Inhibition of growth factor-induced Ras signaling in vascular endothelial cells and angiogenesis by 3,3'-diindolylmethane

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Abbreviations: ATCC, American type culture collection; BP, benzo(a)-pyrene; DIM, 3,3'-diindolylmethane; DMBA, dimethylbenz(a)anthracene; DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; Fli-1, fetal liver kinase-1; Flt-1, fms-like tyrosine kinase; HUVECs, human umbilical vein endothelial cells; I3C, indole-3-carbinol; KDR/Flik-1, kinase insert domain-containing receptor/fetal liver kinase-1; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; PAF, porcine aortic endothelial cell; RBD, Ras binding domain; VEGF, vascular endothelial growth factor.

Introduction

Numerous epidemiological studies have shown that the level of consumption of cruciferous vegetables is inversely associated with incidences of cancers in several sites, including breast, prostate, stomach and lung (1–6). An active component in these plants, indole-3-carbinol (I3C), and its major acid condensation product, 3,3'-diindolylmethane (DIM), were shown to decrease the incidence of 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary tumors in female Sprague–Dawley rats (7–10), and to inhibit benzo[a]pyrene (BP)-induced neoplasia of the forestomach of mice (7). Many possible mechanisms of action of these indoles have been proposed. Early studies demonstrated that I3C induces Phase I, II detoxification enzymes, such as cytochrome P450s, glutathione S-transferases, epoxide hydrolase and NAD(P)H quinone reductase (7,11–13). Activation of detoxification systems may explain in part the protective effect of I3C against carcinogenesis and mutagenesis, along with other forms of toxicity from electrophiles and oxidative stress. In addition, in vitro studies revealed I3C and DIM to be cytotatic agents that induce a G1 arrest in breast and prostate cancer cells independent of estrogen or androgen hormone status, and can result in induction of apoptosis of these cells (14–20). Clinically, I3C and DIM are currently used in therapies for recurrent respiratory papillomatosis (21).

There is increasing evidence supporting a critical role of angiogenesis in tumor growth and metastasis (22,23). A tumor cannot grow beyond a few cubic millimeters unless vascularization has occurred, and metastasis is rare unless proper vasculature is established in primary tumors (24). Vascular endothelial growth factor (VEGF), expressed abundantly in a wide variety of human and animal tumors, is recognized as an important mediator of angiogenesis associated with tumor development. VEGF regulates most of the steps in the angiogenic cascade, including proliferation, migration and tube formation of endothelial cells (25–28). Therapeutic strategies that target VEGF, specifically using anti-VEGF antibodies, soluble VEGF receptors or dominant negative VEGF receptors, have been demonstrated to decrease the vessel density and reduce the growth rate of several tumor types in animal models (29–31).

Aspects of the VEGF signaling pathway are understood in some detail. VEGF can bind with high affinity to its two receptor tyrosine kinases, fms-like tyrosine kinase (Flt-1) and kinase insert domain-containing receptor/fetal liver kinase-1 (KDR/Flik-1), which are expressed on the surface of endothelial cells (32,33). However, most biologically relevant VEGF signaling in endothelial cells is believed to be mediated by KDR/Flik-1 (34–36). The p44/p42 mitogen-activated protein kinases (MAPK, or ERK1/2), which are activated through binding of VEGF to KDR/Flik-1, are linked to the proliferation response of these cells to VEGF (37,38). Classically, ERK1/2 is activated via signal transduction through the Ras-Raf-ERK

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cascade upon binding of growth factors to their individual tyrosine kinase receptor (39,40). Members of the transmembrane growth factor receptor family exhibit intrinsic protein tyrosine kinase activities that can be activated upon binding to a specific ligand. Ras is subsequently activated and the GTP-bound form is generated. Raf binds to Ras–GTP via its Ras-binding domain (RBD) and is brought to the plasma membrane to be activated. Active Raf in turn phosphorylates MAPK/ERK kinase (MEK), and the latter activates ERK1/2 by tyrosine and threonine phosphorylation (41–43). Previously we reported that DIM exhibits antiangiogenic activities by inhibiting human vascular endothelial cell proliferation, migration and tube formation (44). Here we report further evidence of DIM’s antiangiogenic activities by showing that DIM inhibits Ras-Raf-ERK1/2 cascade activation mediated by VEGF and other growth factors in vascular endothelial cells at the level of Ras, and that this inhibition occurs along with growth arrest of vascular endothelial cells and reduced angiogenesis.

Materials and methods

Materials

Dulbecco’s modified Eagles medium (DMEM), Opti-MEM and lipofectin reagent were supplied by Gibco BRL (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Sigma Chemical (St Louis, MO). EGM-MV BulletKit endothelial culture medium (5% FBS in endothelial basic medium with 12 μg/ml bovine brain extract, 10 ng/ml human EGF, 1 μg/ml hydrocortisone and 1 μg/ml GA-1000) was purchased from Clonetics (San Diego, CA). Anti-FLAG M2-agarose and anti-FLAG M2 monoclonal antibodies were from Sigma Chemical. Anti-ERK1/2, phospho-ERK1/2 (Thr202/Tyr204), MEK1/2, phospho-MEK1/2 (Ser-217/221) and phospho-c-Raf antibodies were from Sigma Chemical. Anti-ERK1/2, phospho-ERK1/2 (Thr202/Tyr204), MEK1/2, phospho-MEK1/2 (Ser-217/221) and phospho-c-Raf (Ser-338) antibodies were from Cell Signaling Technology (Beverly, MA). Antibody against total c-Raf was from Transduction Laboratories (Lexington, KY). Ras activation assay kit and anti-Ras antibody (clone RAS10) were from Transduction Laboratories. Upstate Biotechnology (Lake Placid, NY. Cat no. 17-218, Lot no. 25027). Antiphosphotyrosine PY99 and anti-KDR-Fk-1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). ECL™ was from PerkinElmer (Boston, MA). Equal amounts of total protein were fractionated by electrophoresis on polyacrylamide/SDS gels. Proteins were then transferred to PVDF Immobilon-P transfer membranes using transfer buffer (25 mM glycine, 25 mM ethanolamine and 20% methanol) and then blocked 30 min in blocking buffer (1% Tween-20 with 5% w/v non-fat dry milk). Blots were then incubated overnight with antibodies against phospho-ERK1/2 (Thr202/Tyr204), phospho-ERK1/2 (Ser-217/221) and phospho-c-Raf (Ser-338), followed by 1 h secondary antibody incubation, and detected either by ECL™ or SuperSignal® West Duro chemiluminescence. Strips were stripped and re-blotted with antibodies against total ERK1/2, MEK1/2 and c-Raf.

KDR phosphorylation immunoprecipitation assay

The assay was performed as described with minor modifications (50). PAE/KDR cells were cultured to near confluency in 100 mm dishes and were starved for 16 h. Cells were then treated with DMSO or DIM (5, 10 or 25 μM) for indicated times, followed by incubation with 10 ng/ml VEGF or 25 ng/ml EGF, insulin-like growth factor (IGF) or platelet-derived growth factor (PDGF) for 10 min. Cells were then lysed in a buffer (20 m Tris–HCl (pH 7.8), 150 mM NaCl, 1% Triton X-100, 0.5% Nonidet P-40, 50 mM NaF, 10 mM β-glycerophosphate, 1 mM NaVO₃, 5 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml each of aprotinin and leupeptin). Amounts of total protein were fractionated by electrophoresis on polyacrylamide/SDS gel. Proteins were then transferred to PVDF Immobilon-P transfer membranes using transfer buffer (25 mM glycine, 25 mM ethanolamine and 20% methanol) and then blocked 30 min in blocking buffer (1% Tween-20 with 5% w/v non-fat dry milk). Blots were then incubated overnight with antibodies against phospho-ERK1/2 (Thr202/Tyr204), phospho-ERK1/2 (Ser-217/221) and phospho-c-Raf (Ser-338), followed by 1 h secondary antibody incubation, and detected either by ECL™ or SuperSignal® West Duro chemiluminescence. Strips were stripped and re-blotted with antibodies against total ERK1/2, MEK1/2 and c-Raf.

Ras activation assay

Assays were performed using the Ras Activation Assay Kit according to the manufacturer’s instruction. Briefly, HUVECs were grown on 60 mm Petri dishes to confluency and then starved in 1% FBS endothelial basal medium for 6 h. Cells were treated with 25 μM DIM for 12 h. Cells were then treated with 50 ng/ml VEGF for 10 min in the presence or absence of different concentrations of DIM. Whole cell lysates were extracted as described above. Protein (500 μg) extracts were incubated with antiphosphotyrosine PY99 (1:100) at 4°C overnight with gentle agitation. The beads were then washed, collected and reduced in Laemmli sample buffer. Western blot analysis was performed as described above with anti-Ras antibody.

ERK activation trans-reporting assay

Trans-reporting systems were used for ERK activation assay. Assays were performed according to the manufacturer’s instruction. Briefly, the kit provides pFA2-CMV Elk-1 fusion trans-reporter plasmid as transcription factor gene, and pPR-Luc as the reporter plasmid. The Elk-1 fusion trans-reporter plasmid, which consists of a sequence for Elk-1 activation domain and GAL4 binding domain, is constantly expressed and remains inactive unless ERK phosphorylates the Elk-1 domain. After activation by ERK the fusion protein binds to the reporter gene, which contains five tandem repeats of yeast GAL4 binding sites, and expresses the luciferase containing protein. HUVECs at low passages (3–5) were cultured in 6-well plates to ∼70% confluency. The medium was then replaced with 1 ml serum-free Opti-MEM per well. Aliquots of 0.15 μg of each of pFA2-CMV Elk-1 and pPR-Luc with or without 0.2 μg of constitutively active Ras (Ras G12V) plasmid were used to transfect HUVECs with 3 μl of lipofectin reagent for 3 h. After transfection, the medium was replaced with endothelial basal medium with 0.5% FBS and incubated for 12 h. Cells were then treated with DMSO or 5 μM DIM for another 8 h. Luciferase
assay was performed at the end of the treatment. Transfection efficiency was checked with CMV-GAL4-Luc plasmid, and DIM treatment in the above condition did not affect the expression of luciferase.

**Constitutively active Ras and FLAG-ERK2 co-transfection assay**
HUVECs were grown to 70–80% confluency in 100 mm Petri dishes. FLAG-ERK2 (1.5 μg) and 2 μg of constitutively active Ras (G12V) or control vector were used with 24 μl lipofectin reagent to transfect HUVECs. After 3 h, the medium was replaced with complete endothelial culture medium and incubated for another 24 h. Cells were then serum starved in 1% FBS endothelial cell basal medium for 6 h and treated with DIM for 3 h. Some samples were induced with 10 ng/ml VEGF at the end of the treatment. Whole cell lysates were incubated with anti-FLAG M2 antibody conjugated with beads for 2 h at 4°C with gentle agitation. Beads were then collected and washed three times with PBS, and reduced in Laemmli sample buffer. Western blotting was performed with antiphospho-ERK1/2 or anti-FLAG M2 antibody.

**Matrigel plug assay**
The assay was performed on female C57/BL6 mice that were housed in polycarbonate cages kept in rooms maintained at 20–22°C with 12 h light and dark cycles. Mice were acclimated on semi-purified phytoestrogen-free AIN-76A diet ad libitum for 7 days prior to the study and randomly grouped (five/group). Heparin (64 U/ml) with or without 100 ng/ml VEGF or 100 μM DIM was gently mixed with cold liquid Matrigel. The Matrigel solution (0.3 ml) was injected subcutaneously into bilateral flanks of mice. Animals were given free access to either control AIN-76A diet or AIN-76A diet containing 200 mg/kg DIM. After 1 week the mice were killed by CO2 inhalation. The Matrigel plugs were surgically removed and vascularization was quantified by measuring the hemoglobin content using Drabkin reagent kit.

**Statistics**
For statistical analyses, means were compared by one-way analysis of variance (ANOVA) followed by Tukey’s test. SigmaStat 2.03 software was used for all the statistics. Differences were considered significant at the level of P ≤ 0.05. The results are expressed as mean ± SD, unless otherwise stated.

**Results**

**DIM inhibits VEGF-induced proliferation and DNA synthesis of HUVECs**

The proliferation of vascular endothelial cells in response to growth factors is of central importance to the angiogenesis process. To examine the mode of action of DIM as an inhibitor of angiogenesis, we determined whether this indole inhibited VEGF-induced proliferation of cultured vascular endothelial cells. For this assay, we tested the effect of increasing concentrations of DIM (1–10 μM) on proliferation of HUVECs incubated under low serum conditions with or without VEGF. As shown in Figure 1A, cell growth was significantly increased in the presence of VEGF, to ~30–40% over control after 24 h incubation. A DIM concentration as low as 2.5 μM reduced VEGF-induced cell growth to the control level (Figure 1A). This inhibitory effect of DIM on VEGF-induced cell growth was confirmed by [3H]thymidine incorporation assay. For this assay, HUVECs were treated with 2.5 μM DIM with or without 10 ng/ml VEGF for 24 h, and [3H]thymidine was added for the last 6 h of the assay. The results show that 2.5 μM DIM completely blocked VEGF-induced DNA synthesis (Figure 1B).

**DIM inhibits VEGF-induced ERK1/2 phosphorylation in HUVECs**

Since VEGF-induced cell proliferation is ERK1/2 dependent (37,38), we next examined the effect of DIM on VEGF-mediated ERK1/2 phosphorylation in HUVECs. For this experiment, HUVECs were kept in low serum medium (1% FBS) for 6 h and then treated with the indicated concentrations of DIM for various time intervals (Figure 2A). At the end of the treatment, cells were induced with 10 ng/ml VEGF for 10 min. As shown in Figure 2A, VEGF induced a marked increase in ERK1/2 phosphorylation that was ~15-fold greater than the control as determined by densitometry quantification. However, DIM treatment for 3 h produced a concentration-dependent inhibition of VEGF-induced ERK1/2 phosphorylation of 20% at 5 μM and 80% at 25 μM (Figure 2A, upper panel). Results of kinetics studies showed that the inhibitory effects of DIM became obvious as early as 0.5 h for 25 μM DIM treatment. DIM administrations for longer times caused greater inhibition of ERK1/2 phosphorylation (Figure 2A, lower panel). Total ERK1/2 levels remained unchanged with DIM treatment. Viability of HUVECs, or expression of the housekeeping gene, α-tubulin, was not affected by the highest concentration of DIM under the experimental conditions used (data not shown).

Immunostaining of cells with antiphospho-ERK1/2 antibody followed by a secondary antibody conjugated with anti-FITC green fluorescent protein showed that following stimulation with VEGF strong staining of phosphorylated ERK1/2 was present mainly in the nuclei (Figure 2B). This observation is consistent with previous reports that ERK1/2 translocates into the nuclei after being phosphorylated and activated (51). However, 25 μM DIM treatment for 3 h nearly abolished the phospho-ERK1/2 signal (Figure 2B), as evidenced by weak
staining of green fluorescence. Taken together, these results indicate that DIM strongly inhibits VEGF-induced ERK1/2 phosphorylation.

**DIM does not inhibit VEGF receptor KDR phosphorylation**
Because we observed that DIM inhibits ERK1/2 phosphorylation induced by VEGF, we examined whether DIM would directly affect VEGF receptor activity. It has been demonstrated that VEGF induces ERK1/2 activity mainly through its receptor, KDR. To increase the sensitivity of the assay, we examined DIM’s effect on PAE cells stably transfected with KDR (PAE/KDR). In a manner similar to the response of HUVECs, DIM caused a dose-dependent inhibition of VEGF-induced ERK1/2 phosphorylation in PAE/KDR cells, with a 70% reduction of ERK1/2 phosphorylation determined by densitometry quantification (Figure 3). However, DIM did not inhibit KDR phosphorylation induced by VEGF, which suggests that activity of the receptor remains unchanged (Figure 3). Therefore, DIM inhibits ERK1/2 phosphorylation induced by VEGF without blocking VEGF receptor activation.

**DIM inhibits phosphorylations of ERK1/2 and MEK stimulated by multiple growth factors**
The above results suggest that DIM is not a specific VEGF receptor inhibitor, thereby indicating that DIM might affect a

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**Fig. 2.** DIM inhibits VEGF-induced ERK1/2 phosphorylation in HUVECs. HUVECs were stripped in low serum medium (1% FBS) for 6 h and then treated with DMSO or DIM for indicated time intervals. (A) Cells were treated with the indicated concentration of DIM for 3 h (upper panel), or were treated with 25 μM DIM for indicated time intervals (lower panel), and induced with 10 ng/ml of VEGF for 10 min before collection. Phosphorylated and total ERK1/2 were detected by specific antibodies. Western blots were quantified by densitometry and ratio of phosphorylated ERK to total ERK was expressed as fold induction untreated control. Asterisk indicates significant difference compared with control at a level of \( P < 0.05 \). (B) HUVECs grown on coverslips were treated with 25 μM DIM for 3 h and stimulated with 10 ng/ml of VEGF for 10 min before collection. Cells were fixed and stained with anti-phospho-ERK antibody, followed by anti-FITC secondary antibody. Nuclei were visualized with DAPI.
downstream element(s) of the receptor in the signal transduction pathway to ERK1/2 activation. To further confirm this, we first examined whether DIM also inhibits ERK1/2 phosphorylation induced by other growth factors that utilize the Ras-Raf-MEK pathway downstream of their individual receptors to transduce signal to ERK1/2. For these studies, HUVECs or PAE/KDR cells in low serum medium were pretreated with 25 μM DIM for 3 h and then induced with 25 ng/ml EGF, IGF or PDGF for 10 min under conditions described in Materials and methods. The results indicated that ERK1/2 phosphorylation was strongly induced by individual growth factors, and that this activation was reduced in a similar way by DIM in both HUVECs (Figure 4A) and in PAE/KDR cells (Figure 4B).

We observed further that MEK phosphorylation induced by growth factors was also inhibited to the similar degree as that of ERK1/2 (Figure 4A and B). Taken together, these results indicate that DIM disrupts the activity of element(s) downstream of growth factor receptors and upstream of ERK1/2 in the signal transduction pathway.

**DIM inhibits MEK, Raf-1 and Ras activities**

To further define the steps in the signal transduction pathway where inhibition occurs, we next examined in detail DIM’s effect on growth factor-induced MEK1/2, Raf-1 and Ras activation, all of which are upstream elements of ERK1/2 in the signaling pathway. Raf-1 is a protein with multiple phosphorylation sites that regulate its activity (52). Since phosphorylation of serine residue 338 is necessary for the activation of Raf-1 (53), we used a specific antibody to detect phospho-Ser-338 Raf-1. Similarly, activation of MEK1/2 by Raf-1 occurs through phosphorylation of serine residues at positions 217 and 221 (54). Thus, the specific antibody against phospho-Ser-217/221 MEK1/2 was used. We found that VEGF-induced phosphorylation of both MEK1/2 and Raf-1 were inhibited by DIM to the similar degree as phosphorylation of ERK1/2, whereas the total levels of MEK1/2 and Raf-1 were unchanged (Figure 5A). In contrast, U0126, a specific inhibitor of MEK activity, completely blocked ERK1/2 phosphorylation without affecting MEK1/2 (Ser-217/221) or Raf-1 (Ser-338) phosphorylation (Figure 5A).

Since activation of Raf is Ras-dependent following growth factor stimulation, we next examined the effect of DIM on Ras activation. We observed that Ras activity induced by VEGF, EGF or complete medium was strongly inhibited by DIM treatment for 3 h (Figure 5B). The protein extracts from the same samples were blotted with antiphospho-ERK1/2 antibody and the results showed that both EGF- and VEGF-induced ERK1/2 activations were decreased by DIM, which is consistent with decreased Ras activity. In contrast, DIM did not significantly affect ERK1/2 phosphorylation induced by complete medium, even though Ras activation was inhibited under the same conditions (Figure 5B). This observation might be due to the existence of Ras-independent pathways to activate ERK1/2 after the stimulation of complete medium in HUVECs. It further suggests a specific inhibitory effect of DIM on Ras-mediated ERK1/2 activation.

**Overexpression of constitutively active Ras reverses DIM’s inhibitory effect on ERK1/2 phosphorylation**

To confirm the specific role of Ras inhibition in the effects of DIM on ERK activation, we conducted two studies with cells co-transfected with constitutively active Ras (Ras G12V). In the first assay, we co-transfected HUVECs with Ras G12V and ERK-activation reporter plasmids. In this system, luciferase gene expression was driven by a constitutively expressed GAL4 fusion protein (the transcription factor), the activity of which depends on ERK activation (Figure 6A, upper panel). The results show that DIM inhibited VEGF-induced ERK1/2 activity up to 50% based on the luciferase assay. However, when cells were co-transfected with Ras G12V plasmid, the inhibitory effect of DIM was reversed (Figure 6A, lower panel).

In the second study, FLAG-ERK2 was either transfected along with the control vector or with Ras G12V into HUVECs. Cells were then treated with the control vehicle or 25 μM DIM for 3 h, and stimulated with VEGF for 10 min before the assay. FLAG-ERK2 was immunoprecipitated with anti-FLAG antibody followed by western blot with either anti-FLAG or antiphospho-ERK1/2 antibody. As expected, the results showed that VEGF induced a marked phosphorylation of FLAG-ERK2 (Figure 6B, upper panel, lane 3) compared with the untreated control (Figure 6B, upper panel, lane 2), and this effect was blocked by DIM (Figure 6B, upper panel, lane 2).
However, overexpression of Ras G12V largely reversed DIM’s inhibitory effect on FLAG-ERK2 phosphorylation (Figure 6B, upper panel, lanes 5 and 6). These results are quantified in the histogram presented in Figure 6B (lower panel). Taken together, these results confirm that DIM inhibition of VEGF-induced ERK1/2 activation is mediated by blockage of Ras activation.

**DIM inhibits VEGF-induced neovascularization in Matrigel plugs in mice**

To examine whether VEGF-induced angiogenesis could be inhibited by the presence of DIM in vivo, we conducted Matrigel plug assays in mice. Matrigel is a urea extract of Engelbreth–Holm–Swarm (EHS) tumor, which mimics the authentic tumoral basement membrane. After subcutaneous injection, cold liquid Matrigel solidifies and permits penetration by host cells and the formation of new blood vessels. Hemoglobin content of Matrigel parallels blood vessel development in the gel, thereby allowing ready quantification. Matrigel with or without human recombinant VEGF was mixed with DMSO vehicle or DIM. Mice in the DIM group were also given free access to AIN-76 diet containing 200 mg/kg DIM. Mice in other groups were given AIN-76 diet without DIM. Matrigel plugs were removed after 7 days. The results indicate that Matrigel with additional VEGF markedly induced angiogenesis compared with Matrigel by itself, as indicated by the 12-fold increases in hemoglobin concentration (Figure 7). DIM co-treatment, however, reduced VEGF-induced vascularization of the plugs by 74% to approximately the control level. These results indicate that DIM can inhibit VEGF-induced angiogenesis, in vivo, presumably by inhibiting VEGF-induced activation of Ras signaling.

**Discussion**

We demonstrated for the first time that DIM, a chemopreventive dietary indole, strongly inhibits angiogenesis by a mechanism that involves disruption of VEGF-induced mitogenic signal transduction to MAPK44/42 (ERK1/2) in vascular endothelial cells. Binding of VEGF to its receptors induces multiple downstream signaling pathways, with the most potent biological effects mediated through VEGF receptor 2 (KDR/Flik-1). ERK1/2 is activated after binding of VEGF to KDR/Flik-1, and this activation has been shown to be necessary for the mitogenic response of endothelial cells to VEGF (37). Inhibition of MAPK activation by PD98059, a highly specific MEK inhibitor, results in the inhibition of VEGF-induced proliferation of PAE/KDR cells (37). In addition, disruption of signal transduction from KDR to ERK1/2 either by introducing mutant KDR (55) or by incubation with highly specific inhibitors of its upstream activators (56) abolished ERK1/2 phosphorylation and the mitogenic response to VEGF, as well as other growth factors both in HUVECs and in PAE/KDR cells. HUVECs were stripped in low serum medium (1% FBS) for 6 h and treated with DIM for 3 h (A). PAE/KDR cells were stripped for 16 h and treated with DIM for 12 h (B). Cells were then treated with 10 ng/ml VEGF for HUVECs and 50 ng/ml VEGF for PAE/KDR before collection. Phospho-ERK1/2 and phospho-MEK were detected by Western blotting. Western blots were quantified by densitometry and the ratio of phosphorylated protein to total protein was expressed as the fold induction of untreated control. Asterisks indicate significant difference compared with control at a level of $P \leq 0.05$. 

**Fig. 4.** DIM inhibits ERK1/2 and MEK phosphorylation induced by VEGF as well as other growth factors both in HUVECs and in PAE/KDR cells.
VEGF. Consistent with those previous reports, we found that inhibition of VEGF-induced ERK1/2 activation by DIM abolished the effects of the growth factors on cell proliferation and DNA synthesis.

These studies provide strong evidence that DIM inhibition of VEGF-induced ERK activation occurs at the level of Ras. Results from the KDR tyrosine phosphorylation assay exclude the possibility of a specific effect of DIM on KDR activity itself. Even though ERK1/2 phosphorylation was strongly reduced by DIM, KDR phosphorylation was unaffected by DIM following challenge with VEGF. We observed that DIM blocked ERK1/2 activation induced by different growth factors that have no significant structural similarities to each other or in the extracellular domains of their cognate receptors. ERK1/2 activation was inhibited to similar levels regardless of the type of growth factor used. The fact that activations of MEK and Raf-1 were inhibited by DIM in concentration-dependent manners that were similar to the inhibition characteristics of ERK1/2 is consistent with the relationship of these kinases in a single signaling pathway. We demonstrated further that DIM strongly reduced Ras–GTP content induced by treatment with VEGF, EGF or complete culture medium (mixed mitogens). Interestingly, although Ras activation in HUVECs induced by fresh complete medium was inhibited by DIM, ERK1/2 activity was not obviously affected under this condition. This is possibly due to the presence of factors in the medium that can activate ERK1/2 via a Ras-independent pathway(s). Again, this supports the idea that inhibition of ERK1/2 activation by DIM is due to DIM's inhibitory effects at the level of Ras. To confirm that DIM acts on Ras or upstream activators, we overexpressed constitutively active mutant Ras (Ras G12V) in HUVECs and found that inhibition of ERK1/2 activation by DIM was reversed.

The mechanism of activation of the small G-protein Ras has been the subject of extensive investigation. Ras can be activated via the guanine nucleotide exchange factor (GEF), which stimulates the exchange of GTP for GDP on Ras. Ras activation is generally considered to involve recruitment of SOS (Ras–GEF) by GRB2 to the plasma membrane after activation of tyrosine kinase receptors, including the EGF receptor (57,58), and deactivation by the Ras–GTPase-activating protein (Ras–GAP). However, the mechanisms of Ras activation by VEGF and EGF differ since overexpression of the dominant negative Ras mutant (Ras-N17) fails to abolish VEGF- but not EGF-induced ERK1/2 activation. In addition, PKC was consistently shown to be the major mediator of VEGF- but not of EGF-induced ERK1/2 activation (55,56). A recent report

Fig. 5. DIM inhibits ERK1/2 phosphorylation through inhibiting its upstream kinases MEK, Raf-1 and Ras. HUVECs were stripped in low serum medium (1% FBS) for 6 h and treated with DIM for 3 h. (A) Cells were treated with 10 ng/ml VEGF for 10 min before collection. Phospho-ERK1/2, phospho-MEK and phospho-Raf-1 were detected by specific antibodies. Western blots were quantified by densitometry and ratios of phosphorylated protein to total protein were expressed as the fold induction of untreated control. (B) Cells were treated with VEGF (10 ng/ml), EGF (25 ng/ml) or complete culture medium before collection. Whole cell lysates were collected and immunoprecipitated with RBD-agarose beads, followed by Western blot with Ras antibody. The same cell lysates were also subjected directly to Western blot to show the level of total Ras, total ERK1/2 and phospho-ERK1/2. Ras activation was quantified by densitometry and the ratio of Ras–GTP to total Ras was expressed as the fold induction of untreated control. Asterisks indicate significant difference compared with control at a level of P ≤ 0.05.
suggests that Ras is activated by VEGF via a mechanism that involves PKC-mediated Ras–GAP downregulation, rather than through the classical GRB2–SOS. The study also demonstrated that Ras activation is necessary for ERK1/2 activation and the proliferation of endothelial cells in response to VEGF (59). Our work again confirms that VEGF activates Ras and that this activation is necessary for VEGF-stimulation of ERK1/2 activity in HUVECs, since inhibition of VEGF-stimulated Ras activation by DIM treatment results in reduced ERK1/2 activity and decreased DNA synthesis in HUVECs. However, how Ras is inhibited by DIM remains elusive, given the fact that DIM inhibits Ras activation stimulated by both EGF and VEGF, two mitogens that activate Ras by apparently different mechanisms. In addition to the regulatory pathway to Ras, the involvement of GRB2–SOS, Ras–GAP and/or PKC, post-translational modification of Ras protein has been suggested as an additional mechanism for Ras regulation. Thus, membrane anchoring of Ras is achieved through a series of post-translational modifications, the most critical of which is farnesylation of the conserved cysteine catalyzed by farnesyl protein transferase (FTase) (60). Furthermore, it was recently reported that activity of Ras can also be modulated by S-glutathiolation of the Cys118 residue in the presence of reactive oxygen species (ROS) (61). Whether these post-translational mechanisms play roles in DIM’s inhibitory effects on Ras activation is under further investigation in our laboratory.

It is interesting to note that the effects of DIM on HUVEC proliferation and Ras signaling were observed at physiologically relevant concentrations. We estimate that consumption of a 200 g portion of broccoli provides ~12 mg of DIM. With maximum absorption of DIM, the blood concentration of DIM would be expected to reach ~10 μM. Thus, in vivo concentrations of DIM from dietary Brassica vegetables are expected to be similar to the effective levels of DIM in cultured cells.

DIM has been recognized as a chemopreventive agent for many years. Since the growth of vascular endothelial cells is of central importance in tumor angiogenesis, DIM’s novel function as a negative regulator of VEGF-stimulated MAPK activity in endothelial cells has important implications in cancer therapies. Besides proliferation, MAPK signaling has also been suggested to regulate other important processes in angiogenesis such as endothelial cell migration and differentiation (62–67). The ability of DIM to disrupt proper MAPK signaling in vascular endothelial cells might render these cells insensitive to VEGF, leading to reduced establishment of vasculature. Our Matrigel plug assay provides further evidence that DIM weakens VEGF’s ability to induce successful angiogenesis, in vivo. The role of DIM mediated inhibition of Ras signaling in the reduced capacity of vascular endothelial cells for the complex of processes involved in angiogenesis, including cell migration, invasion and tube formation, in addition to proliferation as reported here, is the subject of our further studies.

**Fig. 6.** Constitutively active Ras reverses DIM’s inhibitory effect on ERK activity. (A) HUVECs were transfected with pFA2-CMV and pFR-Luc (upper panel: schematic representation of the plasmids), with or without constitutively active Ras. Cells were then incubated in low serum medium (0.5% FBS) for 12 h before treatment with 5 μM DIM for another 8 h. Luciferase assay was performed at the end of the treatment. (B) HUVECs of 70% confluency were transfected with FLAG-ERK2 with constitutively active Ras (RasG12V) or control vector. Cells were stripped in low serum (1% FBS) medium for 6 h before treatment with DMSO or DIM for 3 h. Results presented in lane 3–6 are for HUVECs stimulated with 10 ng/ml VEGF for 10 min before collection. Cell lysates were immunoprecipitated with anti-FLAG antibody and then subjected to Western blot with phospho-ERK1/2 antibody. The membrane was then stripped and blotted with anti-FLAG antibody. Western blots were quantified by densitometry and the ratio of phosphorylated protein to total protein was expressed as the fold induction of untreated control. Asterisk indicates significant difference compared with control at a level of $P \leq 0.05$. 

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Fig. 7. DIM inhibits VEGF-induced vascularization in Matrigel plugs in mice. Female C57BL/6 mice were injected s.c. with 0.3 ml Matrigel with or without 100 ng/ml VEGF in the presence or absence of 100 µg DIM. After 7 days Matrigel plugs were surgically removed and hemoglobin concentration was quantified by Drabkin reagent. Results were expressed as mean ± SE. ‘a’ indicates $P \leq 0.05$ compared with control group; ‘b’ indicates $P \leq 0.05$ compared with VEGF group.

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