Indole-3-Carbinol Is a Negative Regulator of Estrogen

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ABSTRACT Studies increasingly indicate that dietary indole-3-carbinol (I3C) prevents the development of estrogen-enhanced cancers including breast, endometrial and cervical cancers. Epidemiological, laboratory, animal and translational studies support the efficacy of I3C. Whereas estrogen increases the growth and survival of tumors, I3C causes growth arrest and increased apoptosis and ameliorates the effects of estrogen. Our long-range goal is to best use I3C together with other nutrients to achieve maximum benefits for cancer prevention. This study examines the possibility that induction of growth arrest in response to DNA damage (GADD) in genes by diindolylmethane (DIM), which is the acid-catalyzed condensation product of I3C, promotes metabolically stressed cancer cells to undergo apoptosis. We evaluated whether genistein, which is the major isoflavonoid in soy, would alter the ability of I3C/DIM to cause apoptosis and decrease expression driven by the estrogen receptor (ER)-α. Expression of GADD was evaluated by real-time reverse transcription–polymerase chain reaction. Proliferation and apoptosis were measured by a mitochondrial function assay and by fluorescence-activated cell sorting analysis. The luciferase reporter assay was used to specifically evaluate expression driven by ER-α. The estrogen-sensitive MCF-7 breast cancer cell line was used for these studies. We show a synergistic effect of I3C and genistein for induction of GADD expression, thus increasing apoptosis, and for decrease of expression driven by ER-α. Because of the synergistic effect of I3C and genistein, the potential exists for prophylactic or therapeutic efficacy of lower concentrations of each phytochemical when used in combination. J. Nutr. 133: 2470S–2475S, 2003.

KEY WORDS: • indole-3-carbinol • diindolylmethane • genistein • estradiol

Indole-3-carbinol (I3C)4 and its biologically active dimer diindolylmethane (DIM), which are obtained from the dietary consumption of cruciferous vegetables (Brassicas), are promising agents for the prevention of estrogen-enhanced cancers. A combination of epidemiological and experimental data provides suggestive evidence that a high intake of cruciferous vegetables protects against some cancers at various sites (1). In a nationwide study of postmenopausal women in Sweden, consumption of cruciferous vegetables was inversely associated with breast cancer risk (2). Although cruciferous vegetables have a number of cancer-preventing compounds, I3C alone shows efficacy for the prevention of breast (3), endometrial (4) and cervical cancers (5) in animal models. Importantly, I3C shows efficacy for treatment of precancerous lesions of the cervix in translational human studies (6).

In estrogen-sensitive cells, I3C/DIM and estrogen have opposing activities on cells. Estrogen promotes tumor growth, whereas I3C suppresses it. For example, the K14-HPV16 mouse, which has transgenes for the oncogenes from human papillomavirus type 16, only develops cervical cancer when estrogen is given chronically (7). However, dietary I3C prevents cervical cancer in these estrogen-treated mice (5). This is consistent with many in vitro studies that show that estrogen increases cell proliferation (8,9) and I3C causes growth arrest (10). Immunohistochemistry studies determined (5) that

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PCNA (a component of DNA-δ polymerase) is robustly expressed in the cervical epithelium of estrogen-treated mice (both transgenic and normal mice), and that this increase is reduced by dietary intake of I3C. Studies in breast cancer cells show that estrogen inhibits the effects of a variety of proapoptotic agents (11). In cervical cells, estrogen inhibits apoptosis that is induced by cisplatin, taxol and ultraviolet (UV) radiation, and this inhibition by estradiol is dose dependent (our unpublished data). On the other hand, I3C and DIM induce apoptosis of both breast cancer and cervical cells in vitro (12,13) and induce apoptosis in the cervical epithelium of mice given estrogen (13). When estradiol and I3C are together, the amount of apoptosis depends on the relative concentrations of each (our unpublished data).

A number of mechanisms exist (that are not mutually exclusive) whereby I3C (or DIM) can diminish the effects of estrogen on tumor growth. First, I3C and DIM induce enzymes such as CYP1A1, which converts estrone to 2-hydroxyestrone (14) and ultimately results in metabolites that are antiproliferative and proapoptotic (15,16). Alternative metabolism (16α-hydroxylation) of estradiol results in compounds that increase proliferation and anchorage independent growth (9,17). Second, in the case of genes driven by the estrogen receptor (ER)-α, I3C acts as a negative regulator (18). The tumor suppressor breast cancer 1 (BRCA-1), whose expression is upregulated by I3C/DIM (19), also inhibits the expression of genes driven by ER-α (20). Moreover, I3C and BRCA-1 work together to abrogate ER-α–driven expression (19). Using subtractive hybridization, Chen et al. (21) determined that expression of a battery of genes driven by estrogen was abrogated by DIM. Speculation is that I3C/DIM and estradiol modulate the ER and the aryl hydrocarbon receptor (21). Thus, estrogen could modulate the activity of I3C/DIM as well. Finally, in the absence of estrogen, I3C and DIM induce many genes that have the potential to induce growth arrest and apoptosis and therefore might counteract the effects of estradiol. For example, Cover et al. (10) determined that cyclin-dependent kinase 6 was induced by DIM, which should cause growth arrest of breast cancer cells. In our own studies, we determined that expression of >100 genes was changed by a short (4–6 h) treatment with DIM (22). Many genes that encode for transcription factors and are involved in the endoplasmic reticulum stress response were upregulated, whereas a number of genes involved in proliferation were downregulated.

In this study, we investigated some of the genes [e.g., the growth-arrest genes collectively named for growth arrest in response to DNA damage (GADD)] that were robustly upregulated by DIM (22), because these should provide insight into mechanisms whereby I3C/DIM can overcome the growth and survival effects of estrogen on tumor growth. We hypothesized a mechanism whereby I3C/DIM can specifically target tumor cells, and we have determined that genistein, another phytochemical that is the major isoflavonoid in soy, can act synergistically with I3C/DIM to kill these cells.

### MATERIALS AND METHODS

#### Reagents

17β-Estradiol, I3C, genistein and propidium iodide were purchased from Sigma (St. Louis, MO). DIM was a gift from Dr. M. Zeligs (Bioresponse, Boulder, CO). All the vectors used in this study were from Sigma (St. Louis, MO). DIM was a gift from Dr. M. Zeligs (Bioresponse, Boulder, CO). All the vectors used in this study were from Sigma (St. Louis, MO).

#### Cell lines and cell culture

The breast cancer cell line MCF-7 was purchased from the American Type Culture Collection (Manassas, VA). All cells were maintained as monolayer cultures at 37°C in 7% CO2 and were grown in Dulbecco’s modified Eagle’s medium (DMEM) that contained 4.5 g of glucose and bicarbonate/L (GIBCO-BRL, Gaithersburg, MD) supplemented with 110 mg of sodium pyruvate/L, 200 mmol glutamine/L, 100 mL of fetal bovine serum/L and 100,000 U each of penicillin and streptomycin/L.

#### Mitochondrial function assay for cell viability

Assays were preformed as described previously (13). Cells were trypsinized, seeded at 10,000 cells/well in 96-well plates that contained 100 µL of medium/well and incubated for 16 h. The medium was changed to 200 µL that contained either dimethyl sulfoxide (DMSO) as a solvent control, DIM and/or genistein with 6 replicate wells/condition. Viability was determined after 72 h by reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) using the Cell Titer Aqueous One kit (Promega, Madison, WI) according to the manufacturer’s instructions. Absorbance at 595 nm of the solutions was determined with a multwell plate reader, and protein concentration was measured with the MicroBCA kit (Pierce, Rockford, IL).

#### Fluorescence-activated cell sorting analysis

Subconfluent monolayers were treated with DMSO solvent control or genistein and/or DIM for 48 h, trypsinized, washed in phosphate buffered saline, fixed in 70% ethanol and incubated with 500 U of RNase/mL. The DNA was stained using 50 µg of propidium iodide/mL and sorted by fluorescence using a Becton Dickinson FACScan with CellQuest software (Palo Alto, CA).

#### Real-time reverse transcription–polymerase chain reaction analysis

Total RNA from cells was prepared using reagents from Qiagen (Valencia, CA) followed by cDNA synthesis using a 177–linked oligo(dT) primer (reagents were from GIBCO-BRL, Grand Island, NY). The cDNA were quantified by real-time polymerase chain reaction (PCR) analysis using the TaqMan PCR core reagent kit and the ABI Prism 7700 sequence-detector system (PE Biosystems). In brief, the PCR reaction contained 0.5 µmol primers/L, 0.1 µmol TaqMan probe carboxyfluorescein (FAM)/L, 3.5 mmol MgCl2/L, 0.2 mmol dNTP/L, 0.25 U of uracil DNA glycosylase (AmpErase UNG), 0.625 µM of Taq polymerase (AmpliTaq Gold) and the cDNA in TaqMan buffer. PCR conditions were 50°C for 2 min, 95°C for 10 min and 45 cycles of 95°C for 30 s and 60°C for 1 min. Values were calculated using the software provided with the ABI Prism 7700 system. Each sample was run in triplicate, and mean values were used for data analysis. Results were normalized to β-actin expression and were expressed as a fold change with respect to DMSO–treated values. For GADD-34, the forward primer was cag gta ctc agg ccc c, the reverse primer was cag gta aag act ctt c and the TaqMan probe was 5′–tetrachlorofluorescin phosphorimidite (TET)-cag gcc agg aca aac ctc tca tgc 6-carboxy-tetramethylrhodamine (TAMRA). For GADD-153, the forward primer was ctc aat ctc cag cac gca tga, the reverse primer was cag gtt cgt gtt gcc c and the TaqMan probe was TET-cag tgg gca gaa tca tcc ccc c t TAMRA. For GADD45-α, the forward primer was cag tcc cag cgt cct gaa gta cag tgg gag ttc cct cct cct cca and the TaqMan probe was TET-cag gcc ctc gat ctc ggg g-TAMRA. For β-actin, the forward primer was cct ggc acc cag ccc aat, the reverse primer was gcc gat cca cag gta gta ct and the TaqMan probe was TET-act aag atc att gct cet cct gag cgc-TAMRA.

#### Reporter gene assay for 17β-estradiol–activated ER-α–mediated transcriptional activity

As described previously (18,20), subconfluent cells plated in 24-well culture dishes were cotransfected with the luciferase reporter...
plasmid that contains ERE (ERE-TK-LUC, 0.5 µg/L) and the ER-α expression vector (0.5 µg/L) using the lipofection reagent Lipofectin (GIBCO-BRL, Gaithersburg, MD) according to the manufacturer’s instructions. Cells were cultured an additional 24 h in medium with or without 17β-estradiol, I3C and/or genistein. After lysis with luciferase lysis buffer (Promega), lysates were analyzed for luciferase activity using a liquid scintillation counter (model LS60001C, Beckman, Fullerton, CA), and the data was normalized by protein concentration.

RESULTS

We hypothesized that certain genes may be instrumental in determining how I3C/DIM ameliorates estrogen induction of tumor growth and eventually causes growth arrest and apoptosis of these cells (Fig. 1). We considered the involvement of GADD because of our recent information that their expression is robustly upregulated by DIM (22). GADD are a group of proteins (GADD-153, GADD-45α, β and γ and GADD-34) that induce growth arrest and apoptosis by different pathways. Moreover, BRCA-1, which is induced by I3C/DIM, not only induces expression to GADD-45 (23) but also inhibits estrogen signaling that is dependent on ER-α (20). Finally, as shown below, genistein also can induce expression of GADD and can affect ER signaling.

DIM and genistein synergistically induce GADD

Genistein, an isoflavonoid from soy that is considered to be an anticancer phytochemical (24), inhibits glucose-regulated protein (25). This activity would counteract the protective response to ER stress, and raises the possibility that genistein could be an adjunct to I3C/DIM by modulation of the endoplasmic reticulum stress response in the direction of increased growth arrest and apoptosis. We first asked how genistein might affect the expression of GADD. We evaluated effects of DIM and genistein separately and together on the expression of GADD and used real-time RT-PCR to measure the response in estrogen-sensitive MCF-7 cells. As shown in Figure 2A, a short-time (6-h) treatment with either DIM (100 or 50 µmol/L) or genistein (5 or 25 µmol/L) increases expression of GADD-34. Very little increase is detected using 25 µmol DIM/L. However the combination of 5 µmol genistein/L and 25 µmol DIM/L results in a synergistic increase in the expression of this GADD. Results also are shown for GADD-153 (Fig. 2B) and GADD-45α (Fig. 2C). Similar results occur with the other isoforms of GADD-45 and are virtually identical in C33A cells (unpublished data).

DIM and genistein synergistically increase apoptosis

If DIM and genistein synergistically induce GADD, then growth arrest and apoptosis should be a consequence of this induction. We used two methods [a mitochondrial function assay and fluorescence-activated cell sorting (FACS) analysis] to evaluate growth arrest and apoptosis. In the mitochondrial function assay (Fig. 3), DIM and genistein work together to decrease cell viability. When increasing concentrations of DIM are used together with 5 µmol genistein/L (a concentration of genistein that enhances growth in MCF-7 cells when used alone), increased killing of cells occurs at concentrations of DIM as low as 20 µmol/L (Fig. 3A) compared to the require-
ment for DIM concentrations to be > 50–60 μmol/L when cells are exposed to DIM alone. DIM (50 μmol/L) counteracts the proliferative effect of genistein, and genistein (used at increasing concentrations) potentiates cell killing by DIM (Fig. 3B). Using FACS analysis (Fig. 4A), the profiles of subdiploid (putative apoptotic cells, M1), G1 (M2), S (M3) and G2 (M4) are identical for cells treated for 24 h with DMSO (solvent control), 25 μmol DIM/L or 5 μmol genistein/L. However, the fraction of putatively apoptotic cells is dramatically increased when cells are treated with the combination of both DIM and genistein (Fig. 4, A and B). Results with C33A cells (both assays) are identical to those of MCF-7 cells.

FIGURE 3 DIM and genistein work together to decrease viability. MCF-7 cells were treated for 72 h with DMSO (solvent controls), or 5 μmol genistein/L and increasing amounts of DIM from 0 to 100 μmol/L (A) or 50 μmol DIM/L and increasing amounts of genistein from 0 to 50 μmol/L (B). A minimum of six replicate wells was employed per condition. Viability was determined by a mitochondrial function assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)]. The amount of estrogen in media (10% fetal calf serum) was 10⁻¹⁵ mol/L.

FIGURE 4 DIM and genistein synergistically increase apoptotic cells. MCF-7 cells were treated with DMSO, 25 μmol DIM/L, 5 μmol genistein/L or 25 μmol DIM/L + 5 μmol genistein/L. Cells were treated for 48 h, stained with propidium iodide and sorted by fluorescence (abscissa). (top and middle panels) M1, subN2 cells; M2, G1; M3, S; M4, G2/M. (bottom panel) The gated percent values of M1 cells for the four conditions were 6.5, 4.8, 4.4 and 42.3%.
**DISCUSSION**

We provide insight into additional mechanisms whereby 13C/DIM can counteract the growth and survival of tumors in estrogen-sensitive cells. We performed additional analysis that confirms that not only does DIM induce GADD, but also that DIM and genistein synergistically induce expression of GADD. Consistent with their effects on the induction of GADD proteins, genistein and DIM work better together than alone to increase apoptosis. Another way by which 13C/DIM can lead to the growth arrest of estrogen-sensitive cancer cells is by interferring with estrogen signaling. Here, too, genistein and DIM are synergistic in inhibiting estrogen signaling by ER-α.

Our discovery that DIM induces GADD and other proteins involved in the endoplasmic reticulum stress response (22) not only supports the possibility that GADD contribute to the growth arrest and apoptosis associated with 13C/DIM, but also may answer (at least in part) why 13C/DIM seems to specifically target tumor cells opposed to normal cells. The importance of the tumor microenvironment in malignant progression has received much less attention in the literature than the cellular events that trigger oncogenesis. Tumor cells protect themselves from changes in the microenvironment such as decreased availability of oxygen and nutrients by engaging a biochemical pathway called the metabolic stress response. In vivo cancer cells are likely to be chronically stressed. The cellular response to hypoxia, hypoglycemia and nutrient starvation includes the synthesis of protective proteins and cell cycle arrest, which can lead to apoptosis or survival and also can involve induction of genes that promote angiogenesis and tissue remodeling. In other words, the fate of the stressed cell is survival by adaptation to the stressful conditions or elimination by programmed cell death. Obviously, the desired outcome for cancer prevention is growth arrest and apoptosis.

The fact that genistein works synergistically with 13C/DIM has a number of implications. Importantly (at least for induction of GADD, apoptosis and inhibition of estrogen-increased gene expression), the concentrations of these phytochemicals used in vitro to achieve these activities are more in line with concentrations that people acquire from eating the relevant foods. Additionally, people are exposed to combinations of foods and their bioactive constituents. Although sorting out how diets ultimately may affect a cell necessarily involves evaluating individual nutrients, the study of interactions between nutrients (especially well-studied nutrients) is the next step. Clearly, the Asian diet, which is considered protective against breast and some other cancers, must involve many bioactive compounds and their interactions.

**ACKNOWLEDGMENTS**

We would like to thank Dr. Kai Liu and Kathy Ripali for their technical assistance.

**LITERATURE CITED**


