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Bcl-2 family-mediated apoptotic effects of 3,3′-diindolylmethane (DIM) in human breast cancer cells

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Abstract

3,3′-Diindolylmethane (DIM) is a major *in vivo* derivative of the putative anticancer agent indole-3-carbinol (I3C), which is present in vegetables of the *Brassica* genus. At concentrations above 10 μM, DIM inhibited DNA synthesis and cell proliferation in both estrogen receptor replete (MCF-7) and deficient (MDA-MB-231) human breast cancer cells in a concentration- and time-dependent manner. These antiproliferative effects were accompanied by characteristic indications of programmed cell death in both cell lines, including externalization of phosphatidylserine, chromatin condensation, and DNA fragmentation. Furthermore, Western and Northern blot analyses, as well as coimmunoprecipitation assays, revealed that in both MCF-7 and MDA-MB-231 cells, DIM treatment decreased total transcript and protein levels of the apoptosis inhibitory protein Bcl-2, and the amount of Bcl-2 bound to the pro-apoptotic protein Bax. DIM treatment also caused an increase in Bax protein levels, but did not affect the level of Bax that was bound to Bcl-2. As a functional test of the role of Bcl-2 down-regulation in the DIM-induced apoptotic response, ectopic expression of Bcl-2 in MCF-7 cells was shown to attenuate the apoptotic effect of DIM. These results demonstrate that DIM can induce apoptosis in breast cancer cells independent of estrogen receptor status by a process that is mediated by the modulated expression of the Bax/Bcl-2 family of apoptotic regulatory factors.

Keywords: Dietary indole; Breast cancer; Apoptosis; Bcl-2; Bax; Coimmunoprecipitation

1. Introduction

DIM is a natural product produced during the autolytic breakdown of glucobrassicin, one of over 100 sulfur-containing glucose conjugates, known as glucosinolates, that occur exclusively in the Crucifereae. The best-known food crops in this family occur in the *Brassica* genus and include highly nutrient-rich vegetables such as broccoli, Brussels sprouts, cabbage, and kale, all of which are also good sources of glucobrassicin. The immediate precursor of DIM in the plant is I3C (see structure in Fig. 1), which is liberated from glucobrassicin by the action of myrosinase following disruption of subcellular compartmentalization of enzyme and substrate. I3C is well known to inhibit mammary cancer, as well as to block the cell cycle in several *in vivo* and *in vitro* test systems [1–6]. In some two-stage cancer assays, however, I3C also exhibits tumor-promoting activities in non-reproductive organs [7–10].

The diverse effects of I3C depend on the species, tissue, and route of administration, and it is therefore important to determine the mode of action of this widely consumed phytochemical. We have documented that I3C undergoes extensive and rapid self-condensation reactions under the acidic conditions of the stomach to form several derivatives with distinctive biological activities [11,12]. DIM is a major acid condensation product of I3C, *in vitro* [13]. In addition, DIM is also produced *in vivo* since it has been readily detected in the livers and feces of rodents treated by gavage with I3C, whereas the parent I3C was not detected in these animals [14,15]. DIM is slowly produced from I3C in cell cultures at neutral pH over extended incubation periods, and during the simple mixture and storage of purified diets used in rodent feeding studies. Because of the ready conversion of I3C to DIM and other products under a variety of biologically relevant conditions, the biological effects of I3C may be attributable, at least partially, to DIM.

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Abbreviations: DIM, 3,3′-diindolylmethane; I3C, indole-3-carbinol; Co-IP, coimmunoprecipitation; DMBA, dimethylbenzanthracene; ER, estrogen receptor; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay.

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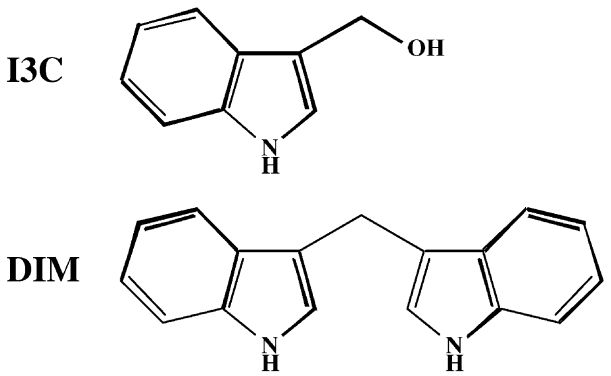

Fig. 1. Molecular structures of I3C and DIM.

Results of several studies indicate that DIM exhibits promising cancer protective activities, especially against mammary neoplasia. Oral administration of DIM in a single dose prior to carcinogen treatment reduced the incidence and multiplicity of DMBA-induced mammary tumors in rats by 70–80% [1], whereas repeated oral administrations during the promotion stage of DMBA-induced mammary tumorigenesis inhibited tumor growth in rodents by as much as 95% [16]. We observed that under conventional cell culture conditions, DIM inhibited the proliferation of breast tumor cell lines, regardless of ER status. Interestingly, DIM exhibited estrogen agonist activity in breast cancer cells cultured under estrogen-depleted conditions, and induced proliferation of ER-replete MCF-7 cells and activated DNA binding of the ER by a mechanism that was independent of ligand binding to the receptor [17]. DIM also increased the level of transcripts for an endogenous estrogen-responsive gene, *pS2*, and activated estrogen-responsive reporter plasmids in transfected MCF-7 cells.

In the present study, we further examined the antiproliferative effects of DIM in estrogen-dependent and -independent cultured human breast tumor cells. Because of the potential involvement of the ER in the effects of DIM, we compared the effects of this indole with those of tamoxifen, an ER antagonist and widely used breast cancer therapeutic agent. We observed that DIM induced apoptosis in a manner that was independent of ER status. Our study provides direct evidence that DIM induces apoptosis in these cells by blocking the expression of the anti-apoptosis factor Bcl-2, and increasing the levels of the pro-apoptotic factor Bax.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), Opti-MEM, and Lipofectamine were supplied by Gibco/BRL. Fetal bovine serum (FBS) and tamoxifen were purchased from the Sigma Chemical Co. DIM was prepared from I3C as described [13,18,19] and recrystallized in toluene. All other reagents were of the highest grade available.

2.2. Cell lines and cell culture

The human breast adenocarcinoma cell lines MCF-7 (ER-positive) and MDA-MB-231 (ER-negative) were obtained from the American Type Culture Collection (ATCC). MCF-7 and MDA-MB-231 cells were maintained in 10% (v/v) FBS–DMEM, supplemented with 3.0 g/L of glucose, and 3.7 g/L of sodium bicarbonate. The cells were cultured as monolayers in a 95% (v/v) air, 5% (v/v) CO_2 water-saturated atmosphere at 37° and passaged at approximately 80% confluency. Cultures of less than 20 passages were used in subsequent experiments.

2.3. Cell counting

Cells were harvested by trypsinization and resuspended in culture medium. Aliquots were diluted 50-fold in Isoton II (Coulter Corp.), and 500 μL duplicates were assessed in a model Z1 Coulter particle counter and averaged.

2.4. Thymidine incorporation

MCF-7 or MDA-MB-231 cells (2.1×10^4/well) were plated onto Corning 24-well tissue culture dishes for 24 hr and treated with DMSO or DIM for 24–72 hr. Three microliters (3 μCi) of [^{3}H]thymidine (NEN) was then added to each well and incubated at 37° for 3 hr. Medium was removed, and the cells were washed three times with 2 mL of cold 10% (w/v) trichloroacetic acid (TCA), followed by the addition of 500 μL of 0.3 N NaOH to each well for 30 min at room temperature. Then 250 μL aliquots were put into scintillation vials with 4 mL of ScintiVerse BD scintillation fluid (Fisher) and assessed for radioactivity by a Beckman liquid scintillation counter.

2.5. Cell viability assay

Cells were seeded at 4.0×10^4/well in Corning 6-well tissue culture dishes and allowed to adhere for 24 hr. After various treatments, cells were trypsinized and stained with a trypan blue solution (Sigma). The results were represented as the number of viable cells/total cells as measured by trypan blue exclusion.

2.6. Annexin V-FITC assay

Annexin V, a calcium-dependent phospholipid-binding protein with a high affinity for phosphatidylserine, was used to detect the early stages of apoptosis. Briefly, MCF-7 cells were grown on cover slips (5×10^4/cover slip) and treated with vehicle control, DIM, or etoposide (the positive control) for various time points. The cells were washed in PBS and resuspended in binding buffer [10 mM HEPES/

0.1 N NaOH (pH 7.4), 140 mM NaCl, 2.5 mM $CaCl_2$]. Annexin V-conjugated with fluorescein isothiocyanate (FITC) (ApoAlert Annexin V Kit, Clontech) was added to a final concentration of 100 ng/mL, and the cells were incubated in the dark for 10 min, washed with PBS, and resuspended in 300 μL of binding buffer. Then 10 μL of propidium iodide (Sigma) was added to each sample. A drop of anti-fade (90% glycerol, 2.3% diazabicyclo[2.2.2]octane) was added to the slide to prevent bleaching of the fluorescence. Cover slips then were mounted onto the slides and observed under a fluorescence microscope.

2.7. DNA staining for chromatin morphological evaluation

Cells were harvested after various treatment protocols. Floating, nonadherent cells in the spent medium and adherent cells were collected by combining the spent medium and trypsin-treated samples, and cells were spun at 3000 *g* for 5 min at room temperature, washed, fixed, and permeabilized with 100% methanol for 15 min. Cells were stained with 1 μg/mL of propidium iodide or bisbenzimide (Hoechst 33342) for 15 min in the dark and viewed using a Zeiss ICM 405 fluorescence microscope with appropriate filters; 600 cells were counted. Cells in which the nucleus contained clearly condensed chromatin or cells exhibiting fragmented nuclei were scored as apoptotic.

2.8. Microscopic detection of DNA fragmentation with the TUNEL assay

Briefly, MCF-7 or MDA-MB-231 cells were seeded on cover slips (5×10^4/cover slip), grown in DMEM with 10% FBS for 24 hr, and then treated with either DMSO (vehicle control), DIM, or etoposide (positive control) for various times. Cells were washed twice with PBS and permeabilized in 0.2% Triton X-100/PBS for 5 min on ice, then washed twice with fresh PBS for 5 min each time at room temperature. The TUNEL assay was performed using the ApoAlert DNA fragmentation assay kit (Clontech), with a final concentration of propidium iodide of 1 μg/mL. A drop of anti-fade solution was added, the cover slip was covered with a slide, and the edges of the slide were sealed with clear nail polish. Slides were viewed immediately under a fluorescence microscope using a dual filter set for green (520 nm) and red (>620 nm) fluorescence. The images were captured and analyzed using NIH image software.

2.9. Western blot analysis

Protein extracts were prepared essentially as described previously [5]. Equal amounts of total protein were fractionated by electrophoresis on 4% polyacrylamide/0.1% SDS stacking gels and 15% polyacrylamide/0.1% SDS resolving gels. Biotinylated molecular weight markers (New England BioLabs) were used as standards. Proteins were electrotransferred to nitrocellulose or PVDF Immobilon-P transfer membranes (Millipore) using transfer buffer [25 mM glycine, 25 mM ethanolamine, and 20% methanol]. The membranes were blocked overnight at 4° with blocking buffer [1 × Tris-buffered saline, 0.1% Tween-20 with 5% (w/v) nonfat dry milk]. Blots were then incubated for 2 hr with primary mouse anti-human Bcl-2 (100) antibody (Santa Cruz Biotechnology, Cat. No. #sc 509) or mouse anti-human Bax (B-9) (Cat. No. #sc 7480) (1 μg/mL), and washed with the wash buffer [10 mM Tris–HCl (pH 9.5), 10 mM NaCl, and 10 mM $MgCl_2$]. The membranes were incubated for 1 hr with secondary anti-mouse IgGs conjugated to alkaline phosphatase (1:1000 dilution) (Santa Cruz Biotechnology or Cell Signaling Technology, Cat. No. #7052-1), together with alkaline phosphatase-conjugated anti-Biotin IgG (New England BioLabs) for detection of the biotinylated molecular weight standards. Blots were treated with the CDK-Star reagent (New England BioLabs), and the proteins were detected by autoradiography. Images were scanned and quantified by densitometer analysis software. Equal protein loading was confirmed by probing for α-tubulin, staining with Coomassie blue solution or with Ponceau S solution.

2.10. Co-IP

Treated cells were processed as described for Western blot analysis. Samples were diluted to 140 μg of protein in 1 mL of Co-IP buffer [10 mM HEPES (pH 7.2), 143 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, and fresh protease inhibitors] and precleared for 60 min at 4° with 20 μL of a 1:1 slurry of protein A/G Plus-Agarose (Santa Cruz Biotechnology, Cat. No. #sc 2003) in Co-IP buffer and 1 μg of mouse IgG. After a brief centrifugation (3000 *g* for 5 min at 4°) to remove precleared beads, 1 μg of primary mouse anti-Bax or rabbit anti-Bcl-2 polyclonal IgG (Cat. No. #sc 493, Santa Cruz Biotechnology) was added to the lysate and incubated for 1 hr at 4°. The antibody–protein complexes were immunoprecipitated with 20 μL of Protein A/G Plus-Agarose. After an overnight incubation at 4° on a rotating device, precipitates were washed four times with Co-IP buffer, resuspended in 40 μL 1 × SDS electrophoresis sample buffer [50 mM Tris–HCl (pH 6.8), 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol], electrophoresed, and analyzed by a Western blotting technique with monoclonal antibodies to the other protein of the complex, Bax or Bcl-2, as above.

2.11. RNA extraction, mRNA purification, and Northern hybridization

After total RNA was extracted as described previously [17], mRNA was isolated from total RNA with oligo(dT)

cellulose columns following a Micro-FastTrack 2.0 user manual (Invitrogen). mRNA (5–10 μg) was electrophoresed on a 1.2% agarose gel containing 3% formaldehyde using MOPS as the running buffer. The gel was then washed gently with 10 × SSC (1.5 M sodium chloride and 0.15 M sodium citrate, pH 7.0) and blotted with a Zeta nylon membrane (Bio-Rad) overnight to transfer the mRNA onto the membrane. The mRNA was fixed to the membrane by UV cross-linking. cDNA probes were biotinylated using an NEBlot Phototope kit (New England BioLabs), purified via precipitation with 3 M sodium acetate (pH 5.2), and washed with 70% ethanol. After hybridization with specific probes, the membrane was incubated with streptavidin and washed twice with solution I (0.5% SDS, 12.5 mM NaCl, 2.5 mM sodium phosphate at pH 7.2). Biotinylated alkaline phosphatase was then incubated with the membrane for 5 min followed by blocking solution. The membranes were washed twice, 15 min each time, with wash solution II (10 mM Tris–HCl, 10 mM NaCl, and 1 mM $MgCl_2$ at pH 9.5), assayed by CDK-Star, and exposed to X-ray film for mRNA detection. The amount of mRNA was quantified by gel densitometry (Bio-Rad) and normalized with β-actin as an internal control.

2.12. Transient transfections with Bcl-2 expression vector or control vector

Transfections were performed using the lipofection method with Lipofectamine (Gibco) as described previously [17] with some variations. Cells were grown in 10% FBS–DMEM to subconfluency and transferred to 6-well or 100-mm (2×10^6 cells/plate) Corning tissue culture dishes 24 hr before transfection. For each dish, 18 μL of Lipofectamine was diluted with 582 μL of OptiMEM serum-free medium (Gibco). The *Bcl-2* gene under the control of the cytomegalovirus promoter (a gift from Dr. S.J. Korsmeyer, Harvard University) or CMV-pCDNA vector (15 μg) was diluted in 600 μL of serum-free medium. Lipid and vector dilutions were combined, mixed gently, and incubated at room temperature for 30–45 min. Meanwhile, the cells were washed with 4 mL of serum-free medium, and 2 mL of serum-free OptiMEM was added to each plate. The lipid–DNA suspension was added to each dish and mixed gently. The dishes were returned to the incubator for 5–6 hr, and 2 mL of medium containing 20% FBS–DMEM was added. The next day, cells were reincubated with fresh 10% FBS–DMEM with or without DIM treatment and harvested after 24 hr.

2.13. Statistical analysis

The statistical differences between groups were determined using ANOVA and Tukey's Studentized Range test. The levels of significance are noted ($P < 0.05$). The results are expressed as means ± SD for at least three replicate determinations for each experiment.

3. Results

3.1. Antiproliferative effects of DIM on human breast cancer cells

We determined the effect of DIM on the proliferative responses of both ER-positive (MCF-7) and ER-negative (MDA-MB-231) human breast cancer cells [20]. The cells were treated with different concentrations of DIM for 24 hr and then pulse-labeled with [^{3}H]thymidine to measure nucleotide incorporation into DNA. The results indicated that DIM induced a steady, concentration-dependent decrease in DNA synthesis in both cell lines (Fig. 2A). For comparison, we examined the effects of the widely used breast cancer therapeutic agent and ER antagonist, tamoxifen, on the proliferative characteristics of breast cancer cells. Results shown in Fig. 2B indicate that, as expected from published work [21], tamoxifen was a more potent inhibitor of DNA synthesis in the ER-positive MCF-7 cells than in the ER-deficient MDA-MB-231 cells. At 50 μM DIM, proliferation of MCF-7 cells was nearly totally inhibited, whereas at 100 μM DIM cell counts clearly declined (Fig. 2C). In MDA-MB-231 cells (Fig. 2D), we observed a similar inhibitory effect of DIM, but it was less effective than in the MCF-7 cells. Results of trypan blue exclusion cell viability studies indicated that concentrations of DIM above 50 μM were increasingly cytotoxic (data not shown).

3.2. DIM-induced apoptotic effects in MCF-7 and MDA-MB-231 cells

To determine whether the cytostatic and cytotoxic effects of DIM were accompanied by apoptosis, we examined several key markers characteristic of this type of cell death in both MCF-7 and MDA-MB-231 cells. Translocation of phosphatidylserine from the inner face of the plasma membrane to the cell surface was observed by staining cells with annexin V conjugated with FITC. Propidium iodide staining was used to distinguish the different stages of apoptosis based on the permeability of the cell membrane. In separate experiments, bisbenzimide (Hoechst 33342) staining of chromatin was used to distinguish and quantify apoptotic cells. DNA fragmentation was determined by 3′-OH DNA end labeling with dUTP conjugated with FITC (TUNEL assay) and observed under a fluorescence microscope.

The results showed that 100 μM DIM produced the halo image that is characteristic of phosphatidylserine redistribution as early as 10 min after treatment, indicating the onset of the early stages of apoptosis. However, no nuclear staining with propidium iodide was apparent at this time, indicating that an increase in plasma membrane permeability had not yet occurred. After 3 hr of treatment with 50 μM DIM, we observed both halo images and the reddish propidium iodide nuclear staining of condensed nuclear chromatin characteristic of late-stage apoptosis (Fig. 3).

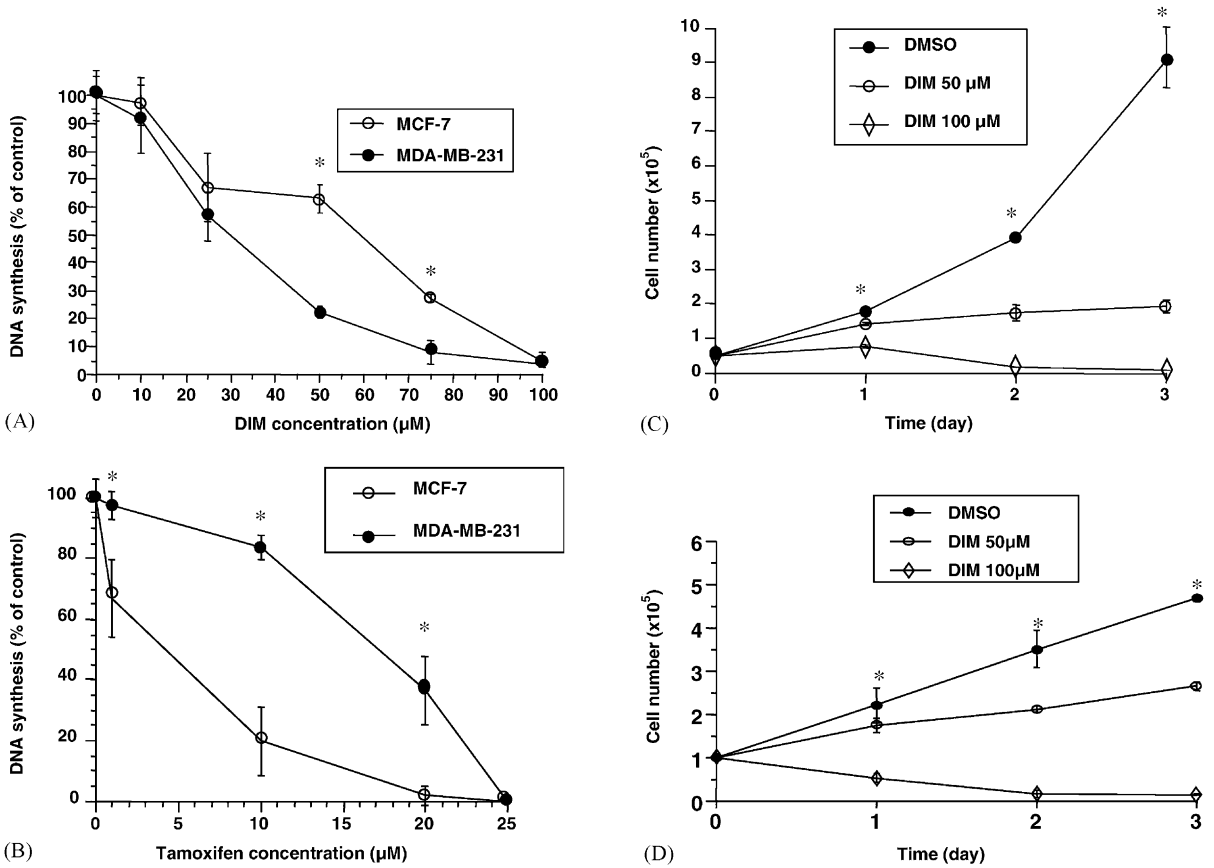

Fig. 2. (A) Concentration response of DIM on [^{3}H]thymidine incorporation in MCF-7 and MDA-MB-231 cells. MCF-7 (○) or MDA-MB-231 (●) cells were plated onto Corning 24-well tissue culture dishes (2.1×10^4 cells/well) for 24 hr and treated with DMSO or DIM (see structure in Fig. 1) for 24 hr. Cells were labeled with [^{3}H]thymidine for 3 hr, and the incorporation into DNA was determined by acid precipitation as described under Section 2. The reported values are averages ± SD from three independent experiments with triplicate samples/experiment. Asterisks indicate a statistically significant difference between cell lines, using ANOVA analysis ($P < 0.05$). (B) Comparison of the effect of tamoxifen concentration on [^{3}H]thymidine incorporation in MCF-7 and MDA-MB-231 cells. MCF-7 (○) and MDA-MB-231 (●) cells were plated onto Corning 24-well tissue culture dishes (2.1×10^4 cells/well) for 24 hr and treated with DMSO or different concentrations of tamoxifen for 24 hr. DNA synthesis was measured as described for panel A, and the values are the averages ± SD from three independent experiments with triplicate samples/experiment. Asterisks indicate a statistically significant difference between cell lines, using ANOVA analysis ($P < 0.05$). (C and D) Time course of the effect of DIM on MCF-7 and MDA-MB-231 cell proliferation. MCF-7 cells (5×10^4/well) (panel C) or MDA-MB-231 cells (10×10^4/well) (panel D) were seeded onto Corning 6-well tissue culture dishes and treated with DMSO (●), 50 μM DIM (○), or 100 μM DIM (◇) for 72 hr. Duplicate aliquots of cells from individual wells and duplicates of each treatment in each experiment were counted. Data from three separate experiments are shown as means ± SD. The asterisks indicate a statistically significant difference at treatment time points, using ANOVA analysis ($P < 0.05$).

Characteristic staining for both phosphatidylserine redistribution and chromatin condensation was obvious following a 3-hr treatment with the positive control apoptotic agent etoposide. Quantification of chromatin condensation and nuclear fragmentation using bisbenzimide staining following 24-hr treatments with DIM indicated a concentration-dependent increase in the proportion of apoptotic cells to as much as 50% in both cell lines following 100 μM DIM (Fig. 4). There were no significant differences between cell types. Similar levels of DNA fragmentation were observed with the TUNEL assay (data not shown).

3.3. Decrease of Bcl-2 expression and increase of Bax expression with DIM treatment in MCF-7 and MDA-MB-231 cells

Because of the essential role of Bcl-2 and Bax proteins in the regulation of apoptosis, we examined the effects of DIM on the expression of these regulatory factors. Breast tumor cells were treated with 50 or 100 μM DIM for 24 hr or with 50 μM DIM for various time periods. Western blot analyses indicated that DIM decreased Bcl-2 protein levels up to 90 and 60% in MCF-7 and MDA-MB-231 cells, respectively (Fig. 5). Incubation of cells with a fully cytostatic concentration of tamoxifen (25 μM), however, produced only about a 30% decrease on Bcl-2 levels in both cell lines.

Results of Northern blot analyses indicated that a 24-hr treatment with DIM reduced the level of Bcl-2 mRNA levels in a concentration-dependent manner in MCF-7 cells, with as much as a 70% decrease observed in cells exposed to 100 μM DIM (Fig. 6A). The inhibitory effect of 50 μM DIM increased approximately 2-fold by 48 hr of treatment, as determined in the kinetics experiment (Fig. 6B). A similar strong reduction in the levels of Bcl-2 transcripts was observed following a 48-hr treatment

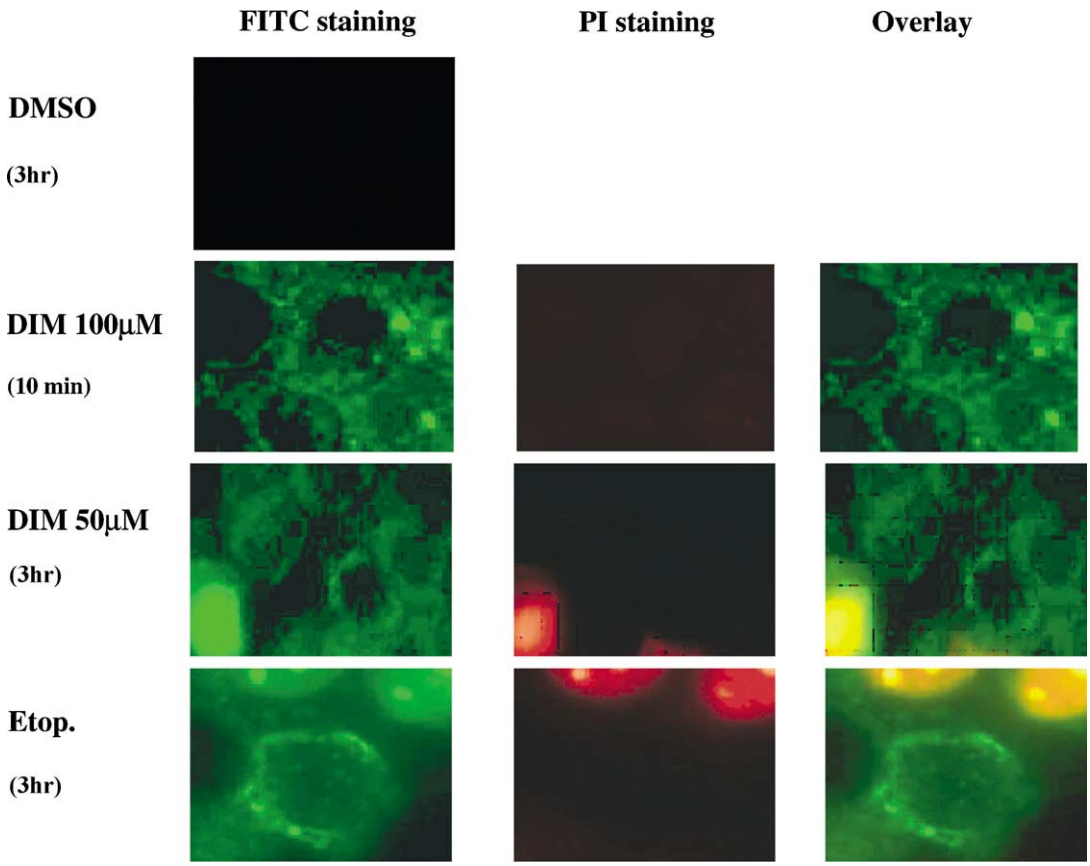

Fig. 3. Effect of DIM on phosphatidylserine externalization and nuclear morphology in MCF-7 cells. MCF-7 cells were grown on cover slips (5×10^4/cover slip) and treated with DMSO (control vehicle), DIM (50 or 100 μM), or etoposide (10 μM) (the positive control) for various times. The cells were incubated with annexin V-FITC and propidium iodide (PI) as described in Section 2 for the annexin V binding assay. The data shown are representative of three independent experiments.

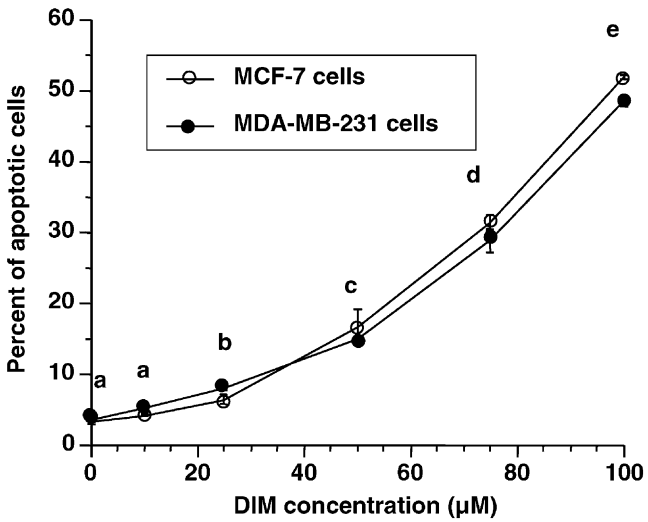

Fig. 4. Effect of DIM on the apoptotic index (AI) in MCF-7 and MDA-MB-231 cells. MCF-7 (○) and MDA-MB-231 (●) cells were harvested following 24-hr treatments with DIM. Floating, non-adherent, and adherent cells were collected, fixed, permeabilized, and stained with bisbenzimide (Hoechst 33342) for viewing under a fluorescence microscope, as described under Section 2. Cells that contained clearly condensed nuclear chromatin or fragmented nuclei were scored as apoptotic. The data shown are averages ± SD from three independent experiments. Different letters indicate a significant difference between treatments ($P < 0.05$).

with tamoxifen. These results suggest that the potent inhibitory effect of DIM on Bcl-2 protein expression can be accounted for by the down-regulation of Bcl-2 transcripts.

Western blot analysis revealed that DIM treatment increased Bax protein levels up to 4- and 6-fold in MCF-7 and MDA-MB-231 cells, respectively (Fig. 7). The maximum effect was observed at 48 hr following DIM treatment. Levels of Bax protein also increased somewhat in both cell lines treated with tamoxifen. Thus, DIM had opposite effects on the regulation of Bcl-2 and Bax expression, consistent with their roles in the regulation of apoptosis.

3.4. Effect of DIM on the interaction of Bcl-2 and Bax protein with Co-IP analysis

Because the apoptosis-modulating activities of Bax and Bcl-2 are neutralized by their association with one another, we conducted Co-IP analyses to examine the potential effect of DIM on the interaction of these two proteins. Western analyses of Bcl-2 protein that coimmunoprecipitated with anti-Bax antibodies demonstrated a decreased association of Bcl-2 with immunoprecipitated Bax in a

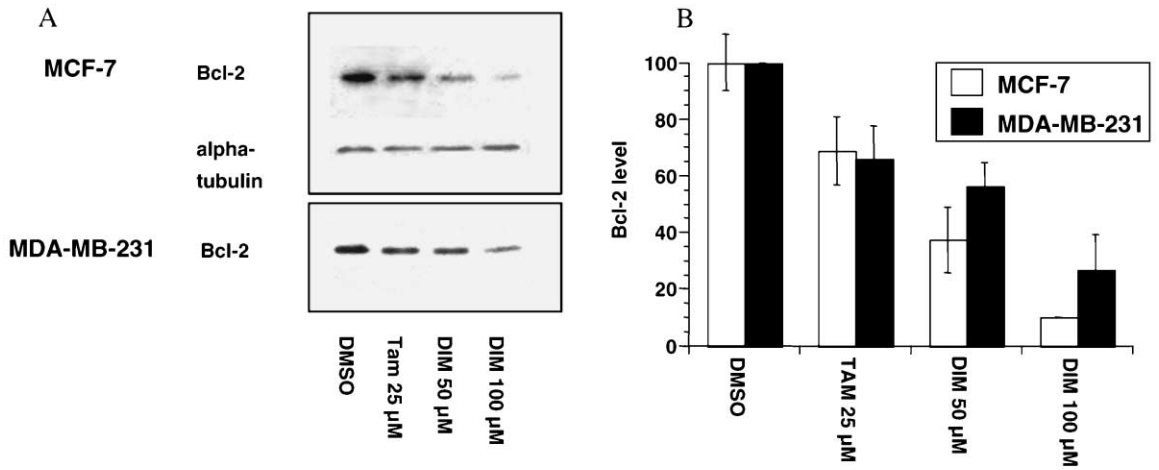

Fig. 5. Effect of DIM on the expression of Bcl-2 protein in MCF-7 and MDA-MB-231 cells. (A) Cells were treated with DMSO, DIM, or tamoxifen (Tam) for 24 hr, and the Bcl-2 protein level was determined by Western blot analysis using specific antibodies. Equal sample loading was confirmed by α-tubulin detection or Ponceau S staining of the Western blots. Experiments were repeated three times with similar results. (B) The relative level of Bcl-2 protein was quantified with densitometry and normalized with α-tubulin as described under Section 2. MCF-7 (open bars), MDA-MB-231 (solid bars). The data are shown as averages ± SD.

time- and concentration-dependent manner in both cell lines (Figs. 8 and 9). A decrease of Bax-associated Bcl-2 of approximately 80% was observed following a 24-hr exposure to 100 μM indole. A similar effect was observed following treatment with a cytostatic concentration of tamoxifen in both cell lines (Fig. 8).

In a kinetics experiment, we observed decreases of approximately 75% in the levels of Bcl-2 that coimmuno-

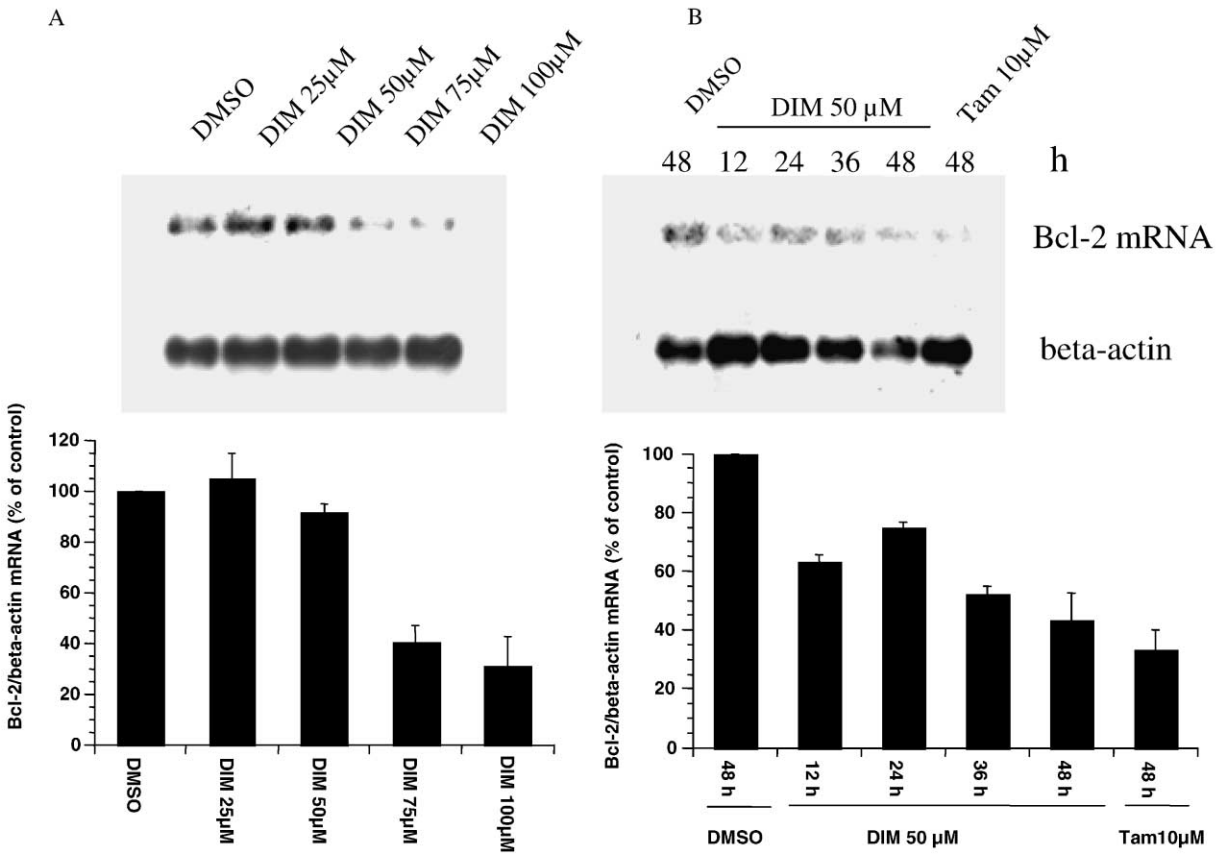

Fig. 6. Effect of DIM on Bcl-2 mRNA transcripts in MCF-7 cells. Cells were treated with different concentrations of DIM for 24 hr (A) or with DIM (50 μM) for the times indicated (B). The isolated poly(A)$^+$ RNA was fractionated electrophoretically, and Northern blots were probed for Bcl-2 transcripts as described under Section 2. As a loading control, the Northern blots were probed for β-actin. The results are shown as averages ± SD from three representative independent experiments.

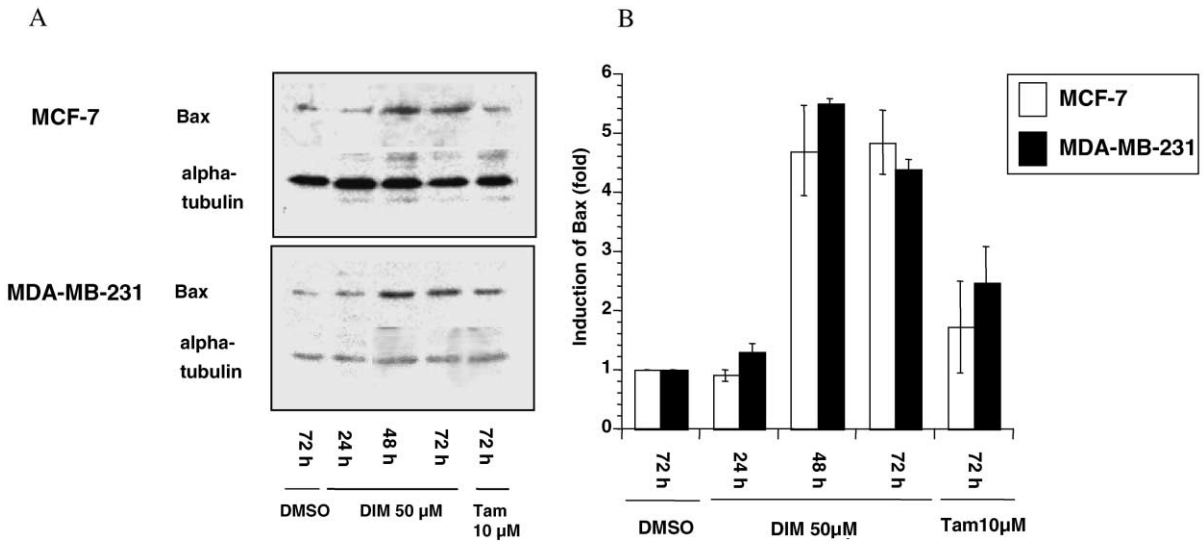

Fig. 7. Kinetic effect of DIM on the expression of Bax protein in MCF-7 and MDA-MB-231 cells. MCF-7 (A, upper panel) or MDA-MB-231 (A, lower panel) cells were treated with DMSO, DIM (50 μM), or tamoxifin (10 μM) for the indicated times, and the Bax protein level was determined by Western blot analysis using specific antibodies. Equal sample loading was confirmed by α-tubulin detection. The blot shown is representative of three independent experiments. (B) The relative level of Bax protein was quantified with densitometry and normalized with α-tubulin as described under Section 2. MCF-7 (open bars), MDA-MB-231 (solid bars). Data are averages ± SD.

precipitated with anti-Bax antibodies in both cell lines after 48 hr of treatment with 50 μM DIM (Fig. 9A and C). Western analysis of proteins that coimmunoprecipitated with anti-Bcl-2 antibodies demonstrated, however, that levels of Bcl-2-associated Bax protein were not modified significantly by DIM treatments in either cell line (Fig. 9B). These results are consistent with a shift in the distribution of Bax from an association with Bcl-2 to an increase in the free homodimeric form of Bax, but no shift in the distribution of Bcl-2 between the free and heterodimeric forms.

3.5. Decreased apoptotic effect of DIM on Bcl-2 overexpressing MCF-7 transfectants

As a functional test for the role of Bcl-2 down-regulation in the apoptotic effect of DIM, we transiently transfected MCF-7 cells with either a Bcl-2 expression vector or with a vector control. Western blot analyses indicated that Bcl-2 protein levels were increased nearly 2-fold in cells transfected with the Bcl-2 expression vector (+Bcl-2) compared with cells transfected with the empty control vector (−Bcl-2) (Fig. 10). DIM treatment decreased the Bcl-2 protein level in the +Bcl-2 cells to the level observed in the untreated −Bcl-2 cells. In a parallel experiment, we determined the effect of constitutive Bcl-2 expression on DIM-induced apoptosis. Measurement of the apoptotic index using bisbenzimide staining demonstrated that by 24 hr of indole treatment, Bcl-2 overexpression attenuated the DIM apoptotic effect in MCF-7 cells by approximately 50% (Fig. 11). Taken together, these results demonstrate that DIM is an apoptotic modulator that functions by strongly regulating the expression of the Bax/Bcl-2 family of apoptotic regulatory factors in breast cancer cells.

4. Discussion

In this study, we observed that DIM could inhibit the proliferation of estrogen-dependent and -independent human breast cancer cells and induce apoptosis in both cell types by decreasing cellular Bcl-2 levels and increasing the levels of free Bax and the Bax/Bcl-2 ratio. Direct evidence is presented showing that Bcl-2 family expression is a major target of DIM action in MCF-7 cells, which demonstrates that DIM is a specific apoptotic modulator in human breast cancer cells.

We characterized the early sequence of apoptotic events induced by DIM using specific cell-staining techniques [22]. Phosphatidylserine externalization, as visualized by annexin V staining, is one of the earliest events in apoptosis [23]. Within 10 min of DIM treatment, apoptosis became apparent with a characteristic halo image of phosphatidylserine relocation without disruption of the cell membrane. With increasing duration of treatment, nuclear staining with propidium iodide became obvious, indicating the onset of the later stages of apoptosis. Analysis of late-stage chromatin condensation (pyknosis) using a high-affinity DNA-binding fluorescent dye, bisbenzimide, clearly indicated that by 24 hr of DIM treatment, 50% of cells exhibited condensed chromatin or fragmented nuclei. The TUNEL assay, which monitors the level of DNA fragmentation catalyzed by released nucleases, confirmed the onset of the final stages of apoptosis.

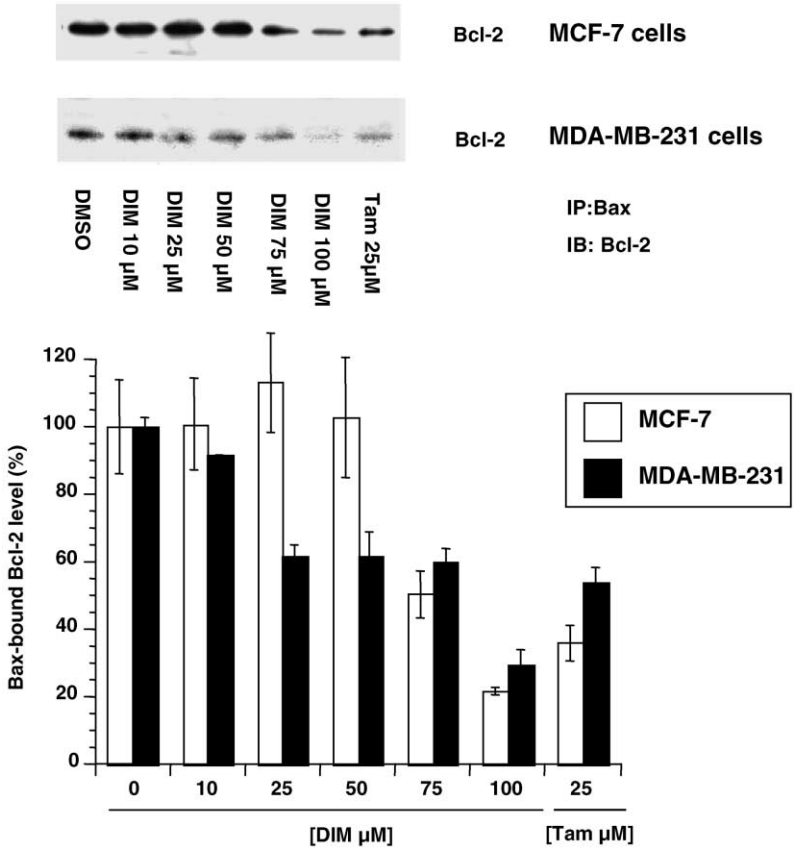

Fig. 8. Concentration response of DIM on Bax-bound Bcl-2 protein levels in MCF-7 and MDA-MB-231 cells. Extracts of cells treated with different concentrations of DIM for 24 hr were immunoprecipitated with anti-Bax antibodies, and the immunoprecipitated complex was immuno-stained with anti-Bcl-2 antibody. The Bcl-2 level was quantified with densitometry, as described under Section 2. (Top panel) Typical Western blot. (Bottom panel) The relative level of Bcl-2 protein was quantified with densitometry and normalized with α-tubulin as described under Section 2. MCF-7 (open bars), MDA-MB-231 (solid bars). The experiments were repeated three times with similar results (shown as averages ± SD).

The major apoptotic signal transduction cascades associated with programmed cell death converge into a common final pathway involving the Bcl-2-related family of proteins. Individual members of this group of proteins either promote cell survival (e.g. Bcl-2 and Bcl-X_L) or induce cell death (e.g. Bax, Bak, and Bcl-X_S) [24]. The relative levels of these factors determine the fate of the cell. Results of studies with several different breast cancer cell lines indicated that the relative levels of Bcl-2 and Bax proteins were the most highly predictive of sensitivity to apoptotic agents compared with other Bcl-2 family members [25]. Bcl-2 prevents apoptosis caused by a variety of stimuli, including growth factor withdrawal, γ-radiation, and chemotherapeutic drugs [26]. Overexpression of anti-apoptotic members of the Bcl-2 family has been implicated in cancer chemo-resistance, whereas ectopic expression of the pro-apoptotic Bax sensitizes breast cancer cells to drug- and radiation-induced apoptosis [27,28]. The low levels of free Bax found in some breast cancers are thought to be responsible for the relative drug resistance in these tumors [29,30]. Furthermore, Bcl-2 overexpression is reported to enhance tumorigenicity and metastatic potential, including invasion, migration, and tumor angiogenesis in breast cancer [31,32].

Important characteristics of the mechanisms of action of the Bcl-2 family of proteins in apoptosis are becoming clear. Anti-apoptotic Bcl-2 factors reside mainly in the mitochondrial outer membrane, nuclear envelope, and endoplasmic reticulum. Although the anti-apoptotic effect of Bcl-2 is observed consistently in different systems in response to various stimuli, the manner by which Bcl-2 inhibits apoptosis is not fully understood, and several mechanisms of action have been proposed. The ratio of levels of anti-/pro-apoptotsis signaling proteins in the mitochondria determines the regulation of mitochondrial release of apoptosis-associated factors, such as Apaf-1 (apoptosis-protease-activated factor 1), Aif (apoptosis-inducing factor), and cytochrome *c*. Bax translocation from the cytosol to the mitochondria results in mitochondrial depolarization and the release of apoptotic factors through outer membrane channels formed by Bax oligomers [33–35]. Although Bcl-2 is thought to inhibit apoptosis by forming heterodimers with Bax, Bcl-2 in its homodimeric form can inhibit cell death by alternate pathways, as well. Indeed, in tranfection experiments with expression vectors of Bcl-2 mutants, the anti-apoptotic effects of Bcl-2 were not diminished in Bcl-2 variants that did not contain the BH dimerization domains [36]. Alternatively, Bcl-2 can

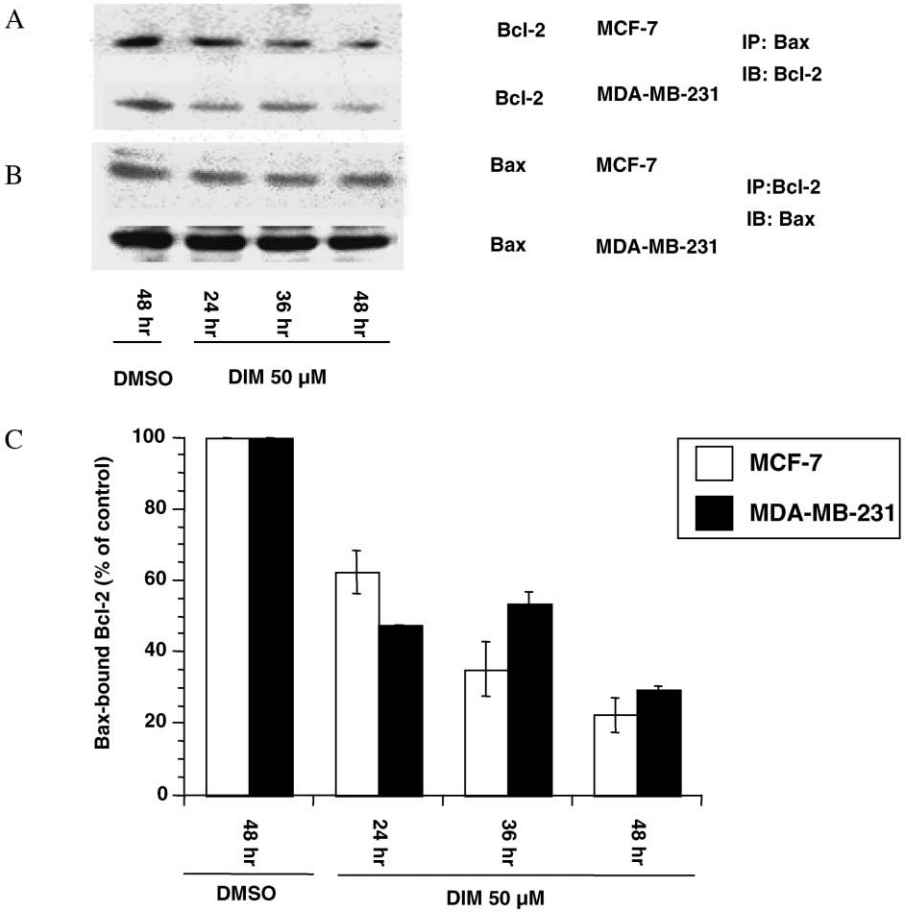

Fig. 9. Kinetic effect of DIM on Bax-bound Bcl-2 and Bcl-2-bound Bax protein in MCF-7 and MDA-MB-231 cells. Cell extracts treated with DIM (50 μM) for the indicated time periods were immunoprecipitated with either anti-Bax or anti-Bcl-2 antibodies. Bax–Bcl-2 complexes were detected with antibodies against the other component of the complex, i.e. Bax or Bcl-2, using anti-Bcl-2 or anti-Bax antibodies, in a quantitative Western blot analysis. (A and B) Typical Western blots from three different experiments. (C) The relative levels of bound Bax and Bcl-2 proteins were quantified with densitometry and normalized with α-tubulin as described under Section 2. MCF-7 (open bars), MDA-MB-231 (solid bars). Data are shown as averages ± SD.

inhibit mitochondrial channel formation independent of its binding to Bax. Bcl-2 can control calcium ion release from stores in the mitochondria and endoplasmic reticulum. The calcium ion is required for activation of nucleases and proteases that mediate cellular destruction during apoptosis [37]. Bcl-2 also can block accumulation of lipid peroxides and control cellular redox reactions that may activate apoptosis [38].

Our results indicating a pronounced DIM-mediated decrease in the proportion of total Bax that is bound to Bcl-2, but little change in the proportion of total Bcl-2 that is bound to Bax, support the significance of increased relative levels of free Bax in the induction of apoptosis. Consistent with this notion, DIM treatment reduced Bcl-2 protein expression to control levels in Bcl-2 transfectants, but reduced apoptosis by only 50%. Induction of free Bax protein with DIM treatment may play an important role in triggering the apoptosis pathway in those transfectants. Ectopic expression of Bcl-2, conceivably, may attenuate DIM-induced apoptosis by neutralization of Bax apoptotic activity through heterodimer formation or through alternate anti-apoptotic effects of the Bcl-2 homodimer. Consistent with the latter possibility is the report that the protection of rodent fibroblasts from apoptosis, provided by the overexpression of Bcl-2, was not accompanied by an increase in the level of Bax–Bcl-2 heterodimers, but was most closely associated with the level of free Bcl-2 [39]. In addition, Bcl-2 is thought to prevent Bax-induced cytochrome *c* release from mitochondria by inhibiting Bax insertion into the outer membrane or by inhibiting Bax-channel activity independent of heterodimer formation [35].

Comparison of our results with published results for the DIM precursor, I3C, suggests that these two indole derivatives induce apoptosis by similar mechanisms. We previously reported that I3C inhibits proliferation of breast cancer cells independent of ER status, as does DIM [5]. A recent study also reported that I3C induces apoptosis in ER-deficient MDA-MB-435 human breast cancer cells by regulating expression of the Bcl-2 family [6]. In this I3C study, apoptosis also was accompanied by increased expression of Bax, decreased expression of Bcl-2, and

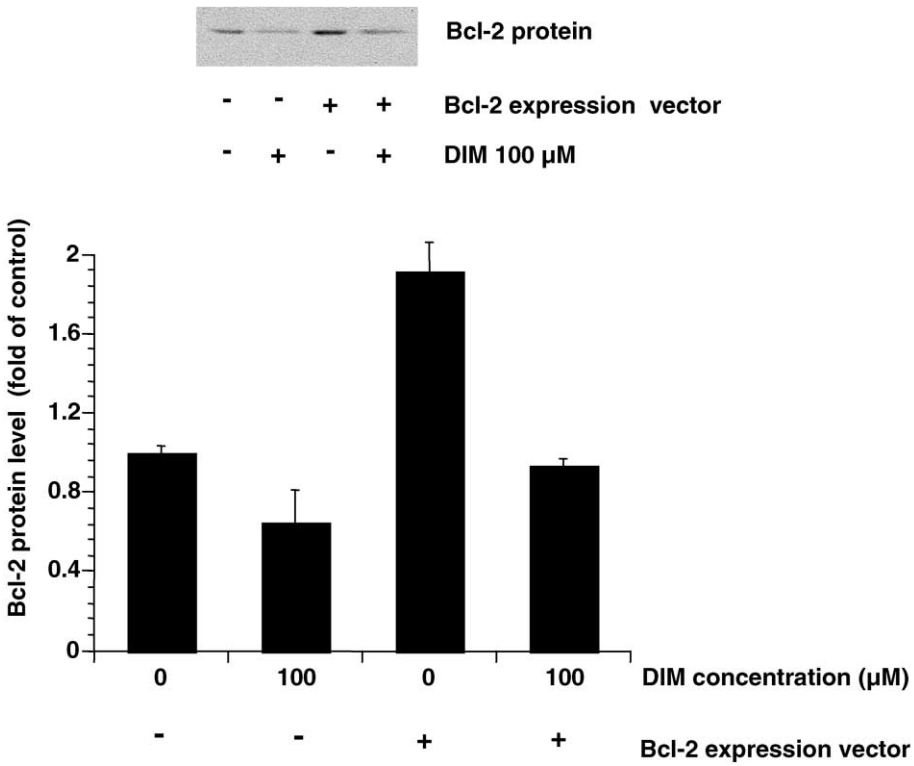

Fig. 10. Expression of Bcl-2 in MCF-7 cells with ectopic expression of Bcl-2. MCF-7 cells were transiently transfected with either a Bcl-2 expression vector or with a control empty vector for 24 hr. Transfectants were treated with control vehicle (DMSO) or DIM (100 μM) for 24 hr, and cell lysates were examined for Bcl-2 protein expression by Western blotting. Data are representative of four separate experiments (Top panel). Typical Western blots (Bottom panel). The levels of Bcl-2 proteins were quantified with densitometry, and the loading was confirmed by Ponceau S staining of the Western blot membrane as described under Section 2. Data are shown as averages ± SD.

an increase in the Bax/Bcl-2 ratio. This similarity in responses of the two substances suggests that DIM and I3C may function similarly. Because I3C can be converted to DIM in cell cultures [40,41], our results suggest further that DIM may contribute to the apoptotic effects observed in I3C-treated cells. In addition, our observation that tamoxifen-induced apoptosis in these cells is accompanied by a down-regulation of Bcl-2 expression is consistent with

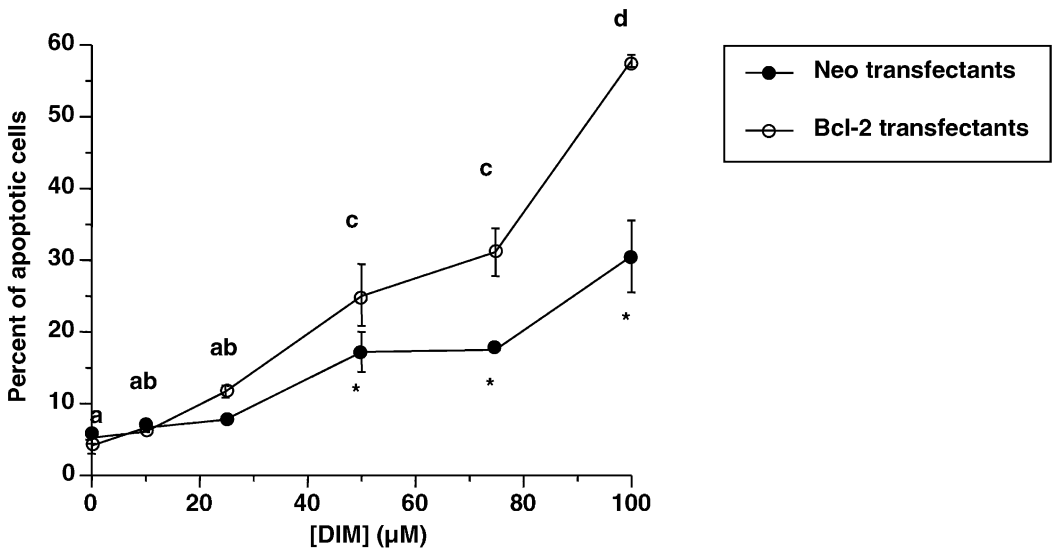

Fig. 11. Effect of ectopic expression of Bcl-2 on DIM-induced apoptosis in MCF-7 cells. Transfected cells were treated with different concentrations of DIM for 24 hr, and the fraction of apoptotic cells was assessed with bisbenzimide staining [transfectants with empty vector only (●), transfectants with Bcl-2 expression vector (○)]. Data are averages ± SD from duplicates/experiment and three individual experiments. Different letters indicate a significant difference ($P < 0.05$) among DIM concentrations. Asterisks indicate a significant difference between transfectant groups at the indicated DIM concentration, using ANOVA analysis ($P < 0.05$).

published findings [42,43] and suggests that the apoptotic pathways triggered by higher concentrations of these substances also appear to be qualitatively similar for the two compounds and do not require the ER. Thus, these effects of DIM appear to be independent of modifications in estrogen metabolism thought to be important in the protective effects of I3C in cervical and respiratory cancers [44,45].

Our studies are the first to characterize a central role for the Bcl-2 family of regulatory factors in the apoptotic effects of DIM. The mechanism by which DIM regulates the expression of Bcl-2 and Bax, and the functional significance of these effects in the regulation of tumor growth and metastasis are the subjects of our continued investigations.

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