Inhibition of angiogenesis and endothelial cell functions are novel sulforaphane-mediated mechanisms in chemoprevention

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Abstract
Sulforaphane, an aliphatic isothiocyanate, is a known cancer chemopreventive agent. Aiming to investigate antiangiogenic potential of sulforaphane, we here report a potent decrease of newly formed microcapillaries in a human in vitro angiogenesis model, with an IC50 of 0.08 μmol/L. The effects of sulforaphane on endothelial cell functions essential for angiogenesis were investigated in HMEC-1, an immortalized human microvascular endothelial cell line. Molecular signaling pathways leading to activation of endothelial cell proliferation and degradation of the basement membrane were analyzed by reverse transcription-PCR. Sulforaphane showed time- and concentration-dependent inhibitory effects on hypoxia-induced mRNA expression of vascular endothelial growth factor and two angiogenesis-associated transcription factors, hypoxia-inducible factor-1α and c-Myc, in a concentration range of 0.8 to 25 μmol/L. In addition, the expression of the vascular endothelial growth factor receptor KDR/flk-1 was inhibited by sulforaphane at the transcriptional level. Sulforaphane could also affect basement membrane integrity, as it suppressed transcription of the predominant endothelial collagenase matrix metalloproteinase-2 and its tissue inhibitor of metalloproteinase-2. Migration of HMEC-1 cells in a wound healing assay was effectively prevented by sulforaphane at submicromolar concentrations, and we determined an IC50 of 0.69 μmol/L. In addition, within 6 hours of incubation, sulforaphane inhibited tube formation of HMEC-1 cells on basement membrane matrix at 0.1, 1, and 10 μmol/L concentrations. These effects were not due to inhibition of HMEC-1 cell proliferation; however, after 72 hours of incubation, sulforaphane nonselectively reduced HMEC-1 cell growth with an IC50 of 11.3 μmol/L. In conclusion, we have shown that sulforaphane interferes with all essential steps of neovascularization from proangiogenic signaling and basement membrane integrity to endothelial cell proliferation, migration, and tube formation. These novel antiangiogenic activities of sulforaphane are likely to contribute to its cancer chemopreventive and therapeutic potential. [Mol Cancer Ther 2006;5(3):575–85]

Introduction
Sulforaphane [1-isothiocyanato-(4R)-(methylsulfinyl)butane: CH2S(O)(CH2)4-N = C = S] is a naturally occurring cancer chemopreventive isothiocyanate found as a glucosinolate precursor in cruciferous vegetables (Brassicaceae; ref. 1). In animal models, sulforaphane prevented 7,12-dimethylbenz[a]anthracene-induced preneoplastic lesions in mouse mammary glands (2) and rat mammary tumorigenesis (3). In addition, sulforaphane treatment inhibited azoxymethane-induced aberrant crypt foci in rat colon (4). Lately, sulforaphane was shown to retard the growth of PC-3 human prostate cancer xenografts in nude mice (5).

Sulforaphane acts through various chemopreventive mechanisms. (a) Sulforaphane modulates carcinogen metabolism by inhibition of phase 1 cytochrome P450 enzymes and benzo[a]pyrene-DNA binding (6). (b) Sulforaphane potently induces enzymes of phase 2 metabolism, including glutathione S-transferases, NAD(P)H:quinone oxidoreductase, UDP-glucuronosyl transferase, and thio- redoxin reductase, in various cancer cell lines and in vivo (2, 7–12). These gene products are regulated by the antioxidant response element and mediate detoxification and/or antioxidant function, thereby protecting cells from genotoxic damage. The transcription of antioxidant response element–driven genes is regulated, at least in part, by nuclear transcription factor Nrf2, which is sequenced in cytoplasm by Kelch-like ECH-associated protein 1. Exposure of cells to antioxidant response element inducers, including sulforaphane, results in the dissociation of Nrf2 from Kelch-like ECH-associated protein 1 and facilitates translocation of Nrf2 to the nucleus, where it binds to antioxidant response element, eventually resulting in the transcriptional regulation of target genes (reviewed in ref. 13). Apparently, this association is not so clear for human Kelch-like ECH-associated protein 1 (14). (c) Sulforaphane also exerts anti-inflammatory properties. Using lipopolysaccharide (LPS)–stimulated murine macrophages, we have described the down-regulation of LPS-mediated expression of inducible nitric oxide synthase (NOS), cyclooxygenase-2,
and tumor necrosis factor-α (15). The major mechanism of sulforaphane action was inhibition of nuclear factor-κB (NF-κB) binding to DNA presumably through modulation of intracellular redox conditions via dithiocarbamoylation of essential thiol groups involved in the activation of NF-κB. Sulforaphane-mediated inhibition of LPS-induced NF-κB-mediated transactivation was recently confirmed in HT-29 cells stably transfected with NF-κB luciferase constructs (16). (d) In addition, sulforaphane possesses antiproliferative and apoptosis-inducing properties as shown in various cancer cell lines and in vivo (17, 18).

Various chemopreventive agents have been shown to inhibit angiogenesis (i.e., the formation of new blood vessels from preexisting microvasculature; ref. 19). As inflammation may play a key role in angiogenesis (20), we were interested whether sulforaphane might possess antiangiogenic properties. Angiogenesis is a physiologic process relevant for tissue growth, remodeling, and wound healing but is also a prerequisite for tumor growth and metastasis (21). A microtumor requires an intact blood vessel system for supply with oxygen and nutrients and the possibility to shed its metabolites to grow beyond a critical size of 1 to 2 mm². Expression of persisting angiogenic activity was described as one of the earliest events in the transformation of a normal to a neoplastic cell (22). In 1971, Folkman first postulated inhibition of angiogenesis as an anticancer strategy (23); nowadays, antiangiogenic strategies are also regarded as a feasible mechanism in chemoprevention, turning cancer into a manageable chronic disease (24, 25).

The initiation of blood vessel formation is the result of a complex series of molecular events (Fig. 1). One of the central factors is the vascular endothelial growth factor (VEGF), which acts as a potent endothelial mitogen and stimulates endothelial cell survival, migration, differentiation, and self-assembly (26). On binding to its receptor, VEGF initiates a signal transduction cascade, which involves activation of multiple downstream protein kinase pathways (27). VEGF mRNA expression is regulated by several transcription factors, including hypoxia-inducible factor-1α (HIF-1α), c-Myc, and NF-κB, but proinflammatory and tumor-promoting mediators, including tumor necrosis factor-α, nitric oxide (released by NOS), prostaglandins (produced by cyclooxygenase-2), and polyamines (generated by ornithine decarboxylase), also contribute to VEGF transcription (27, 28). HIF-1 is an oxygen-dependent transcriptional activator, which plays an important role in gene expression required for angiogenesis, metabolic adaptation to low oxygen, and survival (29, 30). In the presence of oxygen, HIF-1α is readily degraded by the proteasome after post-transcriptional modification. Under hypoxic conditions, however, HIF-1α remains stable and translocates to the nucleus, where it forms heterodimers with constitutively expressed HIF-1β. Individual basic regions of the two subunits then make contact with their corresponding DNA sequence and interact with cofactor CBP/p300 and DNA polymerase II for the transcription of >60 target genes, including VEGF (31). The proto-oncogene c-Myc was recently identified as a master regulator of angiogenic factors essential for the proper expression of many components of the angiogenic network, including both positive (VEGF and angiopoietin-2) and negative (thrombospondin-1) cytokines (32). Interestingly, c-Myc-induced VEGF expression was enhanced in conjunction with hypoxia (33), and a direct correlation between c-Myc overexpression and high levels of VEGF was observed in vivo (34).

Endothelial cells play a crucial role in angiogenesis, bridging the gap between a microtumor and the essential

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**Figure 1.** Scheme of the angiogenic cascade, providing multiple targets for interference with chemopreventive and antiangiogenic agents. Inhibition of proinflammatory and tumor-promoting enzymes (ornithine decarboxylase (ODC), inducible NOS (iNOS), cyclooxygenase-2 (Cox-2), CYP1A1, mediators marked in bold) and/or NF-κB (NF-κB-dependent genes marked in gray) prevents alterations in gene expression of proangiogenic factors (VEGF, basic fibroblast growth factor (bFGF), and platelet-derived growth factor (PDGF)), which potentially stimulate the angiogenic cascade. Further downstream targets include receptor activation and subsequent signal transduction pathways (mitogen-activated protein kinase (MAPK) cascade, phosphatidylinositol 3-kinase (PI3K)-protein kinase B (PKB)/Akt signaling), degradation of the basement membrane by MMPs, endothelial cell proliferation, migration, and differentiation. Reproduced with permission from ref. 42.
Factors for tumor expansion. After mitogenic stimulation by a proangiogenic signal, they proliferate and migrate into the perivascular stroma, initiate capillary sprouting by forming capillary-like tubes, and thus supply a microtumor with essential nutrients and oxygen (35). Endothelial cell migration is controlled by the surrounding extracellular matrix. Therefore, endothelial cells produce type IV collagenase as well as other members of the matrix metalloproteinase (MMP) and serine protease family, which are essentially required for angiogenesis, tumor cell invasion, and metastasis (36, 37). Especially, MMP-2 (gelatinase A) is mostly expressed by microvascular endothelial cells of blood vessels within and surrounding the tumor. Production of MMP precursor enzymes is regulated at the transcriptional level, whereas activation of the proenzymes is tightly controlled by post-transcriptional mechanisms (38). An additional level of control is the interaction with endogenous inhibitory proteins, the tissue inhibitors of metalloproteinases (TIMP), which bind MMPs in a 1:1 stoichiometric fashion and reversibly inhibit MMP enzymatic activity (39).

In this report, we describe novel antiangiogenic properties of sulforaphane. These effects were detected in a human in vitro antiangiogenic assay. In addition, we investigated the influence of sulforaphane on hypoxia-stimulated mRNA expression of VEGF and its receptor KDR/fk-1, HIF-1α, c-Myc, MMP-2, and TIMP-2 in cultured human microvascular endothelial cells (HMEC-1). We further analyzed the effect of sulforaphane on essential endothelial cell functions of HMEC-1 cells, including migration, differentiation, and proliferation. We conclude that these novel antiangiogenic activities of sulforaphane are based on multiple interactions with the angiogenic cascade and might contribute to its chemopreventive and therapeutic potential.

Materials and Methods

Chemicals

All cell culture materials were obtained from Invitrogen (Eggenstein, Germany). Fetal bovine serum was provided by Pan (Aidenbach, Germany). L-arginine, caproic acid, aprotinin, sulforhodamine B, fibronogen, thrombin, and bovine type B gelatin were purchased from Sigma (Taufkirchen, Germany). Matrigel was obtained from BD Biosciences (Heidelberg, Germany). RNaseasy Mini kit, RNase-free Dnase set, and all designed primers were from Qiagen (Hilden, Germany). Moloney murine leukemia virus reverse transcriptase and random primers for the generation of cDNA were provided by Promega (Mannheim, Germany). Euro Taq polymerase was from BioCat (Heidelberg, Germany). RNase inhibitor and deoxynucleotide triphosphates were provided by Eppendorf (Hamburg, Germany). All materials and equipment for gel electrophoresis were purchased from Bio-Rad (Munich, Germany). All other chemicals were obtained from Sigma. Sulforaphane was synthesized as described earlier (15).

Cell Culture

Human microvascular endothelial cells (HMEC-1), estrogen receptor–negative mammary tumor cells (SK-BR3), human colon adenocarcinoma cells (HCT-116), and murine fibroblasts (NIH-3T3) were cultured as described previously (40).

Human In vitro Antiangiogenesis Assay

The assay is based on the culture of human placental blood vessels in fibrin gels described by Brown et al. (41). In brief, superficial vessels of human placentas were cut to fragments of 1 to 2 mm long and embedded in a fibrin gel (1 mL) containing 0.5 unit thrombin, 0.3% fibrinogen, and 5 μg/mL aprotinin in 24-well plates. The gel was overlaid with 1 mL medium mix consisting of 1 part endothelial basal medium MCDB 131 supplemented with 10 mmol/L l-glutamine and 1 part Medium 199 containing 100 units/mL penicillin G sodium, 100 units/mL streptomycin sulfate, and 250 ng/mL amphotericin B and supplemented with 0.1% L-arginine caproic acid and 20% heat-inactivated fetal bovine serum, which was changed twice weekly. The vessels were cultured at 37°C in a humidified 5% CO2 environment for 3 weeks. Resveratrol (1 μmol/L) was used as a positive control. Sulforaphane was dissolved and diluted in 100% DMSO to a final concentration of 0.01 to 20 mmol/L and added to the medium (1 μL/mL, 0.1% final DMSO concentration). Each experiment was repeated at least twice with placentas from different donors. For analysis of microvessel density (MVD), standardized digital images were acquired with a color digital microscopic camera system (Leitz Diavert microscope, Leica, Bensheim, Germany; AxioCam, Carl Zeiss, Göttingen, Germany) with a resolution of 1,300 × 1,030 pixel at ×32 magnification and processed with Axiovision Release 3.1 software package (Carl Zeiss). The measurement of MVD (mm²) was carried out using Adobe Photoshop 7.0 with histogram function to obtain the pixel area of newly formed capillaries in relation to the overall number of pixels in the taken picture, which was then converted to mm². Results are mean ± SD of data originated from three independent experiments (42).

Inhibition of Cell Proliferation

Inhibition of cell proliferation of HMEC-1, SK-BR3, HCT-116, and NIH-3T3 cells as well as flow cytometric analyses of sulforaphane-treated HMEC-1 cells were tested as described previously (40). The influence of sulforaphane on HMEC-1 cell proliferation in assays for endothelial cell functions was also assessed as described before (40).

Endothelial Cell Migration and Tube Formation

HMEC-1 cell migration as well as the formation of capillary-like structures on a basement membrane preparation were measured as described previously (40).

Reverse Transcription-PCR

Total RNA from 3 × 10⁵ HMEC-1 cells (treated as indicated in figure legends) was isolated using Qiagen RNeasy Mini kits for total RNA extraction according to the manufacturer’s manual and treated with Dnase I before use. Experiments under hypoxic conditions were done using chambers for anaerobe bacterial culture (Merck,
Novel Antiangiogenic Properties of Sulforaphane

Darmstadt, Germany). Hypoxia was monitored by indicator sticks and determined as \( pO_2 \leq 3 \text{ mm Hg} \). RNA (0.5 \( \mu \text{g} \)) was transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase and random hexamer primers. Specific primers were designed using the Heidelberg Unix Sequence Analysis Resources computer system at the German Cancer Research Center (Heidelberg, Germany; Table 1). For amplification of cDNA fragments, PCR conditions were 94°C for 5 minutes followed by the indicated number of cycles. Cycling conditions were 94°C for 1 minute followed by 1 minute at the indicated annealing temperature and 72°C for 1 minute. The program was terminated with a 7-minute extension interval at 72°C.

Reaction conditions were optimized for each primer pair. PCR products were separated on 1.8% agarose gels and visualized by ethidium bromide staining. For quantification of mRNA expression, densitometric scans of ethidium bromide-stained gels were acquired using a Herolab EASY RH-3 densitometer (Herolab GmbH, Wiesloch, Germany) with EasyWin 32 software and semiquantitatively evaluated using TINA software version 2.09a (Raytest Isotopenmessgeräte GmbH, Staubenhardt, Germany). Staining intensities were normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA expression, background staining was subtracted, and values were expressed as percentage of induced expression in comparison with maximum control values.

**Gelatin Zymography**

The presence of secreted MMP-2 activity in conditioned medium of HMEC-1 cells was analyzed by gelatin zymography (43). HMEC-1 cells were cultured in serum-free MCDB 131 endothelial basal medium supplemented as described above at 1.8 \( \times 10^6 \) cells/mL/well in 24-well plates. Aliquots from cell culture supernatants were collected in a time- and concentration-dependent manner as indicated in the figure legends, centrifuged for 10 minutes at 2,000 rpm, and sterile filtered. Protein separation was done by electrophoresis on a 7.5% SDS-polyacrylamide gel containing 0.1% gelatin under nonreducing conditions. For molecular weight estimations, individual bands of a prestained protein standard mix (Bio-Rad) were detected and identified by their unique color. After electrophoresis, gels were soaked in 2.5% Triton X-100 for 1 hour and then incubated in renaturation buffer composed of 50 mmol/L Tris-HCl (pH 7.6), 15 mmol/L CaCl\(_2\), 150 mmol/L NaCl, and 0.2% Brij 35 for 48 hours. After staining with 0.1% Coomassie brilliant blue R250 in water, ethanol, acetic acid (55 + 45 + 10) for 20 minutes, gels were destained in water, methanol, acetic acid (80 + 10 + 10) to reveal clear areas corresponding to protein bands with gelatinolytic (i.e., metalloproteinases) activity. Densitometric scans were acquired as described above.

**Statistical Analysis**

Results are mean \( \pm \) SD of data originated from three independent experiments unless stated otherwise. For statistical evaluation Student’s \( t \) test was applied. For the endothelial cell migration assay, paired Student’s \( t \) test was done comparing the migration area after 18 hours to time 0. \( P < 0.05 \) was considered as statistically significant and \( P < 0.005 \) was considered as highly significant.

**Results**

Angiogenesis is a multistep process that offers various targets for intervention: (a) inhibition of release of angiogenic factors or neutralization of released angiogenic mediators; (b) inhibition of synthesis and turnover of vessel basement membrane; and (c) inhibition of vascular endothelial cell migration, proliferation, migration, and differentiation (44).

**Antiangiogenic Activity in the Human In vitro Antiangiogenesis Model**

Based on these strategies, we have established a human in vitro angiogenesis assay, which covers multiple steps of the angiogenic process and is sensitive to known antiangiogenic compounds as well as selected chemopreventive agents (42). Placental vessel fragments were cultured on fibrin gels in the presence or absence of various

### Table 1. Primer sequences for cDNA amplification of selected human genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genbank accession no.</th>
<th>Sequence</th>
<th>( T_m ) (°C)</th>
<th>No. cycles</th>
<th>Size (bp)</th>
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<td>vegf</td>
<td>NM_003376</td>
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<td>40</td>
<td>196</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse 5'-GAAGGCTATCTCTCTGATGTGCAGC-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hif-1a</td>
<td>NM_001530</td>
<td>Forward 5'-CCTGACCCTAATGCCCCAGTGG-3'</td>
<td>64</td>
<td>33</td>
<td>215</td>
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<td>Reverse 5'-GGTGCAACCTGATCGGAAAGCACG-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c-myc</td>
<td>BC008686</td>
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<td>24</td>
<td>226</td>
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<td></td>
<td></td>
<td>Reverse 5'-CTGAGGTTGGTCTACATCGGAAAGCC-3'</td>
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<td></td>
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</tr>
<tr>
<td>kdr</td>
<td>NM_002253</td>
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<td>793</td>
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<tr>
<td></td>
<td></td>
<td>Reverse 5'-CCTGTGGATACACTTTCGCGAT-3'</td>
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<td></td>
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<tr>
<td>mmp-2</td>
<td>NM_004530</td>
<td>Forward 5'-CTATGAGCCTGACACCTAGT-3'</td>
<td>63.5</td>
<td>24</td>
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<td></td>
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<td>Reverse 5'-GAAAGTGAAGGGGAAGACACAG-3'</td>
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<tr>
<td>timp-2</td>
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<td>gapdh</td>
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<td>28</td>
<td>595</td>
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<td></td>
<td></td>
<td>Reverse 5'-GTGTGGCTTGGTAAAGGACGAGG-3'</td>
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Table 2. Inhibitory effects of sulforaphane in the human in vitro antiangiogenesis assay

<table>
<thead>
<tr>
<th>Conc (μmol/L)</th>
<th>Control (mm²)</th>
<th>Sulforaphane (mm²)</th>
<th>P, Student’s t test* (n = 3)</th>
<th>Relative MVD † (% control)</th>
<th>Grand mean † (% inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0.211 ± 0.049</td>
<td>0.007 ± 0.002</td>
<td>&lt;0.001</td>
<td>3.3 ± 1.6</td>
<td>96.9 ± 0.4</td>
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<tr>
<td>20</td>
<td>0.198 ± 0.008</td>
<td>0.005 ± 0.001</td>
<td>&lt;0.001</td>
<td>2.7 ± 0.5</td>
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<tr>
<td>20</td>
<td>0.189 ± 0.017</td>
<td>0.007 ± 0.001</td>
<td>&lt;0.001</td>
<td>3.3 ± 0.6</td>
<td></td>
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<tr>
<td>10</td>
<td>0.155 ± 0.010</td>
<td>0.030 ± 0.006</td>
<td>&lt;0.001</td>
<td>19.6 ± 3.8</td>
<td>77.0 ± 6.1</td>
</tr>
<tr>
<td>10</td>
<td>0.235 ± 0.033</td>
<td>0.046 ± 0.002</td>
<td>&lt;0.001</td>
<td>19.4 ± 5.4</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.232 ± 0.055</td>
<td>0.070 ± 0.013</td>
<td>&lt;0.001</td>
<td>30.0 ± 5.6</td>
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<tr>
<td>1</td>
<td>0.155 ± 0.020</td>
<td>0.039 ± 0.005</td>
<td>&lt;0.001</td>
<td>25.0 ± 2.2</td>
<td>68.0 ± 10.4</td>
</tr>
<tr>
<td>1</td>
<td>0.146 ± 0.017</td>
<td>0.066 ± 0.005</td>
<td>&lt;0.001</td>
<td>44.0 ± 5.0</td>
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<tr>
<td>1</td>
<td>0.158 ± 0.024</td>
<td>0.063 ± 0.006</td>
<td>&lt;0.001</td>
<td>27.1 ± 1.6</td>
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<tr>
<td>0.1</td>
<td>0.904 ± 0.034</td>
<td>0.267 ± 0.019</td>
<td>&lt;0.001</td>
<td>29.6 ± 2.1</td>
<td>59.5 ± 12.4</td>
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<tr>
<td>0.1</td>
<td>0.757 ± 0.041</td>
<td>0.285 ± 0.013</td>
<td>&lt;0.001</td>
<td>37.8 ± 3.0</td>
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<tr>
<td>0.1</td>
<td>0.672 ± 0.015</td>
<td>0.363 ± 0.025</td>
<td>&lt;0.001</td>
<td>54.0 ± 2.6</td>
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<tr>
<td>0.01</td>
<td>0.250 ± 0.047</td>
<td>0.240 ± 0.020</td>
<td>0.432</td>
<td>98.8 ± 19.8</td>
<td>5.5 ± 6.7</td>
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<tr>
<td>0.01</td>
<td>0.236 ± 0.015</td>
<td>0.222 ± 0.019</td>
<td>0.269</td>
<td>94.3 ± 11.2</td>
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<tr>
<td>0.01</td>
<td>0.251 ± 0.010</td>
<td>0.219 ± 0.033</td>
<td>0.700</td>
<td>87.3 ± 14.6</td>
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</table>

NOTE: Resveratrol, used as a positive control, showed a mean inhibitory effect of 51.9 ± 4.9% at a 1 μmol/L concentration.

*For statistical analysis, Student’s t test was done. Differences at P ≤ 0.001 (n = 3) were considered as statistically significant.

†From three experiments, a grand mean ± SD of relative MVD was computed and expressed as percentage of inhibition in comparison with the control group.

cConcentrations of sulforaphane for 3 weeks. We have shown previously that, in the control group, microcapillaries were first detectable after ~7 days of culture and continued to grow until day 21 (42). We observed a donor-dependent variation in MVD of control cultures ranging from 0.146 ± 0.017 to 0.904 ± 0.034 mm² (Table 2). Sulforaphane treatment caused a dose-dependent inhibition of microcapillary growth in comparison with solvent-treated controls in a concentration range of 0.01 to 20 μmol/L. Sulforaphane at 20 μmol/L concentration almost completely prevented microvessel outgrowth, whereas, at 0.1 μmol/L concentration, MVD was still >50% inhibited, and we determined an IC50 of 0.08 ± 0.01 μmol/L.

Influence of Sulforaphane on Hypoxia-Induced Proangiogenic Signaling in HMEC-1 Cells

For investigations of sulforaphane effects on hypoxia-induced gene expression, we used the human microvascular endothelial cell line HMEC-1. Except for constitutive expression of interleukin-6 and granulocyte macrophage colony-stimulating factor absent in primary cells, HMEC-1 cells exhibit major constitutive and inducible morphologic, phenotypic, and functional endothelial cell characteristics, including cobblestone morphology, von Willebrand factor, and factor VIII expression (35) and were therefore selected as a suitable in vitro model.

First, we determined the mRNA expression of VEGF and its transcription factors HIF-1α and c-Myc by reverse transcription-PCR after keeping HMEC-1 cells under hypoxic conditions for up to 24 hours. Because VEGF receptors are mainly expressed on endothelial cells (45), we added KDR/flk-1, a high-affinity receptor tyrosine kinase (VEGF receptor-2), to our investigations. It has been suggested that up-regulation of the KDR gene might be required for endothelial cells to respond to VEGF, whereas other reports indicate KDR mRNA was not directly induced in human umbilical vascular endothelial cells or microvascular endothelial cells under hypoxic conditions (46).

In uninduced HMEC-1 cells, VEGF transcript levels were very low, estimated by weak bands after extensive PCR amplification (Fig. 2A). Under hypoxia, mRNA levels increased continuously up to 12 hours. A similar pattern of induction was observed for HIF-1α mRNA expression, which reached a maximum of expression after 24 hours. In contrast, c-Myc and KDR mRNA expression responded very rapidly to hypoxic treatment, and maximum mRNA levels were detected after 2 hours. Treatment of HMEC-1 cells with sulforaphane at a 10 μmol/L concentration reduced the hypoxia-mediated induction of VEGF mRNA levels by ~50% up to 12 hours of incubation in comparison with the control, whereas, at longer incubation times, transcript level declined in both controls and sulforaphane-treated cells. Although HIF-1α protein expression is mainly regulated at the post-transcriptional level (31), sulforaphane treatment led to a reduction of hypoxia-induced HIF-1α mRNA levels. Strongest effects were seen at earlier time points up to 9 hours. Sulforaphane strongly suppressed c-Myc mRNA induction by ~80% at all time points investigated, whereas its effects on KDR mRNA levels were most pronounced after short-term hypoxic conditions (2–6 hours of treatment; Fig. 2B). In accordance with earlier observations in LPS-stimulated Raw macrophages (15), ornithine decarboxylase mRNA expression, which was rapidly induced in HMEC-1 cells by hypoxia with a maximum at 6 hours, was not inhibited by sulforaphane at a 10 μmol/L concentration (data not shown).

Dose-dependent inhibition was analyzed after 12 hours of incubation under hypoxic conditions. Sulforaphane
potently reduced hypoxia-induced levels of c-Myc, KDR, and VEGF mRNA by >50% at concentrations above 1.56 μmol/L, whereas its effect on HIF-1α mRNA induction was slightly less pronounced, with ~50% reduction at 6.25 μmol/L concentration (Fig. 2C and D). Sulforaphane also very potently reduced the hypoxia-mediated induction of inducible NOS mRNA levels by >50% at a concentration of 0.4 μmol/L (data not shown).

**Sulforaphane-Mediated Effects on Basement Membrane Modulators**

In a recent study with primary human aortic endothelial cells, short-term chronic exposure to hypoxic conditions resulted in an up-regulation of MMP-2 and TIMP-2 mRNA detected after 8 and 24 hours of treatment (47). In HMEC-1 cells, we observed a differential profile of mRNA expression: a maximum of TIMP-2 mRNA expression was seen after 2 hours of hypoxia, which returned to background levels after 12 hours, whereas MMP-2 mRNA levels slowly increased up to 24 hours (Fig. 3A). Inhibitory effects of sulforaphane at a 10 μmol/L concentration were weak. For MMP-2 mRNA levels, we observed stronger inhibition after 6 hours than after 12 or 24 hours of hypoxia. TIMP-2 mRNA expression was reduced by ~20% to 30% after 2 to 9 hours of incubation (Fig. 3B). Concentration-dependent effects were investigated after exposing HMEC-1 cells to hypoxia for 12 hours. At a 12.5 μmol/L concentration, sulforaphane lowered steady-state levels of MMP-2 mRNA to ~50%, whereas lower concentrations were ineffective. TIMP-2 mRNA expression was reduced by 50% at concentrations above 6.25 μmol/L (Fig. 3C and D).

In addition to the expression at the transcriptional level, we analyzed gelatinolytic activity of MMP-2 by SDS-PAGE gelatin zymography in conditioned cell culture supernatants of HMEC-1 cells grown under serum-free conditions. Interestingly, serum withdrawal alone resulted in a continuous increase in MMP-2 activity. Marginal activity was detectable after 4 and 8 hours, and maximal effects were seen after 24 hours of serum withdrawal, which was only partly inhibited by sulforaphane at a 10 μmol/L concentration (Fig. 4A). Consequently, in a concentration range of 0.78 to 25 μmol/L, only the highest sulforaphane concentrations weakly inhibited the induction of MMP-2 activity (Fig. 4B).

**Inhibition of Endothelial Cell Proliferation**

Vasculature in normal adults is generally quiescent, with only 0.01% of endothelial cells undergoing cell division at any given time (48). To investigate whether sulforaphane was able to inhibit endothelial cell proliferation, HMEC-1 cells were cultured in 96-well plates and incubated with sulforaphane for 72 hours in a concentration range of 0.4 to 50 μmol/L. HMEC-1 cell proliferation was inhibited with an IC50 of 11.3 μmol/L (Fig. 5A). Inhibition of cell growth was not selective for
endothelial cells. Sulforaphane also dose-dependently inhibited the proliferation of two epithelial cell lines [i.e., Her-2-overexpressing estrogen receptor–negative SK-BR3 human breast cancer cells (IC$_{50}$ 12.7 μmol/L) and mismatch repair–deficient HCT-116 human colorectal cancer cells (IC$_{50}$ 8.2 μmol/L)] as well as NIH-3T3 murine fibroblasts (IC$_{50}$ 11.9 μmol/L) in a similar concentration range.

To closer investigate the potential mechanism of anti-proliferative activity in HMEC-1 cells, alterations in cell cycle distribution of unsynchronized HMEC-1 cells treated for 24 and 48 hours with sulforaphane were monitored using flow cytometry. As indicated by a sub-G$_1$ peak, sulforaphane treatment at 12.5, 25, and 50 μmol/L concentrations dose-dependently caused a weak induction of apoptosis, which was more prominent after 48 hours than after 24 hours. In contrast to published data in human cancer cell lines, sulforaphane did not arrest HMEC-1 cells in G$_2$–M phase of the cell cycle before induction of apoptosis was observed (Fig. 5B).

**Influence of Sulforaphane on Endothelial Cell Functions**

Proliferation, migration, and tubular formation are essential characteristics of endothelial cells for the generation of new blood vessels. To analyze the effect of sulforaphane on HMEC-1 cell migration, we did a wound healing assay. Confluent monolayers of HMEC-1 cells were disrupted (i.e., wounded) mechanically by scraping them with a pipette tip. Solvent controls reformed a confluent monolayer within 18 hours of incubation. Sulforaphane was tested in a concentration range of 0.1 to 10 μmol/L (Fig. 6A). We detected potent dose-dependent inhibition of HMEC-1 cell migration and determined an IC$_{50}$ of 0.69 ± 0.03 μmol/L (Fig. 6B). Within the observation period of 18 hours, sulforaphane did not influence proliferation of HMEC-1 cells in the concentration range of 0.1 to 10 μmol/L (data not shown).

Similar to primary microvascular endothelial cells, HMEC-1 cells have been shown to form cord-like structures when cultured on Matrigel (35). Untreated controls arranged in a complex network of tubes after a 6-hour incubation period (Fig. 6C). At a 10 μmol/L concentration, sulforaphane reduced tube formation by >80%. Cells treated with 1 μmol/L sulforaphane showed a similar pattern with incomplete tube formation of ~50%. Partial differentiation was observed with 0.1 μmol/L sulforaphane, whereas, at 0.01 μmol/L, no inhibitory activity was detectable, resulting in an extensive tubular network after 6 hours. Within this incubation period, sulforaphane did not influence cell proliferation of HMEC-1 cells in a concentration range of 0.1 to 10 μmol/L (determined in cell culture–coated 96-well microplates without preincubation), indicating that the observed effects were not due to antiproliferative effects of sulforaphane under the experimental conditions.

**Discussion**

Here, we provide first evidence that sulforaphane exerts antiangiogenic properties that could contribute to its cancer chemopreventive and therapeutic potential.

In a human in vitro antiangiogenesis assay, treatment of placental vessel fragments with sulforaphane for 3 weeks potently inhibited angiogenic capillary growth at physiologically relevant concentrations (Table 2).

![Figure 3](image-url)

**Figure 3.** Influence on hypoxia-inducible basement membrane modulators. Time course: A, HMEC-1 cells were treated with DMSO (−) or 10 μmol/L sulforaphane (+) and exposed to hypoxia for 2 to 24 h. mRNA levels were investigated using reverse transcription-PCR. B, semiquantitative analysis of mRNA expression of MMP-2 and TIMP-2, normalized to glyceraldehyde-3-phosphate dehydrogenase, and displayed as a percentage of induced expression in comparison with control values at the transcriptional maximum set as 100%. Stippled columns, sulforaphane treatment. Dose response: C, HMEC-1 cells were treated with DMSO (−) or sulforaphane in a concentration range of 0.78 to 25 μmol/L, respectively, and exposed to hypoxia (+) for 12 h. D, semiquantitative analysis mRNA levels of MMP-2 and TIMP-2 in the presence of sulforaphane, normalized to glyceraldehyde-3-phosphate dehydrogenase, and shown as a percentage of induced expression in comparison with the DMSO control treated with LPS for 12 h, which was set as 100%.
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Under physiologic conditions, isothiocyanates like sulforaphane react rapidly with cysteinyl thiol groups to reversibly form S-thiocarbamoylcysteine derivatives. As an example, interactions of isothiocyanates with sulfhydryl groups of Kelch-like ECH-associated protein 1 are regarded as an important sensor regulating the induction of phase 2 enzymes that protect against carcinogens and oxidants (13). We have reported earlier that sulforaphane prevented active NF-κB from binding to its nuclear DNA site via a thiol-mediated mechanism presumably by dithiocarbamoylation of NF-κB subunits (15). Recently, we could further show that sulforaphane modulates the redox-regulating system composed of thioredoxin and thioredoxin reductase (E.C. 1.8.1.9), a selenocysteine-containing oxidoreductase that catalyzes the NADPH-dependent reduction of thioredoxin. Sulforaphane was shown to inhibit thioredoxin reductase after short-term incubation.

The model covers multiple steps relevant of angiogenesis, including (a) production of growth factors; (b) activation of endothelial cells; (c) production of lytic enzymes to digest the basement membrane and extracellular matrix; and (d) endothelial cell migration, proliferation, and tube formation. Angiogenesis is induced by mechanical damage to the vessels. We have previously identified sulforaphane as a very potent inhibitor of NF-κB-mediated expression of inducible NOS and cyclooxygenase-2 (15). Because nitric oxide and prostaglandins contribute to the expression of VEGF (27), we assume that part of the sulforaphane-mediated inhibitory potential in this model is based on the inhibition of the proangiogenic stimulus triggered by the mechanical stress caused by the preparation of the vessel fragments. In addition, Xu et al. have recently reported that sulforaphane inhibited NF-κB-mediated VEGF expression in human prostate cancer PC-3 C4 cells (49).

Besides the expression of proinflammatory mediators (50), prolonged or severe hypoxia resulting from an inadequate oxygen supply caused by the proliferating tumor cell mass is one of the most important signals in vivo for the induction of proangiogenic genes to regain normoxia. Therefore, we analyzed the influence of sulforaphane on hypoxia-induced transcription of proangiogenic factors in human endothelial cells. Hypoxic or hyperoxic stress can activate or repress the transcription of genes through redox-sensitive transcription factors. It is known that distinct sulfhydryl residues play an essential role in the activation or inactivation of transcription (51–53). DNA binding of activator protein-1, Sp-1, NF-κB, c-Myb, p53, etc., is reduced or lost when critical cysteine residues are oxidized or alkylated (ref. 54 and references therein). In addition, inducible activation of HIF-1α in response to hypoxia is regulated by a thiol-dependent pathway: it was shown that a cysteine residue in the COOH-terminal transactivation domain is modified by the redox regulators Ref-1 and thioredoxin to interact with coactivators like CBP/p300 and enhance transcription. Replacement of the essential cysteine residue with a serine completely abolished transactivation activity (55).

**Figure 4.** Inhibition of MMP-2 gelatinolytic activity. Time course: A, HMEC-1 cells were treated with DMSO (−) or 10 μmol/L sulforaphane (+) and exposed to serum-free conditions for 4 to 24 h. MMP-2 gelatinolytic activity was investigated using gelatin zymography. Dose response: B, HMEC-1 cells were treated with DMSO (−) or sulforaphane in a concentration range of 0.78 to 25 μmol/L, respectively, and exposed to serum-free conditions for 24 h.

**Figure 5.** Inhibition of endothelial cell proliferation in comparison with human cancer cell lines. Cytotoxic effects: A, HMEC-1 (●), SK-BR3 (▲), HCT-116 (▽), or NIH-3T3 (□) cells were incubated for 72 h in 96-well plates in the presence or absence of sulforaphane. IC_{50}s were generated from six serial 2-fold dilutions in a concentration range of 1.6 to 50 μmol/L tested in duplicates. Points, mean of two (SK-BR3 and HCT-116) to three (HMEC-1 and NIH-3T3) independent experiments; bars, SD. Cell cycle distribution: B, flow cytometric analysis of HMEC-1 cells treated with sulforaphane at 12.5 □, 25 □, and 50 □ μmol/L concentrations in comparison with a DMSO control (□). *, P < 0.05; **, P < 0.005, significantly different from control after 24 h (top) and 48 h (bottom) of incubation using Student’s t test (n = 2–4).
This might transiently influence the intranuclear redox potential to disfavor DNA binding of NF-κB and potentially of other redox-responsive transcription factors (56). Consequently, thiol- or redox-regulated mechanisms, including HIF-1 transactivation, involved in proangiogenic signaling under hypoxic conditions might be sensitive to sulforaphane treatment. This could influence not only VEGF mRNA expression (ref. 57; Fig. 2) but also the expression of other HIF-1-regulated genes (summarized in refs. 30, 31), including inducible NOS, cyclooxygenase-2, thioredoxin, c-Jun, c-Fos, HIF-1α itself (Fig. 2), and MMP-2 (Fig. 3). Further experiments have to verify this concept.

Sulforaphane treatment also potently inhibited mRNA expression of c-Myc, which was rapidly enhanced after exposing HMEC-1 cells to hypoxia. Thus, sulforaphane could influence transcription of VEGF also indirectly via inhibition of c-Myc mRNA induction. Knies-Bamforth et al. used an elegant model with c-MycER<sup>TAM</sup> transgenic mice to induce hyperplastic and dysplastic precancerous skin lesions by activation of the c-MycER<sup>TAM</sup> transgene (33). Papilloma formation was accompanied by angiogenesis, and the authors could show that VEGF played a crucial role in mediating these effects. Notably, sulforaphane was recently shown to potentely prevent chemically induced mouse skin carcinogenesis, especially during the promotion stage (58). It is tempting to speculate that the antiangiogenic mechanisms described here might contribute to this cancer preventive effect.

We used a series of test systems to investigate the role of sulforaphane on endothelial cell properties. An imbalance of proangiogenic and antiangiogenic factors leads to an up-regulation of endothelial cell survival accompanied by a significant decrease of apoptotic cells. Sulforaphane dose-dependently inhibited the proliferation of HMEC-1 cells after an incubation time of 72 hours. By comparison with additional cancer cell lines, we confirmed earlier reports indicating that the antiproliferative activity of sulforaphane was not specific for endothelial cells (59). By flow cytometry, we observed a time- and concentration-dependent increase in a sub-G<sub>1</sub> peak indicative of apoptosis induction in HMEC-1 cells (Fig. 5). Sulforaphane was described previously as an inducer of apoptosis in colon cancer cell lines acting by induction of Bax, a proapoptotic molecule, disruption of the mitochondrial membrane potential, and release of cytochrome <em>c</em>, whereas Bcl-2, an antiapoptotic factor, remained unchanged (60). In prostate cancer cells, sulforaphane activated the caspase cascade and caused an irreversible G<sub>2</sub>-M arrest (18). Further studies have to elucidate the apoptosis-inducing mechanisms in HMEC-1 cells.

Notably, endothelial cell migration and differentiation on Matrigel were also potently inhibited by sulforaphane (Fig. 6). These activities were not due to the antiproliferative influence of sulforaphane, because, under the experimental conditions used in these systems, cell proliferation was not impeded. The activation of endothelial cells is closely associated with degradation of the basement membrane following growth factor–mediated stimulation, representing an essential step and target within the angiogenic cascade. Type IV collagenases MMP-2 and MMP-9 may be critical in the digestion of basement membrane and the migration of endothelial cells from the existing blood vessels (38, 61). Expression of MMPs is normally low and is induced when remodeling of extracellular matrix is required. Cleavage of collagen type IV by MMP-2 exposes a cryptic, α<sub>5</sub>β<sub>1</sub> integrin-binding site within the collagen. Blockage of this new site with an
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antibody decreased migration of endothelial cells and in vitro angiogenesis and reduced tumor growth in animal models (36). We could show a weak inhibition of MMP-2 activity after serum withdrawal (Fig. 4). This inhibition is most likely mediated at the transcriptional level (Fig. 3), but we cannot exclude a direct inhibition of zinc-dependent gelatinolytic activity. Inhibition or down-regulation of MMP-2 activity might contribute to the observed inhibitory effects of sulforaphane on endothelial cell differentiation.

In conclusion, our investigations have revealed novel antiangiogenic properties of sulforaphane based on multiple interactions with critical steps in the angiogenic cascade. VEGF expression stimulated by HIF-1 and c-Myc, respectively, as well as endothelial cell migration and differentiation represent important targets of sulforaphane action. These antiangiogenic properties not only might be relevant for the effects of sulforaphane in cancer prevention but also might contribute to its cancer therapeutic efficacy that is presently emerging (5, 62).

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