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* antiangiogenesis model, with an IC_{50} 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 IC_{50} 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 IC_{50} 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-(4*R*)-(methylsulfinyl)-butane: $CH_3S(O)(CH_2)_4\text{-}N = C = S$] 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 *P*450 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 thioredoxin 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 sequestered 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 mm^2. 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
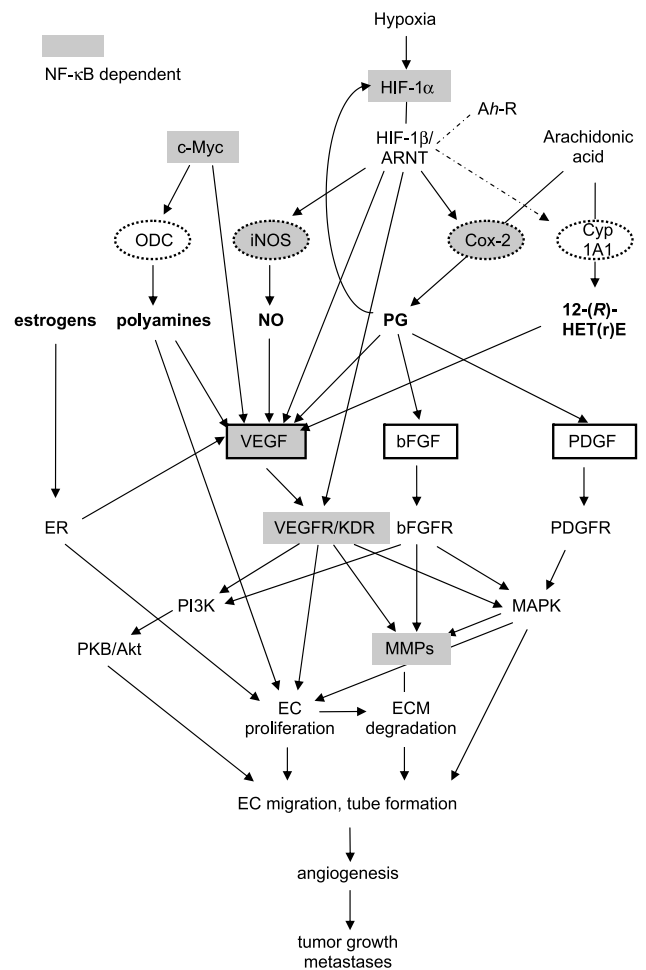
Figure 1. Scheme of the angiogenic cascade, providing multiple targets for interference with chemopreventive and antiangiogenic agents. Inhibition of proinflammatory and tumor-promoting enzymes [ornithine decarboxylase (*ODC*), inducible NOS (*iNOS*), cyclooxygenase-2 (*Cox-2*), CYP1A1, mediators marked 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.

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

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/flk-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). ε-Amino-caproic acid, aprotinin, sulforhodamine B, fibrinogen, thrombin, and bovine type B gelatin were purchased from Sigma (Taufkirchen, Germany). Matrigel was obtained from BD Biosciences (Heidelberg, Germany). RNeasy 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% ε-amino-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% CO_2 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 thrice 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 (mm^2) 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 mm^2. 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 × 10^5 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,

Darmstadt, Germany). Hypoxia was monitored by indicator sticks and determined as $pO_2 \leq 3$ mm Hg. RNA (0.5 μg) 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 1.8×10^6 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 nonre-ducing 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 ± 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 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* antiangiogenesis 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

Gene	Genbank accession no.	Sequence	T_m (°C)	No. cycles	Size (bp)
vegf	NM_003376	Forward 5′-CCTGGTGGACATCTTCCAGGAGTACC-3′ Reverse 5′-GAAGCTCATCTCTCCTATGTGCTGGC-3′	57	40	196
hif-1α	NM_001530	Forward 5′-CCTGAGCCTAATAGTCCCAGTG-3′ Reverse 5′-GGTGACAACTGATCGAAGGAACG-3′	64	33	215
c-myc	BC008686	Forward 5′-ACGCTGACCAAGGTGTTGGTAG-3′ Reverse 5′-CTGAGGTGGTTCATACTGAGCAAG-3′	64	26	226
kdr	NM_002253	Forward 5′-GGAAATCATTATTCTAGTAGGCACGACG-3′ Reverse 5′-CCTGTGGATACACTTTCGCGAT-3′	55	29	793
mmp-2	NM_004530	Forward 5′-CTATGACAGCTGCACCACTGAG-3′ Reverse 5′-GAAAGTGAAGGGGAAGACACAG-3′	63.5	24	163
timp-2	NM_003255	Forward 5′-GGCAGTGTGTGGGGTCTC-3′ Reverse 5′-TCTTCTGGGTGGTGCTCAG-3′	62.2	33	140
gapdh	NM_002046	Forward 5′-CTGAGTACGTCGTGGAGTCCACTG-3′ Reverse 5′-GTGTCGCTGTTGAAGTCAGAGGAG-3′	55	28	595

Table 2. Inhibitory effects of sulforaphane in the human *in vitro* antiangiogenesis assay

Conc (μmol/L)	MVD		*P*, Student's *t* test* (*n* = 3)	Relative MVD [†] (% control)	Grand mean [†] (% inhibition)
	Control (mm^2)	Sulforaphane (mm^2)			
20	0.211 ± 0.049	0.007 ± 0.002	<0.001	3.3 ± 1.6	96.9 ± 0.4
20	0.198 ± 0.008	0.005 ± 0.001	<0.001	2.7 ± 0.5	
20	0.189 ± 0.017	0.007 ± 0.001	<0.001	3.3 ± 0.6	
10	0.155 ± 0.010	0.030 ± 0.006	<0.001	19.6 ± 3.8	77.0 ± 6.1
10	0.235 ± 0.033	0.046 ± 0.002	<0.001	19.4 ± 5.4	
10	0.232 ± 0.055	0.070 ± 0.013	<0.001	30.0 ± 5.6	
1	0.155 ± 0.020	0.039 ± 0.005	<0.001	25.0 ± 2.2	68.0 ± 10.4
1	0.146 ± 0.017	0.066 ± 0.005	<0.001	44.0 ± 5.0	
1	0.158 ± 0.024	0.063 ± 0.006	<0.001	27.1 ± 1.6	
0.1	0.904 ± 0.034	0.267 ± 0.019	<0.001	29.6 ± 2.1	59.5 ± 12.4
0.1	0.757 ± 0.041	0.285 ± 0.013	<0.001	37.8 ± 3.0	
0.1	0.672 ± 0.015	0.363 ± 0.025	<0.001	54.0 ± 2.6	
0.01	0.250 ± 0.047	0.240 ± 0.020	0.432	98.8 ± 19.8	5.5 ± 6.7
0.01	0.236 ± 0.015	0.222 ± 0.019	0.269	94.3 ± 11.2	
0.01	0.251 ± 0.010	0.219 ± 0.033	0.700	87.3 ± 14.6	

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 $P \leq 0.001$ (*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.

concentrations 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 growth 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 mm^2 (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 IC$_{50}$ 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
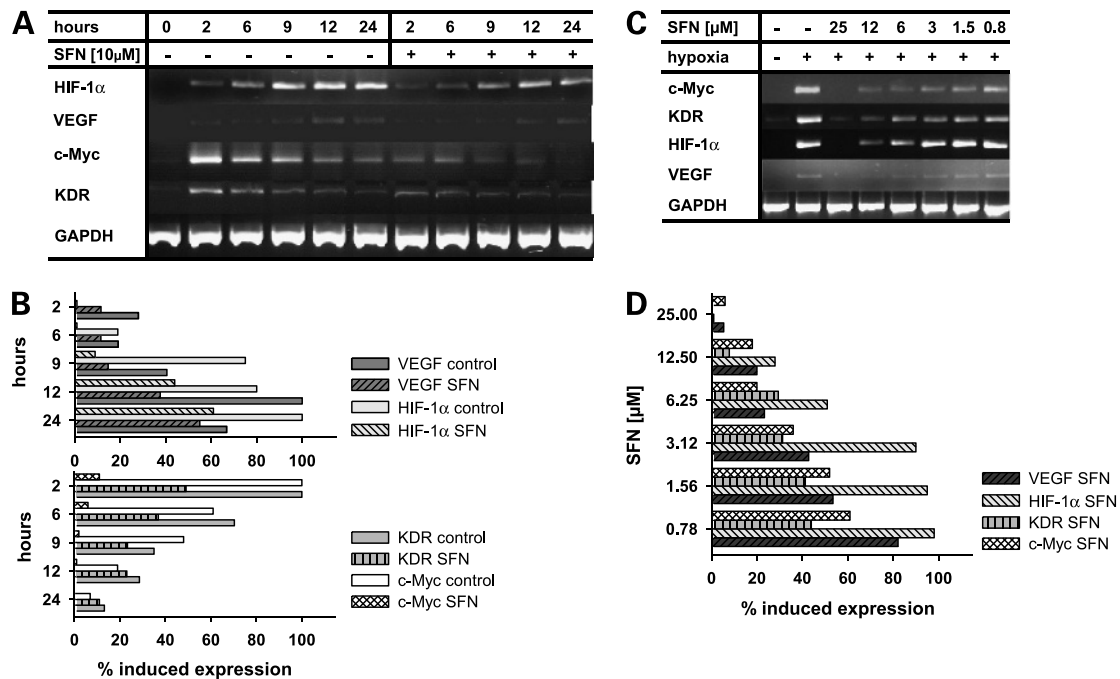
Figure 2. Time- and dose-dependent inhibition of hypoxia-induced proangiogenic factors. Time course: **A,** HMEC-1 cells were treated with DMSO (−) or 10 μmol/L sulforaphane (+) and exposed to hypoxia for 2 to 24 h. mRNA levels were investigated using reverse transcription-PCR. **B,** semiquantitative analysis of mRNA expression of HIF-1 (□) and VEGF (■; *top*) and c-Myc (■) and KDR (■; *bottom*) normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and displayed as a percentage of induced expression in comparison with control values at the transcriptional maximum set as 100%. *Stippled, crossed,* or *striped columns,* sulforaphane treatment. Dose response: **C,** HMEC-1 cells were treated with DMSO (−) or sulforaphane (*SFN*) 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 c-Myc, KDR, HIF-1, and VEGF 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%.

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 IC$_{50}$ 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 antiproliferative 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).
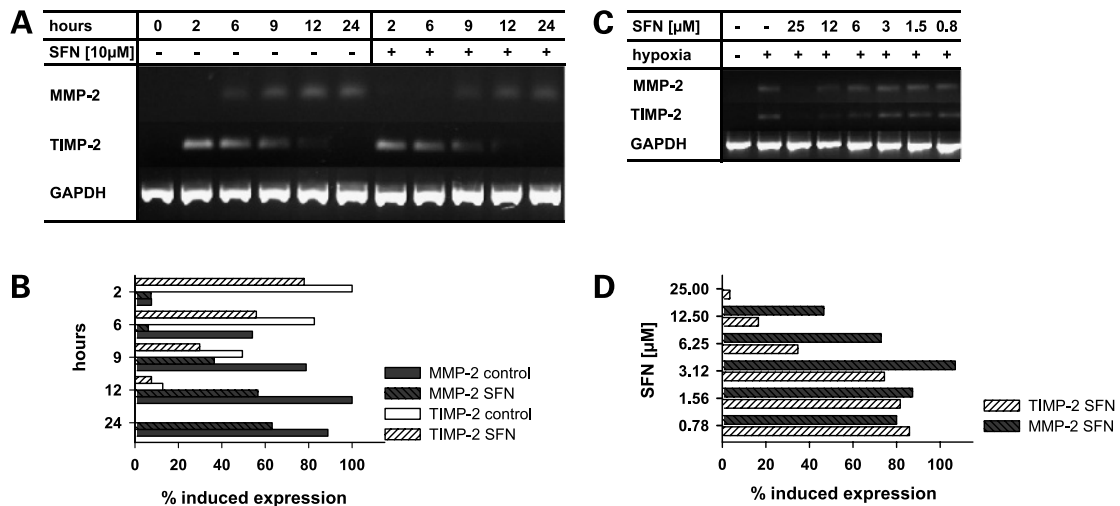
Figure 3. Influence on hypoxia-inducible basement membrane modulators. Time course: **A,** HMEC-1 cells were treated with DMSO (−) or 10 μmol/L sulforaphane (+) and exposed to hypoxia for 2 to 24 h. mRNA levels were investigated using reverse transcription-PCR. **B,** semiquantitative analysis of mRNA 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%.
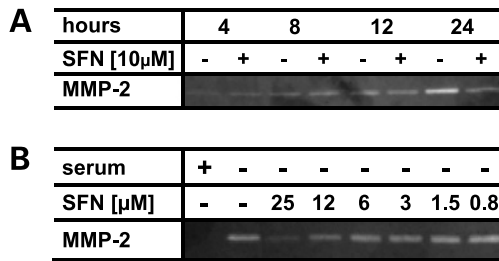
Figure 4. Inhibition of MMP-2 gelatinolytic activity. Time course: **A,** HMEC-1 cells were treated with DMSO (−) or 10 μmol/L sulforaphane (+) and exposed to serum-free conditions for 4 to 24 h. MMP-2 gelatinolytic activity was investigated using gelatin zymography. Dose response: **B,** HMEC-1 cells 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.

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).

Beside 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).

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.
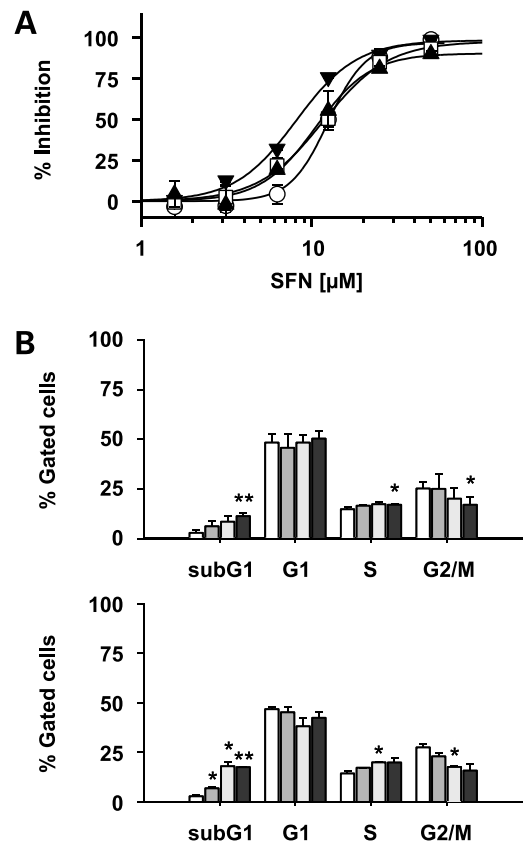
Figure 5. Inhibition of endothelial cell proliferation in comparison with human cancer cell lines. Cytotoxic effects: **A,** HMEC-1 (▲), SK-BR3 (○), HCT-116 (▼), or NIH-3T3 (□) cells were incubated for 72 h in 96-well plates in the presence or absence of sulforaphane. IC$_{50}$s were generated from six 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[TAM] transgenic mice to induce hyperplastic and dysplastic precancerous skin lesions by activation of the c-MycER[TAM] 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 potently 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$_1$ 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 *c*, whereas Bcl-2, an antiapoptotic factor, remained unchanged (60). In prostate cancer cells, sulforaphane activated the caspase cascade and caused an irreversible G$_2$-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,
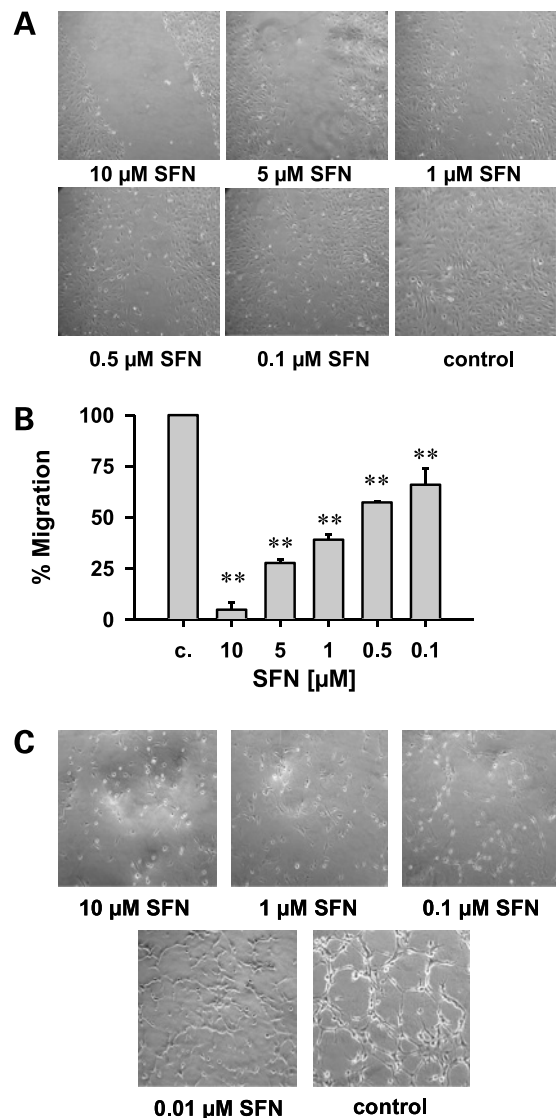
Figure 6. Inhibition of endothelial cell functions. Migration: **A,** HMEC-1 cells were seeded in 24-well plates and allowed to form a monolayer within 72 h. The monolayer was wounded mechanically by scraping with a pipette tip and incubated with sulforaphane at the indicated concentrations for 18 h. *c,* solvent control. **B,** area of migrated cells was acquired by digital image analysis. *, $P < 0.05$; **, $P < 0.005$, significantly different from time 0 using paired Student's *t* test ($n = 3$). Endothelial cell differentiation: **C,** HMEC-1 cells (1×10^5/mL/well) were cultured in 24-well plates coated with Matrigel and treated with sulforaphane at increasing concentrations. Pictures of tube formation were acquired after 6 h of incubation at ×32 magnification.

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$_v$β$_3$ integrin-binding site within the collagen. Blockage of this new site with an

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).

References

1. Zhang Y, Talalay P, Cho CG, Posner GH. A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. Proc Natl Acad Sci U S A 1992;89:2399 – 403.

2. Gerhauser C, You M, Liu J, et al. Cancer chemopreventive potential of sulforamate, a novel analogue of sulforaphane that induces phase 2 drug-metabolizing enzymes. Cancer Res 1997;57:272 – 8.

3. Zhang Y, Kensler TW, Cho CG, Posner GH, Talalay P. Anticarcinogenic activities of sulforaphane and structurally related synthetic norbornyl isothiocyanates. Proc Natl Acad Sci U S A 1994;91:3147 – 50.

4. Chung FL, Conaway CC, Rao CV, Reddy BS. Chemoprevention of colonic aberrant crypt foci in Fischer rats by sulforaphane and phenethyl isothiocyanate. Carcinogenesis 2000;21:2287 – 91.

5. Singh AV, Xiao D, Lew KL, Dhir R, Singh SV. Sulforaphane induces caspase-mediated apoptosis in cultured PC-3 human prostate cancer cells and retards growth of PC-3 xenografts *in vivo*. Carcinogenesis 2004;25:83 – 90.

6. Fahey JW, Talalay P. Antioxidant functions of sulforaphane: a potent inducer of phase II detoxication enzymes. Food Chem Toxicol 1999;37:973 – 9.

7. Basten GP, Bao Y, Williamson G. Sulforaphane and its glutathione conjugate but not sulforaphane nitrile induce UDP-glucuronosyl transferase (UGT1A1) and glutathione transferase (GSTA1) in cultured cells. Carcinogenesis 2002;23:1399 – 404.

8. Hintze KJ, Keck AS, Finley JW, Jeffery EH. Induction of hepatic thioredoxin reductase activity by sulforaphane, both in Hepa1c1c7 cells and in male Fisher 344 rats. J Nutr Biochem 2003;14:173 – 9.

9. Jowsey IR, Jiang Q, Itoh K, Yamamoto M, Hayes JD. Expression of the aflatoxin B1-8,9-epoxide-metabolizing murine glutathione *S*-transferase A3 subunit is regulated by the Nrf2 transcription factor through an antioxidant response element. Mol Pharmacol 2003;64:1018 – 28.

10. Munday R, Munday CM. Induction of phase II detoxification enzymes in rats by plant-derived isothiocyanates: comparison of allyl isothiocyanate with sulforaphane and related compounds. J Agric Food Chem 2004;52:1867 – 71.

11. Svehlikova V, Wang S, Jakubikova J, Williamson G, Mithen R, Bao Y. Interactions between sulforaphane and apigenin in the induction of UGT1A1 and GSTA1 in CaCo-2 cells. Carcinogenesis 2004;25:1629 – 37.

12. Hu R, Hebbar V, Kim BR, et al. *In vivo* pharmacokinetics and regulation of gene expression profiles by isothiocyanate sulforaphane in the rat. J Pharmacol Exp Ther 2004;310:263 – 71.

13. Lee JS, Surh YJ. Nrf2 as a novel molecular target for chemoprevention. Cancer Lett 2005;224:171 – 84.

14. Eggler AL, Liu G, Pezzuto JM, van Breemen RB, Mesecar AD. Modifying specific cysteines of the electrophile-sensing human Keap1 protein is insufficient to disrupt binding to the Nrf2 domain Neh2. Proc Natl Acad Sci U S A 2005;102:10070 – 5.

15. Heiss E, Herhaus C, Klimo K, Bartsch H, Gerhauser C. Nuclear factor κB is a molecular target for sulforaphane-mediated anti-inflammatory mechanisms. J Biol Chem 2001;276:32008 – 15.

16. Jeong WS, Kim IW, Hu R, Kong AN. Modulatory properties of various natural chemopreventive agents on the activation of NF-κB signaling pathway. Pharm Res 2004;21:661 – 70.

17. Kim BR, Hu R, Keum YS, et al. Effects of glutathione on antioxidant response element-mediated gene expression and apoptosis elicited by sulforaphane. Cancer Res 2003;63:7520 – 5.

18. Singh SV, Herman-Antosiewicz A, Singh AV, et al. Sulforaphane-induced G_2-M phase cell cycle arrest involves checkpoint kinase 2-mediated phosphorylation of cell division cycle 25C. J Biol Chem 2004;279:25813 – 22.

19. Losso JN. Targeting excessive angiogenesis with functional foods and nutraceuticals. Trends Food Sci Technol 2003;14:455 – 68.

20. Szekanecz Z, Koch AE. Vascular endothelium and immune responses: implications for inflammation and angiogenesis. Rheum Dis Clin North Am 2004;30:97 – 114.

21. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. Nature 2000;407:249 – 57.

22. Ziche M, Gullino PM. Angiogenesis and neoplastic progression *in vitro*. J Natl Cancer Inst 1982;69:483 – 7.

23. Folkman J. Tumor angiogenesis: therapeutic implications. N Engl J Med 1971;285:1182 – 6.

24. Sharma RA, Harris AL, Dalgleish AG, Steward WP, O'Byrne KJ. Angiogenesis as a biomarker and target in cancer chemoprevention. Lancet Oncol 2001;2:726 – 32.

25. Tosetti F, Ferrari N, De Flora S, Albini A. ''Angioprevention'': angiogenesis is a common and key target for cancer chemopreventive agents. FASEB J 2002;16:2 – 14.

26. Affara NI, Robertson FM. Vascular endothelial growth factor as a survival factor in tumor-associated angiogenesis. *In vivo* 2004;18:525 – 42.

27. Josko J, Mazurek M. Transcription factors having impact on vascular endothelial growth factor (VEGF) gene expression in angiogenesis. Med Sci Monit 2004;10:RA89 – 98.

28. Xie K, Wei D, Shi Q, Huang S. Constitutive and inducible expression and regulation of vascular endothelial growth factor. Cytokine Growth Factor Rev 2004;15:297 – 324.

29. Shi YH, Fang WG. Hypoxia-inducible factor-1 in tumor angiogenesis. World J Gastroenterol 2004;10:1082 – 7.

30. Harris AL. Hypoxia—a key regulator in angiogenesis. Nat Rev Cancer 2002;2:38 – 47.

31. Semenza GL. Targeting HIF-1 for cancer therapy. Nat Rev Cancer 2003;3:721 – 32.

32. Baudino TA, McKay C, Pendeville-Samain H, et al. c-Myc is essential for vasculogenesis and angiogenesis during development and tumor progression. Genes Dev 2002;16:2530 – 43.

33. Knies-Bamforth UE, Fox SB, Poulsom R, Evan GI, Harris AL. c-Myc interacts with hypoxia to induce angiogenesis *in vivo* by a vascular endothelial growth factor-dependent mechanism. Cancer Res 2004;64:6563 – 70.

34. Aref S, Mabed M, Zalata K, Sakrana M, El Askalany H. The interplay between c-Myc oncogene expression and circulating vascular endothelial growth factor (sVEGF), its antagonist receptor, soluble Flt-1 in diffuse

large B cell lymphoma (DLBCL): relationship to patient outcome. Leuk Lymphoma 2004;45:499 – 506.

35. Unger RE, Krump-Konvalinkova V, Peters K, Kirkpatrick CJ. *In vitro* expression of the endothelial phenotype: comparative study of primary isolated cells and cell lines, including the novel cell line HPMEC-ST1.6R. Microvasc Res 2002;64:384 – 97.

36. Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer 2002;2:161 – 74.

37. Rundhaug JE. Matrix metalloproteinases, angiogenesis, and cancer. Clin Cancer Res 2003;9:551 – 4.

38. Nagase H, Woessner JF, Jr. Matrix metalloproteinases. J Biol Chem 1999;274:21491 – 4.

39. Lambert E, Dasse E, Haye B, Petitfrere E. TIMPs as multifacial proteins. Crit Rev Oncol Hematol 2004;49:187 – 98.

40. Bertl E, Becker H, Eicher T, et al. Inhibition of endothelial cell functions by novel potential cancer chemopreventive agents. Biochem Biophys Res Commun 2004;325:287 – 95.

41. Brown KJ, Maynes SF, Bezos A, Maguire DJ, Ford MD, Parish CR. A novel *in vitro* assay for human angiogenesis. Lab Invest 1996;75:539 – 55.

42. Bertl E, Klimo K, Heiss E, et al. Identification of novel inhibitors of angiogenesis using a human *in vitro* anti-angiogenic assay. Int J Cancer Prev 2004;1:47 – 61.

43. Tremblay P, Houde M, Arbour N, et al. Differential effects of PKC inhibitors on gelatinase B and interleukin 6 production in the mouse macrophage. Cytokine 1995;7:130 – 6.

44. Gastl G, Hermann T, Steurer M, et al. Angiogenesis as a target for tumor treatment. Oncology 1997;54:177 – 84.

45. Terman BI, Carrion ME, Kovacs E, Rasmussen BA, Eddy RL, Shows TB. Identification of a new endothelial cell growth factor receptor tyrosine kinase. Oncogene 1991;6:1677 – 83.

46. Gerber HP, Condorelli F, Park J, Ferrara N. Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. Flt-1, but not Flk-1/KDR, is up-regulated by hypoxia. J Biol Chem 1997;272:23659 – 67.

47. Ning W, Chu TJ, Li CJ, Choi AM, Peters DG. Genome-wide analysis of the endothelial transcriptome under short-term chronic hypoxia. Physiol Genomics 2004;18:70 – 8.

48. Keshet E, Ben-Sasson SA. Anticancer drug targets: approaching angiogenesis. J Clin Invest 1999;104:1497 – 501.

49. Xu C, Shen G, Chen C, Gelinas C, Kong AN. Suppression of NF-κB and NF-κB-regulated gene expression by sulforaphane and PEITC through IκBα, IKK pathway in human prostate cancer PC-3 cells. Oncogene 2005;24:4486 – 95.

50. Naldini A, Carraro F. Role of inflammatory mediators in angiogenesis. Curr Drug Targets Inflamm Allergy 2005;4:3 – 8.

51. Rupec RA, Baeuerle PA. The genomic response of tumor cells to hypoxia and reoxygenation. Differential activation of transcription factors AP-1 and NF-κB. Eur J Biochem 1995;234:632 – 40.

52. Michiels C, Minet E, Mottet D, Raes M. Regulation of gene expression by oxygen: NF-κB and HIF-1, two extremes. Free Radic Biol Med 2002;33:1231 – 42.

53. Maulik N. Redox signaling of angiogenesis. Antioxid Redox Signal 2002;4:805 – 15.

54. Wu X, Bishopric NH, Discher DJ, Murphy BJ, Webster KA. Physical and functional sensitivity of zinc finger transcription factors to redox change. Mol Cell Biol 1996;16:1035 – 46.

55. Ema M, Hirota K, Mimura J, et al. Molecular mechanisms of transcription activation by HLF and HIF1α in response to hypoxia: their stabilization and redox signal-induced interaction with CBP/p300. EMBO J 1999;18:1905 – 14.

56. Heiss E, Gerhauser C. Time-dependent modulation of thioredoxin reductase activity might contribute to sulforaphane-mediated inhibition of NF-κB binding to DNA. Antioxid Redox Signal. Antioxid Redox Signal 2005;7:1601 – 11.

57. Forsythe JA, Jiang BH, Iyer NV, et al. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol 1996;16:4604 – 13.

58. Gills JJ, Jeffery EH, Matusheski NV, Moon RC, Lantvit DD, Pezzuto JM. Sulforaphane prevents mouse skin tumorigenesis during the stage of promotion. Cancer Lett 2005 (doi:10.1016/j.canlet.2005.05.007).

59. Wu X, Kassie F, Mersch-Sundermann V. Induction of apoptosis in tumor cells by naturally occurring sulfur-containing compounds. Mutat Res 2005;589:81 – 102.

60. Gamet-Payrastre L, Li P, Lumeau S, et al. Sulforaphane, a naturally occurring isothiocyanate, induces cell cycle arrest and apoptosis in HT29 human colon cancer cells. Cancer Res 2000;60:1426 – 33.

61. Schnaper HW, Grant DS, Stetler-Stevenson WG, et al. Type IV collagenase(s) and TIMPs modulate endothelial cell morphogenesis *in vitro*. J Cell Physiol 1993;156:235 – 46.

62. Pham NA, Jacobberger JW, Schimmer AD, Cao P, Gronda M, Hedley DW. The dietary isothiocyanate sulforaphane targets pathways of apoptosis, cell cycle arrest, and oxidative stress in human pancreatic cancer cells and inhibits tumor growth in severe combined immunodeficient mice. Mol Cancer Ther 2004;3:1239 – 48.