Antiangiogenic Effects of Indole-3-Carbinol and 3,3′-Diindolylmethane Are Associated with Their Differential Regulation of ERK1/2 and Akt in Tube-Forming HUVEC[1–3]

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Abstract

We previously reported that indole-3-carbinol (I3C), found in cruciferous vegetables, suppresses angiogenesis in vivo and in vitro. However, the underlying molecular mechanisms still remain unclear. Antiangiogenic effects of its major metabolite, 3,3′-diindolylmethane (DIM), also have not been fully elucidated. In this study, we investigated the effects of these indoles on angiogenesis and tested a hypothesis that I3C and DIM inhibit angiogenesis and induce apoptosis by affecting angiogenic signal transduction in human umbilical vein endothelial cells (HUVEC). We found that I3C and DIM at 25 μmol/L significantly inhibited tube formation and only DIM induced a significant increase in apoptosis in tube-forming HUVEC. DIM showed a stronger antiangiogenic activity than I3C. At the molecular level, I3C and DIM markedly inactivated extracellular signal-regulated kinase 1/2 (ERK1/2) and the inhibitory effect of DIM was significantly greater than that of I3C. DIM treatment also resulted in activation of the caspase pathway and inactivation of Akt, whereas I3C did not affect them. These results indicate that I3C and DIM had a differential potential in the regulation of the 2 principal survival signals, ERK1/2 and Akt, in endothelial cells. We also demonstrated that pharmacological inhibition of ERK1/2 and/or Akt was enough to inhibit tube formation and induce \caspase-dependent apoptosis in tube-forming HUVEC. We conclude that both I3C and DIM inhibit angiogenesis at least in part via inactivation of ERK1/2 and that inactivation of Akt by DIM is responsible for its stronger antiangiogenic effects than those of I3C.   J. Nutr. 140: 1–6, 2010.

Introduction

Epidemiological studies have shown that consumption of cruciferous vegetables, such as broccoli, cabbage, and cauliflower, is associated with lower cancer risk (1). Indole-3carbinol (I3C)[7] is a naturally occurring compound abundantly found in such vegetables and appears to contribute to the anticancer effects of the vegetable family (2). It has been reported that I3C causes significant regression of cervical and vulvar intraepithelial neoplasia in clinical trials and prevents chemically induced and spontaneous tumorigenesis in several animal studies (3–6). Thus, I3C is regarded as a promising chemopreventive agent against various cancers (7,8). However, anticancer mechanisms of I3C have not been fully elucidated.

I3C is unstable in acidic milieu such as gastric juice, thereby converting into several condensation products (9). One of its major metabolites, 3,3′-diindolylmethane (DIM), is detected in plasma and various organs after oral administration of I3C (**Supplemental Fig. 1**) (10,11). Thus, to elucidate the anticancer effects of I3C, it is important to assess the biological activities of DIM as well and to compare the effects of these indoles at both the cellular and molecular levels.

We and others have previously reported that I3C and DIM inhibit angiogenesis (12–16). We also observed that I3C induces apoptosis in endothelial cells, but their molecular mechanisms are poorly understood. Angiogenesis is defined as the process in which a network of new blood vessels emerges from preexisting vessels. Endothelial cells play a central role in the formation of such neovessels (17). Angiogenesis is essential for tumor growth and metastasis, which are 2 major factors that hinder cancer therapy (18). Hence, tumor angiogenesis can be a very effective target for cancer prevention and treatment (19).

Induction of apoptosis in endothelial cells is reported to be one of the major antiangiogenic mechanisms by many angiogenesis inhibitors (20). Apoptosis is a genetically programmed form of cell death, which is strictly regulated by a balance between apoptotic signals and survival signals. Apoptotic stimuli are known to activate a caspase cascade in a cell that ultimately leads to the oligonucleosomal fragmentation of DNA and the cleavage of proteins such as poly ADP-ribose polymerase (PARP), thus irreversibly committing the cell to die (21). In contrast, angiogenic stimuli such as vascular endothelial growth factor and basic fibroblast growth factor (bFGF) are known to activate extracellular signal-regulated kinase 1/2 (ERK1/2) and Akt, which transduce survival signals in endothelial cells and prevent apoptosis by inactivating proapoptotic proteins (17,22).

In this study, we investigated and compared antiangiogenic activities of I3C and DIM using tube formation of human umbilical vein endothelial cells (HUVEC), an in vitro angiogenesis model. We hypothesize that the 2 indoles inhibit tube formation and induce apoptosis by affecting angiogenic signal transduction in HUVEC. We tested this hypothesis by assessing their effects on survival signals and the apoptotic pathway.

Materials and Methods

Materials. DIM was purchased from LKT Laboratories, MCDB-104 medium from Nihon Pharmaceutical, fetal bovine serum (FBS) from Moregate, Atelocollagen Bovine Dermis (type I collagen) from Koken, epidermal growth factor from BD Biosciences, human bFGF (recombinant) from Austral Biologicals, U0126 from Calbiochem, and LY294002 from Cell Signaling Technology. Unless otherwise stated, all chemicals, including I3C, were purchased from Sigma. Antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was obtained from Santa Cruz Biotechnology and all the other antibodies were from Cell Signaling Technology. I3C, DIM, U0126, and LY294002 were dissolved in dimethylsulfoxide and the final concentration of dimethylsulfoxide was <0.2% in all experiments.

Cell culture. HUVEC were grown in HUVEC growth medium (MCDB-104 medium supplemented with 10 μg/L epidermal growth factor, 100 mg/L heparin, 100 μg/L endothelial cell growth factor, and 10% FBS) as previously reported (23) and incubated at 37°C. The cells were seeded on plates coated with 0.1% gelatin and allowed to grow to subconfluence before the experimental treatment.

Tube formation assay. Capillary tube-like structures formed by HUVEC in collagen gel were prepared as previously described (24). Briefly, HUVEC (6.0×10^4 cells/cm^2) were seeded between 2 layers of collagen gel (0.21% collagen) and incubated in MCDB-104 medium with 0.5% FBS supplemented with 10 μg/L bFGF, 8 nmol/L phorbol 12-myristate 13-acetate, and 25 mg/L ascorbic acid for up to 30 h. The cells were also treated with vehicle, I3C, DIM, U0126, and/or LY294002. Tube area (area ratios of the formed tubes per pictured field) was quantified in a blinded manner by determining the pixel numbers of tubes and the entire pictured field in each image using the NIH Image program.

For Western blot analysis, cells (2.4×10^5 cells/48-well plate) were suspended 3-dimensionally in collagen gel (instead of being sandwiched between 2 layers of collagen gel) for 6, 12, and 24 h.

Apoptosis. Observation and quantification of apoptosis were conducted as previously described (25). Briefly, tube-forming HUVEC after experimental treatment were fixed with 1% glutaraldehyde overnight at 4°C and stained with 500 μg/L 4′,6-diamidino-2-phenylindole overnight at room temperature. Cells exhibiting chromatin condensation and/or cell nuclear fragmentation were counted as apoptotic cells. The rates of apoptosis were quantified in a blinded manner as percentage of condensed and fragmented cell nuclei against total cell nuclei. A total of >500 cells from 6 fields were counted for each treatment.

Western blot analysis. Western blotting was carried out as previously described (26). Briefly, tube-forming HUVEC after experimental treatment were treated with SDS sampling buffer (0.05 mol/L Tris-HCl, pH 6.8, 2% SDS, 5.88% 2-mercaptoethanol, 10% glycerol) with 1× protease inhibitor cocktail, 1 × phosphatase inhibitor cocktail I, 1× phosphatase inhibitor cocktail II, 1 mmol/L β-glycerophosphate, and 2.5 mmol/L sodium pyrophosphate. Each sample was electrophoresed in a 6–12% SDS-PAGE under reducing conditions. Immunoreactive protein bands were visualized using an enhanced chemiluminescence or enhanced chemiluminescence plus detection system and their signal intensities were quantified using the NIH Image program. Changes in ERK1/2, Akt, caspase-3, and PARP were determined by calculating the
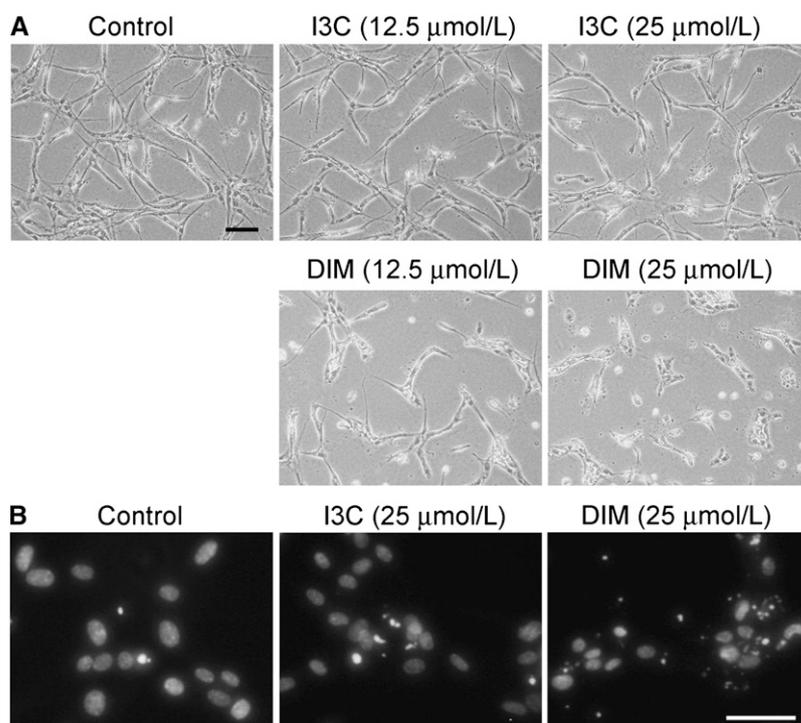
FIGURE 1 Inhibition of tube formation and induction of apoptosis in tube-forming HUVEC by DIM and I3C. Endothelial cells were induced to form blood vessel-like tubes and were treated with vehicle, I3C (12.5 and 25 μmol/L), or DIM (12.5 and 25 μmol/L) for 30 h. (Upper panel) Stronger fragmentation of the tube network by DIM than I3C. Bar indicates 100 μm. (Lower panel) Induction of chromatin condensation and nuclear fragmentation by DIM. Bar indicates 50 μm.

TABLE 1 Inhibition of tube formation and induction of apoptosis in tube-forming HUVEC by I3C and DIM[1,2]

Treatment	Tube area	Apoptosis
	%	
Control	31 ± 1[a]	15 ± 2[c]
I3C		
12.5 μmol/L	24 ± 2[ab]	19 ± 3[bc]
25 μmol/L	21 ± 1[b]	22 ± 2[bc]
DIM		
12.5 μmol/L	14 ± 4[bc]	30 ± 3[ab]
25 μmol/L	10 ± 2[c]	40 ± 3[a]

[1] Values are means ± SEM, $n = 3$. Means in a column without a common letter differ, $P < 0.05$.
[2] After 30-h treatment with vehicle, I3C, or DIM, the areas of formed tubes and the rates of apoptotic cells were measured.

ratios of phospho-ERK1/2 (Thr202/Tyr204):total ERK1/2, phospho-Akt (Ser473):total Akt, cleaved caspase-3:GAPDH (an internal control), and cleaved PARP:GAPDH, respectively.

Statistical analysis. Results are expressed as means ± SEM obtained from 3 independent experiments. Differences were ascertained by ANOVA. Multiple comparisons among treatments were checked with Tukey's test.

Results

Inhibition of tube formation and induction of apoptosis in tube-forming HUVEC by I3C and DIM. We first investigated and compared the effects of I3C and DIM on angiogenesis in vitro. After induction of tube formation, the endothelial cells formed a network of capillary-like tubes, which were composed of multiple cells that gathered together and adhered to each other. I3C slightly, but not significantly, reduced the formed tube area at 12.5 μmol/L and inhibited it at 25 μmol/L ($P < 0.05$), whereas DIM reduced the tube area even at 12.5 μmol/L and had a stronger inhibitory effect on tube formation at 25 μmol/L than I3C ($P < 0.05$) (Fig. 1A; Table 1). Thus, DIM had a stronger inhibitory activity on tube formation of endothelial cells than I3C.

Inhibition of tube formation by these indoles was accompanied by partial fragmentation of endothelial cells, indicating that they induced cell death. The observation of cell nuclear morphology by 4′,6-diamidino-2-phenylindole staining revealed that I3C and DIM induced chromatin condensation and nuclear fragmentation, morphological markers of apoptosis, during inhibition of tube formation (Fig. 1B; Table 1). I3C at 12.5 and 25 μmol/L had only an increased tendency in apoptosis induction, whereas DIM at 25 μmol/L had a stronger proapoptotic effect on tube-forming HUVEC compared with control (time matched and vehicle treated) ($P < 0.01$) and I3C at 25 μmol/L ($P < 0.01$), consistent with the in vitro tube formation assay (Table 1).

Activation of caspase pathway and inactivation of survival signals in tube-forming HUVEC by I3C and DIM. We further analyzed how activation of caspase-3 and cleavage of PARP, molecular markers of apoptosis, were affected by these indoles using Western blotting. Although I3C tended to induce the activation of caspase-3 and cleavage of PARP, only DIM at 25 μmol/L showed significant changes in both caspase-3 activation ($P < 0.01$) and cleavage of PARP ($P < 0.01$) compared with the control (time matched and vehicle treated) group (**Supplemental Fig. 2; Table 2**). Further analysis of changes in survival signals, ERK1/2 and Akt, revealed that DIM induced inactivation of ERK1/2 more potently than I3C ($P < 0.05$). DIM also suppressed Akt activation compared with the control group ($P < 0.01$), whereas I3C in concentrations tested did not block it (Supplemental Fig. 2; Table 2).

Inhibition of tube formation and induction of apoptosis through ERK1/2 and Akt inactivation in tube-forming HUVEC by U0126 and LY294002. We next investigated whether inactivation of ERK1/2 and Akt was actually involved in the regulation of tube formation inhibition using U0126, a specific mitogen-activated protein kinase/ERK kinase 1/2 inhibitor, and LY294002, a specific phosphatidylinositol 3-kinase inhibitor. Because mitogen-activated protein kinase/ERK kinase 1/2 and phosphatidylinositol 3-kinase are responsible for phosphorylation of ERK1/2 and Akt, respectively, U0126 at 10 μmol/L and LY294002 at 5 μmol/L effectively prevented ERK1/2 and Akt activation in tube-forming HUVEC, respectively (**Supplemental Fig. 3; Table 3**). These inhibitors alone or in combination lowered the tube areas ($P < 0.01$) (Fig. 2A; Table 4). Moreover, ERK1/2 and/or Akt inactivation by these inhibitors caused a marked induction of chromatin condensation and nuclear fragmentation at the cellular level (Fig. 2B; Table 4) and a significant increase in the amounts of cleaved forms of

TABLE 2 Effects of I3C and DIM on activation of caspase-3, cleavage of PARP, and inactivation of ERK1/2 and Akt in tube-forming HUVEC[1,2]

Treatment	Cleaved caspase-3:GAPDH	Cleaved PARP:GAPDH	Phospho-ERK1/2:total ERK1/2	Phospho-Akt:total Akt
	% of Control			
Control	100 ± 3[c]	100 ± 9[b]	100 ± 5[a]	100 ± 5[a]
I3C				
12.5 μmol/L	129 ± 25[bc]	138 ± 27[b]	61 ± 5[b]	102 ± 2[a]
25 μmol/L	133 ± 14[bc]	147 ± 14[b]	37 ± 4[c]	101 ± 2[a]
DIM				
12.5 μmol/L	195 ± 15[ab]	191 ± 38[b]	14 ± 5[d]	69 ± 2[b]
25 μmol/L	245 ± 10[a]	217 ± 22[a]	10 ± 3[d]	65 ± 4[b]

[1] Values are expressed as percentages of the ratio relative to the control condition as indicated and are means ± SEM, $n = 3$. Means in a column without a common letter differ, $P < 0.05$.
[2] After 24-h (cleaved caspase-3 and cleaved PARP) or 12-h (ERK1/2 and Akt) treatments with vehicle, I3C, or DIM, changes in cellular proteins were analyzed by Western blotting.

TABLE 3 Effects of U0126 and LY294002 on activation of caspase-3, cleavage of PARP, and inactivation of ERK1/2 and Akt in tube-forming HUVEC[1,2]

Treatment	Phospho-ERK1/2:total ERK1/2	Phospho-Akt:total Akt	Cleaved caspase-3:GAPDH	Cleaved PARP:GAPDH
	% of Control			
Control	100 ± 5[a]	100 ± 7[a]	100 ± 10[b]	100 ± 17[b]
U0126	10 ± 3[c]	98 ± 6[a]	270 ± 11[a]	256 ± 30[a]
LY294002	78 ± 3[b]	36 ± 9[b]	337 ± 20[a]	299 ± 16[a]
U0126 + LY294002	6 ± 2[c]	29 ± 7[b]	367 ± 52[a]	349 ± 18[a]

[1] Values expressed as percentages of the ratio relative to the control condition as indicated and are means ± SEM, $n = 3$. Means in a column without a common letter differ, $P < 0.05$.

[2] After 6-h (ERK1/2 and Akt) or 24-h (cleaved caspase-3 and cleaved PARP) treatments with vehicle, U0126 (10 μmol/L), and/or LY294002 (5 μmol/L), changes in cellular proteins were analyzed by Western blotting.

caspase-3 and PARP at the molecular level compared with the control (time matched and vehicle treated) group ($P < 0.05$) (Table 3).

Discussion

We recently reported that I3C suppresses tumor-induced angiogenesis in vivo and tube formation of HUVEC in vitro, which seemed to be due to its ability to induce apoptosis in endothelial cells (12). In this report, we compared the antiangiogenic activities of I3C and its major metabolite DIM at the cellular and molecular levels and showed that the effect of the latter was stronger than that of the former. We confirmed that angiogenesis suppression by I3C and DIM was associated at least in part with their inhibitory effects on a survival signal ERK1/2. We also demonstrated that inactivation of another survival signal, Akt, by DIM, but not by I3C, was a possible cause of DIM's stronger antiangiogenic effects (**Supplemental Fig. 4**).

I3C exhibits biological activities such as antiproliferative and proapoptotic against tumor or endothelial cells, but the effective concentrations of I3C used in those experiments were relatively high (>100 μmol/L in most cases) (14,27–31). Pharmacokinetic studies in mice and humans demonstrated that the concentration of I3C in plasma fell below the limit of detection within 1 h, whereas that of DIM remained detectable considerably longer after oral administration of I3C (10,11). It was also reported that I3C exerted its activity only when administered orally and not by intraperitoneal injection (32,33). Other researchers also reported that DIM has stronger effects than I3C to prevent proliferation or to induce apoptosis in various tumor cells (28,29,34–36). In this study, we showed that DIM possessed stronger antiangiogenic activities than I3C, further confirming that I3C may exert its biological activities in vivo by being converted into more effective DIM with a longer half-life in blood.

We and other researchers recently reported that I3C induces apoptosis in human or bovine endothelial cells (12,27). In this report, we showed that not only I3C but also DIM induces apoptosis in endothelial cells. Both ERK1/2 and Akt have been reported to play essential roles in endothelial cell survival (25). Inactivation of one of these survival signals is known to trigger apoptosis induction through activation of the caspase pathway in endothelial cells (25). Thus, our results suggested that I3C and DIM induced caspase-dependent apoptosis in endothelial cells, mainly through inactivation of ERK1/2, and that inactivation of Akt by DIM was responsible for its stronger antiangiogenic effects than that of I3C. In contrast, the 2 indoles have been

reported to induce caspase-dependent apoptosis in tumor cells, mainly through inactivation of Akt but not of ERK1/2 (34,37–40), which indicates that proapoptotic mechanisms of I3C and DIM might be cell type specific. We need to further investigate
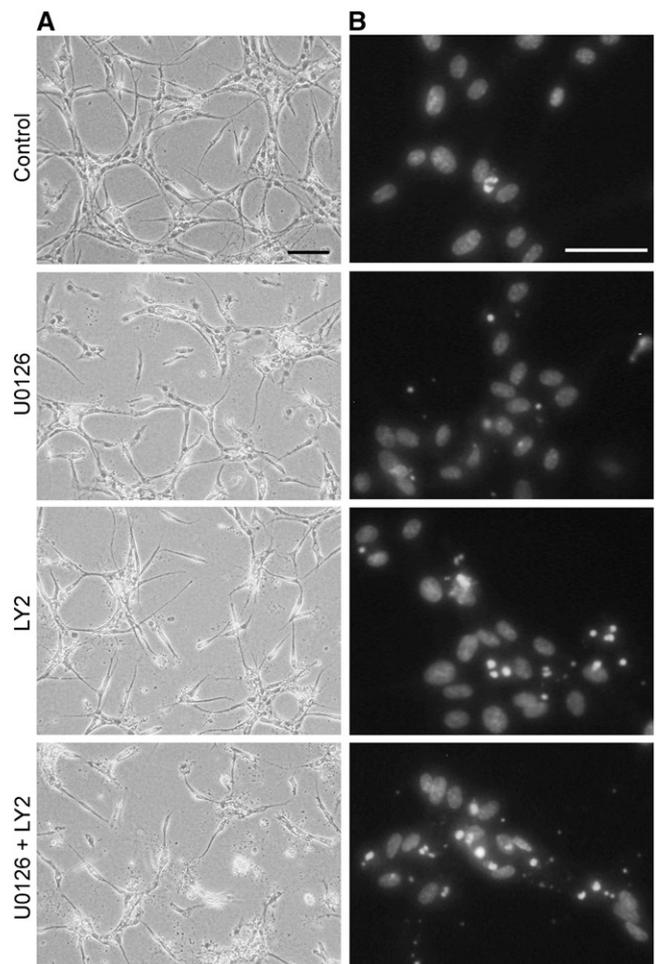

FIGURE 2 Inhibition of tube formation and induction of apoptosis in tube-forming HUVEC by ERK1/2 inactivation (U0126) and Akt inactivation (LY294002). Endothelial cells were induced to form blood vessel-like tubes and were treated with vehicle, U0126 (10 μmol/L), LY294002 (LY2) (5 μmol/L), or U0126 (10 μmol/L) + LY294002 (5 μmol/L) for 24 h. (Left panel) Reduction of the tube network by U0126 and LY294002. Bar indicates 100 μm. (Right panel) Induction of chromatin condensation and nuclear fragmentation by U0126 and LY294002. Bar indicates 50 μm.

TABLE 4 Inhibition of tube formation and induction of apoptosis in tube-forming HUVEC by U0126 and LY294002[1,2]

Treatment	Tube area	Apoptosis
	%	
Control	27 ± 2^a	9 ± 2^d
U0126	14 ± 3^b	21 ± 2^c
LY294002	12 ± 3^b	37 ± 1^b
U0126 + LY294002	8 ± 1^b	49 ± 3^a

[1] Values are means $\pm$ SEM, $n = 3$. Means in a column without a common letter differ, $P < 0.05$.
[2] After 24-h treatment with vehicle, U0126 (10 μmol/L), and/or LY294002 (5 μmol/L), the areas of formed tubes and the rates of apoptotic cells were measured.

how the 2 indoles inactivate these survival signals and induce apoptosis in endothelial cells at the molecular level.

In this article, we showed that both I3C and DIM inhibit angiogenesis, at least in part, through their abilities to inactivate ERK1/2. DIM was also shown to inactivate Akt, which seemed to be the reason why DIM had stronger antiangiogenic effects than I3C. Further investigation is necessary to determine whether these indoles inhibit tube formation of endothelial cells through mechanisms other than the induction of apoptosis. I3C and DIM may prove useful in the development of agents and foods with therapeutic or preventive activity against tumor angiogenesis and angiogenesis-related diseases. We hope our findings on antiangiogenic effects of I3C and DIM will help us improve medical prevention and treatment of human cancer and other angiogenesis-related diseases in the near future.

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