Carcinogenesis vol.22 no.11 pp.1809–1817, 2001

Cytostatic effects of 3,3′-diindolylmethane in human endometrial cancer cells result from an estrogen receptor-mediated increase in transforming growth factor-α expression

Hoyee Leong, Gary L.Firestone[1] and Leonard F.Bjeldanes[2]

Department of Nutritional Sciences and Toxicology and [1]Department of Molecular and Cell Biology, University of California-Berkeley, Berkeley, CA 94720, USA

[2]To whom correspondence should be addressed
Email: lfb@nature.berkeley.edu

3,3′-Diindolylmethane (DIM), a major *in vivo* product of indole-3-carbinol (I3C), is a promising anticancer agent derived from vegetables of the *Brassica* genus including broccoli, Brussels sprouts and cabbage. We report here that DIM has a potent cytostatic effect in cultured human Ishikawa endometrial cancer cells. A combination of northern blot and quantitative PCR analyses revealed that DIM induced the level of TGF-α transcripts by ~4-fold within 24 h of indole treatment. DIM also induced a 4-fold increase in the activity of the estrogen response marker, alkaline phosphatase (AP). Co-treatment of cells with the estrogen receptor (ER) antagonist ICI, or with the inhibitor of PKA-mediated activation of the ER, H89, ablated the DIM induction of both TGF-α expression and AP activity. Furthermore, DIM increased the maximum stimulatory effect of estrogen on TGF-α expression. Co-treatment with the protein synthesis inhibitor, cycloheximide, abolished the inductive effects of DIM, indicating differences in the mechanistic requirements of DIM and estrogen. DIM treatment also stimulated levels of secreted TGF-α protein by >10-fold. The ectopic addition of TGF-α inhibited the growth of Ishikawa cells, whereas incubation with a TGF-α antibody partially reversed the growth inhibitory effects of DIM. Taken together, these results extend our previous findings of the ligand independent estrogen receptor agonist activity of DIM, and uncover an essential role for the stimulation in TGF-α expression and the TGF-α activated signal transduction pathway in the potent cytostatic effects of DIM in endometrial cancer cells.

Introduction

Endometrial cancer is the most common cancer of the female reproductive tract and is ranked as the fourth most common cancer in women (1). Most risk factors for endometrial cancer are associated with reproductive characteristics that result in increased exposures to estrogen (2). Estrogen is a potent mitogen in the uterus, and prolonged or increased exposure to estrogen by early menarche and late menopause, or by hormone replacement therapy, increases cancer risk. However, such risk factors do not adequately explain the increasing incidence of endometrial cancer among migrants to western countries. Such an increase may be attributed to the adaptation to a western diet (3).

Little is known of the modulation of uterine carcinogenesis by micronutrients. Epidemiological evidence suggests, however, that vegetable-rich diets are associated with a lower risk for other estrogen-responsive neoplasias such as breast cancer (4,5). Dietary compounds such as indole-3-carbinol (I3C), an autolysis product of glucobrassicin found in vegetables of the *Brassica* genus including broccoli, Brussels sprouts, and others, have been implicated as chemopreventive agents (6–10). Several studies have demonstrated that dietary exposure to I3C has a pronounced effect against tumor development in estrogen-responsive tissues (8–10). For example, dietary administration of I3C to rodents prior to treatment with a carcinogen decreased the incidence of DMBA-induced mammary tumors by 70–90% (8). Spontaneous mammary tumor development in rodents was also reduced by 50% following long-term dietary I3C administration (9). Additionally, dietary I3C decreased the incidence of spontaneous endometrial tumors in rodents by 24% (10).

The observed *in vivo* biological activity of I3C is most likely due to the effects of its acid-catalyzed self-condensation products. Following ingestion, I3C is rapidly converted into several dimeric and oligomeric compounds due to its instability in gastric acid. Indolo[3,2-*b*]carbazole (ICZ), a cyclic trimer (CTr), and DIM are the most biologically active products examined so far (11–13). ICZ binds to the aryl hydrocarbon receptor (AhR) with high affinity and exerts antiestrogenic effects including inhibition of estrogen-induced growth and estrogen-responsive reporter gene activity in MCF-7 human breast cancer cells (14–16). CTr binds strongly to ER and activates estrogen-responsive gene expression and proliferation of MCF-7 cells (13). DIM has low affinity for the Ah and estrogen receptors and ligand independently activates the ER in a promoter and cell-specific manner (17,18). Although we determined that DIM exerts estrogenic effects under certain conditions in cultured cells, the chemoprotective effects of DIM in estrogen responsive tissues are well established (18,19). For example, oral administration of DIM inhibited the growth of DMBA-induced rat mammary tumors by >75% (19). Whether such a protective effect involves the ligand independent activation of the ER is unclear.

With evidence that dietary I3C has a protective effect on uterine carcinogenesis, we investigated the effect of DIM on human endometrial cancer cells. We demonstrate that whereas DIM was a strong inhibitor of tumor cell growth in culture, this indole also exhibited potent estrogen agonist activities, most notably, in the induction of TGF-α gene expression. We demonstrate further that the DIM-mediated induction of TGF-α expression contributes to the cytostatic effects of DIM.

Abbreviations: AhR, aryl hydrocarbon receptor; AP, alkaline phosphatase; CTr, cyclic trimer; DIM, 3,3′-diindolylmethane; E_2, estradiol; EGFR, epidermal growth factor receptor; ER, estrogen receptor; I3C, indole-3-carbinol; TGF-α, transforming growth factor-α; ICZ, indolo[3,2-*b*]carbazole; JNK, c-Jun NH_2 terminal kinase; MAPK, mitogen activated protein kinase.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM), Opti-MEM and lipofectamine were supplied by Gibco BRL (Grand Island, NY, USA). Phenol red-free

DMEM, fetal bovine serum (FBS), calf serum, 17β-estradiol (E_2), tamoxifen and *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H89) were supplied by Sigma Chemical (St Louis, MO, USA). ICI-182780 (ICI) was supplied by Tocris (Ballwin, MO, USA). DIM was prepared and purified from I3C as described (11,12). All other reagents used in these studies were of highest commercial grade available.

Cell culture

The Ishikawa human endometrial adenocarcinoma cell line was obtained from B.A. Lessey (Chapel Hill, NC, USA). Ishikawa cells were grown as adherent monolayers in DMEM supplemented with 10% FBS, 4.0 g/l glucose and 3.7 g/l sodium bicarbonate. Cells were maintained at 37°C in humidified air containing 5% CO_2 and passaged at 80% confluency. Cultures used in subsequent experiments were at <35 passages.

Cell proliferation

For the indicated experiments, cells were depleted of estrogen for 4 days prior to treatment in DMEM without phenol-red (Sigma Chemical) supplemented with 5% calf serum doubly stripped in dextran-coated charcoal (Gibco BRL), 4.0 g/l glucose, 3.7 g/l sodium bicarbonate, 10 ng/ml insulin and 2 mM glutamine (stripped medium). Medium was changed every other day during the depletion period. DIM, E_2, tamoxifen, H89 and ICI were dissolved in DMSO (99.9% HPLC grade, Aldrich) at 1000-fold higher concentrations than the final medium concentration. For all experiments, treatments were administered for the indicated time by addition of 1 µl of the concentrated agent per 1 ml of medium. Control cells were treated with an equal volume of DMSO. Following the initial treatment, medium was renewed every other day. Cells were harvested by trypsinization and counted in a model Z1 Coulter particle counter.

Alkaline phosphatase activity

AP activity was determined by the hydrolysis of *p*-nitrophenylphosphate to *p*-nitrophenol as described previously (20). Briefly, cells were depleted of estrogen for 4 days and seeded in parallel 96-well plates in 200 µl of medium. Following overnight incubation, cells were treated with test compounds for 72 h. Following treatment, cells were washed with PBS and exposed to *p*-nitrophenyl phosphate (Sigma). *p*-Nitrophenol production was monitored spectrophotometrically at 405 nM. Protein content was estimated by a methylene blue dye microculture assay as described previously (21).

cDNA probe construction

The TGF-α cDNA clone (pINCY vector) was obtained from Genome Systems (St Louis, MO, USA). Plasmid DNA was isolated using the Wizard Plus Minipreps DNA Purification System (Promega, Madison, WI, USA). The cDNA insert was PCR amplified using primers previously designed in our laboratory (5′-GCTGGCGAAAGGGGATG- TGC-3′ and 5′-GAAAGCT-GGTACGCCTGCAGGTACC-3′) and purified using the Wizard PCR Preps DNA Purification Kit (Promega). The β-actin cDNA clone (pBluescript vector) was obtained from ATCC (Rockville, MD, USA). Plasmid DNA was isolated as described, restriction digested by *Eco*RI (Gibco BRL) and subjected to electrophoresis on a 1% agarose gel. The cDNA insert was excised and purified using the QIAEX II Gel Extraction Kit obtained from Qiagen (Chatsworth, CA, USA). Purified cDNAs were biotin labeled using the NEBlot Phototope Kit obtained from New England Biolab (Beverly, MA, USA).

RNA isolation and northern blot analysis

Following the indicated treatments, total RNA was isolated from cells using Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA) as described previously (18). Subsequently, poly(A)$^+$ RNA was isolated from total RNA using the mRNA Separator Kit (Clontech Laboratories, Palo Alto, CA, USA). Poly(A)$^+$ RNA (10 µg) was subjected to electrophoresis on a 1.2% agarose gel containing 3% formaldehyde, transferred overnight to a Zeta nylon membrane (Bio-Rad, CA, USA) with 20× SSC and UV cross-linked. The membrane was pre-hybridized for 4 h in hybridization buffer (0.4 M sodium phosphate pH 7.2, 7% SDS, 1% bovine serum albumin, 0.1 M EDTA) at 65°C, hybridized with cDNA probe in hybridization buffer overnight, and detected by autoradiography using the Phototope-STAR Kit obtained from New England Biolab. The membrane was stripped (0.1× SSC, 0.5% SDS) at 95°C and rehybridized with the β-actin probe for normalization. Poly(A)$^+$ RNA was scanned and quantitated using the NIH Image program.

RT–PCR

Following the indicated treatments, total RNA was isolated as described and reverse transcribed into cDNA using the Superscript Preamplification System (Gibco BRL). Briefly, 2 µg total RNA was reverse transcribed at 42°C for 50 min using random oligomers in a 50 µl reaction mixture. The resultant cDNA (2 µl) was subjected to 25 rounds of PCR (94°C for 45 s, 60°C for 45 s, 72°C for 2 min) using 18S primers obtained from Ambion (Austin, TX, USA) and TGF-α primers synthesized by Genosys (The Woodlands, TX, USA). The sequences of the TGF-α primers, adapted from Imai *et al*., were as follows: TGF-α-1, 5′-ATGGTCCCCTCGGCTGGACA; TGF-α-2, 5′-CTGCAGGTTCCA- TGGAAGCA (22). Amplified products were visualized following electophoresis on a 1.8% agarose gel with ethidium bromide staining. Band intensities were scanned and quantitated using the NIH Image program, and the ratio between 18S and TGF-α was plotted.

Western blot analysis

Following the indicated treatment, cells were harvested in lysis buffer (10 mM Tris, 150 mM NaCl, 0.1% SDS, 1% deoxycholate, 1 mM EDTA, 1% Nonidet P-40) with protease inhibitors (10 µg/ml aprotinin, 10 µg/ml leupeptin, 5 µg/ml pepstatin, 50 µg/ml phenylmethylsulfonylfluoride). Total cellular protein (100 µg) was fractionated by electrophoresis on 10% polyacrylamide, 0.1% SDS resolving gels and electrically transferred overnight to nitrocellulose membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 5% non-fat dry milk in wash buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween 20) for 4 h and incubated with ER-α antibody, sc-543 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or TGF-α antibody, Ab-1 (Oncogene Research Products, Uniondale, NY, USA), at 2 µg/ml wash buffer with 5% non-fat dry milk for 1.5 h. Membranes were subsequently washed and incubated for 1 h with alkaline phosphatase (AP)-conjugated secondary antibody, anti-mouse IgG, (New England Biolabs) diluted 1000-fold in wash buffer. Protein was detected by autoradiography using the Phototope-STAR Western Blot Detection Kit (New England Biolabs). Blotted membranes were stained with Coomassie blue to determine protein loading.

Enzyme-linked immunosorbent assays

Conditioned medium was collected from cell culture following the indicated treatment and concentrated 10-fold using a Centriprep YM-3 Centrifugal Filter Device (Millipore). Because the level of immunoreactive protein detected in complete medium was not significantly different from the level detected in stripped medium, complete medium was routinely used for the assay. Sandwich enzyme-linked immunosorbent assays (ELISA) for TGF-α were performed as described previously (23). Briefly, 100 ng/well of mouse monoclonal TGF-α antibody, Ab-1, was coated overnight. Wells were blocked with bovine serum albumin and incubated with conditioned medium followed by goat TGF-α polyclonal antibody, C-18 (1:100 dilution, Santa Cruz Biotechnology), and finally anti-goat IgG AP conjugate (1:2000 dilution, Sigma). *p*-Nitrophenylphosphate was added to the wells, and the reaction was allowed to proceed for 2–8 h before spectrophotemetrically monitoring the absorbance at 405 nm. The concentration of TGF-α in conditioned medium was measured by comparison to a standard curve generated from human recombinant TGF-α (Oncogene Research Products).

Results

Regulation of cell growth

The effect of DIM on cell proliferation was examined in cultured Ishikawa human endometrial cancer cells. Cells were plated at 60% subconfluency and treated with a range of concentrations of DIM for 24 h, or with 10 µM DIM for up to 6 days. The combined results (Figure 1) indicated that after 24 h of treatment, DIM suppressed growth significantly compared with the vehicle (DMSO)-treated controls at concentrations at or above 10 µM. Cell growth inhibitions of 20 and 40% were observed with 10 and 30 µM treatments, respectively. The results indicated further that 10 µM DIM inhibited proliferation by 55% after 4 days and by ~60% after 6 days of treatment. Treatments with DIM at concentrations of 50 µM or greater induced apoptosis as determined by DNA staining and TUNEL assay (data not shown). A minimal effective concentration of 10 µM DIM was routinely used in subsequent experiments, as this dose did not affect cell viability.

The growth response of Ishikawa cells to estrogen (E_2) was also examined. Cells were depleted of E_2, plated at subconfluency and treated with 10 nM E_2 for up to 20 days. No significant differences between the growth rates of E_2 and DMSO-treated controls were observed up to 8 days of treatment (Figure 2). The results indicated, however, that following 8 days of treatment, or post-confluency, both the growth rate and maximum cell density reached were significantly lower in

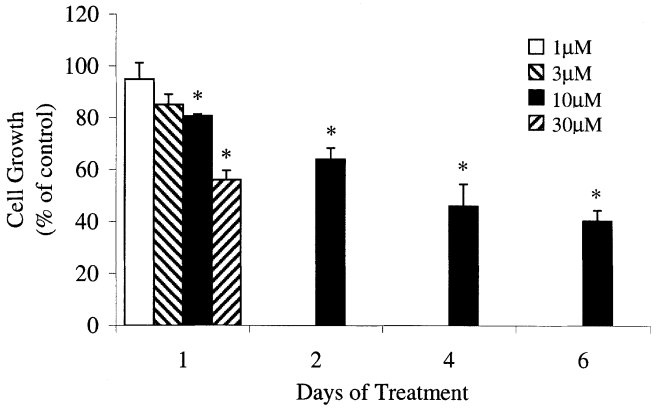


Fig. 1. Effects of DIM on proliferation of Ishikawa endometrial cancer cells. Ishikawa cells were plated in 24 well tissue culture plates (forming a subconfluent monolayer). Cells were plated at 40 000 cells/well and treated with vehicle control (DMSO) or the indicated concentrations of DIM for 24 h, or plated at 10 000 cells/well and treated every 2 days with 10 μM DIM. Cells were counted after the indicated times, and the reported values are expressed as a percentage of DMSO-treated controls. Data represent the mean ± SD of triplicate samples. Asterisks indicate a significant difference from DMSO control ($P < 0.05$) using the Student *t*-test for data analysis.

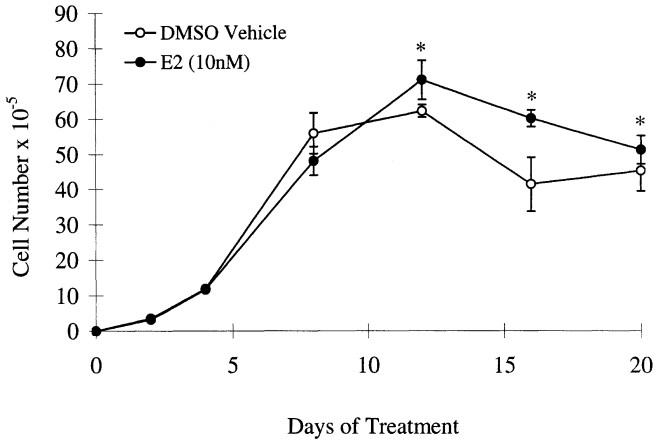


Fig. 2. Effects of E_2 on proliferation of Ishikawa cells. Ishikawa cells were plated in 6 well tissue culture plates at 115 000 cells/well and treated with vehicle control (DMSO) or 10 nM E_2 every 2 days. Cells were counted after the indicated times, and the reported values are expressed as the mean ± SD of triplicate samples. Asterisks indicate a significant difference from DMSO control ($P < 0.05$) using the Student *t*-test for data analysis.

the absence of E_2. The growth rate of cells in the absence or presence of E_2 subsided similarly after the maximum cell density was attained. These results suggest that the growth-promoting effects of E_2 are evident primarily in post-confluent Ishikawa cells as reported by others (24).

Induction of gene expression

The effect of DIM on TGF-α gene expression was examined by northern blot analyses, and findings were confirmed and extended by quantitative RT–PCR analyses. Results of northern blot analysis indicated that DIM increased TGF-α transcript levels by 3.5-fold after 24 h, compared with the vehicle-treated control (Figure 3A). Quantitative RT–PCR measurements using 18S rRNA as an internal control indicated that DIM induced both concentration (1–50 μM) and time (2–48 h)-dependent increases in TGF-α transcript levels. A maximum induction of 6-fold was reached after 24 h at 50 μM relative to vehicle-treated controls (Figure 3B). DIM (10 μM) increased TGF-α transcripts by almost 2-fold after 2 h, and a maximum induction of nearly 4-fold was reached after 16 h of treatment (Figure 3C).

As a control for E_2 agonist effects of DIM in Ishikawa cells, we examined the effect of DIM on the activity of the estrogen-responsive marker enzyme, AP. We observed that following 72 h treatments, 10 μM DIM-induced AP enzyme activities by almost 4-fold relative to controls (Figure 4), a level that was ~50% of the maximal stimulation obtained by 10 nM E_2. DIM did not increase the maximum activity induced by E_2.

DIM-induced expression of TGF-α is mediated by the ER

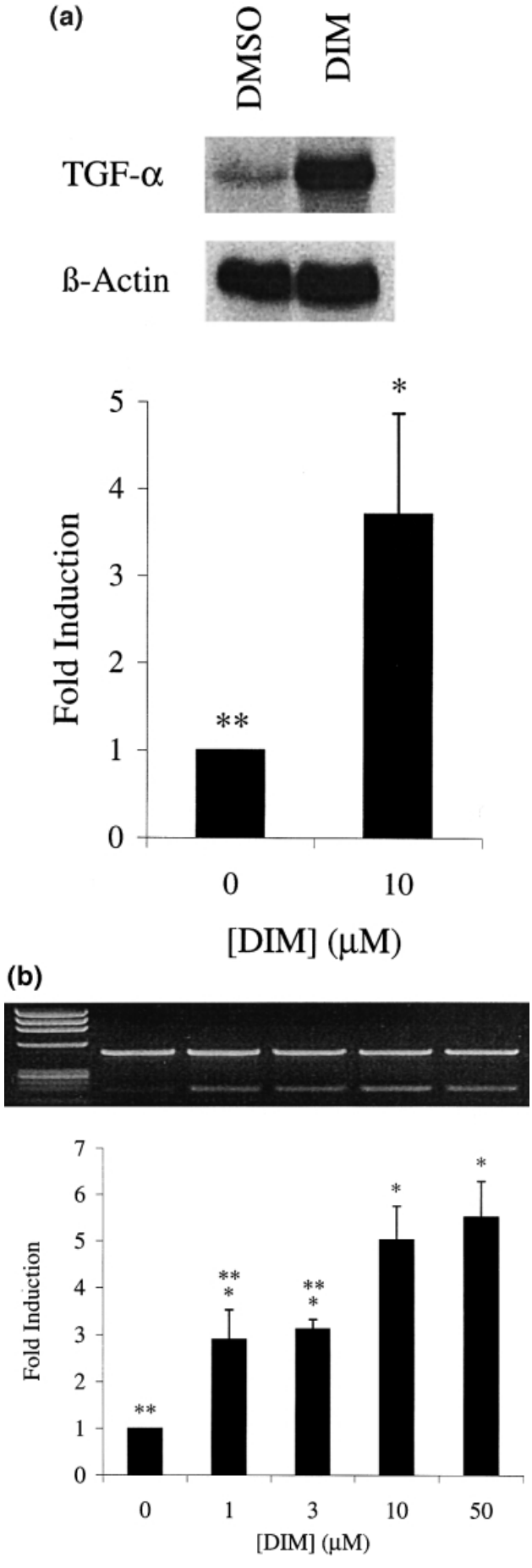
To assess whether DIM induction of TGF-α expression requires interaction with the ER, cells were co-treated with DIM, E_2, the anti-estrogen, ICI or the inhibitor of PKA-mediated activation of the ER, H89. The combined results of these experiments (Figure 5) indicated that co-treatment of cells with DIM (10 μM) and E_2 (10 nM) induced TGF-α expression to an even greater extent than did either compound alone. ICI and H89 inhibited the stimulatory effects of both E_2 (data not shown) and DIM on TGF-α expression. Co-treatment with cycloheximide also reduced TGF-α transcript levels to control levels. As a control, we examined the effects of ICI and H89 on E_2 and DIM stimulation of AP. The combined results indicated that these treatments also blocked the inducing effects of both E_2 (data not shown) and DIM on AP activity (Figure 6). In addition, ER expression in Ishikawa cells was verified by western analysis (data not shown). Taken together these results indicate that induction of TGF-α gene expression is an indirect ER agonist effect of DIM that requires protein synthesis and PKA activation.

Regulation of TGF-α protein level

We determined next whether DIM stimulation of TGF-α gene expression was accompanied by a corresponding increase in TGF-α protein levels. Following treatments with DIM, total cellular and secreted protein fractions were isolated and examined by western analysis and ELISA respectively. Treatment with up to 50 μM DIM for 24 h had no effect on cellular TGF-α protein levels relative to DMSO-treated controls (Figure 7A). Analyses of concentrated conditioned media using a sandwich ELISA assay, however, indicated that DIM induced the secretion of TGF-α by ~5-fold at 10 μM and as much as 13-fold at 50 μM compared with untreated controls. The results of kinetics studies indicated that levels of secreted TGF-α were maximized by 36 h and then decreased to basal levels by 48 h of DIM treatment (Figure 7B), suggesting that excess TGF-α was either degraded or taken up by the cells. Due to cell-to-cell variability, the ELISA results were difficult to obtain as reflected by large standard deviations. However, these results showing similar, strong DIM-induced increases in TGF-α protein secretion and gene expression suggest that protein secretion is regulated at the level of gene expression.

Effect of exogenous TGF-α on cell proliferation

To determine whether an increase in TGF-α expression could mediate the cytostatic effects of DIM in Ishikawa cells, we examined the effect of ectopic addition of TGF-α on cell growth. Cells were cultured at subconfluency and treated with TGF-α in the presence or absence of DIM. The results showed that addition of 1.8 nM TGF-α significantly reduced cell growth by 35 and 25% following 4 and 6 days of treatment, respectively (Figure 8). Treatment of cells with a 10-fold greater concentration of TGF-α (18 nM) gave similar results, whereas a 10-fold lower concentration (180 pM) had no effect



on cell number (data not shown). The inhibitory effect of TGF-α after 6 days of treatment was ~50% that of 10 μM DIM. Co-treatment of cells with DIM and TGF-α did not significantly increase growth inhibition compared with DIM alone.

As a functional test of the role of TGF-α expression in the cytostatic effects of DIM, we conducted growth experiments using a TGF-α neutralizing antibody. Subconfluent cultures were treated with various concentrations of antibody and DIM for 72 h. The results showed that lower concentrations of antibody (0.02, 0.05 μg/ml) markedly stimulated cell growth by as much as 2-fold, whereas the higher concentrations (0.1, 0.2 μg/ml) had lesser effects (Figure 9). Antibody concentrations >1.0 μg/ml inhibited growth (data not shown). Co-treatments with a range of antibody concentrations partially reversed the effects of DIM from 80% inhibition in the 0.02 μg/ml groups to ~60% in the 0.2 groups. Taken together, the

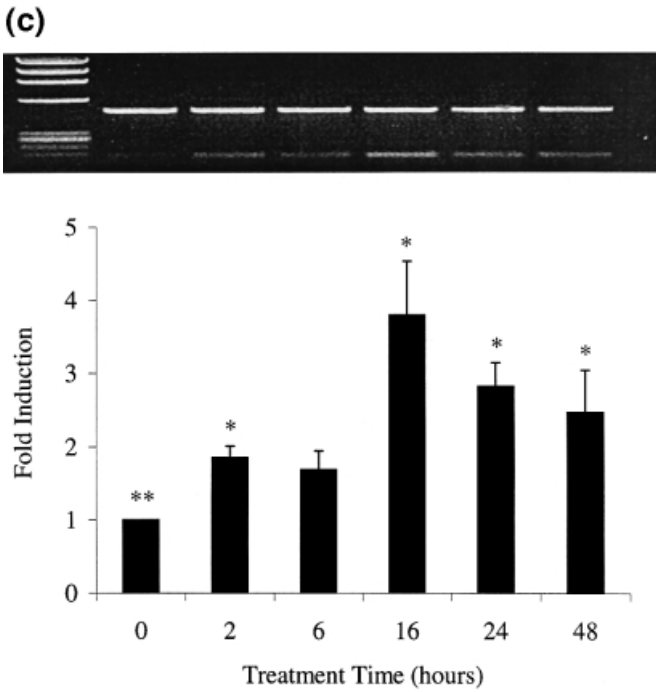


Fig. 3. Effects of DIM on expression of TGF-α transcript in Ishikawa cells. (**A**) Poly(A)$^+$ RNA was isolated from Ishikawa cells treated with vehicle control (DMSO) or 10 μM DIM for 24 h. The RNA was electrophoretically fractionated, and a northern blot was probed for TGF-α transcript as described under 'experimental procedures'. The northern blot was reprobed for β-actin as a loading control. The band intensities of TGF-α and β-actin transcripts were quantitated as described under Materials and methods. TGF-α mRNA level was normalized to β-actin mRNA level by dividing the band intensity of TGF-α by β-actin. The reported values are expressed as fold induction relative to the DMSO control and represent the mean ± SD of three independent experiments. Total RNA was isolated from Ishikawa cells treated with vehicle control (DMSO) or the indicated concentrations of DIM for 24 h (**B**), or 10 μM DIM for the indicated times (**C**). Total RNA was reverse transcribed and PCR-amplified with TGF-α primers and 18S primers as an internal control as described under Materials and methods. The PCR products were resolved and visualized on 1.8% agarose gels with ethidium bromide. X174/*Hae*III DNA marker bands are shown in the first lane. The band intensities corresponding to TGF-α and 18S were quantified as described under Materials and methods. TGF-α expression level was normalized to 18S by dividing the band intensity of TGF-α by 18S. The reported values are expressed as fold induction relative to DMSO-treated controls and represent the mean ± SD of three independent experiments. Asterisks indicate a significant difference from DMSO control ($P < 0.05$), and double asterisks indicate a significant difference from 10 μM DIM at 24 h using the Student *t*-test for data analysis.

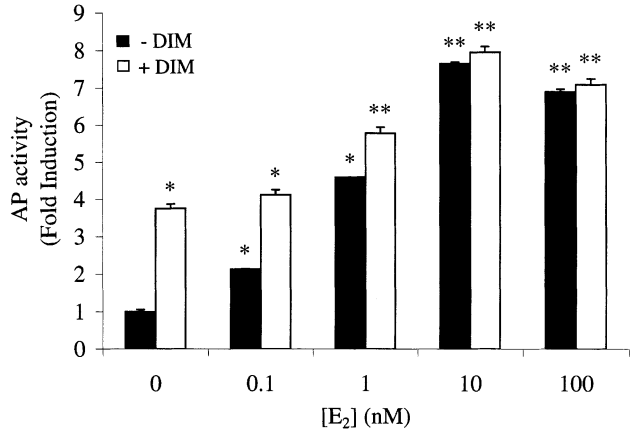


Fig. 4. Effects of DIM on AP activity in Ishikawa cells. Estrogen depleted Ishikawa cells were plated at 20 000 cells/well in parallel 96 well flat-bottom microtiter plates. Cells were treated with E_2 in the presence or absence of 10 μM DIM. Enzyme activity and protein content were determined after 72 h as described under Materials and methods. Enzyme activity was normalized to protein content by dividing the absorbance of AP by that of methylene blue. The reported values are expressed as fold induction relative to DMSO-treated controls and represent the mean ± SD of quadruplicate samples. Asterisks indicate a significant difference from DMSO control, and double asterisks indicate a significant difference from both DMSO control and DIM alone ($P < 0.05$) using the Student *t*-test for data analysis.

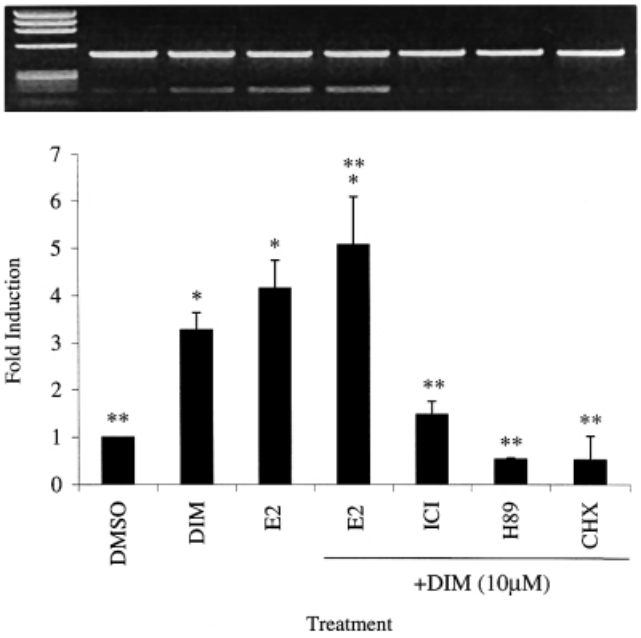


Fig. 5. Effects of DIM, E_2, ICI, cycloheximide and H89 on expression of TGF-α transcript in Ishikawa Cells. Total RNA was isolated from Ishikawa cells treated with vehicle control (DMSO) or 10 μM DIM in the presence or absence of 10 nM E_2, 0.1 μM ICI, 50 μM cycloheximide (CHX) or 10 μM H89 for 24 h. Total RNA was reverse transcribed and PCR-amplified with TGF-α primers and 18S primers as an internal control as described under Materials and methods. The PCR products were resolved and visualized on 1.8% agarose gels with ethidium bromide. X174/*Hae*III DNA marker bands are shown in the first lane. The band intensities corresponding to TGF-α and 18S were quantified as described under Materials and methods. TGF-α expression level was normalized to 18S by dividing the band intensity of TGF-α by 18S. The reported values are expressed as fold induction relative to DMSO-treated controls and represent the mean ± SD of three independent experiments. Asterisks indicate a significant difference from DMSO control ($P < 0.05$), and double asterisks indicate a significant difference from DIM alone using the Student *t*-test for data analysis.

results of these experiments show that the effects of DIM are most pronounced when background TGF-α activity is reduced and that inactivation of released TGF-α partially reverses the cytostatic effects of DIM.

Discussion

Our results demonstrate that DIM has a strong anti-proliferative effect on Ishikawa human endometrial cancer cells. DIM, at a concentration of 10 μM, inhibited cell growth and concurrently activated the expression of two estrogen-responsive genes, AP and TGF-α. That the activation of expression of these proteins was mediated by the ER was indicated by our observations that the inducing effects of DIM could be blocked by co-treatments with an ER antagonist and with an inhibitor of PKA-mediated ER phosphorylation. In addition, the induction of TGF-α by DIM was completely inhibited by the addition of cycloheximide, suggesting that protein synthesis is required for increased TGF-α gene expression. Our results showed that the ectopic addition of TGF-α inhibited Ishikawa cell proliferation. Furthermore, the addition of a TGF-α neutralizing antibody stimulated growth of otherwise untreated cells and partially reversed the inhibitory effect of DIM. Thus, in the presence of neutralizing antibody, the anti-proliferative effect of DIM was increased to a nearly 80% growth inhibition when compared with controls. Altogether, our results indicate that DIM exhibits both strong ER agonist and cytostatic activities in Ishikawa cells, and that the anti-proliferative effects of DIM are partially mediated by the ER dependent activation of TGF-α transcription.

We have reported previously the anti-proliferative and ER agonistic effects of DIM in MCF-7 human breast cancer

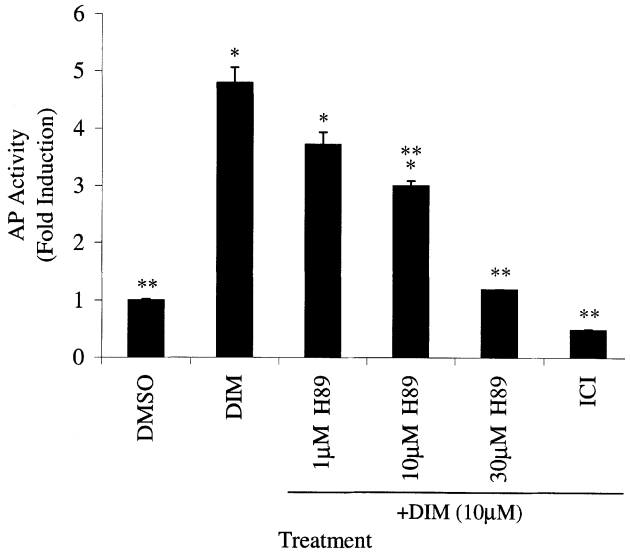


Fig. 6. Effects of DIM, H89 and ICI on AP activity Ishikawa cells. Estrogen depleted Ishikawa cells were plated at 20 000 cells/well in parallel 96 well flat-bottom microtiter plates. Cells were treated with vehicle control (DMSO) or 10 μM DIM in the presence or absence of the indicated concentrations of H89 or 0.1 μM ICI. Enzyme activity and protein content was determined after 72 h as described under Materials and methods. Enzyme activity was normalized to protein content by dividing the absorbance of AP by that of methylene blue. The reported values are expressed as fold induction relative to DMSO-treated controls and represent the mean ± SD of quadruplicate samples. Asterisks indicate a significant difference from DMSO control, and double asterisks indicate a significant difference from DIM alone ($P < 0.05$) using the Student *t*-test for data analysis.

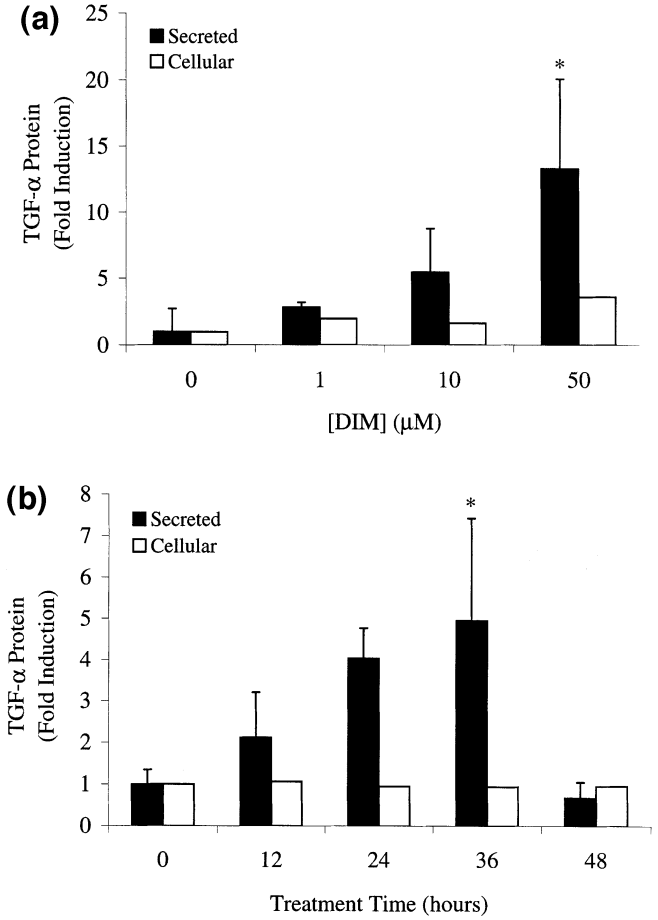


Fig. 7. Effects of DIM on TGF-α protein levels in Ishikawa cells. Ishikawa cells were treated with vehicle control (DMSO) or the indicated concentrations of DIM for 24 h (**A**) or 10 μM DIM for the indicated times (**B**). Conditioned media was collected and concentrated to determine levels of secreted TGF-α by a sandwich ELISA method as described under Materials and methods. Detected levels of secreted TGF-α were normalized against cell number. The reported values are expressed as fold induction relative to controls and are representative of three independent experiments. Data represent the mean ± SD of quadruplicate samples. Asterisks indicate a significant difference from DMSO control ($P < 0.05$) using the Student *t*-test for data analysis. Levels of cellular TGF-α protein were determined by western blot analysis as described under Materials and methods. Equal sample loading was confirmed by Coomassie blue staining of the western blot membrane. The band intensities of TGF-α protein were quantitated as described under Materials and methods. The reported values are expressed as fold induction relative to the DMSO control and are representative of three independent experiments.

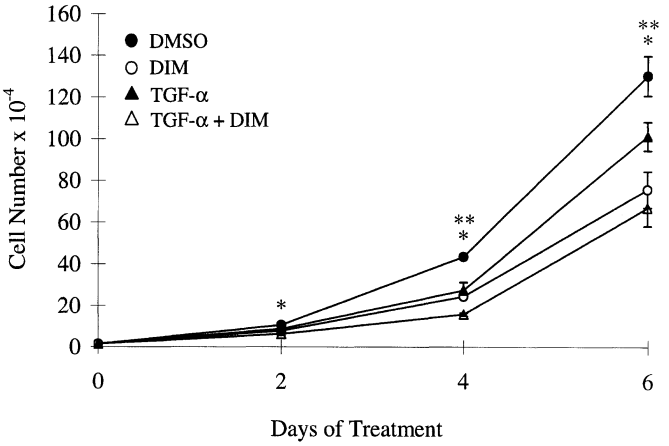


Fig. 8. Effects of TGF-α and DIM on proliferation of Ishikawa cells. Ishikawa cells were plated at 10 000 cells/well in 24 well tissue culture plates and treated with vehicle control (DMSO), 10 μM DIM, or TGF-α in the presence or absence of 10 μM DIM every 2 days. Cells were counted after 2, 4 and 6 days of treatment. Data represent the mean ± SD of triplicate samples. Asterisks indicate a significant difference between DIM and DMSO control, and double asterisks indicate a significant difference between TGF-α and DMSO control ($P < 0.05$) using the Student *t*-test for data analysis.

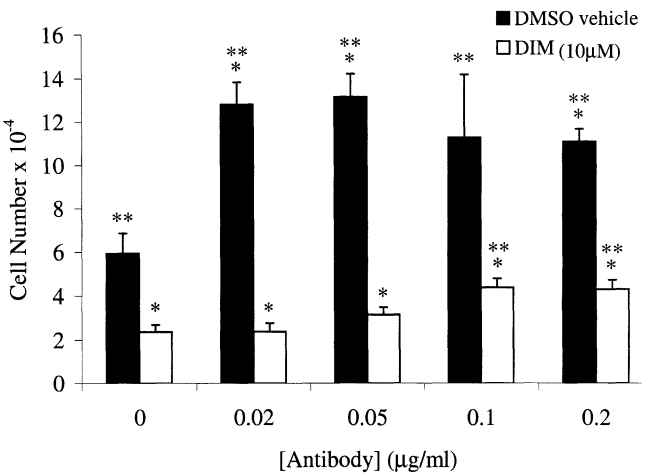


Fig. 9. Effects of TGF-α neutralizing antibody on proliferation of Ishikawa cells. Ishikawa cells were plated at 10 000 cells/well in 96 well flat bottom microtiter plates. Cells were treated with the vehicle control (DMSO) or the indicated concentrations of TGF-α neutralizing antibody in the presence or absence of 10 μM DIM and counted after 72 h. Data represent the mean ± SD of triplicate samples. Asterisks indicate a significant difference from DMSO control, and double asterisks indicate a significant difference from DIM alone ($P < 0.05$) using the Student *t*-test for analysis.

cells (18). In the presence of E_2, 10 μM DIM reduced the proliferation of MCF-7 cells by 40% after 7 days, while in the absence of E_2, DIM stimulated cell growth. We also reported the agonist activity of DIM on the transcriptional activation of estrogen-responsive endogenous and transfected reporter genes in MCF-7 cells. We found, further, that DIM promoted the binding of the ER to its consensus DNA-responsive sequence, and that this ER activation was independent of DIM binding to the ER. In Ishikawa cells, 10 μM DIM in complete medium reduced cell proliferation by 60% after only 6 days and had no stimulatory effect in the absence of E_2. These observations indicate that the growth inhibitory effects of DIM are more pronounced in the endometrial cancer cells and demonstrate that the proliferative effects of DIM are cell-specific. The estrogenic effects of DIM, however, are analogous in both cell types.

Several studies have demonstrated that ligand-independent ER activation may be mediated by activators of PKA (25,26). Stimulation of the PKA signaling pathway has been found to induce ER-mediated transcriptional activation in cell type and reporter-specific manners (25). Furthermore, stimulation of the PKA signaling pathway is accompanied by increased phosphorylation of the ER, which presumably produces a conformational change in the ER that facilitates interaction with transcriptional machinery (25,26). We have previously demonstrated that ER activation by DIM required PKA activity in the MCF-7 cells (18). Similarly, we demonstrate in the Ishikawa cells that PKA activity is necessary for the estrogenic effect of DIM. Such observations suggest that DIM may

activate the ER by enhancing ER phosphorylation by a mechanism that requires protein synthesis.

That anti-proliferative effects of DIM in endometrial tumor cells are mediated through the TGF-α signal transduction pathway is consistent with the well established effects of growth factors in the normal physiology of the endometrium. The human endometrium expresses TGF-α, epidermal growth factor (EGF) and their receptor, the epidermal growth factor receptor (EGFR), in a menstrual cycle-dependent manner, consistent with the notion that endometrial proliferation may be mediated by such growth factors (22,27). The levels of TGF-α and EGF in the endometrium are significantly higher during the late follicular and luteal stages than during the early follicular stage (22,27). E_2 levels are elevated during the late stages, as well, compared with the early follicular stage (27). The menstrual cycle-dependent expression of EGF and TGF-α is consistent with the hypothesis that steroid hormones regulate the growth of endometrial cells by stimulation of expression of these peptide growth factors (28,29).

These EGFR ligands are also well known autocrine/paracrine modulators of abnormal cell growth (30,31). Increases in expression of TGF-α and the EGFR are often characteristic of epithelial neoplasia including breast, lung and bladder cancers (32). Results of studies with transgenic animals have also demonstrated the growth-enhancing role of TGF-α in hepatocellular carcinomas, pancreatic metaplasia, mucosal hyperplasia of the colon and mammary neoplasia (31,33). Similarly, the growth-enhancing effect of EGF has been demonstrated in several cancer cell lines including breast and endometrial cancer cells (34,35). In contrast, and in accord with our observations with Ishikawa cells, exogenous TGF-α significantly inhibited the growth of cultured RL95-2 human endometrial carcinoma cells and MDA-MB-468 human breast cancer cells by 90% or greater (23,36). Furthermore, the inhibitory effect on MDA-MB-468 cells was reversed by an EGFR antibody (23). Similarly, treatment with TGF-α antibody has been reported to increase proliferation of cultured thyroid tumor cells, presumably by inactivating TGF-α in serum (37). The 2-fold increase in growth that we observed on treatment of Ishikawa cells with low concentrations of TGF-α antibody presumably had the same cause. Previous studies have also demonstrated the inhibitory effect of EGF on the growth of thyroid, breast, endometrial and vulvar cancer cell lines (37–40). Taken together, these observations indicate that EGFR ligands exhibit divergent, cell-specific effects on cancer cell growth.

The mechanisms by which TGF-α exerts growth inhibitory effects are unknown. It has been suggested that the inhibitory effects are mediated by low affinity receptors, which are activated only when the ratio of TGF-α to EGFR is high (36). This hypothesis was proposed based on the observation of cell growth inhibition with only high concentrations of TGF-α. We observed a similar cytostatic effect with high concentrations of TGF-α in the Ishikawa cells. TGF-α at nanomolar concentrations inhibited cell growth by up to 35%, while picomolar concentrations had no effect. Alternatively, long-term activation of the EGFR has been reported to lead to receptor internalization and down regulation, and thus, abrogation of normal growth stimulatory signals via the EGFR (37). Our results indicate that EGFR gene expression levels are similar to basal levels after DIM treatment of up to 6 days, suggesting that DIM does not promote down regulation of EGFR expression (data not shown).

Other possible growth inhibitory mechanisms of TGF-α may involve activation of the mitogen activated protein kinase (MAPK) and c-Jun NH_2 terminal kinase (JNK) pathways via the EGFR (41,42). Activation of the MAPK pathway following the binding of TGF-α to the EGFR may result in either increased cell proliferation or differentiation. Hence, high concentrations of TGF-α may indirectly mediate inhibitory effects by activating differentiation factors. Alternatively, TGF-α may exert inhibitory effects via activation of the JNK pathway, which plays a role in the initiation of apoptosis by cytokines such as tumor necrosis factor (43). Whether the inhibitory effects of TGF-α involve activation of the MAPK pathway and/or activation of the JNK pathway will be a focus of our future studies.

The growth inhibitory effect of TGF-α is important to consider given the identification of the EGFR and its associated growth factors as targets for cancer therapy (32,44). Destruction of the EGFR with immunotoxin-linked monoclonal antibodies, targeting of EGFR ligands with radioisotopes, and blockage of downstream signal transduction pathways by inhibitors of phosphorylation are examples of current strategies being developed as potential therapeutic measures. Given the inhibitory potential of EGFR ligands observed in the Ishikawa and other cell lines, EGFR-targeted therapies are likely to be highly tissue specific.

There is considerable precedent in the literature for an influence of estrogen and certain xenobiotics on TGF-α expression. For example, administration of E_2 increased the transcription of TGF-α in the rodent uterus and human breast cancer cells (28,29). A previous study has also demonstrated the activation of TGF-α expression and secretion in breast cancer cells by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), presumably by activation of the Ah receptor pathway (23). In contrast, AhR agonists have been found to exhibit antiestrogenic activity in both breast cancer cells and Ishikawa cells (45). Our results demonstrate that the effect of DIM on TGF-α expression in Ishikawa cells is comparable to the effect of E_2. We also found in these studies, however, that the AhR ligand, ICZ, had no effect on TGF-α expression in Ishikawa cells, indicating that a DIM-mediated activation of the AhR pathway would not lead to an increase in TGF-α expression. Whether AhR agonists would antagonize a DIM or E_2-mediated induction of TGF-α expression requires further investigation.

The TGF-α inducing and anti-proliferative effects of DIM in the Ishikawa cells were observed at physiologically relevant concentrations. A heavy eater of *Brassica* vegetables, for example, may consume 200 g of Brussels sprouts daily, which would provide ~12 mg of DIM. Assuming maximum absorption of DIM, the blood concentration of DIM will reach nearly 10 μM in a man of average weight. Thus, dietary *Brassica* vegetables can provide *in vivo* concentrations of DIM found to be effective *in vitro* in the present study.

Our results demonstrate that DIM, in doses that may be obtained from the diet, shows promise as a therapeutic agent for cancers of the female reproductive organs. A comparison between the effects of DIM on breast and endometrial cancer is important to consider given the association of increased risk for endometrial cancer in women receiving tamoxifen therapy for breast cancer. In a recent case-control study conducted by the National Cancer Institute, women using tamoxifen for >5 years for the treatment of breast cancer had a >4-fold risk for endometrial cancer compared with non-users (46). Despite the fact that the benefits of tamoxifen are considered to far

outweigh the associated risks, a therapeutic agent effective against both breast and endometrial cancer is desired. DIM, or related substances, may provide this advantage.

Taken together, our results indicate that DIM is a strong anti-proliferative agent in human endometrial cancer cells, and that this growth inhibitory effect is partially mediated by the induction of TGF-α expression and secretion. Whereas the effect of DIM on TGF-α gene expression is indirect (i.e. requires protein synthesis) and involves the activation of the ER, the regulation of TGF-α secretion by DIM is less clear. DIM may induce the expression or activation of proteases involved in the cellular release of TGF-α. Alternatively, induced TGF-α secretion may simply be a consequence of increased TGF-α gene expression. In addition, the mechanisms by which TGF-α exerts its anti-proliferative effects remain unclear, but are likely to involve signaling pathways that induce differentiation and/or apoptosis. We are examining further the signaling pathways involved in DIM-mediated activation of the ER and associated cytostatic activity in endometrial tumor cells.

Acknowledgements

This research was supported by the Department of Defense, Army Breast Cancer Research Program Grant DAMDI7-96-1-6149, and by Grant CA69056 from the National Institutes of Health.

References

1. Burke,T.W., Fowler,W.C.Jr and Morrow,C.P. (1995) Clinical aspects of risk in women with endometrial carcinoma. *J. Cell. Biochem.*, **23S**, 131–136.
2. Hulka,B.S. and Brinton,L.A. (1995) Hormones and breast and endometrial cancers: preventive strategies and future research. *Environ. Health Perspect.*, **103S**, 185–189.
3. Tzonou,A., Lipworth,L., Kalandidi,A., Trichopoulou,A., Gamatsi,I., Hsieh,C.C., Notara,V. and Trichopoulos,D. (1996) Dietary factors and the risk of endometrial cancer: a case-control study in Greece. *Br. J. Cancer*, **73**, 1284–1290.
4. Steinmetz,K.A. and Potter,J.D. (1996) Vegetables, fruit and cancer prevention: a review. *J. Am. Dietetic Assoc.*, **96**, 1027–1039.
5. Freudenheim,J.L., Marshall,J.R., Vena,J.E., Laughlin,R., Brasure,J.R., Swanson,M.K., Nemoto,T. and Graham,S. (1996) Premenopausal breast cancer risk and intake of vegetables, fruits and related nutrients. *J. Natl Cancer Inst.*, **88**, 340–348.
6. Wattenberg,L.W. and Loub,W.D. (1978) Inhibition of polycyclic aromatic hydrocarbon-induced neoplasia by naturally occurring indoles. *Cancer Res.*, **38**, 1410–1413.
7. Dashwood,R.H. (1998) Mechanisms of anti-carcinogenesis by indole-3-carbinol: detailed *in vivo* DNA binding dose-response studies after dietary administration with aflatoxin B1. *Carcinogenesis*, **9**, 427–432.
8. Grubbs,C.J., Steele,V.E., Casebolt,T., Juliana,M.M., Eto,I., Whitaker,L.M., Dragnev,K.H., Kellof,G.J. and Lubet,R.L. (1995) Chemoprevention of chemically-induced mammary carcinogenesis by indole-3-carbinol. *Anticancer Res.*, **15**, 709–716.
9. Bradlow,H.L., Michnovicz,J., Telang,N.T. and Osborne,M.P. (1991) Effects of dietary indole-3-carbinol on estradiol metabolism and spontaneous mammary tumors in mice. *Carcinogenesis*, **12**, 1571–1574.
10. Kojima,T., Tanaka,T. and Mori,H. (1994) Chemoprevention of spontaneous endometrial cancer in female Donryu rats by dietary indole-3-carbinol. *Cancer Res.*, **54**, 1446–1449.
11. Bjeldanes,L.F., Kim,J.Y., Grose,K.R., Bartholomew,J.C. and Bradfield,C.A. (1991) Aromatic hydrocarbon responsiveness-receptor agonists generated from indole-3-carbinol *in vitro* and *in vivo*: comparisons with 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Proc. Natl Acad. Sci. USA*, **88**, 9543–9547.
12. Grose,K.R. and Bjeldanes,L.F. (1992) Oligomerization of indole-3-carbinol in aqueous acid. *Chem. Res. Toxicol.*, **5**, 188–193.
13. Riby,J.E., Feng,C., Chang,Y.C., Schaldach,C.M., Firestone,G.L. and Bjeldanes,L.F. (2000) The major cyclic trimeric product of indole-3-carbinol is a strong agonist of the estrogen receptor signaling pathway. *Biochemistry*, **39**, 910–918.
14. Liu,H., Wormke,M., Safe,S.H. and Bjeldanes,L.F. (1994) Indolo[3,2-*b*]carbazole: a dietary-derived factor that exhibits both antiestrogenic and estrogenic activity. *J. Natl Cancer Inst.*, **86**, 1758–1765.
15. Chen,Y-H., Riby,J., Srivastava,P., Bartholomew,J., Denison,M. and Bjeldanes,L.F. (1995) Regulation of CYP1A1 by indolo[3,2-*b*]carbazole in murine hepatoma cells. *J. Biol. Chem.*, **270**, 22548–22555.
16. Jellinck,H., Forkert,P.G., Riddick,D.S., Okey,A.B., Michnovicz,J.J. and Bradlow,H.L. (1993) Ah receptor binding properties of indole carbinols and induction of hepatic estradiol hydroxylation. *Biochem. Pharmacol.*, **45**, 1129–1136.
17. Chen,I., Safe,S. and Bjeldanes,L.F. (1996) Indole-3-carbinol and diindolylmethane as aryl hydrocarbon (Ah) receptor agonists and antagonists in T47D human breast cancer cells. *Biochem. Pharm.*, **51**, 1069–1076.
18. Riby,J.E., Chang,G.H., Firestone,G.L. and Bjeldanes,L.F. (2000) Ligand-independent activation of estrogen receptor function by 3, 3′-diindolylmethane in human breast cancer cells. *Biochem. Pharm.*, **60**, 167–77.
19. Chen,I., McDougal,A., Wang,F. and Safe,S. (1998) Aryl hydrocarbon receptor-mediated antiestrogenic and antitumorigenic activity of diindolylmethane. *Carcinogenesis*, **19**, 1631–1639.
20. Littlefield,B.A., Gurpide,E., Markiewicz,L., McKinley,B. and Hochberg, R.B. (1990) A simple and sensitive microtiter plate estrogen bioassay based on stimulation of alkaline phosphatase in Ishikawa cells: estrogenic action of delta 5 adrenal steroids. *Endocrinology*, **127**, 2757–2762.
21. Finlay,G.J., Baguley,B.C. and Wilson,W.R. (1984) A semiautomated microculture method for investigating growth inhibitory effects of cytotoxic compounds on exponentially growing carcinoma cells. *Anal. Biochem.*, **139**, 272–277.
22. Imai,T., Kurachi,H., Adachi,K. *et al.* (1995) Changes in epidermal growth factor receptor and the levels of its ligands during menstrual cycle in human endometrium. *Biol. Reprod.*, **52**, 928–938.
23. Wang,W.L., Porter,W., Burghardt,R. and Safe,S.H. (1997) Mechanism of inhibition of MDA-MB-468 breast cancer cell growth by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Carcinogenesis*, **18**, 925–933.
24. Holinka,C.F., Hata,H., Gravanis,A., Kuramoto,H. and Gurpide,E. (1986) Effects of estradiol on proliferation of endometrial adenocarcinoma cells (Ishikawa line). *J. Steroid Biochem.*, **25**, 781–786.
25. Katzenellenbogen,B.S. (1996) Estrogen receptors: bioactivities and interactions with cell signaling pathways. *Biol. Reprod.*, **54**, 287–293.
26. Weigel,N.L. and Zhang,Y. (1998) Ligand-independent activation of steroid hormone receptors. *J. Mol. Med.*, **76**, 469–479.
27. Niikura,H., Sasano,H., Kaga,K., Sato,S and Yajima,A. (1996) Expression of epidermal growth factor family proteins and epidermal growth factor receptor in human endometrium. *Hum. Pathol.*, **27**, 282–289.
28. Nelson,K.G., Takahashi,T., Bossert,H.L., Walmer,D.K. and MacLachlan, J.A. (1991) Epidermal growth factor replaces estrogen in the stimulation of female genital-tract growth and differentiation. *Proc. Natl Acad. Sci. USA*, **88**, 21–25.
29. Nelson,K.G., Takahashi,T., Lee,D.C., Luetteke,N.C., Bossert,N.L, Ross,K., Eitzman,B.E. and McLachlan,J.A. (1992) Transforming growth factor-alpha is a potential mediator of estrogen action in the mouse uterus. *Endo.*, **131**, 1657–1664.
30. Derynck,R. (1992) The physiology of transforming growth factor-alpha. *Adv. Cancer Res.*, **58**, 27–52.
31. Kumar,V., Bustin,S.A. and McKay,I.A. (1995) Transforming growth factor alpha. *Cell Biol. Int.*, **19**, 373–388.
32. Rusch,V., Mendelsohn,J. and Dmitrovsky,E. (1996) The epidermal growth factor receptor and its ligands as therapeutic targets in human tumors. *Cytokine Growth Factor Rev.*, **7**, 133–141.
33. Sandgren,E.P., Luetteke,N.C., Palmiter,R.D., Brinster,R.L. and Lee,D.C. (1990) Overexpression of TGF alpha in transgenic mice: induction of epithelial hyperplasia, pancreatic metaplasia and carcinoma of the breast. *Cell*, **61**, 1121–1135.
34. El-Tanani,M.K. and Green,C.D. (1997) Interaction between estradiol and growth factors in the regulation of specific gene expression in MCF-7 human breast cancer cells. *J. Steroid Biochem. Mol. Biol.*, **60**, 269–276.
35. Gershtein,E.S., Shatskaya,V.A., Kostyleva,O.I., Ermilova,V., Kushlinsky, N.E. and Krsil'nikov,M.A. (1995) Comparative analysis of the sensitivity of endometrial cancer cells to epidermal growth factor and steroid hormones. *Cancer*, **76**, 2524–2529.
36. Korc,M., Haussler,C.A. and Trookman,N.S. (1987) Divergent effects of epidermal growth factor and transforming growth factors on a human endometrial carcinoma cell line. *Cancer Res.*, **47**, 4909–4914.
37. Broecker,M., Hammer,J. and Derwahl,M. (1998) Excessive activation of tyrosine kinases leads to inhibition of proliferation in a thyroid carcinoma cell line. *Life Sci.*, **63**, 2373–2386.

38. Korc,M., Padilla,J. and Grosso,D. (1986) Epidermal growth factor inhibits the proliferation of a human endometrial carcinoma cell line. *J. Clin. Endocrinol. Metab.*, **62**, 874–880.
39. Imai,Y., Leung,C.K.H., Friesen,G.H. and Shiu,R.P. (1982) Epidermal growth factor receptors and effect of epidermal growth factor on growth of human breast cancer cells in long-term tissue culture. *Cancer Res.*, **42**, 4394–4398.
40. Barnes,D.W. (1982) Epidermal growth factor inhibits growth of A431 human epidermoid carcinoma in serum-free cell culture. *Cell Biol.*, **93**, 1–4.
41. Wells,A. (1999) EGF receptor. *J. Biochem. Cell Biol.*, **31**, 637–643.
42. Rosette,C. and Karin,M. (1996) Ultraviolet light and osmotic stress: activation of the JNK cascade through multiple growth factor and cytokine receptors. *Science*, **274**, 1194–1197.
43. Pimentel-Muinos,F.X. and Seed,B. (1999) Regulated commitment of TNF receptor signaling: a molecular switch for death or activation. *Immunity*, **11**, 783–793.
44. Aaronson,S.A. (1991) Growth factors and cancer. *Science*, **254**, 1146–1153.
45. Wormke,M., Castro-Rivera,C., Chen,I. and Safe,S. (2000) Estrogen and aryl hydrocarbon receptor expression and crosstalk in human Ishikawa endometrial cancer cells. *J. Steroid Biochem. Mol. Biol.*, **72**, 197–207.
46. Bernstein,L., Deapen,D., Cerhan,J.R., Schwartz,S.M., Liff,J., McGann-Maloney,E., Perlman,J.A. and Ford,L. (1999) Tamoxifen therapy for breast cancer and endometrial cancer risk. *J. Natl Cancer Inst.*, **91**, 1654–1662.

Received May 29, 2001; revised August 7, 2001; accepted August 24, 2001