

Differential effects of sulforaphane in regulation of angiogenesis in a co-culture model of endothelial cells and pericytes

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Abstract. Aberrant neovascularization supports nutrients and the oxygen microenvironment in tumour growth, invasion and metastasis. Recapitulation of functional microvascular structures *in vitro* could provide a platform for the study of vascular conditions. Sulforaphane (SFN), an isothiocyanate, has been reported to possess chemopreventive properties. In the present study, the effects of SFN on cell proliferation and tubular formation have been investigated using endothelial cells (ECs) and pericytes in coculture. SFN showed a dose-dependent inhibition on the growth of ECs and pericytes with IC_{50} values 46.7 and 32.4 μ M, respectively. SFN (5–20 μ M) inhibited tube formation in a 3D coculture although a lower dose (1.25 μ M) promoted 30% more endothelial tube formation than control. Moreover, SFN affected intercellular communication between ECs and pericytes via inhibition of angiogenic factor such as vascular endothelial growth factor (VEGF) expression in pericytes. However, the expression of its receptor (VEGFR-2) was found significantly increased in ECs. These effects were associated with downregulation of prolyl hydroxylase domain-containing protein 1 and 2

(PHD1/2) and activation of hypoxia-inducible factor-1 (HIF) pathway by SFN. Furthermore, thioredoxin reductase-1 was also upregulated by SFN treatment, suggesting that anti-oxidant and redox regulation are involved in angiogenesis. Taken together, the results of the present study suggest that SFN differentially regulates endothelial cells and pericytes disrupting their interplay through the VEGF-VEGFR signaling pathway. Anti-angiogenesis property of SFN indicates that it has potential role as an anticancer agent.

Introduction

Angiogenesis, the growth of new capillary blood vessels, is a normal and vital process in growth, development and wound healing. However, it is also a fundamental step in the growth of tumours. Nutrients and oxygen, supplied by the blood vessels into the tumours, are essential for the growth and progression of malignant tumours beyond the size of 1–2 mm³ (1). Newly formed blood vessels can facilitate cells escaping through leakage from primary tumour sites to metastasize, the major cause of mortality for cancer patients. Anti-angiogenesis has been recognized as valuable therapy in treatment of various metastatic cancers since the theory was first proposed by Folkman (2,3). Tumour angiogenesis is believed to be regulated by the interactions between pro-angiogenic and anti-angiogenic factors in the tumour microenvironment (4). Angiogenic response is a dynamic process requiring a series of fine-tuned angiogenic signalling and molecular events. Hypoxia is the common inducer of angiogenesis in the core of large tumours stimulating the release of pro-angiogenic factors to promote endothelial cell proliferation, migration, differentiation and self-assembly into vascular-like structures. Subsequently, perivascular cells are recruited to form mature and stable vessels (5).

The interaction between endothelial cells (ECs) and pericytes (PVCs) has gained increasing attention as a central process in the regulation of blood microvascular structures as well as in their stabilization and maturation. Aberrant interplays between the two cell types have been observed in a multitude of human pathological conditions, including cancer angiogenesis (6). Targeting one cell type, either ECs or PVCs may produce limited effects. The most effective therapies will probably involve targeting multiple mediators,

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Abbreviations: DMSO, dimethyl sulfoxide; ECs, endothelial cells; EGM-2; endothelial growth medium-2; FBS, fetal bovine serum; HIF-1 α , hypoxia-inducible factor-1 α ; HUVECs, human umbilical vein endothelial cells; ITCs, isothiocyanates; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide; Nfr2, NF-E2-related factor 2; PHD, prolyl hydroxylase; PVC, pericytes; ROS, reactive oxygen species; SFN, sulforaphane; TrxR1, thioredoxin reductase 1; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor

Key words: sulforaphane, angiogenesis, VEGF, HUVEC, pericyte

and will require improving the efficiency of drug delivery to the tumour microenvironment (7,8). Therefore, improving the therapeutic response will require consideration of various signalling pathways and cell types involved in the vascular component of cancer.

Hypoxia-inducible factor-1 (HIF-1 α) dependent hypoxia-induced response is tightly controlled by HIF-prolyl hydroxylase domain (PHD) which targets HIF-1 α for degradation. Oxygen-dependent PHDs negatively regulate HIFs and, crucially, confer its oxygen sensitivity. In the presence of oxygen, PHD2 hydroxylates HIF-1 α on two specific proline residues, which results in its destruction. In hypoxia, PHD2 is missing its co-substrate (oxygen), rendering it inactive. HIF-1 α then becomes stabilized, and results in the upregulation of angiogenic factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF)-2 and angiopoietin-2 thereby promoting neovascularization. Hypoxia also inactivates PHDs, causing accumulation of HIF-1 α which in turn further transactivates PHDs. This feedback loop ensures the homeostasis of HIF-1 α activity caused by hypoxia (9,10).

After VEGF is released from tumour cells, it binds to two cognate VEGF receptors, VEGF receptor 1 (VEGFR1) and VEGF receptor 2 (VEGFR2/KDR/flk-1), which are expressed on local vascular ECs (11). Signalling through VEGFR1/2 drives the process of angiogenesis, which involves dissolution of the vascular basement membrane, endothelial cell proliferation and formation of new blood vessels (12). The binding to VEGF receptor is a crucial step in initiation of EC proliferation, migration and differentiation during angiogenesis (13,14). The VEGF signalling system has been suggested as a highly 'druggable' target and potent inhibitors of the VEGF signalling pathway have been used clinically including bevacizumab, sunitinib and sorafenib (15-17). To date, targeting the HIF/VEGF-VEGFR axis has been a promising strategy for cancer therapy (18). Anti-VEGF-VEGFR therapies may also have immunological effects (19). Since HIF signalling contributes to the acquisition of resistance against anti-VEGF therapy, the combined blockade of VEGF and HIF-1 α is being explored as a cancer treatment strategy (5). Despite the indisputable success of anti-angiogenic drugs in the clinical treatment for some advanced solid cancers, there are difficulties with regard to the control of the activities of these drugs and of the identification of patients who are sensitive to them (20).

Epidemiological studies have demonstrated that cruciferous vegetables can reduce the risk of various types of cancers in humans (21,22). Sulforaphane (SFN) is one of the most extensively studied isothiocyanates (ITCs) from broccoli and cauliflower. SFN has been found to suppress tumour cell growth through multiple molecular mechanisms including induction of cell cycle arrest and apoptosis in many types of tumour cells (23-26). SFN has been shown to inhibit ECs proliferation via apoptosis and autophagy (27-29) and suppress VEGF and MMP-2 expression (30,31), the latter being associated with the inhibition of FOXO1/AKT pathways (32). Moreover, SFN is a known inducer of both thioredoxin reductase (TrxR1) and thioredoxin (26,33), which are involved in inflammation, apoptosis, angiogenesis, embryogenesis and cardiovascular disease involved in angiogenesis (34). The molecular mechanisms of SFN suppression of angiogenesis, in particular the effects on signalling

pathways between ECs and pericytes in response to SFN treatment, are not fully understood. In the present study a coculture of primary human umbilical vein endothelial cells (HUVECs) and pericytes in a 3D collagen gel model were used to dissect the mechanism by which SFN interacts with crosstalk between these two key cell types in angiogenesis. Understanding the interactions between HUVECs and pericytes under SFN treatment may contribute to the development of novel agents in anti-angiogenic therapy.

Materials and methods

Reagents. SFN (4-methylsulfinylbutyl isothiocyanate) was purchased from Toronto Research Chemicals, Inc. (North York, ON, Canada). High concentration rat tail type-I collagen solution and purified mouse anti-human CD31/PECAM (555444) antibody were both purchased from BD Biosciences (Oxford, UK). Polyclonal donkey anti-mouse Cy3 was purchased from Abcam (Cambridge, UK). Growth factors PDGF-BB and bFGF were obtained from Gibco/Life Technologies (Paisley, UK). The primary antibodies against HIF-1 α (ab2185), VEGF (sc7269), Flk1 (sc6251), TrxR1 (sc20147) were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). The primary antibodies against PHD1 (ab80361) and PHD2 (ab83560) were purchased from Abcam. Secondary antibodies were from Santa Cruz Biotechnology. siRNA for TrxR1 and AllStars (AS) negative control were all purchased from Qiagen (West Sussex, UK). Electrophoresis and western blotting supplies were obtained from Bio-Rad Laboratories (Hemel Hempstead, UK) and the chemiluminescence kit was from GE Healthcare (Little Chalfont, UK).

Cell culture. HUVECs were obtained from TCS Cellworks and used between passages 2 and 8 for all experiments. The cells were grown in plastic flasks pre-treated with 10 μ g/ml type-I collagen (BD Biosciences) in phosphate-buffered saline (PBS) for 30 min in 37°C incubator. Endothelial growth medium-EGM2 (C22011; PromoCell, Birmingham, UK) with supplements was used as culture medium for HUVECs according to the manufacturer's protocols. Murine perivascular cells (PVC) were isolated as previously described and used between passages 32 and 38 (35). Pericytes were routinely incubated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). All cells were incubated at 37°C in 95% humidified air containing 5% CO₂.

Cell viability assay. The MTT assay was used to examine the toxicity of SFN in HUVECs and pericytes. Cells were seeded in 96-well plates and cultured in an incubator at 37°C to ~70-80% confluence. Cultured cells were treated with concentrations (1.25-160 μ M) of SFN or DMSO (0.1% as control) for 24 h with four replicate wells per treatment. After all treatments, the medium was removed and fresh medium (100 μ l) was added together with 10 μ l MTT solution (5 mg/ml), then incubated at 37°C for 1 h to allow the MTT to be metabolized. The formazan produced was re-suspended in 100 μ l of dimethyl sulfoxide (DMSO)/well. The final absorbance in the wells was quantified using a microplate reader (BMG Labtech Ltd., Aylesbury, UK) at a test wavelength of 570 nm and a reference wavelength of 670 nm. Viability of treated cells was expressed

as a percentage of control as follows: (A570 nm-A670 nm) sample/(A570 nm-A670 nm) control \times 100. The IC_{50} was determined using CalcuSyn software version 2.0 (Biosoft, Cambridge, UK).

3D co-culture in collagen gel. Capillary-like tube formation in 3D collagen matrices of co-culture with HUVEC and pericytes was used to test the angiogenic effects of SFN. Collagen type I gels (2 mg/ml) were prepared in 1X DMEM medium from concentrated rat tail type I collagen solution (>8 mg/ml in 0.02 M acetic acid; BD Biosciences) at 4°C, supplemented with final concentrations of 2% FBS, 22.5 mM $NaHCO_3$, 1 mM sodium pyruvate and neutralized with 0.1N NaOH according to the supplier's instructions. Cells were trypsinised, washed with PBS, counted and desired cell numbers were collected by centrifugation. Cells were suspended in the collagen I gel solution at 4°C and 400 μ l suspension added per well into 24-well plates. After an initial incubation at 37°C for 20 min, 400 μ l EGM2 culture medium containing SFN (0.6-20 μ M) or 0.1% DMSO (control) was added to solidified collagen I gels with supplements to achieve final concentrations of 10 ng/ml for VEGF and PDGF, respectively, and 250 μ g/ml ascorbic acid phosphate. Typical experiments contained 2.5×10^5 HUVEC and/or 0.5×10^5 PVC/well, unless otherwise stated. Medium was changed every 24 h and cultures were maintained for up to 5 days.

Immunostaining of HUVEC-pericyte coculture model. Immunohistochemical analyses of cell cultures were performed as described by Brachvogel *et al* (36) and Zhou *et al* (37). Whole mount immunohistochemistry of 3D collagen cultures was performed as described by Bader *et al* (38). Briefly, gels were washed in PBS, fixed with 80% methanol/20% DMSO for 30 min at 20°C (or 16 h at 4°C), then rehydrated in 50% methanol/PBS, 20% methanol/PBS and PBS-T (PBS, 0.1% Tween-20) for 1 h each and then incubated with blocking buffer (10% FBS, 5% BSA in PBS) for 2-4 h at room temperature or 16 h at 4°C. Gels were incubated with primary antibodies in blocking buffer for 16 h at 4°C and then washed 7 times for 1 h each in TBS-T (TBS, 0.1% Tween-20) followed by incubation with fluorescently labelled secondary antibodies in blocking buffer for 2-16 h and then washed again as described above. After nuclear staining, samples were mounted in Gelvatol and examined by fluorescence microscopy (SteREO LumarV12 and Axioplan2; Carl Zeiss, Oberkochen, Germany). Pictures of fluorescent signals were captured by a black-white camera and colour-coded by AxioVision software (version 4.5).

Western blot analysis of protein expression. HUVECs and PVC cells were treated with SFN (1-20 μ M) or DMSO (0.1% as control) at 70-80% confluence. Total protein was extracted by washing cells twice with ice-cold PBS and harvested by scraping in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Nonidet P-40 (NP-40) containing mini-complete proteinase inhibitor. The cell suspension/lysate was placed in an ice bath for 20 min and then centrifuged at 12,000 \times g for 15 min at 4°C. Protein concentrations were determined using the Brilliant Blue G dye-binding assay of Bradford using BSA as a standard. Equivalent aliquots of protein were mixed with 4X SDS-PAGE sample buffer

and DTT reducing agent (to 50 nmol/l) and were heated to 95°C for 5 min. Equal amounts of samples were loaded onto SDS-PAGE gel and subsequently transferred to PVDF (polyvinylidene difluoride) membranes (Bio-Rad Laboratories). The membrane was washed three times for 45 min with PBST and then incubated with the secondary antibody diluted with 5% milk in PBST for 1 h. After further washing the membrane three times for 45 min with PBST, antibody binding was determined by a chemiluminescence detection kit and densitometry was measured by Fluor ChemImager (Alpha Innotech, San Leandro, CA, USA).

Statistical analysis. All experiments were independently repeated at least three times. Data are means \pm SD. Student's t-test was applied for differences between groups using SPSS software. Significant differences among groups were calculated and $P < 0.05$ was viewed as statistically significant.

Results

Effect of SFN on cell proliferation and viability in HUVECs and PVC. As a viable and adequate population of HUVECs and PVC is essential for angiogenesis, the effect of SFN on cell proliferation and viability of both cell types were measured by treating the cells with SFN over a range of concentrations (0.625-160 μ M) for 24 and 48 h followed by an MTT assay. At low doses, from 0.625 to 5 μ M, SFN has no toxic effect on cell viability. SFN at 2.5 μ M (24 h) promoted cell proliferation to 116 and 136% in HUVECs and PVC, respectively (Fig. 1). However, a dose-dependent effect on cell viability was observed following treatments with SFN between 20 and 160 μ M, i.e. cell viability decreased to 83.8% and $<10\%$ respectively, IC_{50} , 46.7 μ M. In parallel, the influence of SFN on PVC was also determined, and a similar dose-dependent effect on cell viability was observed. In both cell types, there was no significant difference in cell viability after exposure to 10 μ M SFN at 24 and 48 h although the PVCs are slightly more sensitive to SFN with IC_{50} , 32.4 μ M. Based on these results, 10 μ M SFN was chosen as an optimum dosage for the tube formation experiments and 1-20 μ M were used for mechanistic studies.

SFN suppresses capillary formation in 3D collagen model of HUVECs and PVC co-culture. The tube formation assay in 3D collagen gel is a well-established procedure for the evaluation of angiogenic capacities. To detect the angiogenic effect of SFN, a co-culture model consisting of HUVECs and pericytes was used to mimic more realistically the *in vivo* angiogenic process. Angiogenic growth factors were present in the collagen matrix in order to more closely represent a tumour microenvironment to promote capillary-like tube formation through ECs alignment with supporting pericytes. In the vehicle control (0.1% DMSO), HUVECs and pericytes formed robust capillary structures. The addition of SFN to the growth medium of the co-culture collagen gel, led to a concentration-dependent disruption of the tube structure. The formation of tube structures were only partially inhibited at moderate SFN concentrations (2.5-10 μ M) whilst higher dosages (20 μ M) completely inhibited the formation of tube structures (Fig. 2A). Notably, SFN at low concentrations from 0.625 to 1.25 μ M promoted the formation of tubes by 115-120% of the

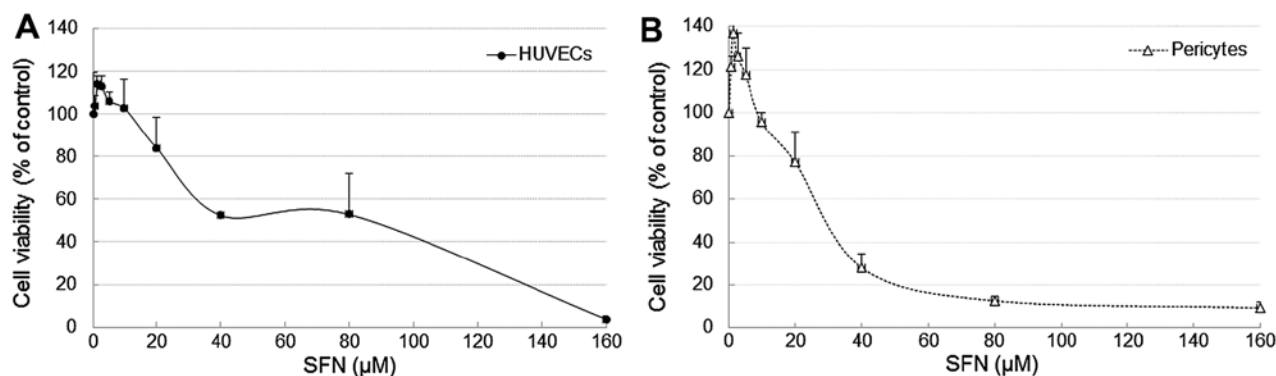


Figure 1. Effect of SFN on cell viability in HUVECs and pericytes. Dose-dependent effect of SFN on cell viability in (A) HUVECs and (B) pericytes. Exponentially growing cells were treated with SFN (0-160 μM) for 24 and 48 h. Cell viability was measured by MTT assay. Each concentration was tested in quadruplicate and the experiment was repeated twice. Results were present as % of control. * $P < 0.05$ compared with control.

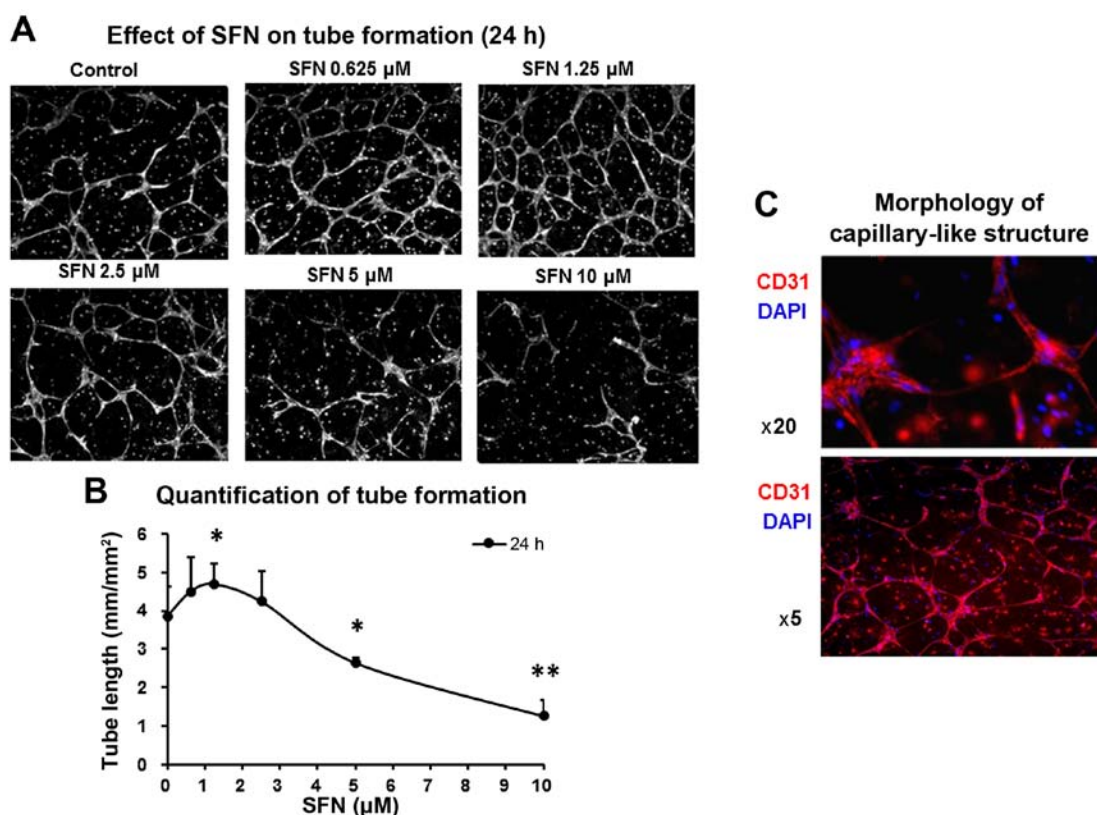


Figure 2. Effect of SFN on capillary formation in 3D culture of HUVECs and pericytes. (A) Representative images show dose-dependent effect of SFN for 24 h in the 3D tube model of co-culture HUVECs with pericytes (magnification, $\times 5$). Images were taken by optical micrographs after 24 h. (B) Line chart represents cumulative tube length of 3D co-culture model exposed to SFN (0-20 μM). (C) Morphology of capillary-like structures in untreated control. Data are representative of three independent experiments. * $P < 0.05$; ** $P < 0.01$.

control, i.e. total tube length was 3.86 mm/mm² in control and 4.69 mm/mm² in SFN (1.25 μM) treated cells (Fig. 2B). Immunostaining of control cells with anti-CD31 and DAPI showed capillary-like morphology in the untreated HUVECs and PVCs (Fig. 2C).

Effect of SFN on VEGF and VEGFR2 expression. To evaluate whether SFN inhibits VEGF expression, PVC were cultured in EGM2 medium for 24 h followed by a dose-dependent (1.25-20 μM), or a time course (0-24 h) of treatment with SFN (10 μM). Western blot analysis for VEGF protein

expression demonstrated that SFN inhibits VEGF in PVC in a dose- and time-dependent manner (Fig. 3A and B). SFN inhibited VEGF expression to 60 and 30% of the control after the treatments with 5 and 20 μM SFN (24 h), respectively. An inhibitory effect of 10 μM SFN was also observed after 3 h (70% of control), 12 h (48% of control) and 24 h (36% of control) treatment (Fig. 2B). However, VEGF was not at detectable level in similarly treated HUVECs (data not shown). To further investigate VEGF and its receptor signalling pathway, the effect of SFN on VEGFR2 protein expression was determined. Not surprisingly, PVCs do not

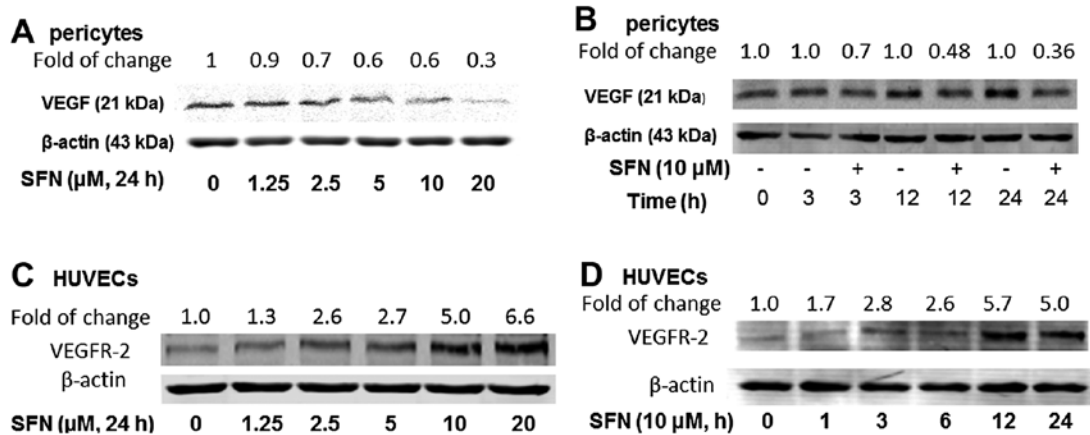


Figure 3. Effect of SFN on VEGF and VEGFR2 expression in HUVECs and pericytes. (A) Pericytes were treated with SFN (1.25-20 μ M) for 24 h; Control was DMSO at 0.1%. (B) Cells were treated with SFN 10 μ M for 1, 3, 6, 12 and 24 h. Time 0 was control (DMSO 0.1%). All pericytes were cultured in DMEM medium then replaced by EGM2 medium 24 h before SFN treatments. (C) HUVECs were treated with SFN (1.25-20 μ M) for 24 h; SFN 0 was control (DMSO 0.1%). (D) HUVECs were treated with SFN 10 μ M for 1, 3, 6, 12 and 24 h. Equal amounts of cellular protein (80 μ g) were loaded, and resolved by SDS/10% PAGE and western blot analysis. Data are representative of three independent experiments.

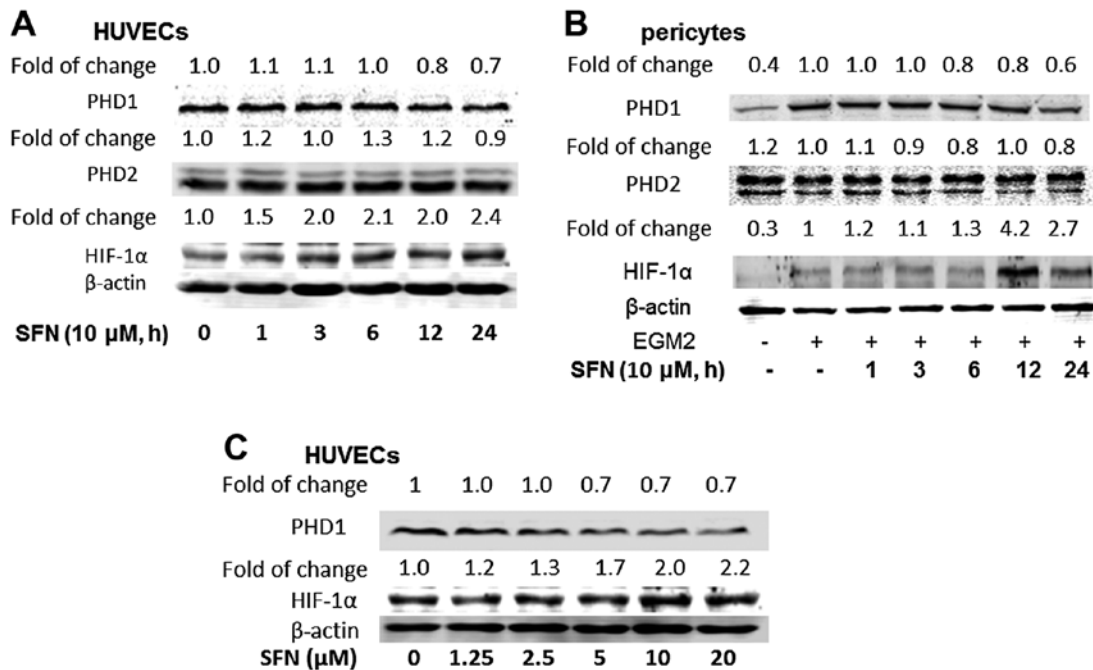


Figure 4. Effect of SFN on HIF-1 α , PHD1 and PHD2 expression in HUVECs and pericytes. (A) HUVECs were treated with SFN (10 μ M) and whole protein was isolated at 1, 3, 6, 12 and 24 h. Time 0 cells was treated with DMSO (0.1%) as control. (B) HIF-1 α , PHD1 and PHD2 were detected in pericytes after exposure to SFN (10 μ M for 1 to 24 h) using western blot analysis. Cells grown to ~50-60% confluence in DMEM medium, then replaced by EGM2 medium and incubated for further 24 h before SFN treatment. In the DMEM only group, cells were cultured and treated with SFN (10 μ M). (C) HUVECs were treated with SFN (1.25-20 μ M) for 24 h. Data are representative of three independent experiments.

express detectable levels of VEGFR2 (data not shown), whilst HUVECs showed a significant upregulation of VEGF-R2 level after SFN treatment (Fig. 3C and D).

Differential regulation of HIF signalling pathway after SFN exposure. HIF pathway is an important signaling pathway for HIF secretion especially in tumour micro-environments. In the present study, HIF-1 α , PHD1 and PHD2 protein expression in both HUVECs and PVC were quantified by western blot assay. Both cell types were treated with 10 μ M SFN for different time periods (1, 3, 6, 12 and 24 h) and HIF-1 α and

the relative expressions in both types of cells were measured. SFN increased HIF-1 α expression in both cell types, i.e. 2.0- to 2.4-fold in HUVECs and 4.2-2.7-fold in PVC at 12 and 24 h, respectively (Fig. 4A and B). As expected, the effect of SFN on the expression of PHD1 was suppressed by SFN, 30% in HUVECs and 40% in pericytes at 24 h of treatment. Moreover, increasing SFN concentration in HUVECs from 1.25 to 20 μ M for 24 h, had no significant effect on PHD1 protein expression, but again increased HIF-1 α expression (Fig. 4C). Activation of HIF-1 α may associate with the low-dose promotion effect of SFN on cell growth.

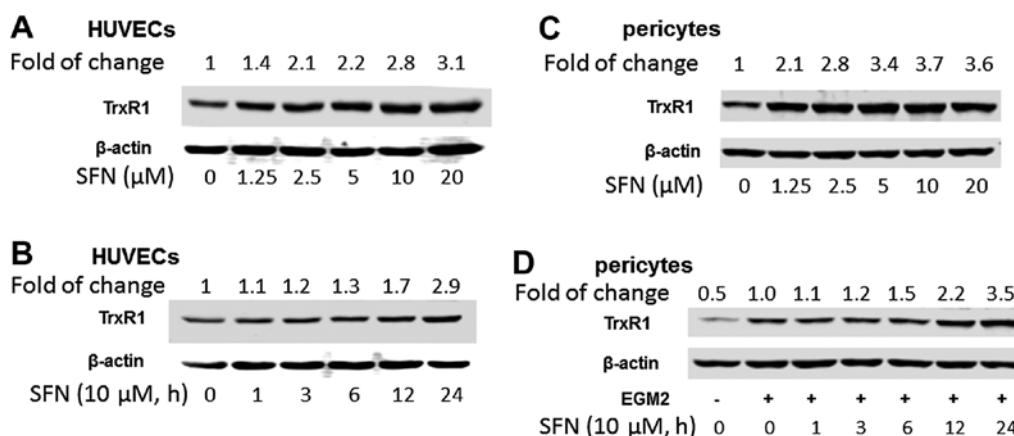


Figure 5. Effect of SFN on TrxR1 protein expression in HUVECs and pericytes. (A) HUVECs were treated with SFN (1.25-20 μ M) for 24 h. (B) HUVECs were treated with SFN 10 μ M for different times (1, 3, 6, 12 and 24 h). (C) Pericytes were treated with SFN (1.25-20 μ M) for 24 h. (D) Pericytes were treated with SFN (10 μ M) for 1, 3, 6, 12 and 24 h. All pericytes were cultured in DMEM medium (50-60%) until being replaced by EGM2 medium and incubated for 24 h before SFN treatment for another 24 h.

Effect of SFN on TrxR1 expression. Inhibition of thioredoxin reductase (TrxR1) has been shown to regulate angiogenesis by increasing endothelial cell-derived VEGF (39). SFN increased TrxR1 expression in both HUVECs and PVC dose- and time-dependently, i.e. SFN at 10 μ M induced TrxR1 2.8- and 3.7-fold in HUVECs (Fig. 5A and B) and PVC (Fig. 5C and D), respectively. TrxR1 plays an important role in SFN inhibition of angiogenesis. Knockdown of TrxR-1 abolished the downregulation of PHD1 by SFN treatment (10 μ M, 24 h), suggesting a deficiency of TrxR-1 may cause the accumulation of PHD1. However, knockdown of TrxR1 only attenuated ~20% of the SFN-inhibited tube formation (data not shown) suggesting that the inhibition of tube formation by SFN is only partly TrxR1-dependent.

Discussion

In the present study, we demonstrated that SFN exhibited differential effect on the regulation of angiogenesis in ECs and pericytes which may interrupt the crosstalk mechanism between them leading to reduced angiogenic capacity. Conventional planar cultures fail to recreate the *in vivo* physiology of the microvasculature with respect to 3D geometry (lumens and axial branching points) and interactions of endothelium with perivascular cells, extracellular tissue and blood flow (40,41). There is strong evidence that the 3D *in vitro* model consisting of multiple stromal cells is not only cheaper but also provides quicker results than animal models. Additionally, 3D models are more analogous to pathological progression of angiogenesis than 2D cell culture models. Herein, we set up a 3D collagen model of HUVECs and pericytes in co-culture with growth factors to closely mimic the angiogenesis process *in vivo* microenvironment, and to allow the detection of the effects of dietary bioactives such as SFN, and the delineation of the molecular mechanisms of SFN in the interactions between HUVEC and PVCs.

SFN affected the network structure of neovascularization, causing disruption at concentrations >5 μ M dose- and time-dependently. A marked increase in microvessel formation

at lower doses between 0.625-1.25 μ M was also observed. This bell-shaped effect (hormesis) is common in anticancer agents (15,42) which suggests the importance of maintaining the SFN at high concentrations (>5 μ M) for cancer chemoprevention or treatment. These tube formation results were consistent with the cell viability data that showed that SFN inhibited the growth of ECs at concentrations of 5-20 μ M while at low concentrations of 0.625-2.5 μ M, SFN promoted cell growth. Our findings suggest that the multi-targeted effect of SFN in EC-pericyte coculture model and on proliferation of ECs, demonstrates that its mechanism of action is complex. The biphasic effects of SFN on angiogenesis indicate that lower concentrations may provide benefit in conditions where the formation of an inadequate number of new blood vessels prevent an adequate blood supply, such as many cardiovascular diseases. In contrast, high concentrations of SFN (in the present study, 5-20 μ M) are needed for the anti-angiogenic effects.

At the level of protein expression, SFN treatment was found to inhibit VEGF secretion from pericytes but upregulate VEGF receptor-2 expression in ECs. VEGF, one of the major angiogenesis factors, is induced in growing tumours and stimulates EC proliferation and migration primarily through the VEGFR2 (Flk1/KDR) pathway (43). HIF-1 is overexpressed in many human cancers and it regulates the expression of VEGF. Yao *et al* (23) demonstrated the inhibitory effect of SFN on HIF-1 α and VEGF expression in human SCCs and prostate cancer cells via multiple pathways. Bertl *et al* (31), reached a similar conclusion in their study. The HIF pathway is also responsible for acquisition of resistance against anti-VEGF therapy. Our results contradict some other findings that SFN was found to have unique character by activating HIF-1 α pathway in both HUVECs and PVC but suppressing VEGF expression in PVC. The exact mechanism is not clear but early research has revealed other transcription factors are also involved in VEGF expression, such as Sp1/Sp3, AP2, Egr-1 and STAT-2 (44). For example, SFN was found to be able to inhibit STAT and SP1 pathways (45,46). Our finding may suggest that SFN has a potential role in attenuating anticancer resistance.

TrxR1, an Nrf2-driven selenoprotein, was upregulated by SFN and may increase the anti-oxidant and redox regulation capability. The increase in TrxR1 expression following SFN treatment (10 μ M) was found time- and dose-dependently (Fig. 4).

TrxR1 is involved in cell proliferation, redox regulation of gene expression and signal transduction, protection against oxidative stress, anti-apoptotic functions and regulation of the redox state of the extracellular environment (47). Upregulation of TrxR1 plays a role in protection against free-radical mediated cell death (48). In this study, knockdown of TrxR1 attenuated ~20% SFN inhibition of tube formation, and co-treatment of HUVECs/PVC with SFN and N-acetylcysteine (NAC) at 2 mM abolished the promoting effect of low dose SFN on tube formation (data not shown). This indicates the involvement of reactive oxygen species (ROS) (49,50). However, excessive ROS production may play a role in microvascular instability (51).

It is well accepted that angiogenesis is a critical, rate-limiting step in the development of cancers and its inhibition suppresses tumour growth, progression and metastases. Antiangiogenic therapy using dietary compounds represents a new cost-effective approach to the early intervention and prevention of cancer (52). There are many natural and synthetic antioxidants such as curcumin, tea polyphenols and vitamins E and C that possess anti-angiogenic activities (53) and there is significant potential in the further investigation of their interactions in modulation of angiogenesis. Dietary isothiocyanates such as SFN, derived from glucoraphanin from cruciferous vegetables, have attracted much attention for their potential to prevent various types of cancers. SFN may protect endothelium from oxidative stress by inducing TrxR1 expression and activity and also by suppressing the activation of MAPKs (38,54). When 5-20 μ M SFN was used, the expression of TrxR1 was increased and this is associated with resistance to inflammation (55). The downregulation of angiogenic signalling pathways by SFN could offer a new therapeutic strategy in suppressing malignant tumour progression, especially if multiple molecules were targeted and suppressed in cells responsive to SFN potentiated modulation of the angiogenic process. The results from the present study strongly indicate a potential role of SFN at higher doses in anti-angiogenesis cancer therapy. The action of SFN in targeting HIF, PHD and VEGF in the interplay between ECs and pericytes may shed insights into new treatment strategies. Therefore, the therapeutic potential of SFN as a potential alternative anti-VEGF agent in chemoprevention is worthy of further exploration in animal models and small scale human trials.

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