ORIGINAL ARTICLE

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Suppression of VEGFR2 Expression in Human Endothelial Cells by Dimethylfumarate Treatment: Evidence for Anti-Angiogenic Action

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The association between angiogenesis and chronic inflammatory diseases, such as psoriasis, seems to be an important phenomenon implicated in the pathogenesis of these medical conditions. Recent studies provide evidence that dimethylfumarate (DMF) has a profound anti-inflammatory as well as anti-tumorigenic action, although the effect of DMF on angiogenesis is unknown. Signaling via the vascular endothelial growth factor receptor-2 (VEGFR2) pathway is critical for angiogenic responses. Therefore, we explored whether the known anti-inflammatory and anti-tumorigenic properties of DMF might be mediated in part by anti-angiogenic effects through the reduction in VEGFR2 expression. In this study, DMF was found to inhibit endothelial VEGFR2 expression; time- and concentration-dependent inhibition was demonstrated both at the level of protein and mRNA expression. This blockade was coincident with the inhibition of the formation of capillary-like structures. The DMF-dependent inhibition of VEGFR2 transcription was found to be mediated by an element located between base pairs −60 and −37, which contains two adjacent, consensus Sp1 transcription factor-binding sites, and the constitutive formation of complexes containing Sp1 at this site is decreased by DMF treatment. Inhibition of VEGFR-2 is shown to be one critical aspect in DMF-mediated anti-angiogenic effects.

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INTRODUCTION

In the last two decades, a plethora of evidence has shown the association between inflammation and angiogenesis in pathological conditions. There are many chronic inflammatory diseases, such as rheumatoid arthritis, psoriasis, diabetes, metabolic syndrome-associated disorders, arteriosclerosis, and cancer, where inflammation and angiogenesis are mutually dependent upon each other. In fact, chronic inflammation and angiogenesis are two processes that can develop concurrently (Heidenreich *et al.*, 2009). Hence, targeting angiogenesis may lead to the suppression of inflammation by decreasing the number of invading immune cells, preventing the supply of nutrition, or reducing inflammatory and proteolytic mediators. Conversely, targeting inflammation may also negatively affect angiogenesis. Therefore, a compound capable of inhibiting both processes may be efficacious in the treatment of various chronic inflammatory diseases and tumor entities.

Fumaric acid esters (FAEs) have been successfully used in the treatment of psoriasis for more than 40 years (Schweckendiek, 1959). To date, several clinical trials have proven the efficacy of FAEs, especially dimethylfumarate (DMF), in the treatment of this chronic inflammatory disease (Yazdi and Mrowietz, 2008). In addition, a recent study by Kappos *et al.* (2008) impressively demonstrated that DMF might be effective in the treatment of relapsing-remittent multiple sclerosis, which is another inflammatory disease.

To date, multiple mechanisms of action have been defined for FAEs against inflammatory cells. Stoof *et al.* (2001) showed that IL-8, Gro-alpha, IP-10, and Mig are effectively downregulated during treatment with DMF, which switches the cytokine profile of these cells toward one that characterizes Th2 cells. In addition, DMF treatment has been shown to cause leukocytopenia, with apoptosis causing a reduction in CD8+ and CD4+ T cells in psoriasis lesions (Sebök *et al.*, 1994; Treumer *et al.*, 2003).

Leukocytes are recruited to the site of inflammation by multiple adhesion molecules on endothelial cells, including ICAM-1, vascular cell adhesion molecule-1, and E-selectin. In Th1 cytokine-dominated diseases, such as psoriasis, the expression of these receptors enables leukocytes to transmigrate the endothelium. Vandermeeren *et al.* (1997) convincingly demonstrated that DMF is an effective inhibitor of

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Abbreviations: DMF, dimethylfumarate; HUVEC, human umbilical vein endothelial cell; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor

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cytokine-induced vascular cell adhesion molecule-1, ICAM-1, and E-selectin expression in human endothelial cells. Further investigations revealed that DMF inhibits the nuclear translocation of activated NF-κB in endothelial cells and suppresses the activation of NF-κB in human dendritic cells (Loewe *et al*., 2001, 2002; Yamazoe *et al*., 2009). The NF-κB transcription factor is known to have a central role in immune and inflammatory responses that regulate a large number of proinflammatory Th1 cytokines and adhesion molecules. Therefore, it has been suggested that NF-κB is one of the central molecular targets of DMF.

Loewe *et al*. (2002) demonstrated that DMF reduces melanoma metastasis in a severe combined immunodeficient mouse model. These data were supported by the data of Yamazoe *et al*. (2009), who showed that DMF reduces cell invasion and metastasis by inhibiting metalloproteases. Other than the anti-inflammatory and anti-tumorigenic properties of DMF, the influence of this compound on angiogenesis is unknown.

Vascular endothelial growth factor (VEGF; VEGF-A) is an endothelial cell-specific growth factor that is essential for endothelial cell differentiation (vasculogenesis), the sprouting of new capillaries from pre-existing vessels (angiogenesis), vasodilatation, and vascular permeability. VEGF is also a key regulator of angiogenic sprouting in cancer, ischemic and inflammatory diseases, and wound repair (Carmeliet and Jain, 2000; Yancopoulos *et al*., 2000). These functions are primarily mediated through the interaction of VEGF with three structurally related tyrosine kinase receptors: VEGF receptor-1 (VEGFR-1; Flt-1), VEGFR-2 (KDR/Flk-1), and VEGFR-3. There is increasing evidence that VEGFR-1 is a regulator of VEGFR-2 signaling by functioning as a decoy receptor or by directly regulating the VEGFR2 signaling pathway (Cao, 2009). Although VEGFR-1 is required for normal blood vessel development during embryogenesis, VEGFR-2 is thought to mediate the major growth effects and permeability of VEGF (Olsson *et al*., 2006). VEGFR-3 seems to be essential for lymphatic vessel formation. VEGFR2 is only detectable at proportionally low levels in the normal adult vasculature; however, during chronic inflammation, wound repair, or tumor growth, its expression is upregulated in the involved blood vessels (Olsson *et al*., 2006). VEGF is secreted by a variety of different cell types, including macrophages and tumor cells. However, VEGFR2 expression is restricted mainly to vascular endothelial cells. Therefore, the suppression of VEGF/VEGFR2 signaling is being intensely investigated as a therapeutic option to prevent new blood vessel formation (Ellis and Hicklin, 2008).

Taking into account that angiogenesis is an essential part of chronic inflammatory diseases and cancer, we hypothesized that DMF, a known effective anti-inflammatory compound, would also suppress angiogenesis and therefore might be an effective new anti-angiogenic drug.

To test this hypothesis, human umbilical vein endothelial cells (HUVECs) were treated with DMF and subjected to long-term endothelial tube formation assays. Herein, we demonstrate a profound anti-angiogenic effect that exceeds the effects of suramin, a known anti-angiogenic drug. This suppressive action was coincident with a profound downregulation of VEGFR2 at the transcriptional and translational levels.

These results provide to our knowledge the first evidence that DMF is an effective anti-angiogenic drug and define a mechanism of action for this compound in the treatment of chronic inflammatory diseases and cancer. In addition, we provide basic research evidence for the use of DMF in the treatment of other diseases associated with angiogenesis, such as cancer.

RESULTS

Dimethylfumarate treatment inhibits basal and VEGF-induced endothelial cell function

Angiogenesis is an important component of chronic inflammatory processes. To analyze whether DMF exhibits distinct anti-angiogenic effects in addition to its known anti-inflammatory action, we performed long-term tube formation assays investigating the effect of DMF on the ability of HUVECs to form capillary-like structures.

We tested the effect of various concentrations of DMF on cell proliferation and cytotoxicity using human endothelial cells; DMF inhibited HUVEC cell proliferation in a dose-dependent manner, as determined by the BrdU assay (Figure 1a). This effect could not be abrogated by the addition of VEGF or basic fibroblast growth factor (Supplementary Figure S1a online), indicating that angiogenic signal-transduction pathways might be affected by DMF. Interestingly, DMF did not significantly increase HUVEC cytotoxicity even at high concentrations (Figure 1b), whereas DMF did induce apoptosis (Figure 1c).

Next, VEGF-induced capillary-like structures formed by endothelial cells were co-incubated with DMF and analyzed 11 days after seeding onto Matrigel. DMF treatment significantly inhibited VEGF-induced capillary-like structure formation, as well as basal tube-like formation, of HUVECs in Matrigel (Figure 1d and e). The suppressive effect of DMF was comparable with that of suramin, a known anti-angiogenic compound. Comparable results were also demonstrated with basic fibroblast growth factor treatment (Supplementary Figure S1b and c online). These data suggest that DMF directly affects endothelial cell capabilities, which most likely involves the inhibition of VEGFR2 expression, which is the most important angiogenic receptor.

DMF suppresses VEGFR2 protein expression in HUVECs

We next determined whether the anti-angiogenic effects of DMF correspond with suppression of VEGFR2 protein expression, as measured by western blot analysis. Interestingly, DMF suppressed VEGFR2 protein expression in HUVECs in a time- and concentration-dependent manner (Figure 2a and b). In contrast, DMF treatment did not influence the expression of neuropilin-1 and VE-cadherin (Figure 2a and b).

Cycloheximide, a eukaryotic protein synthesis inhibitor, was then used to analyze whether VEGFR2 downregulation was due to a decrease in protein stability (Figure 3). In the presence of cycloheximide, DMF treatment did not affect the half-life of VEGFR2 protein, which argues against any

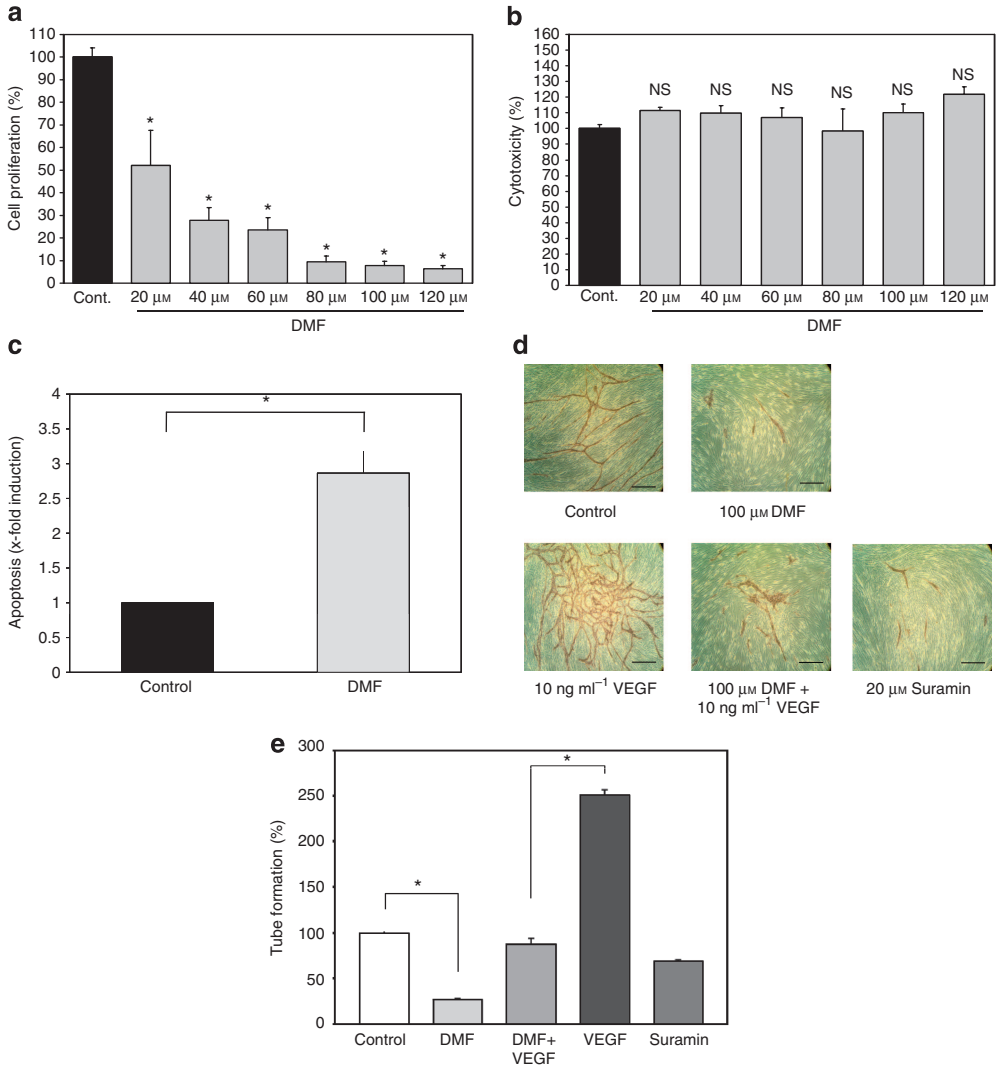

Figure 1. Analysis of endothelial cell function and apoptosis in DMF-treated HUVECs. (**a**) HUVEC proliferation assay. Cells were treated with the indicated concentrations of DMF for 24 hours. Data are expressed as the percentage of cell proliferation in relation to vehicle-treated samples (100%; 0.1% DMSO). Data displayed are representative of at least three experiments that were performed with comparable results. *$P<0.05$ was considered significant. (**b**) Colorimetric assay for the quantification of plasma membrane damage based on the measurement of lactatedehydrogenase activity released from the cytosol of damaged cells into the supernatant. After seeding HUVECs in 96-well plates, cells were incubated with the indicated concentrations of DMF for 24 hours. Average absorbance values (mean ± SD) from quadruplicate replicates per experimental condition were calculated. Data are expressed as the cytotoxicity (%), as determined according to the manufacturer's recommendations. Data displayed are representative of at least three experiments that were performed with comparable results. *$P<0.05$ was considered significant. (**c**) For apoptosis determination, we used a colorimetric assay that quantified histone-complexed DNA fragments. Cells were incubated with 100 μM DMF or a solvent control (0.1% DMSO) for 24 hours. Average absorbance values (mean ± SD) from quadruplicate replicates per experimental condition were calculated. The data are expressed as percent apoptosis (%), as determined according to the manufacturer's recommendations. The data displayed are representative of at least three experiments that were performed with comparable results. *$P<0.05$ was considered significant. (**d**) Two-dimensional, long-term (11 days) *in vitro* angioassay of HUVECs that were treated with vehicle (0.1% DMSO), DMF (100 μM), or suramin (20 μM) in the absence or presence of rhVEGF165 (10 ng ml^{-1}). After 11 days, the cells were fixed and stained for CD31. Images of five representative fields corresponding to the experimental procedures are presented. Data displayed are representative of three experiments that were performed with comparable results. Bar = 100 μM. (**e**) For proliferating vessel quantification, we performed a CD31 ELISA using the culture wells of the angioassay according to the manufacturer's procedures. The data displayed are representative of three experiments that were performed with comparable results. Average absorbance values (mean ± SD) from quadruplicate replicates per experimental condition were calculated. *$P<0.05$ was considered significant. DMF, dimethylfumarate; HUVEC, human umbilical vein endothelial cell; NS, not significant; VEGF, vascular endothelial growth factor.

post-translational mechanism of VEGFR2 downregulation. To determine whether DMF influenced the VEGFR2 phosphorylation, we used a phospho-specific VEGFR2 antibody to analyze the receptor phosphorylation status by western blot analysis; no changes were detected in the phosphorylation status of the receptor (Supplementary Figure S2 online).

DMF suppresses VEGFR2 steady-state mRNA expression

We then determined whether DMF-induced protein downregulation was due to the suppression of VEGFR2 steady-state mRNA levels by reverse transcription (RT)-PCR analysis. Consistent with our protein expression data, DMF suppressed VEGFR2 mRNA expression in a concentration- and

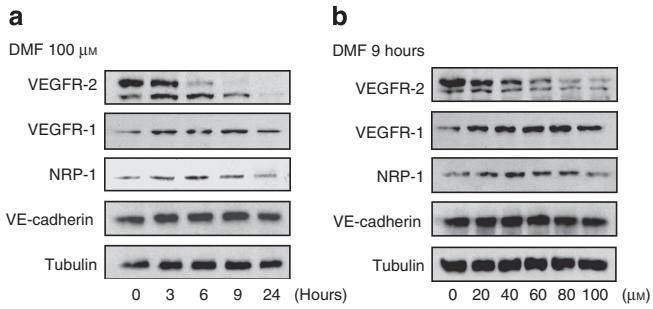

Figure 2. Analysis of endothelial VEGFR2 protein expression in DMF-treated HUVECs. Representative western blot analyses of HUVECs that were treated with vehicle or DMF (100 μM) for varying times (**a**) or concentrations (**b**), as indicated. Total cellular protein was resolved by 8% SDS-PAGE. VEGFR1, VEGFR2, neuropilin-1 (NRP1), VE-cadherin, and tubulin proteins were detected by enhanced chemiluminescence. Comparable results were obtained from at least three independent experiments. DMF, dimethylfumarate; HUVEC, human umbilical vein endothelial cell; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

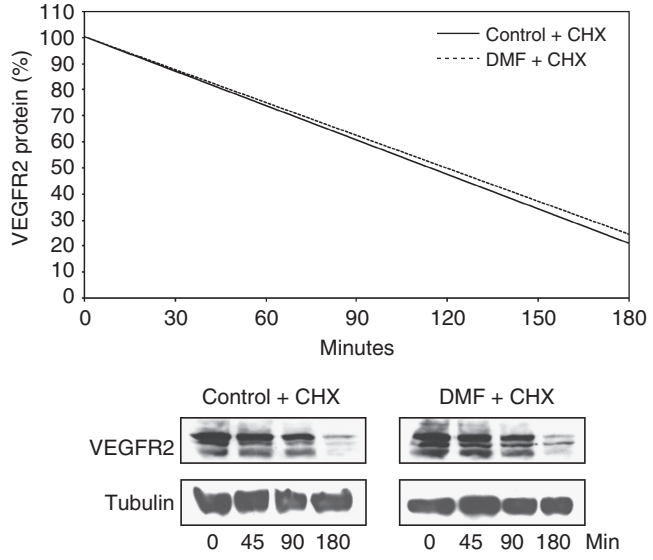

Figure 3. Analysis of VEGFR2 protein half life in DMF-treated HUVECs. HUVECs were preincubated with vehicle or DMF (100 μM) for 2 hours, followed by the addition of cylcohexamide (CHX; 10 μg ml^{-1}) for 0, 45 min, 1.5 and 3.0 hours.Total cellular protein was resolved by 8% SDS-PAGE. VEGFR2 and tubulin were detected by enhanced chemiluminescence. VEGFR2 bands were quantified by densitometric scanning and normalized to tubulin. Comparable results were obtained from at least three independent experiments. DMF, dimethylfumarate; HUVEC, human umbilical vein endothelial cell; VEGFR, vascular endothelial growth factor receptor.

time-dependant manner (Figure 4a and b). Recently, it was demonstrated that VEGFR2 expression is also regulated by a reduction in VEGFR2 mRNA stability (Meissner *et al.*, 2009). We therefore used actinomycin-D, a transcription inhibitor, to determine whether DMF treatment decreased VEGFR2 mRNA stability in HUVECs. In the presence of actinomycin-D, there was no difference in VEGFR2 mRNA half-life between the DMF- and vehicle-treated HUVECs (Figure 4c). Therefore, we determined that there might be additional control mechanisms at the transcriptional level.

Inhibition of VEGFR2 promoter activity is regulated by a cluster of two Sp1 transcription factor-binding sites

We then employed luciferase reporter assays to delineate the underlying molecular mechanisms that mediate DMF-induced downregulation of VEGFR2 mRNA expression. Luciferase reporter constructs containing the 5′-region of the VEGFR2 promoter and a series of deletion and mutation constructs were transiently transfected into vehicle- and DMF-treated HUVECs.

The analysis of luciferase expression in vehicle- and DMF-treated cells revealed that basal luciferase activity was suppressed by approximately 60–80% with the −3.2/+268, −164/+268, and −60/+268 VEGFR2 luciferase constructs (Figure 5). The −60/+268 M1, −60/+268 M2, and −60/+268 M3 constructs, which harbor site-directed mutations in the Sp1 sites at −58 bp, −48 bp, or at both sites, respectively, displayed no significant suppression compared with the non-mutated reporter plasmids (Figure 5). In addition, mutational analysis revealed that both Sp1 sites were essential for the DMF-induced inhibition of VEGFR2 mRNA expression. Mutation of the SP1 sites resulted in the loss of the DMF-induced suppression of VEGFR2 reporter gene expression. These results provide strong evidence that VEGFR2 promoter suppression occurs in an Sp1-dependent manner.

DMF treatment reduces constitutive Sp1-dependent binding to the VEGFR2 promoter

To determine which nuclear factors bind to the Sp1 binding site, we performed electromobility shift assays using nuclear extracts from HUVECs and a ^{32}P-labeled oligonucleotide probe corresponding to the −85/−31 bp VEGFR2 promoter sequence containing the Sp1 binding site cluster. In untreated HUVECs, a distinct complex was observed binding and shifting the migration pattern of the oligonucleotide (Figure 6a, lane 1); however, a significant decrease in DNA binding activity was observed in the lysates of cells treated with DMF (Figure 6a, lane 2). Competition assays using excess unlabeled oligonucleotides supported the assumption that nuclear proteins bind to the −85/−31 bp VEGFR2 promoter sequence in an Sp1 site-specific manner (Figure 6a, lanes 3 and 4). A supershift was observed with addition of Sp1 or Sp3 antibody (Figure 6a, lanes 5 and 6), confirming that SP1 and SP3 bind at the VEGFR2 promoter.

We then determined whether DMF modulated the expression of Sp1 or Sp3 to inhibit VEGFR2 expression. Western blot analysis was performed to analyze the expression of Sp1 and Sp3 protein in DMF-treated cells, and we found that Sp1 and Sp3 protein expression remained unchanged after DMF treatment (Figure 6b). Therefore, these findings collectively provide strong evidence that the DMF-mediated suppression of VEGFR2 transcription is mediated by one distinct mechanism; Sp1-dependent transactivation of VEGFR2 is repressed through the reduction of Sp1 binding at the VEGFR2 promoter.

DISCUSSION

FAEs have been effectively used for the treatment of psoriasis for decades, and several clinical trials have proven the

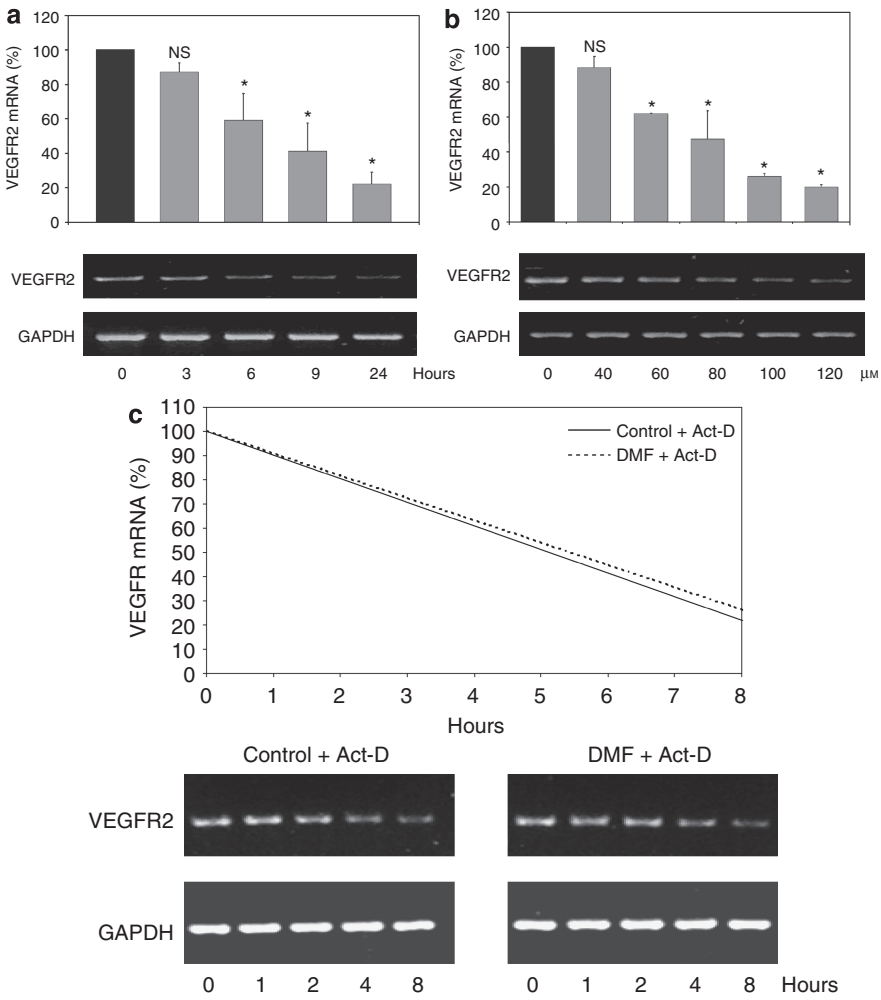

Figure 4. Analysis of endothelial VEGFR2 mRNA expression in DMF-treated HUVECs. Reverse transcription (RT)-PCR analysis of total mRNA extracted from endothelial cells that were treated with vehicle or DMF (100 μM) for varying times (**a**) or varying concentrations (**b**), as indicated. The RT-PCR bands were quantified by densitometry. Optical densities of VEGFR2 bands were normalized based on the corresponding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) bands. The results were confirmed in at least three independent sets of experiments. *$P<0.05$ was considered significant; NS, not significant. (**c**) HUVECs were incubated with vehicle or DMF (100 μM) for 1 hour followed by the addition of actinomycin-D (Act-D; 10 μg ml^{-1}) for 0, 1, 2, 4, and 8 hours. RT-PCR analyses for VEGFR2/GAPDH of total RNA extracted from sub-confluent cell cultures were performed. The PCR products were resolved by 2% agarose gel electrophoresis, and ethidium bromide-stained bands were visualized using an ultraviolet transilluminator. VEGFR2 bands were quantified by densitometric scanning, and the results were normalized to the amount of GAPDH mRNA. Comparable results were obtained from three independent experiments. DMF, dimethylfumarate; VEGFR, vascular endothelial growth factor receptor.

efficacy of FAEs in the treatment of this chronic inflammatory disease, although the definite mechanisms of action are still unknown (Yazdi and Mrowietz, 2008). Recent studies have shown that the anti-inflammatory effects of DMF are also successful in the prevention of relapsing-remitting multiple sclerosis or the suppression of melanoma growth and metastasis (Kappos *et al.*, 2008; Loewe *et al.*, 2006). These results highlight the growing importance and the expanding applicability of DMF for clinical use.

Our data has shown that DMF has distinct anti-angiogenic properties that are in part due to the suppression of VEGFR2 expression. As VEGFR2 is the main receptor involved in endothelial cell survival, proliferation, and vascular permeability, VEGFR2 suppression by DMF may represent a critical mechanism by which its anti-angiogenic effects are mediated.

We provide solid evidence that DMF downregulates VEGFR2 mRNA expression in HUVECs through a distinct mechanism; thus, DMF effectively inhibits angiogenesis and cell proliferation. In addition, we demonstrated that another pro-angiogenic cytokine, basic fibroblast growth factor, failed to stimulate angiogenesis during DMF treatment, possibly revealing another additional mechanism of DMF-dependent anti-angiogenic action. The expression of other major angiogenic receptors, such as neuropilin-1 or VE-cadherin, seems to be insensitive to DMF. VEGFR2 is an important anti-apoptotic receptor in human endothelial cells; therefore, receptor downregulation could explain the DMF-dependent increase in HUVEC apoptosis that we observed. Of course, it cannot be excluded that further apoptotic pathways are induced by DMF intensifying apoptosis. Comparable results were also demonstrated in melanoma cells;

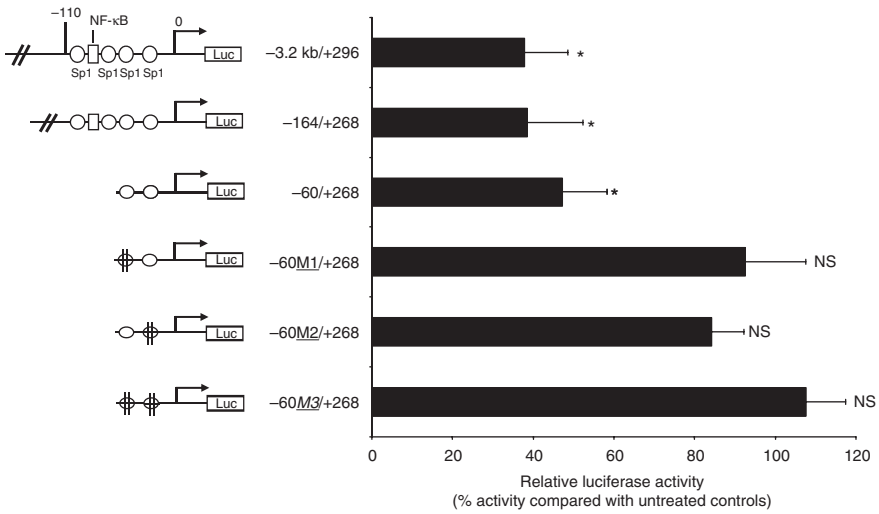

Figure 5. Analysis of 5′ deletion and mutation vascular endothelial growth factor receptor-2 luciferase (Luc) reporter constructs in human umbilical vein endothelial cells. Schematic representation of the respective reporter gene constructs (left panel) with respect to the transcription start site (center panel) and the relative Luc activities (expressed as % activity compared to untreated controls, right panel; mean ± SD of five independent assays performed in triplicate). Data displayed are representative of at least three experiments that were performed with comparable results. *$P<0.05$ was considered significant; NS, not significant.

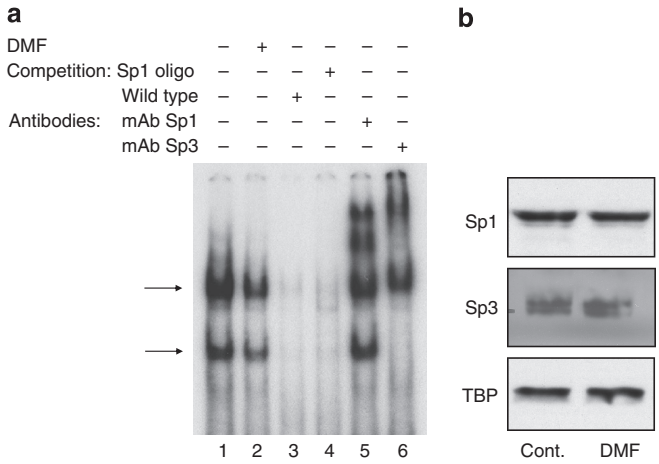

Figure 6. Analysis of transcription factor binding activity in DMF-treated human endothelial cells. (**a**) Representative electromobility shift assays using nuclear extracts of vehicle- and dimethylfumarate (DMF)-treated (100 μmol l^{-1}, 1 hour) human umbilical vein endothelial cells (HUVECs). Competition with unlabeled DNA (−63/−31 bp; lane 3; 100 molar excess) or with unlabeled excess double-stranded Sp1 consensus oligonucleotides (lane 4; 0.35 μmol l^{-1}). A representative autoradiography from three independent experiments is shown. Supershift analyses were performed by the addition of specific Sp1 (lane 5) or Sp3 antibody (lane 6; 100 ng μl^{-1}). Arrows to the left indicate the formation of Sp-dependent binding complexes. Data displayed are representative of at least three experiments that were performed with comparable results. (**b**) Representative western blot analysis of HUVECs that were treated with vehicle or DMF (100 μM) for 24 hours. Nuclear protein was resolved by 8% SDS-PAGE. Sp1, Sp3, and TATA binding protein was detected by enhanced chemiluminescence. Comparable results were obtained from at least three independent experiments.

Loewe *et al.* (2006) showed an increase in apoptosis in DMF-treated melanoma cells *in vitro* and *in vivo*. Interestingly, the melanoma cell line A375 used by Loewe *et al.* (2006) overexpresses VEGF and VEGFR2, favoring cell growth and survival comparable with human endothelial cells that also stimulate each other by an autocrine loop (Graells *et al.*, 2004). The pro-apoptotic action of DMF in these tumor cells might in part be explained by the suppression of VEGFR2.

Sp1 is a ubiquitously expressed transcription factor that is particularly important for the regulation of TATA-less genes that encode housekeeping proteins. This gene family also regulates most growth factors and receptors (Wierstra, 2008). Our data indicate that DMF suppresses VEGFR2 transcription via two Sp1 binding sites in the proximal promoter (between −60 and −30 bp), thus repressing Sp1-dependent DNA binding and transactivation. A similar mode of action has recently been described for the anti-angiogenic effects of proteasome inhibitors, which mediate suppression by a single Sp1 site located at the −58-bp position in the VEGFR2 promoter (Meissner *et al.*, 2009). To date, there is no evidence that DMF can prevent Sp1 binding to target genes; however, there are reports describing the DMF-induced regulation of other transcriptional factors. DMF was described to be sufficient to suppress the DNA-binding ability of NF-κB and the nuclear accumulation of the p50 and p65 subunits (Loewe *et al.*, 2002; Gesser *et al.*, 2007). Recently, Seidel *et al.* (2009) demonstrated that DMF suppresses the tumor necrosis factor-α-mediated phosphorylation of the p65 subunit and NF-κB nuclear entry, as well as the NF-κB/DNA complex formation in airway smooth muscle cells. These events were partly mediated by the inhibition of mitogen stress kinase-1 and the subsequent dephosphorylation of Sp1. These effects might suppress the important inflammatory effects of NF-κB in asthma and seem to explain the potentially beneficial effects of DMF on inflammatory diseases (Seidel *et al.*, 2009).

To date, little is known about the effects of DMF on human endothelial cells. The published literature mainly concentrates on DMF regulation of different endothelial cell

adhesion molecules, such as ICAM-1, E-Selectin, vascular cell adhesion molecule-1, or CD62E, whose expression was suppressed by DMF treatment (Vandermeeren *et al.*, 1997; Loewe *et al.*, 2001). These reports demonstrate that cell adhesion molecules are important targets of DMF in the therapy of chronic inflammatory diseases such as psoriasis. Rubant *et al.* (2008) demonstrated that DMF inhibits the rolling of human peripheral blood mononuclear cells *in vivo,* mainly through a P- and E-selectin-dependent manner. Loewe *et al.* (2002) analyzed these effects in more detail and demonstrated that DMF appears to selectively prevent nuclear entry of activated NF-κB.

Reliable data regarding the effects of DMF on angiogenesis have not been published. The results of our study demonstrate that DMF treatment has a significant anti-angiogenic concentration-dependant effect on human endothelial cells. Angiogenesis is a key process in the evolution and maintenance of psoriasis and other chronic inflammatory diseases, such as inflammatory bowel disease, chronic obstructive pulmonary disease, and rheumatoid arthritis (Siafakas *et al.*, 2007; Pousa *et al.*, 2008; Paleolog, 2009). Interestingly, Sauder *et al.* (2002) observed that anti-angiogenic therapy with neovastat, which targets the expression of VEGF and matrix metalloproteinases, is an effective treatment option for psoriasis. Therefore, the results of our study with DMF fit well into the picture of treatment of chronic inflammatory diseases and broaden the knowledge of the mode of action of DMF.

Recently, DMF was demonstrated to exhibit profound anti-tumor effects; Yamazoe *et al.* (2009) convincingly showed that DMF inhibits tumor cell invasion and metastasis in a B16B6 mouse model by suppressing the expression and activity of matrix metalloproteinases in melanoma cells. Similar results were presented by Loewe *et al.* (2006), showing that DMF is anti-proliferative, pro-apoptotic, and reduces melanoma growth and metastasis in a severe combined immunodeficient mouse model. Whether DMF-dependent anti-angiogenic mechanisms are involved *in vivo* in its anti-tumor effects has only recently been addressed by Valero *et al.* (2010), who showed that lymphangiogenesis is inhibited by DMF in a melanoma xenotransplantation model. Additional *in vivo* analyses of the inflammatory process and different tumor diseases are needed to identify the setting, in which angiogenesis has an important role.

The inhibition of angiogenesis is a key process not only in preventing inflammation but also in the prevention of tumorigenesis, and clinical trials targeting angiogenesis have already begun using tyrosine kinase inhibitors or anti-VEGF antibodies (Loges *et al.*, 2009; Ribatti, 2009). Therefore, our study analyzing the impact of DMF on angiogenesis also delivers previously unreported information regarding the anti-tumor action of this compound. It can be hypothesized that the anti-angiogenic action of DMF, conveyed by the inhibition of VEGFR2 expression, explains the anti-tumor effects of this compound. Whether this compound might be beneficial in the treatment of human cancer should be further evaluated in animal and clinical studies because of the very low cytotoxic effects seen with DMF.

DMF is a proven anti-psoriatic and anti-inflammatory compound that has been in clinical use for more than 30 years. In addition to the known anti-inflammatory and anti-tumor mechanisms mediated by DMF, our study provides to our knowledge a previously unreported mechanism of action, and knowing the mechanism by which DMF works might help in the treatment of diseases associated with increased angiogenesis.

MATERIALS AND METHODS

Reagents

Recombinant human $VEGF_{165}$ and basic fibroblast growth factor were purchased from R&D Systems (Minneapolis, MN). DMF, cycloheximide, and actinomycin-D were obtained from Sigma-Aldrich (Hamburg, Germany).

Cell culture

HUVECs were purchased from PromoCell (Heidelberg, Germany) and were cultured until the fifth passage at 37 °C and 5% CO_2 in Endothelial Cell Growth Medium (Lonza, East Rutherford, NJ).

Cell proliferation and cytotoxicity assay

The effect of DMF on cell proliferation was measured by quantifying BrdU via a cell proliferation immunoassay from Roche Diagnostics (Grenzach, Germany). Twenty-four hours after seeding, cells were serum-starved for 24 hours and incubated with BrdU and DMF at the indicated concentrations for 24 hours. The cytotoxic potential of DMF was determined using a lactatedehydrogenase-based cytotoxicity detection kit from Roche. Twenty-four hours after seeding, the cells were incubated with DMF for 24 hours at the indicated concentrations.

Apoptosis assay

The effect of DMF on apoptosis was analyzed using a Cell Death Detection ELISA PLUS-Kit from Roche Diagnostics. The assay was carried out according to the manufacturers' instructions. Briefly, synchronized cells (10^5 cells per 100 µl) were incubated with 100 µM DMF or a solvent control (0.3% DMSO) for 24 hours as indicated. Cell lysates were placed into a streptavidin-coated microtiter plate followed by the addition of anti-histone-biotin and anti-DNA-peroxidase The quantification of the amount of nucleosomes retained in the immunocomplexes was determined photometrically using 2,29-azino-bis-3-ethylbenzthiazoline-6-sulfuric acid as the substrate.

Tube-formation assay

HUVECs and human diploid fibroblasts were co-cultured in 24-well plates at day 0 (AngioKit, TCS CellWorks, Buckinghamshire, UK). The cells were then treated with vehicle (0.1% DMSO), DMF (100 µM), VEGF (10 ng ml^{-1}), VEGF and DMF, or suramin (20 µM) for 11 days; the compound concentrations were maintained during the course of the experiment. Tube formation was assayed at day 11 following fixation and tubule staining with CD31 (platelet/endothelial cell adhesion molecule-1) according to the manufacturer's instructions. Images of five representative fields corresponding to the experimental procedures are shown. Proliferating vessels were quantified by a CD31 ELISA using the samples from the culture wells of the AngioKit according to the manufacturer's procedures.

Western blot analysis

Protein extracts were prepared as described previously (Meissner *et al.*, 2008). Following SDS-PAGE and electroblotting, membranes were incubated with the following primary antibodies: anti-VEGFR2 and anti-neuropilin-1 (R&D Systems), anti-Sp1, clone PEP2, anti-VE-cadherin, phospho-VEGFR2 and anti-tie-2 (Santa Cruz, Heidelberg, Germany), and anti-tubulin (LabVision, Fremont, CA). Primary antibody application was followed by incubation with horseradish peroxidase-conjugated secondary antibodies (anti-mouse and anti-rabbit IgG, Amersham, Uppsala, Sweden; anti-goat, Dako, Glostrup, Denmark). Blots were developed using an enhanced chemiluminescence detection system (Amersham) according to the manufacturer's instructions.

RNA extraction and RT-PCR

RT-PCR analysis was performed on total RNA (150 ng) extracted from sub-confluent cell cultures. Total cellular mRNA was isolated by the RNeasy Mini Procedure (Qiagen, Hilden, Germany) after DNase digestion. RT-PCR analyses for VEGFR2 and glyceraldehyde-3-phosphate dehydrogenase were performed with the One Step RT-PCR Kit (Qiagen). PCR products were resolved by 1–2% agarose gel electrophoresis, and ethidium bromide-stained bands were visualized using an ultraviolet transilluminator. The densitometric analysis was used to quantify band intensities using the public domain Java image-processing program ImageJ (v1.29 s). Optical densities of the VEGFR2 bands were corrected for loading differences based on the corresponding glyceraldehyde-3-phosphate dehydrogenase bands. The primer sets for VEGFR2 and glyceraldehyde-3-phosphate dehydrogenase were previously published (Meissner *et al.*, 2008).

Transient transfection and analysis of reporter gene expression

HUVECs (1.0×10^5 cells per well in 12-well plates) were transfected with 0.5 μg of the appropriate *firefly* luciferase construct and 0.1 μg phRG-TK vector (Promega, Madison, WI) using the SuperFect transfection reagent (Qiagen). Human VEGFR2 reporter gene constructs were generously provided by Dr C Patterson (University of North Carolina, Chapel Hill, NC). Twenty-four hours after transfection, cells were treated with vehicle (0.3% DMSO) or with DMF for 24 hours. Luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega).

Preparation of nuclear extracts and gel mobility shift analysis

HUVECs were treated with vehicle (0.1% DMSO) or DMF for 30 minutes. Nuclear proteins were extracted as described previously. DNA-binding reactions were performed with or without excess unlabeled competitor, Sp1 consensus oligonucleotide (Promega), and Sp1 and Sp3 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) as described previously (Meissner *et al.*, 2009).

Statistical analysis

The data are expressed as the mean ± SD/SE from at least three independent experiments. Statistical analyses were performed using the Student's *t*-test.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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