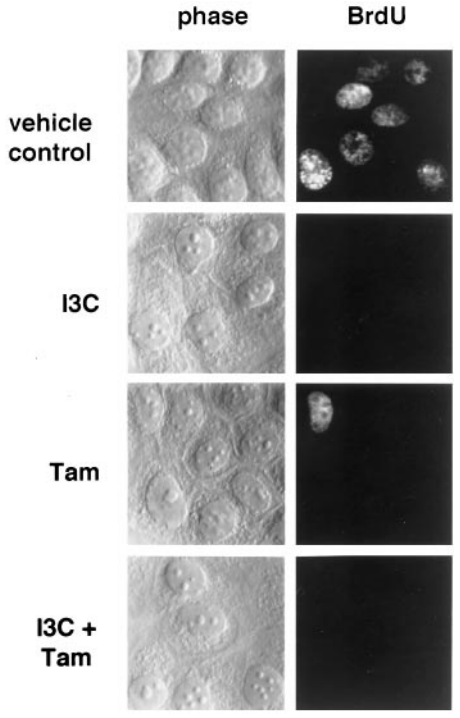

FIG. 7. **Single cell analysis of the effects of I3C and tamoxifen on DNA synthesis in MCF7 breast cancer cells.** MCF7 cells cultured on eight-well slides were treated with the indicated combinations of 100 μM I3C and/or 1 μM tamoxifen (*Tam*) or with only Me_2SO (vehicle control) for 96 h. Cells were then labeled with 100 μM BrdUrd for 2 h, fixed in paraformaldehyde, and incubated with mouse anti-BrdUrd antibodies. BrdUrd-incorporating cells were then visualized by fluorescence microscopy using anti-mouse rhodamine-conjugated secondary antibodies as described under "Experimental Procedures."

to a 2-h pulse of BrdUrd, which was used as a measure of DNA synthesis on a single cell level. The incorporation of BrdUrd was monitored by indirect immunofluorescence using BrdUrd-specific antibodies. Representative photographs are shown in Fig. 7. The number of BrdUrd-incorporating cells was quantitated by examining approximately 500–1000 cells/condition. In an asynchronous growing population, 21.5% of MCF7 cells incorporated BrdUrd. Treatment with either I3C or with tamoxifen suppressed BrdUrd incorporation to 4.7 and 12.3%, respectively, whereas treatment with both reagents induced a more effective growth suppression (3.8%) compared with either I3C or tamoxifen alone. Treatment with I3C or tamoxifen caused the cells to show a more flattened morphology, which is consistent with the effects of other antiproliferative agents (47, 48).

Because I3C and tamoxifen both inhibited cell growth, the effects of these two antiproliferative agents on CDK6 production were examined. MCF7 cells were treated with combinations of I3C and tamoxifen over a 96-h time course, and cell extracts were analyzed for CDK protein by Western blots. I3C, but not tamoxifen, reduced the level of CDK6 protein, whereas no effects were observed on CDK4 or CDK2 production with either reagent (Fig. 8). A combination of I3C and tamoxifen reduced CDK6 protein levels to approximately the same extent as I3C alone. These results indicate that the inhibition of CDK6 expression is a specific I3C response and not a general consequence of growth arrest in MCF7 cells. Because the effects of the antiestrogen tamoxifen and I3C differ, these results suggest that I3C may act, in part, through an ER-independent pathway to suppress breast cancer cell growth.

I3C Can Suppress the Growth and Reduce CDK6 Production in an ER-deficient Human Breast Cancer Cell Line—The ER-deficient MDA-MB-231 cells were utilized to demonstrate that I3C can suppress the growth of human breast cancer cells independent of any effects on ER responsiveness. Western blot analysis using antibodies to the human ER confirmed that the MDA-MB-231 breast cancer cells do not express ER protein, while MCF7 cells produce ER (Fig. 9, *upper panel*). Moreover, consistent with the lack of ER protein, neither estrogen nor

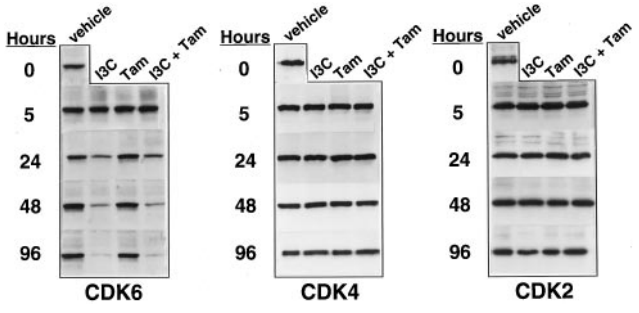

FIG. 8. **Effects of I3C and tamoxifen on the expression of G_1 CDK proteins in MCF7 breast cancer cells.** MCF7 cells were treated with combinations of 100 μM I3C and/or 1 μM tamoxifen or with the vehicle control (Me_2SO) for the indicated times. The protein production of CDK6, CDK4, and CDK2 was determined by Western blot analysis using specific antibodies. The same cell extracts were utilized for the analysis of each CDK protein, and equal sample loading was confirmed by Ponceau S staining of the Western blot membrane.

tamoxifen had any effects on ERE-vit-CAT reporter activity in transiently transfected MDA-MB-231 cells (data not shown). To test whether I3C can suppress the growth of human breast cancer cells in an ER-independent manner, MCF7 cells, which contain ERs, and MDA-MB-231 cells, which do not contain ERs, were treated with combinations of I3C and tamoxifen for 48 h, and DNA synthesis was assayed as a measure of the incorporation of [^{3}H]thymidine. As shown in Fig. 9, *middle panel*, treatment with either I3C or tamoxifen inhibited MCF7 DNA synthesis by approximately 70 and 60%, respectively, compared with vehicle controls. Consistent with a more stringent growth-inhibitory effect of both reagents, a combination of both I3C and tamoxifen inhibited [^{3}H]thymidine incorporation by more than 90%. In contrast, I3C, but not tamoxifen, strongly

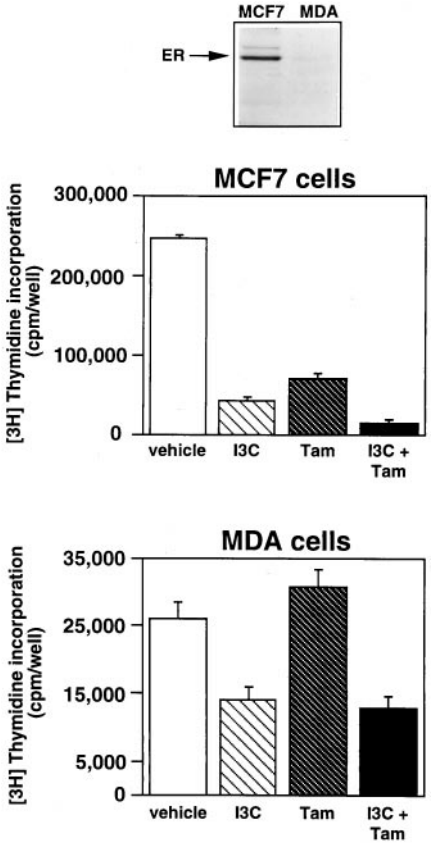

FIG. 9. **Effects of I3C and tamoxifen on DNA synthesis in ER-containing and ER-deficient breast cancer cells.** *Upper panel*, expression of ER was confirmed in ER-containing MCF7 cells and ER-deficient MDA-MB-231 (*MDA*) cells. ER protein expression was determined by Western blot analysis using specific antibodies, and equal sample loading was confirmed by Ponceau S staining of the Western blot membrane. *Lower panels*, MCF7 and MDA cells were plated at 20,000 cells/well on 24-well tissue culture dishes and treated with the indicated combinations of 100 μM I3C and/or 1 μM tamoxifen or with the vehicle control (Me_2SO) for 48 h. Cells were labeled with [^{3}H]thymidine for 3 h, and the incorporation into DNA was determined by acid precipitation as described under "Experimental Procedures." The reported values are an average of triplicate samples.

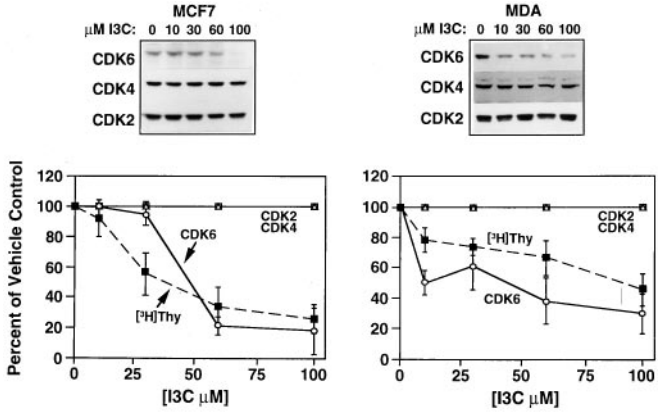

FIG. 10. **Dose-response effects of I3C on the expression of G_1 CDK proteins and DNA synthesis in ER-containing and ER-deficient breast cancer cells.** *Upper panels*, ER-containing MCF7 (*upper left*) and ER-deficient MDA-MB-231 (*upper right*) cells were treated with the indicated concentrations of I3C for 48 h. The protein production of CDK6 (○), CDK4 (△), and CDK2 (□) was determined by Western blot analysis using specific antibodies. The same cell extracts were utilized for the analysis of each CDK protein, and equal sample loading was confirmed by Ponceau S staining of the Western blot membrane. *Lower panels*, the relative level of each CDK shown in the representative Western blots in the upper panels, as well as from other Western blots, were quantitated as described under "Experimental Procedures." The reported values were calculated as the percentage of vehicle control-treated growing cells (0 μM I3C) by dividing the densitometry measurements of I3C-treated cells by the measurements of vehicle control cells for each assay. In parallel with the Western blots, triplicate sets of MCF7 and MDA-MB-231 cells were plated at 20,000 cells/well on 24-well tissue culture plates and treated with the indicated concentrations of I3C for 48 h. Cells were labeled with [^{3}H]thymidine for 3 h, and the incorporation into DNA was determined by acid precipitation as described under "Experimental Procedures" (*[^{3}H]Thy*, ■).

inhibited [^{3}H]thymidine incorporation in the ER-deficient MDA-MB-231 cells (Fig. 9, *lower panel*). A combination of I3C and tamoxifen suppressed DNA synthesis to approximately the same extent as I3C alone. This result demonstrates that I3C can suppress breast cancer cell growth independent of ER-mediated events.

The ER-positive MCF7 cells and ER-negative MDA-MB-231 cells were utilized to examine the relationship between the estrogen-independent suppression of cell growth by I3C and the reduction in CDK6 protein. Cells were treated with increasing concentrations of I3C for 48 h, and the level of G_1-acting CDK proteins was analyzed by Western blots. As shown in Fig. 10, *upper panels*, this indole can dose-dependently reduce CDK6 production in both cell lines, while in the same extracts, CDK4 and CDK2 protein levels remained unaffected. Quantitative analysis of the Western blots and a parallel analysis of [^{3}H]thymidine incorporation of the I3C dose response revealed that the inhibition of CDK6 protein levels approximately correlated with the I3C-mediated decrease in DNA synthesis (Fig. 10, *lower panels*). Consistent with the MDA-MB-231 cells not being as stringently growth-suppressed, I3C reduces CDK6 levels to a lesser extent compared with the MCF7 cells. Thus, I3C can suppress the growth and reduce CDK6 production in an ER-independent manner.

DISCUSSION

Extracellular regulators of cell proliferation, such as steroid and protein hormones, can transduce an intricate network of growth-inhibitory and -stimulatory signals that converge on specific sets of cell cycle components, which through their concerted actions, either drive cells through critical cell cycle transitions or inhibit cell cycle progression (3, 21). Dietary and environmental compounds that alter cell growth are likely to mediate many of their effects through signal transduction pathways analogous to the known mechanisms of hormone receptor signaling. Our results demonstrate the existence of a distinct growth-inhibitory pathway that establishes a direct link between the regulation of cell cycle control by the dietary indole I3C and the selective control of cell cycle components. The unique feature of this response is that the I3C-mediated growth arrest is accompanied by the specific inhibition of expression of CDK6 transcripts, protein, and activity. The selective regulation of CDK6 in mammary epithelial cells by I3C provides the basis to propose a biological mechanism by which dietary indoles potentially control the emergence and proliferation of breast cancer cells. In this regard, several recent studies have demonstrated that CDK6 expression and activity is altered in a manner that correlates with the transformed state. For example, tumor-specific amplification of CDK6 has recently been observed in human gliomas (49), and the activity of CDK6 is amplified in certain human squamous cell carcinoma lines (50).

Changes in cyclin or CDK inhibitor expression have been thought to be the key regulatory mechanisms controlling CDK function. A few recent studies have started to uncover examples of changes in expression of the G_1-acting CDKs under conditions that either inhibit or stimulate cell cycle progression (23, 51, 52). For example, staurosporine treatment of human

breast cancer cells causes a minor reduction in CDK6 protein levels that accompanies a G_1 cell cycle arrest (53). Treatment of T cells with the herbimycin A tyrosine kinase inhibitor reduces the stability of the CDK6 protein (54), while both CDK4 and CDK6 protein production were suppressed by the apopototic signaling through the sIgM surface antigen receptor in B cells (55).

CDK6 functions during progression through the G_1 phase of the cell cycle (56). However, relatively little is known about the influence on CDK6 expression by regulators of cell growth. In our studies with human MCF7 breast cancer cells, time course and dose-response assays with I3C revealed that the loss of CDK6 production and activity closely coincided with the reduction in cell proliferation, which implicates CDK6 as a direct target for cell cycle control in human breast cancer cells. The rapid I3C effect on CDK6 gene expression is unique, because it is the first report of a growth inhibitor decreasing the mRNA for CDK6. One other study has shown that the level of another G_1-acting CDK can be reduced in response to a specific antiproliferative signal. Under conditions in which retinoic acid suppresses the growth of MCF7 cells, the level of CDK2 mRNA was decreased within 8 h of retinoic acid treatment (57). In our study, the CDK6 transcript levels were significantly reduced between 5 and 15 h of I3C treatment, which appears to account for the decrease in CDK6 protein production. In contrast, no obvious changes were observed in the production of the other G_1-acting CDKs and cyclins.

Our results have established that I3C signaling can induce the G_1 cell cycle arrest of cultured human MCF7 breast cancer cells in an estrogen-independent manner. Most strikingly, the I3C-mediated cell cycle arrest and repression of CDK6 production were observed in ER-deficient MDA-MB-231 human breast cancer cells under conditions in which the antiestrogen tamoxifen had no effect on cell growth. In ER-containing MCF7 cells, tamoxifen suppressed DNA synthesis to approximately the same extent as I3C but had no effect on CDK6 expression. Moreover, while tamoxifen reduced the activity of an ERE-containing reporter plasmid in cells cultured with FBS-containing endogenous estrogens, I3C had no effect on ER responsiveness under these same culture conditions. Consistent with I3C and tamoxifen acting through distinct antiproliferative pathways, a combination of I3C and tamoxifen inhibited MCF7 cell growth to a greater extent compared with the effects of either agent alone.

Tamoxifen has been a clinically useful antiestrogen (58–60); however, 60–75% of patients with metastatic breast cancer have ER-positive tumors, and only approximately half of those patients will respond to tamoxifen therapy. Therefore, only 35% of metastatic breast cancer patients actually benefit from tamoxifen therapy (61). All patients who initially respond to therapy will eventually develop acquired tamoxifen resistance following prolonged administration. The cellular and molecular mechanisms underlying the development of acquired resistance to antiestrogens is unclear, but it has been proposed that continued exposure of cells to tamoxifen may select for hormone-independent, resistant cells (62). Our results suggest that I3C, in combination with tamoxifen, could prove to be an effective therapy. Patients could receive intermittent pulses of tamoxifen or lower doses of tamoxifen, both of which are proposed methods of circumventing tamoxifen resistance, while on I3C treatment. I3C has been shown to reduce the formation of both spontaneous and carcinogen-induced mammary tumors in rodents with no apparent side effects (9, 63–65), and human subjects treated with I3C also had no side effects (13, 66).

The ER-dependent pathway presumably involves the activation of the Ah receptor by the acid-catalyzed products of I3C

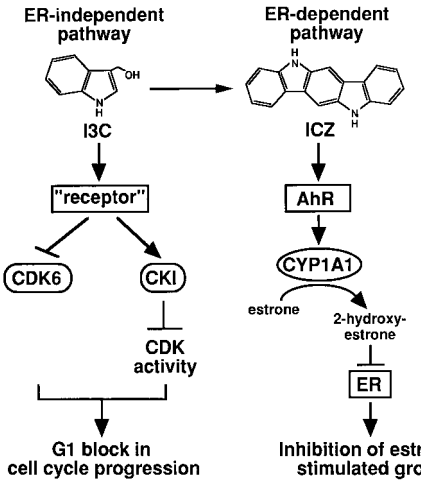

FIG. 11. **Model for the ER-independent and ER-dependent antiproliferative effects of I3C in breast cancer cells.** For the ER-independent pathway, I3C suppresses breast cancer cell growth by a rapid inhibition of CDK6 expression and activity and a later stimulation of CDK inhibitor (*CKI*) production. We propose that this effect causes an inhibition of the activity of G_1-acting CDKs, resulting in decreased retinoblastoma protein phosphorylation, and thereby induces a G_1 block in cell cycle progression. We also propose that I3C is mediating these effects through a putative cellular receptor (*"receptor"*). In contrast, the ER-dependent pathway is mediated by the I3C acid-catalyzed product ICZ, which binds to and activates the aromatic hydrocarbon receptor (*AhR*). The aromatic hydrocarbon receptor transcriptionally activates cytochrome P4501A1 (*CYP1A1*)-dependent monooxygenase, which inactivates estrone and thereby prevents the estrogen-stimulated growth of breast cancer cells.

and subsequent regulation of estrogen metabolism (17). In our studies, no effects on growth were observed within 48 h of treatment with ICZ or DIM. However, it is likely that the ER-dependent effects of these indoles require treatment periods longer than a week (9). The regulation of CDK6 expression appears to be specific for the I3C-mediated ER-independent pathways. Several studies with human breast cancer cells have shown that the ER pathway stimulates cell proliferation by targeting the expression and/or activity of G_1-acting cell cycle components (3, 52, 67). Although the immediate promoter targets of the ER are unknown, in MCF7 cells the estrogen-induced activation of CDK4 and CDK2 kinase activity was shown to be accompanied by the increase in cyclin D1 expression and decreased CDK inhibitor association with the cyclin E-CDK2 protein complex (36). No changes in CDK6 expression were reported in these studies, although in other human breast cancer cells, antiprogestin or antiestrogen treatment was shown to increase production of p21 (42).

Taken together with previous studies on the acid-catalyzed products of I3C (18, 68), our results with I3C indicate that dietary indoles are likely to work through both ER-independent and ER-dependent pathways (see Fig. 11). It is tempting to consider that I3C, or perhaps a cellular metabolite of I3C, interacts with a putative indole receptor to reduce the expression and activity of the CDK6 cell cycle component, resulting in the G_1 arrest of breast cancer cells by an ER-independent pathway. The I3C-mediated decrease in the amount of CDK6 protein may have multiple effects on the cell cycle control of breast cancer cells. The inhibited production of active CDK6 could contribute to the observed decrease in endogenous phosphorylation of Rb and thereby directly influence the cell cycle arrest. It is also possible that the decreased production of CDK6 protein could indirectly affect the stoichiometry of the other G_1-acting CDK complexes by releasing bound CDK inhibitors and cyclins. For example, the activities of CDK2 and/or CDK4 could then be affected, although the expression of these

CDKs remains constant. Furthermore, I3C treatment resulted in a 50% increased expression of the p21 and p27 CDK inhibitors after the cells reached their maximal cell cycle arrest. We are currently attempting to determine whether I3C alters the expression and/or function of other G_1-acting cell cycle components that may also contribute to the reduction in endogenous Rb phosphorylation. Another of our future goals will be to identify the putative indole receptor and to characterize the ER-independent pathway by which I3C regulates CDK6 gene expression and cell cycle control in human breast cancer cells.

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