Abstract. Recent studies have suggested that sulforaphane, a compound found largely in cruciferous vegetables, could inhibit tumor growth through regulation of angiogenesis. However, the molecular mechanism by which it inhibits angiogenesis has not been reported. In this study, we examined the molecular mechanisms by which sulforaphane (SNF) inhibits angiogenesis through regulation of FOXO transcription factor in human umbilical vein endothelial cells (HUVECs). Inhibition of MEK/ERK and PI3K/AKT pathways synergistically inhibited cell migration and capillary tube formation by HUVECs and further enhanced the antiangiogenic effects of sulforaphane. Inhibitors of MEK and AKT kinases synergistically enhanced nuclear translocation of FOXO3a. Inhibition of the MEK/ERK and PI3K/AKT pathways synergistically induced FOXO transcriptional activity and inhibited cell migration and capillary tube formation; these events were further enhanced in the presence of sulforaphane. Phosphorylation deficient mutants of FOXO enhanced antiangiogenic effects of sulforaphane by activating the FOXO transcription factor. In conclusion, activation of FOXO transcription factor by sulforaphane could be an important physiological process to inhibit angiogenesis which may ultimately control tumor growth. These novel antiangiogenic activities of sulforaphane are likely to contribute to its cancer chemopreventive and therapeutic potential.

Introduction

Epidemiological studies have shown that people who eat cruciferous vegetables have reduced incidence of cancer. Isothiocyanates (ITCs) are sulfur-containing compounds that are broadly distributed among cruciferous vegetables such as cabbages and broccoli (1-3). The consumption of ITCs is expected to rise due to the use of dietary supplements and public health initiatives promoting the consumption of more fruits and vegetables. SFN possesses antioxidant, anti-proliferative, chemopreventive and anti-carcinogenic properties (1-3). SFN inhibits growth of prostate and breast cancer in vivo (4-9), and also prevents chemically induced breast (10,11), stomach (12) and colon (13) cancers in rats. SFN inhibits osteoclastogenesis by inhibiting NFκB (14). It is rapidly absorbed and shows an absolute bioavailability of 82% (15). SFN induces a phase 2 enzyme, thereby neutralizing carcinogens before they can damage DNA (16,17). SFN is extensively metabolized and can therefore compete with other substrates of phases I, II, III enzymes and transporters. It inhibits benzo[a]pyrene-DNA and 1,6-dinitropyrene-DNA adducts formation. However, the molecular mechanisms by which sulforaphane inhibits angiogenesis are not well understood.

FOXO subfamily of forkhead transcription factors include FOXO1a/FKHR, FOXO3a/FKHRL1, and FOXO4/AFX (18). The phosphoinositides 3-kinase (PI3K) pathway, via activation of its downstream kinase AKT, phosphorylates each of the FOXO proteins (19). Inhibition of the PI3K pathway leads to dephosphorylation and nuclear translocation of FOXO3a. Inhibition of the MEK/ERK and PI3K/AKT pathways synergistically induced FOXO transcriptional activity and inhibited cell migration and capillary tube formation; these events were further enhanced in the presence of sulforaphane. Phosphorylation deficient mutants of FOXO enhanced antiangiogenic effects of sulforaphane by activating the FOXO transcription factor. In conclusion, activation of FOXO transcription factor by sulforaphane could be an important physiological process to inhibit angiogenesis which may ultimately control tumor growth. These novel antiangiogenic activities of sulforaphane are likely to contribute to its cancer chemopreventive and therapeutic potential.

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mycinitreated cells were fixed with 90% methanol and stained with Giemsa. Cell migration through the membrane was determined after 24 h of incubation at 37˚C. Cells remaining on the topside of the transwell membrane were removed using a cotton swab. The membrane was washed with ice-cold PBS. Cells that had migrated to the underside were fixed with 90% methanol and stained with Giemsa. Cell migration was quantified by counting the number of cells per field in five random fields.

The purpose of this study was to examine whether the inhibition of MEK/ERK and PI3K/akt pathways enhanced the antiangiogenic effects of sulforaphane through activation of FOXO transcription factors. We have demonstrated that inhibition of MEK/ERK and PI3K/akt pathways acted synergistically to induce FOXO transcription activity, capillary tube formation and migration. Furthermore, antiangiogenic effects of sulforaphane were regulated through activation of FOXO transcription factors. Thus, sulforaphane may regulate tumor angiogenesis through activation of FOXO transcription factors.

Materials and methods

Reagents. Sulforaphane was purchased from LKT Laboratories, Inc. (St. Paul, MN). MEK inhibitor (PD98059), and AKT inhibitor-IV were purchased from EMD Biosciences (San Diego, CA). Dual Luciferase Reporter Assay kit was purchased from Promega Corporation (Madison, WI).

Cell culture and cell proliferation assay. Human umbilical vein endothelial cells (HUVECs) were cultured in endothelial cell growth factor medium-2 (EGM2 MV SingleQuots, Clonetics) supplemented with 5% FBS. For cell proliferation analysis, HUVECs were seeded in 24-well plates and treated with sulforaphane or DMSO (control). The plates were incubated at 37˚C in a humidified atmosphere of 95% air and 5% CO2. Capillary tube formation was assessed after 24 h by counting the total number of capillary-like tubular structures from three randomly chosen fields using an inverted microscope.

 Luciferase assay. HUVECs were transfected with empty vector, FOXO1-TM, or FOXO3a-TM along with reporter plasmids, p6xDBE-luc and pRL-TK. The FOXO expression vectors (wild-type and phosphorylation deficient mutants) and FOXO-luciferase constructs have been described elsewhere (30,32,33). After 24 h, transfection medium was replaced with culture medium and cells were treated with sulforaphane (10-20 μM). After incubation of 24 h, the relative luciferase activity, i.e., firefly enzyme activity divided by that of the Renilla enzyme, was determined using Dual Luciferase Reporter Assay System (Promega) according to the manufacturer’s protocol.

Immunofluorescence analysis. For visualization of FOXO3a, HUVEC cells were transiently transfected with plasmids encoding GFP-FOXO3a and grown in multiple well chamber slides. After transfection, cells were pretreated with AKT inhibitor IV (1 μM) and/or MEK1/2 inhibitor PD98059 (10 μM) for 2 h, followed by treatment with or without sulforaphane (20 μM) for 24 h. Cells were stained with DAPI (50 ng/ml), and visualized under a fluorescence microscope. The slides were viewed at x1000 magnification on an Olympus fluorescence microscope (BX40 with MagnaFire CCD camera).

Statistical analysis. The mean and SD were calculated for each experimental group. Differences between groups were analyzed by one or two-way ANOVA using PRISM statistical analysis software (GraphPad Software, Inc., San Diego, CA). Significant differences among groups were calculated at P<0.05.

Results

Inhibitors of AKT and ERK synergistically/additively inhibit migration and capillary tube formation of HUVEC cells, and further enhance the inhibitory effects of sulforaphane on migration and capillary tube formation. We first measured the effect of SFN treatment on invasion potential (migration) of HUVEC cells using a modified Boyden Chamber assay (Fig. 1A). In DMSO-treated controls, a large fraction of HUVEC migrated to the bottom face of the membrane. Treatment of chambers with AKT inhibitor, ERK inhibitor (MEK1/2 inhibitor PD98059) or sulforaphane resulted in inhibition of migration of HUVECs. The combination of AKT inhibitor and PD98059 synergistically/additively inhibited...
cell migration. Interestingly, the inhibitory effects of SFN on cell migration were further enhanced in the presence of AKT inhibitor and/or PD98059.

We next examined the effects of PI3K/AKT and MEK/ERK pathway on capillary tube formation by HUVEC on growth factor-reduced matrigel. The data revealed that AKT inhibitor, PD98059 and SFN alone inhibited capillary tube formation (Fig. 1B). The combination of AKT inhibitor and PD98059 synergistically inhibited capillary tube formation. Interestingly, the inhibitory effects of SFN on capillary tube formation were further enhanced in the presence of AKT inhibitor and/or PD98059. These results indicated that PI3K/AKT and MEK/ERK pathways act synergistically/additively to inhibit migration and capillary tube formation by HUVEC cells.

FOXO1-TM and FOXO3A-TM (phosphorylation deficient mutants of FOXO) enhance the inhibitory effects of sulforaphane on migration and capillary tube formation by HUVEC cells. We next examined the involvement of FOXO transcription factors in SFN-induced migration and capillary tube formation by HUVEC cells (Fig. 2). Overexpression of phosphorylation deficient mutants of FOXO (FOXO1-TM and FOXO3a-TM) inhibited migration and capillary tube formation by HUVEC cells. Furthermore, overexpression of FOXO1-TM and FOXO3a-TM further enhanced the inhibitory effects of SFN on migration and capillary tube formation. These data suggest that FOXO transcription factors may play major role in angiogenesis.

Inhibitors of AKT and ERK synergistically/additively induce FOXO transcriptional activity, and further enhanced FOXO transcriptional activity induced by sulforaphane. We next examined whether inhibition of PI3K/AKT and MEK/ERK pathways synergistically act to induce FOXO transcriptional activity, and inhibition of these two pathways further enhance SFN-induced FOXO activity (Fig. 3). AKT inhibitor, MEK1/2 inhibitor PD98059 and SFN alone induced FOXO transcriptional activity. The combination of AKT inhibitor and PD98059 synergistically induced FOXO activity. Furthermore, the combination of AKT inhibitor and/or PD98059 with SFN further enhanced FOXO transcriptional activity. Inhibitors of AKT and MEK synergistically enhanced nuclear translocation of FOXO3a in HUVECs (data not shown). These data suggest that inhibition of PI3K/AKT and MEK1/2/ERK pathways act synergistically to induce FOXO transcriptional activity, and inhibition of these two pathways further enhance SFN-induced FOXO activity.
Phosphorylation deficient mutants of FOXO enhance sulforaphane-induced FOXO transcriptional activity in HUVEC cells. FOXO transcriptional activity was assessed using a dual-luciferase reporter assay system (Promega). After transfection, HUVEC cells were pretreated with sulforaphane (20 μM) for 2 h, followed by treatment with or without sulforaphane (20 μM) for 24 h. Cells were harvested for firefly/Renilla luciferase assays. Data represent the mean ± S.D., ** significantly different from respective controls, P<0.05.

Discussion

The consumption of cruciferous vegetables has long been associated with a reduced risk in the occurrence of cancer at various sites, including the prostate, lung, breast and colon (3). This protective effect is attributed to isothiocyanates present in these vegetables (3). Recent studies have demonstrated that sulforaphane (SFN) present in broccoli inhibit tumor growth and angiogenesis (6,9,34,35). The major mechanism by which SFN protects cells was traditionally thought to be through Nrf2-mediated induction of phase 2 detoxification enzymes that elevate cell defense against oxidative damage and promote the removal of carcinogens. However, it is becoming clear that there are multiple mechanisms activated in response to SFN, including suppression of cytochrome P450 enzymes, induction of apoptotic pathways, suppression of cell cycle progression, inhibition of angiogenesis and anti-inflammatory activity. Moreover, these mechanisms seem to have some degree of interaction to synergistically afford chemoprevention.
strated that another chemopreventive agent EGCG can modulate the expression of genes known to play a role in the cancer progression, invasion, metastasis and angiogenesis (31,36). We have also demonstrated that inhibition of PI3K/AKT and MEK/ERK pathways converge to inhibit angiogenesis through activation of FOXO transcription factors, and anti-angiogenic effects of EGCG were regulated by FOXO (30).

Angiogenesis is a critical step in tumor growth and metastasis (37,38), which are regulated closely by the local increase of the activity of a variety of angiogenic factors. During angiogenesis, endothelial cell migrate, proliferate, organize into tube-like structures, and play an active role in tissue remodeling. Substantial evidence also suggests that stroma cells adjacent to the cancer cells, including fibroblasts and inflammatory cells such as macrophages, neutrophils, and lymphocytes, can interact with cancer cells and express angiogenic factors (39,40). We have recently demonstrated that SFN can inhibit the activation of NFκB and its gene products such as VEGF, HIF-1α, MT1-MMP, MMP-2, MMP-9, COX-2, IL-6 and IL-8 in an orthotopic model of prostate cancer (9). These tumor derived factors can affect angiogenesis and metastasis. In the present study, SFN inhibited cell migration and capillary tube formation which are important events for angiogenesis.

FOXO transcription factors suppress tumor growth because of their effects on cell-cycle, apoptosis, angiogenesis, DNA-damage and reactive oxygen species. FOXO proteins have also been implicated in the negative regulation of signaling by the hypoxia-inducible factor 1 (HIF-1) during vascular development, raising the possibility that the FOXO proteins suppress not only tumor formation but also tumor angiogenesis. FOXO1 and FOXO3a are the most abundant FOXO isoforms in mature endothelial cells and that over-expression of constitutively active Foxo1 or Foxo3a significantly inhibits endothelial cell migration and tube formation in vitro (41). Silencing of either FOXO1 or FOXO3a gene expression led to a profound increase in the migratory and sprout-forming capacity of endothelial cells. The FOXO1-deficient mice died around embryonic day 11 because of defects in the branchial arches and impaired vascular development of embryos and yolk sacs (32). Gene expression profiling showed that FOXO1 and FOXO3a specifically regulate a non-redundant but overlapping set of angiogenesis- and vascular remodeling-related genes. FOXO1 regulates angiopoietin-2 (Ang-2), whereas eNOS, which is essential for postnatal neovascularization, is regulated by FOXO1 and FOXO3a. Consistent with these findings, constitutively active FOXO1 and FOXO3a repressed eNOS protein expression. In vivo, Foxo3a deficiency increased eNOS expression and enhanced postnatal vessel formation and maturation. Thus, our data suggest an important role for FOXO transcription factors in the regulation of angiogenesis.

In conclusion, we have demonstrated that inhibition of PI3K/AKT and MEK/ERK pathways interact synergistically to activate FOXO transcription factors which, in turn, inhibit angiogenesis. Furthermore, inhibition of both of these pathways further enhances the anti-angiogenic effects of sulforaphane. The activation of FOXO transcription factors through inhibition of MEK/ERK and PI3K/AKT pathways may have physiological significance for tumor growth through regulation of angiogenesis. Our data strongly support the role of SFN as an antiangiogenic agent.

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