

# ABSTRACT

Dietary indole-3-carbinol (I3C) has clinical benefits for both cervical cancer and laryngeal papillomatosis, and causes apoptosis of breast cancer cells in vitro. We asked whether I3C and its major acid-catalyzed condensation product diindolylmethane (DIM), which is produced in the stomach after consumption of cruciferous vegetables, could induce apoptosis of cervical cancer cell lines. We also asked whether this effect could be observed in vivo. In vitro, both I3C and DIM caused accumulation of DNA strand breaks in three cervical cancer cell lines. Induction of apoptosis was confirmed by nuclear morphology, nucleosome leakage, altered cytoplasmic membrane permeability and caspase 3 activation. Neither I3C nor DIM caused apoptotic changes in normal human keratinocytes. In C33A cervical cancer cells, DIM was more potent than I3C [dose at which the number of viable cells was 50% of that in untreated cultures ( $LD_{50}$ ) = 50–60  $\mu\text{mol/L}$  for DIM and 200  $\mu\text{mol/L}$  for I3C in a mitochondrial function assay] and faster acting. Furthermore, I3C reduced Bcl-2 protein in a time- and dose-dependent manner. In HPV16-transgenic mice, which develop cervical cancer after chronic estradiol exposure, apoptotic cells were detected in cervical epithelium by TdT-mediated dUTP nick-end labeling staining and by immunohistochemical staining of active caspase 3 only in mice exposed to 17 $\beta$ -estradiol ( $E_2$ ) and fed I3C. Rare apoptotic cells were also observed by hematoxylin and eosin staining in the spinous layer of the cervical epithelium in both control and transgenic mice. Estradiol reduced the percentage of these late-stage apoptotic cells in the cervical epithelium of transgenic,  $E_2$ -treated mice, but this reduction was prevented by I3C. These data confirm the proapoptotic action of I3C on transformed cells in vitro, extend the observations to cervical cancer cells and to DIM and show for the first time that dietary I3C results in increased apoptosis in target tissues in vivo.

**KEY WORDS:** • cervical cancer • indole-3-carbinol • diindolylmethane • apoptosis • mice

## INTRODUCTION

Indole-3-carbinol (I3C),<sup>4</sup> a common phytochemical in the human diet, is present in all members of the cruciferous vegetable family, which includes cabbage, broccoli, Brussels sprouts, cauliflower and kale. Recently, it has become clear that I3C has the potential to prevent and even to treat a number of common cancers, especially those that are estrogen-related. Pure I3C taken as a dietary supplement (the equivalent of one third of a head of cabbage per day) reverses precancerous changes in women with stage II and stage III cervical dysplasia (1\*). A diet rich in cruciferous vegetables (2\*) or supplements of I3C (3\*) causes regression of tumors or decreases their rate of growth or recurrence in two thirds of patients with recurrent laryngeal papillomatosis. Clinical trials are planned to test the efficacy of I3C as a preventive treatment for breast cancer (4\*). Laboratory studies suggest that this phytochemical can act in several different ways to prevent transformation and/or tumor progression, as well as to kill transformed cells selectively.

I3C is rapidly converted in the stomach to a variety of condensation products, chiefly diindolylmethane (DIM) (5\*). Plasma from humans and rats fed I3C contains no detectable I3C, but large amounts of DIM, as well as other metabolites, some of which remain uncharacterized (6\* ; L. Bjeldanes, University of California at Berkeley, personal communication). Thus DIM, rather than I3C, is probably the major compound initially available to cells after ingestion of I3C. I3C is also converted slowly to DIM at neutral pH (5\*), with the result that either compound is active in vitro. For example, both I3C and DIM induce apoptosis in MCF-7 breast carcinoma cells growing in culture (7\*, 8\*).

We showed previously that dietary I3C prevents the appearance of cervical cancer after chronic estrogen exposure in transgenic mice expressing the type 16 human papillomavirus (HPV) oncogenes (9\*). This effect is accompanied by a shift in estrogen metabolism to favor the production of 2-OH estrone rather than the 16 $\alpha$ -OH metabolite, which is associated with prolonged estrogenic activity and carcinogenesis (our unpublished data)(10\*, 11\*). Thus, it is likely that one of the major pathways by which I3C and its derivatives prevent the onset of cervical cancer involves alteration of estrogen metabolism by inducing specific cytochrome P<sub>450</sub> isoforms via the aryl hydrocarbon receptor (9\*, 10\*) for which DIM is a weak ligand (12\*, 13\*).

the American Type Culture Collection (ATCC, Manassas, VA). C33AE6 cells (15 $\star$ ) were stably transfected with the HPV16 E6 gene (pLXSN16E6 from D. Galloway, Fred Hutchinson Cancer Research Center, Seattle, WA) and express low levels of E6 transcripts (15 $\star$ ). All cells were maintained as monolayer cultures at 37°C, 7% CO<sub>2</sub>. Cervical cancer cells and 3T3 fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose and bicarbonate (GIBCO-BRL, Gaithersburg, MD), supplemented with 110 mg/L sodium pyruvate, 200 mmol/L glutamine, 100 mL/L fetal bovine serum, and 1 x 10<sup>5</sup> U/L each of penicillin and streptomycin. Normal human foreskin keratinocytes were grown in F12-DMEM on feeder layers by the method of Rheinwald and Green (16 $\star$ ) as described previously (16 $\star$ , 17 $\star$ ).

#### ***Mice.***

K14-HPV16 transgenic mice were derived and described by Arbeit et al. (18 $\star$ ), and were characterized and maintained by us as described previously (9 $\star$ , 17 $\star$ ). Control and experimental groups were as described previously (9 $\star$ ). Virgin normal and transgenic mice (4–5 wk old) were implanted subcutaneously with 0.25 mg/d release pellets of E<sub>2</sub> and fed diets with or without I3C as described below. Implants were repeated every 60 d until the end of the study. Mice were housed in groups of 5/cage. All experiments involving mice were done in strict adherence to IACUC-approved procedures.

#### ***Diet.***

Mice consumed ad libitum the AIN76a diet or AIN76a diet (19 $\star$ ) enriched with 0.1 g/kg I3C. Diets were prepared by Ziegler (Gardner, PA). The AIN76a diet contains 5% corn oil and supplies a total of 18.5 MJ/kg, with 22% of energy from protein, 11% from fat and 67% from carbohydrates.

#### ***Cell viability.***

C33A Cells were trypsinized, seeded at 10<sup>4</sup> cells/well in 96-well plates containing 100  $\mu$ L medium/well and incubated over night. The next day, the medium was changed to 200  $\mu$ L containing either DIM or dimethyl sulfoxide (DMSO) as solvent control, employing a minimum of four replicate wells per condition. Viability was determined at indicated times by a mitochondrial function assay [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 solution in individual wells was determined with a multiwell plate reader. Data were analyzed by plotting the mean and SD of cell viability vs. DIM concentration. Protein concentration was measured with the MicroBCA kit (Pierce, Rockford, IL) using a bovine serum albumin (BSA) standard.

#### ***Nucleosomal leakage apoptosis assay.***

Nucleosomal leakage was monitored with a Cell Death Detection ELISA<sup>PLUS</sup> kit from Roche Molecular Biochemicals (Mannheim, Germany) that detects histones and DNA in cytoplasmic extracts. Cells were grown in 96-well plates as described above in replicates of 6 wells/condition. Cells in duplicate wells were lysed and the postnuclear supernatant solution was analyzed for nucleosomal leakage according to manufacturer's instructions. Results were determined by measuring absorbance at 405 nm with a microwell plate reader as above. The second set of 4 wells from the same plate was analyzed for viability using the MTS assay by absorbance at 595 nm, and the ELISA results were normalized to this value ( $A_{405}/A_{595}$ ) to correct for reduction in viable cell number after treatment with I3C and DIM. The mean and SD of the normalized data were plotted vs. DIM concentration.

#### ***Western blotting.***

Cells treated with I3C, or vehicle controls, each with or without estradiol, were lysed at room temperature in buffer containing 10 mmol/L NaH<sub>2</sub>PO<sub>4</sub>, 20 g/L triton X-100, 12g/L SDS, 10g/L sodium deoxycholate supplemented just before use with 2  $\mu$ mol/L aprotinin, 100  $\mu$ mol/L phenylmethylsulfonyl fluoride and 0.1 mmol/L EDTA, boiled for 2 min, and centrifuged for 10 min at 12,000 x g at 4°C. Supernatant solutions were stored at -80°C until use.

and centrifuged at 1600 x g for 5 min. The supernatant solution was incubated with (5 mg/L) RNase A for 2 h at 56°C and then digested with proteinase K (2.5 mg/L) at 37°C for 16 h. DNA was precipitated with an equal volume of 10 mol/L ammonium acetate and 2.5 volumes of ethanol. The precipitates were rinsed with 700 g/L ethanol, air dried, dissolved in Tris/EDTA buffer (10 mmol/L Tris-HCl, pH 7.5, 1 mmol/L EDTA), electrophoresed through a 1.5% agarose gel, stained with ethidium bromide and photographed on a UV transilluminator.

### ***Immunohistochemistry.***

Tissues were procured, fixed and processed for immunostaining as described (9\*). For detection of activated caspase 3, both cells in culture and tissue slices were fixed in 10 g/L paraformaldehyde for 60 min at room temperature, followed by three washes in PBS. After being blocked overnight with 150 g/L BSA in PBS, the cells or tissue slices were incubated with a polyclonal antibody specific for the activate form of caspase 3 (Promega) overnight at 4°C. After three washes in PBS, samples were incubated with peroxidase-conjugated goat anti-rabbit second antibody (Santa Cruz Biochemicals); the signal was developed according to the manufacturer's instructions. For TUNEL staining of tissue sections, paraffin-embedded tissues were sectioned at a thickness of 5 µm and processed using the Complete ApopTag in situ hybridization kit (Intergen, Purchase, NY). Hematoxylin and eosin (H&E) staining was accomplished as described (9\*).

### ***Cytologic detection of apoptosis.***

Two groups of mice ( $n = 50$  transgenic and 50 nontransgenic) were treated continuously with  $E_2$  starting at 4–5 wk of age. Half of each group was fed a diet containing 2 g/kg I3C and the other half a control diet without I3C. At 6 mo, when the majority of  $E_2$ -treated transgenic mice fed the control diet were found to have cervical tumors (9\*), all mice were killed by  $CO_2$  inhalation and their cervical tissue examined by H&E staining as described (9\*). Samples were coded and the determination of apoptotic cells was carried out by a single individual unaware of treatment groups (M.Q.).

### ***Fluorescence staining of nuclei.***

C33A cells and normal human keratinocytes growing in monolayer were fixed in paraformaldehyde as above, followed by 10 µg/L 4,6-diamidino-2-phenylindole (DAPI) in methanol (Boehringer Mannheim, Germany) for 30 min at 37°C. Stained cells were mounted with Aqueous Mounting Medium (Biomedica, Loomis, CA) before fluorescence microscopy.

### ***Fluorescence-activated cell sorting analysis.***

To determine altered permeability, C33A cells were treated with or without 200 µmol/L I3C for 48 h and trypsinized. After washing with PBS, the cells were incubated in PBS containing 8 mg/L of 7-amino actinomycin D (7-AAD)-fluorescein isothiocyanate (Sigma) in the dark at 4°C for 20 min and then washed in PBS + 150 g/L BSA + 0.2 g/L  $NaN_3$  containing 20 mg/L of nonfluorescent actinomycin D (AD; Sigma). The resulting cell suspension was then analyzed cytofluorometrically on a Coulter Elite flow cytometer, set for single color, ungated fluorescence intensity.

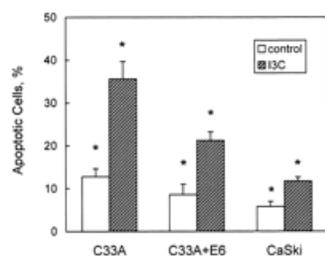
### ***Statistical analysis of data.***

Standard deviations were calculated for all quantitative data as indicated in the figures and figure legends. Significant differences ( $P < 0.05$ ) were determined using Student's  $t$  test.

## **RESULTS**

$\mu\text{mol/L}$ ; lanes 2 and 7, 50  $\mu\text{mol/L}$ ; lanes 3 and 8, 100  $\mu\text{mol/L}$ ; lanes 4 and 9; 200  $\mu\text{mol/L}$ ; lanes 5 and 10, 300  $\mu\text{mol/L}$ . (B) DIM concentrations: lane 1, 0  $\mu\text{mol/L}$ ; lane 2, 50  $\mu\text{mol/L}$ ; lane 3, 100  $\mu\text{mol/L}$ .

We next used TUNEL to determine the fraction of cells undergoing apoptosis. I3C treatment substantially increased the fraction of apoptotic cells in each of three cell lines (C33A, C33AE6 and CaSki), from a 100% increase with CaSki cells to a >200% increase with C33A (**Fig. 2**). C33AE6, which express the HPV16 E6 oncogene (15), showed an intermediate response (Fig. 2). Thus, it appeared that the induction of DNA strand breaks by I3C was a general phenomenon for cervical cancer cell lines and was independent of viral gene expression because C33A cells do not express detectable viral gene products (20), whereas both CaSki and C33AE6 do (15, 21).



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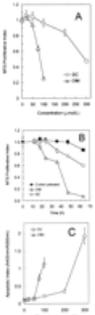
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**Figure 2.** TdT-mediated dUTP nick-end labeling (TUNEL) staining of human cervical cancer cells exposed to indole-3-carbinol (I3C). C33A, C33A/E6 and CaSki cells in monolayer culture were exposed to 200  $\mu\text{mol/L}$  I3C for 48 h and then fixed and stained for DNA strand breaks by TUNEL as described in Methods and Materials. Cells from each culture and/or condition ( $n = 2000$ ) were examined and the result expressed as a percentage of TUNEL-positive cells. Open bars, solvent control. Gray bars, I3C. Values are means  $\pm$  SD,  $n = 3$  replicate cultures. \*Untreated control and I3C-treated cultures differed,  $P < 0.002$ .

**Cell death induced by I3C in C33A cervical cancer cells has the characteristics of apoptosis.**

If the DNA fragmentation caused by I3C were the result of late-stage apoptosis, then it should be possible to detect early apoptotic changes in these cells. We next determined whether I3C could cause the cytoplasmic membrane changes characteristic of this process. Extraversion of phosphatidylserine during apoptosis (22) is accompanied by altered membrane permeability such that a fluorescent derivative of AD, namely, 7-AAD, can enter apoptotic cells and bind to DNA in the nucleus (23, 24). Fluorescence-activated cell sorting (FACS) analysis of 7-AAD-stained C33A cells that had been treated with 200  $\mu\text{mol/L}$  I3C confirmed that the compound increased the percentage of stained cells from 9.8% of the total (**Fig. 3A**) to 55% of the total (Fig. 3B) after 48 h of exposure to I3C.

If the DIM, the dimeric adduct of I3C, were the active molecular form, or more proximate to the active form, then DIM would be expected to act faster and at a lower concentration than I3C because the latter is converted only slowly to DIM in cell culture. **Figure 4A\*** shows the effect of increasing concentrations of DIM and I3C on C33A cells after a 48-h exposure, using a mitochondrial function assay (MTS assay) as an indirect measure of cell viability. We observed a reduction in the number of viable cells in cultures treated with as little as 40  $\mu\text{mol/L}$  DIM, whereas I3C had no observable effect at concentrations  $<100 \mu\text{mol/L}$  (Fig. 4A\*). The dose at which the number of viable cells is 50% of that in untreated cultures ( $\text{LD}_{50}$ ) for DIM was  $\square 60 \mu\text{mol/L}$ , compared with  $\square 200 \mu\text{mol/L}$  for I3C. We next compared the rate of cell killing by DIM and I3C; 100  $\mu\text{mol/L}$  DIM began to have an observable effect between 16 and 20 h after addition to growing cells, whereas 300  $\mu\text{mol/L}$  I3C had no observable effect until  $\square 36$  h (Fig. 4B\*). To confirm that DIM was in fact inducing apoptosis, the dose-response experiment in Figure 4A\* was repeated, and ELISA was used to detect nucleosomal leakage into the cytoplasm. Histones and DNA were detected in the cytoplasm of cells treated with 75  $\mu\text{mol/L}$  DIM, whereas release in I3C-treated cells required concentrations several fold higher (Fig. 4C\*).



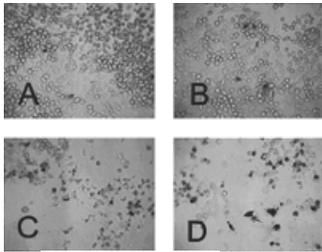
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**Figure 4.** Human C33A cell killing and nucleosomal leakage induced by indole-3-carbinol (I3C) and diindolylmethane (DIM). Cells were exposed to varying concentrations of I3C or DIM for indicated periods of time and the relative number of viable cells was determined by measuring absorbance at 595 nm as described in Methods and Materials (*panels A and B*). (*A*) Dose response curves for 48-h treatment; (*B*) kinetics of cell killing. Values are means  $\pm$  SD,  $n = 4$  independent measurements. (*C*) Cytoplasmic extracts were assayed for DNA and histones by ELISA as described in Methods and Materials and expressed as absorbance at 405 nm. A duplicate 96-well plate was analyzed for cell viability as in *panels A and B*, and results are expressed in *panel C* as the ratio of absorbance at 405 and 595 nm. MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.

Two additional hallmarks of apoptotic cell death are nuclear condensation and activation of caspases. **Figure 5\*** shows an immunofluorescence photomicrograph of DAPI-stained C33A cells (*panels A and B*) and normal human keratinocytes (*panels C and D*) treated with DIM. Because DAPI stains only DNA and chromatin, the outline of the stained region indicates the relative size and shape of the nucleus, as well as the distribution of DNA within it. At concentrations as low as 30  $\mu\text{mol/L}$ , DIM caused observable changes in the nuclear morphology of virtually all of the C33A cells (data not shown); at 60  $\mu\text{mol/L}$  DIM, condensed, fragmented late-stage apoptotic nuclei were observed in C33A cells and virtually all of the nuclei were contracted compared with those in untreated cells (*panel B*, white arrows). At 100  $\mu\text{mol/L}$ , few C33A cells remained on the cover slip after staining (not shown). In contrast, normal keratinocytes did not exhibit these changes even at 100  $\mu\text{mol/L}$  DIM (compare Fig. 5C\* and D\*). When duplicate cultures of C33A cells were stained for activated caspase 3 by immunohistochemistry, a similar dose response was observed (**Fig. 6\***).



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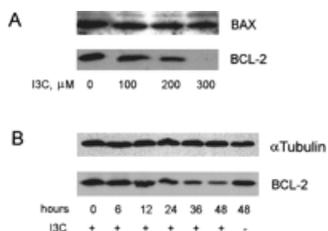
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**Figure 6.** Caspase 3 activation in human C33A cells after diindolylmethane (DIM) treatment. Monolayer cultures were exposed to increasing concentrations of DIM for 48 h, fixed and immunostained for activated caspase 3. (A) No DIM; (B) 30  $\mu\text{mol/L}$  DIM; (C) 60  $\mu\text{mol/L}$  DIM; (D) 100  $\mu\text{mol/L}$  DIM.

***Bcl-2 is reduced in cells treated with I3C and DIM.***

One of the points at which several pathways of apoptotic induction converge is the breakdown of mitochondrial membrane integrity. This process is thought to be controlled in part by the relative abundance of various members of the Bcl-2 family of proteins, notably BCL-2 itself and BAX, the former acting as an antiapoptotic agent and the latter as an apoptotic inducer [reviewed in (15 $\star$ )]. I3C caused a time- and dose-dependent reduction in the amount of Bcl-2 protein detected by Western blot (Fig. 7A $\star$  and B), whereas BAX was not affected by treatment with 300  $\mu\text{mol/L}$  I3C for 72 h. DIM reduced the amount of BCL-2 to undetectable levels by 72 h.



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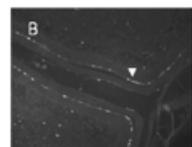
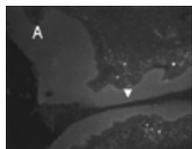
**Figure 7.** BCL-2 and BAX levels in indole-3-carbinol (I3C)-treated human C33A cells. C33A monolayers were treated with increasing amounts of I3C for 48 h (panel A) or with 300  $\mu\text{mol/L}$  I3C for different lengths of time (panel B). After treatment, 30  $\mu\text{g}$  of whole-cell protein was analyzed for BAX, BCL-2 and  $\alpha$ -tubulin content by Western blot.

***Indole-3-carbinol increases apoptosis in cervical epithelium of HPV16 transgenic mice.***

The observation that I3C and its major condensation product DIM can induce apoptotic changes in cervical cancer cells in vitro led us to ask whether I3C had the same effect in vivo. Transgenic mice expressing the human HPV E6 and E7 oncogenes under control of the keratin 14 promoter all develop cervical cancer when exposed chronically to estradiol (9 $\star$ , 18 $\star$ ), but a diet supplemented with I3C protects nearly all of these mice (9 $\star$ ). We examined sections of cervical epithelium from normal and transgenic mice by H&E staining in a double-blind protocol, scoring for apoptotic cell nuclei (Fig. 8A $\star$

treated mice were fed indole-3-carbinol (I3C) or a diet without I3C. After 6 mo, cervical tissue was examined by hematoxylin and eosin staining as described in Material and Methods. (A) A representative area of cervical epithelium from  $E_2$ -treated, nontransgenic (control) mice fed I3C. (B) An area similar to that in *panel A* from  $E_2$ -treated HPV16-transgenic mice fed I3C. Arrows indicate late-stage apoptotic cells. (C) Number of late apoptotic cells in a complete epidermal layer cross section from each of 5 mice for each condition indicated in the figure (transgenic or nontransgenic, with or without estradiol or I3C). Values are means  $\pm$  SD,  $n = 5$ . \*I3C-treated and untreated, transgenic mice fed I3C differed,  $P < 0.05$ .

We next used TUNEL as an alternative method to detect apoptotic cells in sections of cervical tissue. **Figure 9** shows clear evidence of stained cells in cervical epithelium only from transgenic mice chronically exposed to estrogen, i.e., those expected to develop cervical cancer but also fed I3C. We did not observe TUNEL staining cells in the cervical epithelium of normal mice or in transgenic mice exposed to amounts of estrogen that induce epithelial hypertrophy and dysplasia.



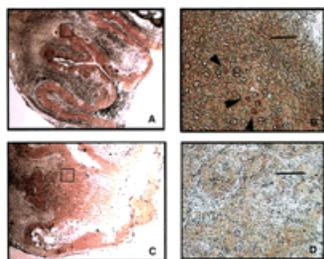
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**Figure 9.** TdT-mediated dUTP nick-end labeling (TUNEL) staining of cervical sections from human papillomavirus (HPV)16 transgenic mice fed indole-3-carbinol (I3C). Cervical sections from HPV16 transgenic mice treated as in Figure 8 were stained by immunofluorescence for DNA strand breaks. Arrows indicate the cervical epithelium. (A) Non-17 $\beta$ -estradiol ( $E_2$ )-treated mice fed I3C; (B)  $E_2$ -treated mice fed I3C.

Finally, we asked whether activation of caspase 3 could be detected in the cervical epithelium of the transgenic mice fed I3C. Using the immunohistochemical staining method described above for Figure 6C, individual cells stained positively for active caspase 3 in the suprabasal epithelium of the transgenic mice fed I3C (**Fig. 10B**), but not of placebo-fed controls (Fig. 10D).



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the active compound or is further metabolically converted to the active compound(s). However, serum levels of DIM in rats fed I3C were only  $\approx$  10% of those required to induce apoptosis in vitro in our experiments (6 $\star$ ). The fact that relatively high concentrations of DIM (and I3C), pharmacologic as opposed to dietary levels, were required to obtain biologic effects in vitro is consistent with a requirement for metabolic conversion of DIM to a secondary active compound. However, it is now widely recognized that serum levels of bioactive compounds are often uninformative because other factors such as carrier proteins, intracellular accumulation and localized concentration in specific tissues may all come into play. It is therefore not surprising that the concentrations of DIM required to produce effects in vitro are higher than those normally attainable in vivo. Similar observations have been made for other bioactive natural products, for example isoflavones from soy (26 $\star$ , 27 $\star$ ). In any case, the fact that dietary I3C appeared to induce apoptosis in the cervical epithelium of HPV16 transgenic mice suggests that this compound or its active metabolites do indeed reach effective intracellular levels in vivo.

Detection of apoptotic cells in cervical epithelia was undoubtedly facilitated by our experimental model system. In the HPV transgenic mouse, just as in human cervical cancer, nearly every cell is expressing HPV oncogenes and is thus initiated for transformation. If, as our results suggest, transformed cells or cells undergoing preneoplastic conversion are differentially sensitive to I3C/DIM, then the enrichment for these cells in cervical epithelium as a result of VP6 and VP7 gene expression would explain our ability to detect what in other tissues or experimental systems would be a rare event. In cervical epithelium from HPV16 mice, chronic exposure to elevated  $E_2$  is required for expression of the transformed phenotype (9 $\star$ , 18 $\star$ ). The observed reduction in late-stage apoptotic cells identifiable by routine histopathology in samples from HPV16 mice exposed to  $E_2$  (Fig. 8C $\star$ ) is therefore consistent with the generally observed phenomenon that apoptosis is inhibited in neoplastic and preneoplastic cells [reviewed in (25 $\star$ )]. By contrast, when  $E_2$ -treated HPV transgenic mice were also fed I3C, we did not observe the  $E_2$ -related reduction in apoptotic cells. This suggests that I3C either prevented transformation or killed the cells as they became transformed. Our in vitro data are consistent with the latter explanation.

We do not yet know either the mechanism by which these phytochemicals induce apoptosis or what determines their apparent specificity for transformed cells. Our data and published reports from other laboratories rule out both HPV and estrogen effects as requirements for induction of apoptosis by I3C/DIM, although both mechanisms may play ancillary roles in specific instances. Apart from sex, two differences of potential relevance between cervical cancer cells, which are sensitive to DIM, and foreskin keratinocytes, which are not, are the presence of HPV oncogenes and estrogen receptors in the former. However C33A cells, unlike C33AE6 and CaSki cells, do not express HPV oncogenes or any other viral genes at detectable levels (15 $\star$ ); therefore, it is unlikely that viral gene products account for the sensitivity of cervical cancer cells to I3C and DIM. The reduced sensitivity of C33AE6 cells to killing by I3C compared with the parental C33A cell line (Fig. 2) $\star$  may have been caused by the reported antiapoptotic effect of E6 (28 $\star$ –30 $\star$ ). However, even the protection afforded by expression of this viral oncogene was not complete.

Cell killing by DIM or I3C similarly does not seem to require estrogen; in fact, I3C and  $E_2$  have opposing effects on cell survival. Although C33A cells possess estrogen receptors (our unpublished data), and estrogen induces proliferation of epithelial cells in the cervix, estradiol interferes with induction of apoptosis caused by a number of agents, including I3C (our unpublished observations). Conversely, I3C interferes with signaling from the estrogen receptor (31 $\star$ ). The cytotoxic specificity of I3C/DIM for transformed cells also appears to be reflected in cervical epithelium in vivo, regardless of whether mice were exposed chronically to elevated estrogen (Figs. 8 $\star$  9 $\star$  10) $\star$ . During the course of multiple studies spanning  $>2$  y, we have never seen apoptotic cells in cervical epithelium or any evidence of cervical histopathology in normal mice fed a diet containing DIM. We conclude that induction of apoptosis in cervical cancer cells exposed to I3C or DIM both in vitro and in vivo occurs by a pathway separate from, and independent of estrogen-responsive mechanisms, and that it is specific for transformed cells.

How does I3C/DIM induce apoptosis of cancer cells? Several possibilities are suggested by published work from our laboratory and others. One mechanism that could account for the sensitivity of transformed cells is cell cycle inhibition. I3C inhibits cdk6 expression in MCF-7 cells (32 $\star$ ) by interfering with transcription (33 $\star$ ). Thus it might be that proliferating cells are more sensitive to I3C and DIM due to a need for cdk6 activation, which

diindolylmethane; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; E<sub>2</sub>, 17β-estradiol; H&E, hematoxylin and eosin; HPV, human papillomavirus; HRP, horseradish peroxidase; I3C, indole-3-carbinol; LD<sub>50</sub>, dose at which the number of viable cells is 50% of that in untreated cultures; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; POD, peroxidase; TUNEL, TdT-mediated dUTP nick-end labeling. [+](#)

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## LITERATURE CITED

1. Bell, M. C., Crowley-Nowick, P., Bradlow, H. L., Sepkovic, D. W., Schmidt-Grimminger, D., Howell, P., Mayeaux, E. J., Tucker, A., Turbat-Herrera, E. A. & Mathis, J. M. (2000) Placebo-controlled trial of indole-3-carbinol in the treatment of CIN. *Gynecol. Oncol.* 78:123-129. [\[Medline\]](#)
- 2. Auborn, K., Abramson, A., Bradlow, H. L., Sepkovic, D. & Mullooly, V. (1998) Estrogen metabolism and laryngeal papillomatosis: a pilot study on dietary prevention. *Anticancer Res* 18:4569-4574. [\[Medline\]](#)
- 3. Rosen, C. A., Woodson, G. E., Thompson, J. W., Hengesteg, A. P. & Bradlow, H. L. (1998) Preliminary results of the use of indole-3-carbinol for recurrent respiratory papillomatosis. *Otolaryngol. Head Neck Surg.* 118:810-815. [\[Medline\]](#)
- 4. Anon, A. (1996) A clinical development plan: indole-3-carbinol. *J. Cell. Biochem.* 26:127-136.
- 5. Grose, K. R. & Bjeldanes, L. F. (1992) Oligomerization of indole-3-carbinol in aqueous acid. *Chem. Res. Toxicol.* 5:188-193. [\[Medline\]](#)
- 6. Stresser, D. M., Williams, D. E., Griffen, D. A. & Bailey, G. S. (1995) Mechanisms of tumor modulation by indole-3-carbinol. disposition and excretion in male Fisher. 344 rats. *Drug Metab. Disp.* 23:966-975.
- 7. Ge, X., Yannai, S., Rennert, G., Gruener, N. & Farres, F. A. (1996) 3,3'-Diindolylmethane induces apoptosis in human cancer cells. *Biochem. Biophys. Res. Commun.* 228:153-158. [\[Medline\]](#)
- 8. Ge, X., Fares, F. A. & Yannai, S. (1999) Induction of apoptosis in MCF-7 cells by indole-3-carbinol is independent of p53 and bax. *Anticancer Res* 19:3199-3203. [\[Medline\]](#)
- 9. Jin, L., Qi, M., Chen, D.-Z., Arbeit, J. M., Yang, G.-Y. & Auborn, K. J. (1999) Indole-3-carbinol prevents cervical cancer in HPV16 transgenic mice. *Cancer Res* 59:1693-1699.
- 10. Auborn, K. J., Woodworth, C., DiPaolo, J. & Bradlow, H. L. (1991) The interaction between HPV infection and estrogen metabolism in cervical carcinogenesis. *Int. J. Cancer* 49:867-869. [\[Medline\]](#)
- 11. Yuan, F., Chen, D.-Z., Liu, K., Sepkovic, D. W., Bradlow, H. L. & Auborn, K. (1999) Anti-estrogenic activities of indole-3-carbinol in cervical cancer cells: implication for cervical cancer. *Anticancer Res* 19:1673-1680. [\[Medline\]](#)

- 19.Dillehay, D. L., Webb, S. K., Schmelz, E. M. & Merrill, A. H., Jr (1994) Dietary sphingomyelin inhibits 1,2-dimethylhydrazine-induced colon cancer in CF1 mice. *J. Nutr.* 124:615-620.
- 20.Crook, T., Wrede, D. & Vousden, K. H. (1991) p53 point mutation in HPV negative human cervical carcinoma cell lines. *Oncogene* 6:873-875.[\[Medline\]](#)
- 21.Smotkin, D. & Wettstein, F. O. (1986) Transcription of human papillomavirus type 16 early genes in a cervical cancer and a cancer-derived cell line and identification of the E7 protein. *Proc. Natl. Acad. Sci. U.S.A.* 83:4680-4684.[\[Abstract/Free Full Text\]](#)
- 22.Fadok, V. A., Voelker, D. R., Campbell, P. A., Cohen, J. J., Bratton, D. L. & Henson, P. M. (1992) Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J. Immunol.* 148:2207-2216.[\[Abstract\]](#)
- 23.Schmid, I., Uittenbogaart, C. H. & Giorgi, J. V. (1994) Sensitive method for measuring apoptosis and cell surface phenotype in human thymocytes by flow cytometry. *Cytometry* 15:12-20.[\[Medline\]](#)
- 24.Lecoeur, H., Ledru, E., Prevost, M. C. & Gougeon, M. L. (1997) Strategies for phenotyping apoptotic peripheral human lymphocytes comparing ISNT, annexin-V and 7-AAD cytofluorometric staining methods. *J. Immunol. Methods* 209:111-123.[\[Medline\]](#)
- 25.Strasser, A., O'Connor, L. & Dixit, V. M. (2000) Apoptosis signaling. *Annu. Rev. Biochem.* 69:217-245.[\[Medline\]](#)
- 26.Frey, R. S., Li, J. & Singletary, K. W. (2001) Effects of genistein on cell proliferation and cell cycle arrest in non-neoplastic human mammary epithelial cells: involvement of Cdc2, p21(waf/cip1), p27(kip1), and Cdc25C expression. *Biochem. Pharmacol.* 61:979-989.[\[Medline\]](#)
- 27.Constantinou, A. I., Kamath, N. & Murley, J. S. (1998) Genistein inactivates bcl-2, delays the G2/M phase of the cell cycle, and induces apoptosis of human breast adenocarcinoma MCF-7 cells. *Eur. J. Cancer* 34:1927-1934.
- 28.Park, J. S., Kim, E. J., Lee, J. Y., Sin, H. S., Namkoong, S. E. & Um, S. J. (2001) Functional inactivation of p73, a homolog of p53 tumor suppressor protein, by human papillomavirus E6 proteins. *Int. J. Cancer.* 91:822-827.[\[Medline\]](#)
- 29.Butz, K., Denk, C., Ullmann, A., Scheffner, M. & Hoppe-Seyler, F. (2000) Induction of apoptosis in human papillomavirus positive cancer cells by peptide aptamers targeting the viral E6 oncoprotein. *Proc. Natl. Acad. Sci. U.S.A.* 97:6693-6697.[\[Abstract/Free Full Text\]](#)
- 30.Kamradt, M. C., Mohideen, N., Krueger, E., Walter, S. & Vaughan, A. T. (2000) Inhibition of radiation-induced apoptosis by dexamethasone in cervical carcinoma cell lines depends upon increased HPV E6/E7. *Br. J. Cancer* 82:1709-1716.[\[Medline\]](#)
- 31.Meng, Q., Yuan, F., Goldberg, I. D., Rosen, E. M., Auborn, K. & Fan, S. (2000) Indole-3-carbinol is a negative regulator of estrogen receptor-alpha signaling in human tumor cells. *J. Nutr.* 130:2927-2931.[\[Abstract/Free Full Text\]](#)
- 32.Cover, C. M., Hsieh, S. J., Tran, S. H., Hallden, G., Kim, G. S., Bjeldanes, L. F. & Firestone, G. L. (1998) Indole-3-carbinol inhibits the expression of cyclin-dependent kinase-6 and induces a G1 cell cycle arrest of human breast cancer cells independent of estrogen receptor signaling. *J. Biol. Chem.* 273:3838-3847.

