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# Inhibition of Cell Proliferation and in Vitro Markers of Angiogenesis by Indole-3-carbinol, a Major Indole Metabolite Present in Cruciferous Vegetables

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A variety of studies have suggested a cancer protective role of cruciferous vegetables. In the present study, we investigated the effect of indole-3-carbinol (I3C), a major indole metabolite in cruciferous vegetables, on cell proliferation and in vitro markers of angiogenesis in phorbol myristate acetate (PMA)-stimulated endothelial EA hy926 cells. The results showed that I3C inhibited the growth of EA hy926 cells in a concentration-dependent manner. The capillary-like tube formation by PMA-activated endothelial cells was significantly suppressed by I3C, and such inhibition was associated with decreased vascular endothelial growth factor (VEGF) and increased interleukin-8 (IL-8) secretion, but not with the expression of VEGF receptor-2 protein. Additionally, gelatin zymography analysis indicated that I3C suppressed activities of matrix metalloproteinases-2 (MMP-2) and MMP-9 stimulated by PMA. These results suggest that the dietary I3C may be useful in the treatment of human cancers and angiogenic diseases.

**KEYWORDS: Cruciferous vegetables; indole-3-carbinol; angiogenesis; tube formation; vascular endothelial growth factor; matrix metalloproteinases**

## INTRODUCTION

Angiogenesis, the formation of new capillaries from preexisting blood vessels, is essential in several physiological situations, including embryo development, female reproductive cycle, and wound healing, during which the process of angiogenesis is under strict control. The angiogenic process is regulated by a variety of endogenous molecules (including vascular endothelial growth factor [VEGF], basic fibroblast growth factor [bFGF], transforming growth factors, and cytokines interleukin [IL]-2 and IL-8) (*1*, *2*) and has the following steps: proliferation of endothelial cells, degradation of vascular basement membrane, migration of endothelial cells, and tube formation. On the other hand, deregulated angiogenesis is related to a number of pathological diseases, such as rheumatoid arthritis, cardiovascular diseases, and cancer (*2*−*4*). Therefore, compounds that suppress proliferation and endothelial cell angiogenesis have been proposed as potential therapies for cancer and other angiogenesis-related diseases (*3*, *5*, *6*).

Cruciferous vegetables, including broccoli, cauliflower, Napa cabbage, and other cabbages, have anticarcinogenic activity, which is thought to be due to their high content of glucosinolates and glucosinolate derivatives (*7*−*9*). Indole-3-carbinol (I3C) is one of the major autolytic breakdown products of indole glucosinolates in *Brassica* plants. Several mechanisms have been suggested to contribute to the anticarcinogenic activities of I3C, but its role in angiogenesis has not been discussed yet. Because I3C inhibits the growth of several lines of cells (*10*, *11*) and suppresses the production of inducible nitric oxide (NO) (*12*), a small molecule known to affect angiogenesis, this study examined the effects of I3C on angiogenesis in a cell culture model (i.e., the permanent endothelial cell line EA hy926 in the presence or absence of phorbol myristate acetate [PMA], a potent angiogenesis stimulator). To understand the effects of I3C on angiogenesis, cell proliferation and tube formation were evaluated. To determine the antiangiogenic mechanism of I3C, angiogenesis-related factors, including VEGF, IL-8, VEGF receptor (VEGFR), matrix metalloproteinase (MMP)-2, and MMP-9, were analyzed. Results from this study may increase understanding of how compounds from cruciferous vegetables suppress angiogenesis on the molecular level and of how these compounds might be used in cancer therapy.

## MATERIALS AND METHODS

**Chemicals and Biochemicals.** I3C, PMA, dimethyl sulfoxide (DMSO), Tween 20, phenylmethylsulfonyl fluoride (PMSF), and gelatin were purchased from Sigma Chemical (St. Louis, MO). Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were from GIBCO (Grand Island, NY). Protease inhibitor cocktail was from Roche (Mannheim, Germany). All other laboratory chemicals were the highest quality available and purchased from Sigma and USB (Cleveland, OH).

**Cell Culture.** EA hy926, a human endothelium-derived permanent cell line, was kindly provided by Dr. Cora-Jean Edgell (University of North Carolina, Chapel Hill). This permanent cell line was used because

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it possesses endothelial characteristics including the formation of tubelike structures (*13*), and the difficulty in obtaining larger numbers of uncontaminated cells as well as the requirement of expensive growth factors associated with the used primary endothelial cells may be avoided. Cells were grown as monolayers in DMEM supplemented with 10% FBS at 37 °C in 95% air and 5% $CO_2$. I3C and PMA dissolved in DMSO were added to the medium; the concentration of DMSO in the medium never exceeded 0.2% (v/v).

**Cell Growth and Cell Proliferation Assays.** To evaluate the effects of I3C on the growth of cultured EA hy926 cells, various concentrations of I3C and/or 50 nM PMA were incubated with $5 \times 10^4$ cells/well, plated in six-well plates. Viable cells were estimated by the trypan blue dye-exclusion method, and numbers of cells were counted with a Coulter counter. Cell proliferation was evaluated using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI). Basically, cells ($3 \times 10^3$/well) were plated in 96-well plates and treated with I3C in the presence or absence of PMA for 24 h. The rate of proliferation was then determined by measuring the absorbance of the formazan product of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) reduction by live cells with a microplate reader at $OD_{490\ nm}$.

**Tube Formation Assay.** To understand whether I3C affected the angiogenic process, tube formation was evaluated using the BD BioCoat Angiogenesis System: Endothelial Cell Tube Formation Assay kit (BD Biosciences, Bedford, MA). In brief, cells ($2 \times 10^4$/well) were cultivated in BD Matrigel Matrix coated 96-well plates for 30 min followed by incubating with PMA and various concentrations of I3C in a serum-free condition for 24 h. After staining with Calcein AM (Molecular Probes, Eugene, OR), the networks of vessel-like tubes were observed and photographed under a fluorescent microscope.

**Assays for Production and Secretion of VEGF and IL-8.** The VEGF and IL-8 released into culture medium were determined by commercial human ELISA assay systems (Amersham Biosciences, Buckinghamshire, England). Basically, cells were treated with various concentrations of I3C or PMA or both for 24 h, and the culture medium was then collected for the analysis of VEGF and IL-8 according to the manufacturers' instructions.

**Western Blot Analysis.** Because VEGF acts through VEGFR to activate angiogenesis, the expression of membrane VEGFR was determined. Cell membrane was isolated by treating cells with HES buffer (0.25 M sucrose, 10 mM HEPES, 10 mM $MgCl_2$, and protease inhibitor cocktail). Cell lysates were centrifuged at 80 000*g* for 1 h to obtain the cell membrane proteins, and 70 $\mu$g of these were separated by 7.5% sodium dodecyl sulfate (SDS)−polyacrylamide gel electrophoresis (SDS−PAGE) and then electroblotted onto a nitrocellulose membrane. The VEGFR-2 protein was detected by monoclonal antimouse VEGFR Flk-1 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and peroxidase-conjugated IgG (Novocastra Laboratories Ltd., Newcastle upon Tyne, UK). The specific binding of the VEGFR-2 was detected using an enhanced chemiluminescent ECL Western detection system (Amersham Biosciences, Buckinghamshire, England) and observed after Scientific Imaging film (Eastman Kodak, Rochester, NY) was exposed to the membrane. The visualized bands were quantitated using the Image-Pro Plus software package (Media Cybernetics, Silver Spring, MD).

**Gelatin Zymography.** To understand the effect of I3C on PMA-induced MMP activities, gelatin zymography was performed according to procedures described by Birkedan-Hansen and Taylor (*14*). Briefly, the treated cultured medium containing 5 $\mu$g of protein was separated on a 10% SDS−PAGE gel that was copolymerized with 1 mg/mL of gelatin. After removing SDS with 2.5% Triton-X100 solution, the gel was incubated with Zymogen developing buffer (Bio-Rad Laboratories, Hercules, CA) and then stained with 0.5% Coomassie Blue R-250. The enzyme-digested regions, observed as clear bands in destained gel against a blue background, indicated the presence of MMP-2 (72 kDa) and MMP-9 (92 kDa). The visualized bands were also quantitated using the Image-Pro Plus software package (Media Cybernetics, Silver Spring, MD).

**Statistical Analysis.** Values were expressed as the mean ± SD. One-way ANOVA followed by Fisher's least significant difference test and Student's *t* test were performed to determine statistical differences between groups with the aid of SAS software version 6.12 (SAS Institute, Cary, NC). Differences between means were considered significant at $p < 0.05$.

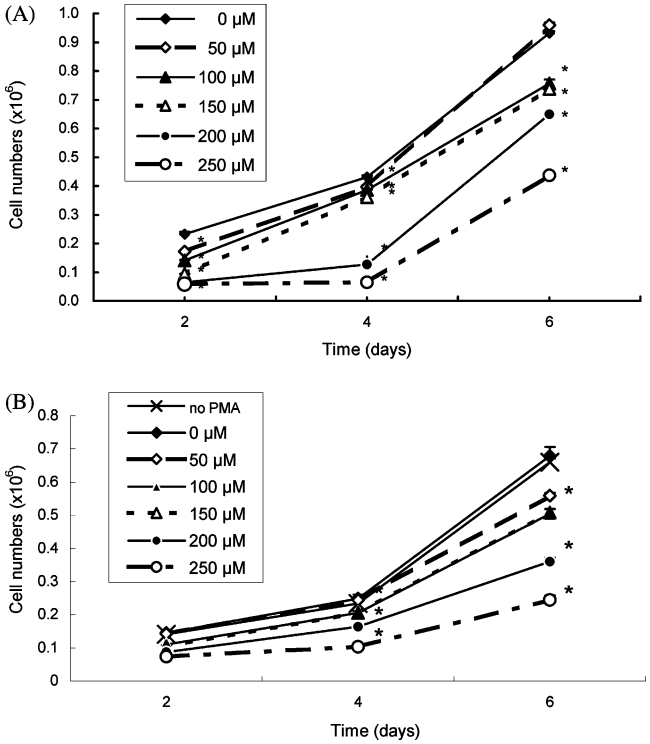


**Figure 1.** Effects of I3C on the growth of EA hy926 cells. Cells were treated with various concentrations of I3C in the absence (**A**) or presence of PMA (50 nM, **B**). Cells were then collected and counted with a Coulter counter at the times indicated. Values represent the mean ± SD from three measurements. * $p < 0.05$ compared with the control at the same time point.

## RESULTS

**Effects of I3C on Cell Growth and Cell Proliferation.** EA hy926 cells were treated with various concentrations of I3C with or without 50 nM PMA, and numbers of cells were determined after 2, 4, and 6 days. **Figure 1** indicates that I3C inhibited the growth of EA hy926 cells in a concentration-dependent manner after 6 days treatment regardless of the presence of PMA, and 250 $\mu$M I3C produced 53−65% growth inhibition after 6 days of incubation. On the other hand, PMA did not enhance the growth of EA hy926 cells, but 50 $\mu$M I3C (**Figure 1B**) inhibited to a significantly greater extent the growth of PMA-treated than non-PMA treated cells (**Figure 1A**), suggesting that the PMA-treated cells were more sensitive to I3C treatment. MTS assay results also showed that I3C inhibited in a concentration-dependent manner, but the inhibition was not as great in the PMA-treated group as in the PMA-untreated group (**Figure 2**). These 24-h MTS results indicated that the angiogenic-inhibitory effects of I3C in the presence of PMA was not apparently due to cytotoxicity, because all the other assays were performed 24 h after treatment of I3C and PMA. Additionally, the contradictory results presented in cell growth and in cell proliferation as measured by MTS assays may be explained by different incubation time, since only 24 h was used in MTS assays.

**Effects of I3C on Tube Formation.** Tube formation is one of the key steps in the process of angiogenesis. As shown in **Figure 3**, PMA (50 nM) markedly induced tube formation in EA hy926 cells, whereas cotreatment of I3C inhibited such induction. Additionally, the extent of inhibition by 250 $\mu$M I3C

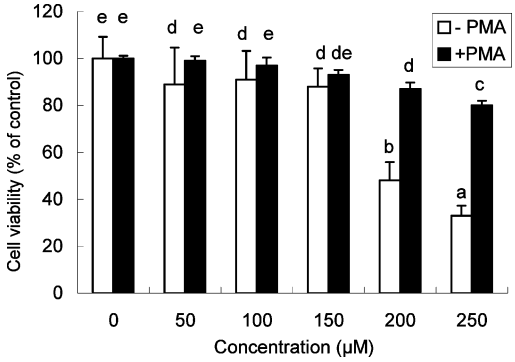


**Figure 2.** Effects of I3C on the proliferation of EA hy926 cells. Cells were treated with various concentrations of I3C in the absence or presence of PMA (50 nM) for 24 h, and cell viability was measured with the MTS assay kit. Values represent the mean ± SD from six measurements. Data with different superscripts significantly differ ($p < 0.05$).

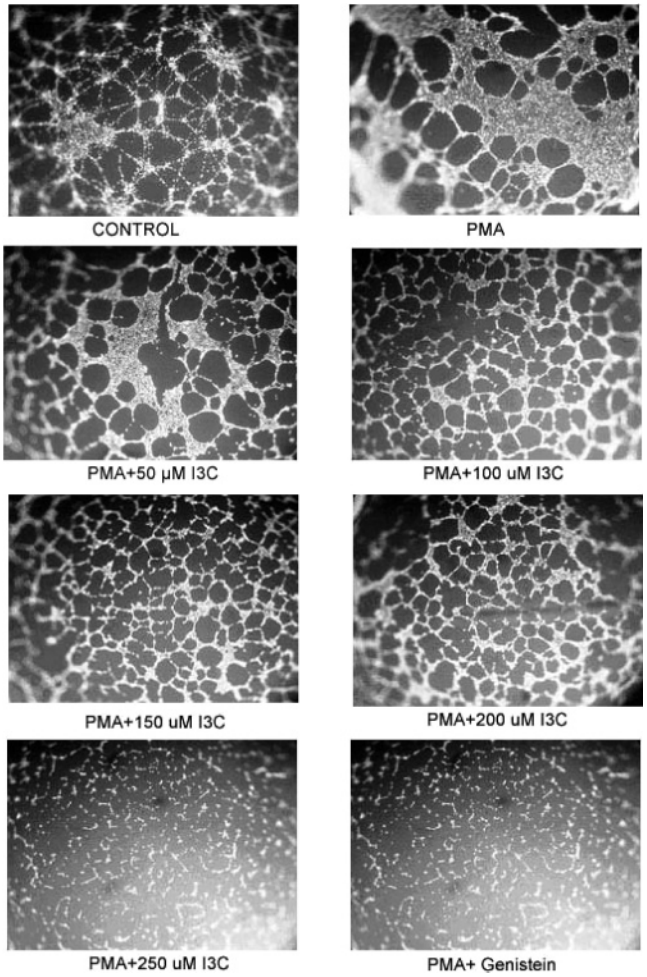


**Figure 3.** Effects of I3C on vascular tube formation in PMA-stimulated EA hy926 cells. (A) Cells were cultivated on Matrigel-coated plate treated with PMA (50 nM) and different concentrations of I3C or genistein (100 $\mu$M) in serum-free medium for 24 h. Tube formation was analyzed by the BD BioCoat angiogenesis system and photographed under a fluorescence microscope (100×). Pictures are representative of three independent experiments.

was similar to that of 100 $\mu$M genistein, a compound that has been shown to inhibit in vitro angiogenesis (*15*).

**Effects of I3C on Angiogenesis-Related Factors.** Because I3C inhibited in vitro angiogenesis, the effect of I3C on several angiogenesis-related factors, including VEGF, IL-8, VEGFR, and MMP, were examined to gain further insight into this inhibition. **Figure 4** indicates that 100−250 $\mu$M I3C did not affect the secretion of VEGF and IL-8. However, treatment with PMA significantly enhanced the secretion of both VEGF and IL-8, and treatment with I3C significantly suppressed the PMA-stimulated VEGF levels (**Figure 4A**), but enhanced the PMA-stimulated IL-8 levels (**Figure 4B**) in a concentration-dependent manner. Additionally, because VEGF-induced activation of endothelial cells is mediated by the binding of VEGF to VEGFRs, the expression of membrane VEGFR-2 was examined by Western blot analysis. As shown in **Figure 5**, neither PMA nor PMA plus I3C affected the expression of membrane VEGFR-2 protein in EA hy926 cells.

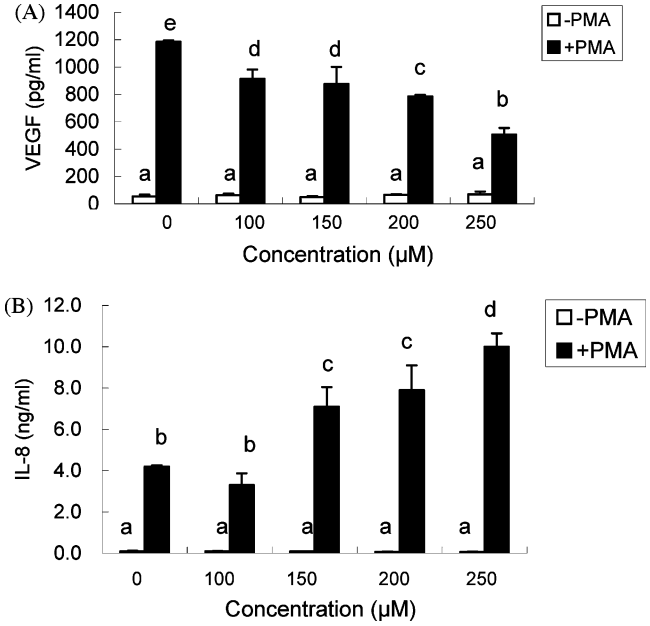


**Figure 4.** Effects of I3C on secretion of VEGF (**A**) and IL-8 (**B**) in EA hy926 cells. Cells were treated various concentrations of I3C in the absence or presence of PMA (50 nM) for 24 h. VEGF and IL-8 secreted into the cultured medium were then determined by ELISA kits. Values are the mean ± SD from three measurements. Data with different superscripts significantly differ ($p < 0.05$).

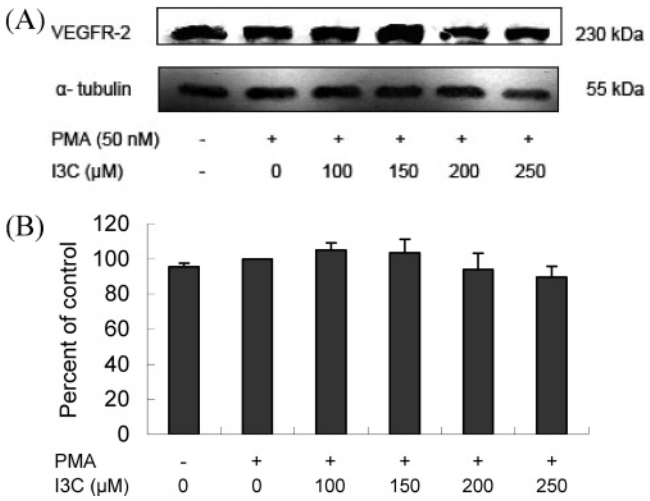


**Figure 5.** Effects of I3C on the expression of membrane VEGFR-2 in EA hy926 cells. (**A**) Cells were treated various concentrations of I3C in the presence of PMA (50 nM) I3C for 24 h. The membrane proteins were then separated by SDS–PAGE, and VEGFR-2 protein was detected. (**B**) Relative density of bands normalized for α-tubulin. Values are the mean ± ranges from two measurements.

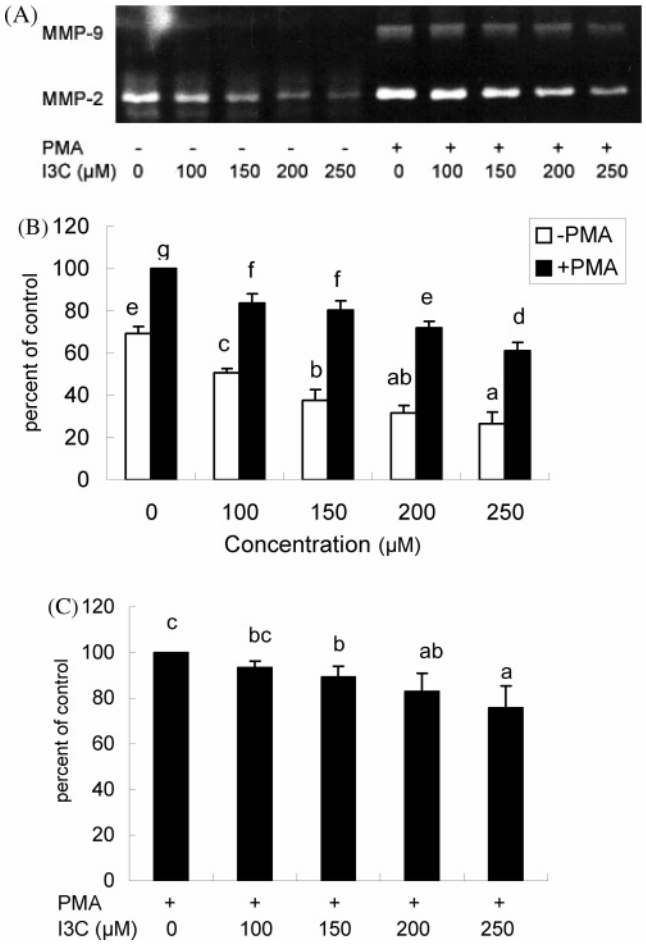


**Figure 6.** Effects of I3C on MMP-2 and MMP-9 activities in EA hy926 cells. (**A**) Cells were treated various concentrations of I3C in the absence or presence of PMA (50 nM) for 24 h. MMP-2 and MMP-9 secreted into the cultured medium were determined by gelatin zymography. (**B**) Relative density of MMP-2. (**C**) Relative density of MMP-9. Values are the mean ± SD from three measurements. Data with different superscripts significantly differ ($p < 0.05$).

Since gelatinase and collagenase MMPs are also involved in the angiogenic process (*16*), we utilized gelatin zymography to determine the activities of secreted MMP-2 and MMP-9. Like tube formation, the activities of MMP-2 (72 kDa) and MMP-9 (92 kDa) were significantly stimulated by PMA and concentration-dependently inhibited by I3C (**Figure 6**). Meanwhile, the results also indicated that EA hy926 cells released basal levels of MMP-2, but not MMP-9, when cultured in serum-free medium.

## DISCUSSION

In the present study, we first demonstrated that I3C inhibits the proliferation and tube formation, an in vitro marker of angiogenesis, of PMA-activated EA hy926 endothelial cells, and such suppression is associated with decreased secretion of VEGF, MMP-2, and MMP-9, but increased secretion of IL-8. I3C is one of the bioactive components in cruciferous vegetables that exhibit anticarcinogenic activities (*7*, *17*, *18*). I3C has been shown to modulate the activity of xenobiotic-metabolizing enzymes (*19, 20*), to induce apoptosis in cancer cells (*10, 21*), to inhibit lipopolysaccharide-induced nitric oxide production (*12*), and to regulate estrogen activity (*22*), pathways that have been suggested to be involved in the anticarcinogenic process. Accumulating evidence indicates that angiogenesis inhibitors are potential candidate drugs for cancer chemoprevention and therapy (*2*, *3*, *5*). Because both VEGF and MMPs act as angiogenic factors during the process of angiogenesis or tube formation, the results of this study suggest that the chemopreventive effect of I3C may be mediated through suppression of angiogenesis by inhibiting the secretions of VEGF and MMP-2/MMP-9. However, more in vivo experiments are required to confirm this possibility.

The process of angiogenesis is always accompanied by increased endothelial cell proliferation, and antiangiogenic therapy can inhibit further endothelial cell proliferation or induce their apoptosis (*23*, *24*). I3C has been shown to inhibit proliferation or induce apoptosis in various lines of cancer cells, including breast (*10*), human prostate (*21*), and non small-cell lung cancer cells *(25)*, through inactivation of Akt activity (*26*, *27*), an event that is also involved in the angiogenesis. Therefore, the inhibitory effects of I3C on PMA-stimulated in vitro angiogenesis may be partly due to the induction of apoptosis of EA hy926 cells. On the other hand, I3C suppressed the growth of the cells without PMA treatment, but did not affect the secretions of VEGF and IL-8, indicating I3C may not affect the cellular homeostasis under physiological conditions. The growth suppressive effect of I3C may be explained by its apoptosis-inducing activity.

VEGF is one of the most potent and extensively studied angiogenic stimulators, and it has been shown to promote endothelial cell proliferation, migration, and tube formation (*6*). After being released, VEGF binds to the VEGFR on the endothelial cell membrane and initiates a cascade of signals for vessel formation. There are indications that the induction of endothelial cell proliferation and migration by VEGF and/or PMA is dependent upon the activation of phosphatidyl inositol 3-kinase (PI3-kinase) and stimulation of phosphorylation of Akt (*28*−*31*). Because I3C has been shown to inhibit Akt phosphorylation in human prostate PC-3 cancer cells (*26*, *27*), it is plausible to hypothesize that I3C inhibited PMA-stimulated tube formation by decreasing VEGF secretion and thereby inactivating Akt. Furthermore, VEGF mediates its signals through tyrosine kinase VEGFRs (*31*). Binding of VEGF to VEGFR-2, one of the VEGFRs involved in angiogenic responses, leads to receptor autophosphorylation and then to a cascade of angiogenic signals. Although neither PMA nor I3C affected the expression of membrane VEGFR-2 in our study, their effects on active phosphorylated VEGFR-2 cannot be ruled out. Additionally, although the membrane, instead of total cellular VEGFR-2, was determined, Western blot analysis may not be sensitive enough to detect the changes of VEGFR-2 protein on the membrane.

IL-8 has also been shown to enhance endothelial cell proliferation and angiogenesis (*32*−*35*) and to be overexpressed in several human cancers (*36*−*38*). Although our study showed that I3C inhibited PMA-stimulated tube formation, it also found that I3C enhanced PMA-induced IL-8 production, suggesting IL-8 may not play a significant role in angiogenesis in the system we used. Presta et al. (*39*) indicated that IL-8 differentially regulates bFGF-activated proliferation of different endothelial cell types. The proliferation of primary HUVEC, bovine aortic endothelial cells (BAECs), 1G11, and immortalized mouse brain microvascular endothelial cells (MBECs) was not affected by IL-8 treatment regardless of the presence of bFGF, whereas the bFGF-elicited mitogenic activity in primary sponge-induced mouse endothelial cells (SIEC) and the fetal bovine aortic endothelial GM 7373 cell line was abolished by IL-8 treatment. Therefore, the enhanced IL-8 secretion by I3C in EA hy926 cells may also be explained by cell specificity. On the other hand, IL-8 is affected by several other cytokines,

and the secretion of cytokines could be altered by the consumption of cruciferous vegetables by animals (*40*, *41*), so it is possible that the enhanced IL-8 was due to the regulation of other cytokines by I3C. Further experiments are required to identify the precise role of IL-8 in I3C-inhibited angiogenesis.

MMPs are a group of Zn-containing enzymes that degrade various components in extracellular matrix (ECM), including collagens, proteoglycans, gelatin, fibronectin, and glycoproteins (*16*). Evidence indicates that deregulation of MMP expression contributes to invasion, metastasis, and tumor angiogenesis (*16*). For example, overexpression of MMP-2 and MMP-9 has been reported to be positively associated with the progression of human cancers (*42*−*44*). Our results demonstrated that MMP-9 is not constitutively expressed in EA hy926 cells cultured in serum-free medium, whereas treatment with PMA significantly increased EA hy926 cell expression of both MMP-2 and MMP-9. Moreover, I3C inhibited the PMA-stimulated activities of MMP-2 and MMP-9 in a concentration-dependent manner. Because increased activities of MMPs are closely associated with the angiogenic pathway, the inhibition of tube formation by I3C may thus be due to the decreased activities of MMPs. Consistent with our results, those of Meng et al. (*45*, *46*) indicated that I3C can inhibit invasion and migration of breast cancer cells. Therefore, I3C, a compound from natural sources, may be an angiogenesis inhibitor and therefore a potential chemopreventive and therapeutic agent in human cancer as well as other angiogenic diseases. Several other dietary phytochemicals have been identified to be angiogenesis inhibitors, and most of them are polyphenols (*47*), such as epigallocatechin gallate (*48*), resveratrol (*49*), and genistein (*15*). Thus, our study results may suggest a new group of antiangiogenesis compounds.

In the UK, where the cruciferous vegetables tend to be dietary staples, the estimated average daily intake is at the equivalent of 6.4 mg of I3C (*50*). Ideally, without considering digestion and absorption, this dose of I3C would produce approximately 9 $\mu$M of plasma concentration in a 70-kg person based on a blood volume in the adult human that comprises 7% of body weight. Hence, the plasma concentration of I3C from ordinary intake of cruciferous vegetables is much lower than the concentrations we used in this study, but higher plasma concentrations may be achieved by taking I3C dietary supplements. Alternatively, several acid-catalyzed compounds formed following oral consumption of I3C have been identified may play protective roles in carcinogenesis (*51*−*53*). Among these derivatives, 3,3′-diindolylmethane (DIM) has been reported to inhibit angiogenesis (*53*). Therefore, the effects of I3C acts in vivo require further investigation.

In conclusion, we have demonstrated that I3C derived from cruciferous vegetables inhibits proliferation and in vitro markers of angiogenesis of PMA-stimulated EA hy926 cells. Such inhibition is associated with decreased VEGF secretion, decreased MMP-2 and MMP-9 activities, and increased IL-8 secretion. However, the expression of membrane VEGFR-2 protein was not affected by I3C treatment. These findings suggest that dietary I3C could have a role in the treatment of human cancers and angiogenic diseases.

## ABBREVIATIONS USED

I3C, indole-3-carbinol; PMA, phorbol myristate acetate; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; IL, interleukin; MMP, metalloproteinases; bFGF, basic fibroblast growth factor; NO, nitric oxide; DMSO, dimethyl sulfoxide; PMSF, phenylmethylsulfonyl fluoride; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; ECM, extracellular matrix

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