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The Role of Vascular Endothelial Growth Factor in Systemic Sclerosis

Abstract

The pathological hallmarks of Systemic sclerosis (SSc) constitute an inter-related triad of autoimmunity, vasculopathy and tissue remodeling. Many signaling mediators have been implicated in SSc pathology; most focusing on individual components of this pathogenic triad and current treatment paradigms tend to approach management of such as distinct entities. The present review shall examine the role of vascular endothelial growth factor (VEGF) in SSc pathogenesis. We shall outline potential mechanisms whereby differential vascular endothelial growth factor-A (VEGF-A) isoform expression (through conventional and alternative VEGF-A splicing,) may influence the relevant burden of vasculopathy and fibrosis offering novel insight into clinical heterogeneity and disease progression in SSc. Emerging therapeutic approaches targeting VEGF signaling pathways might play an important role in the management of SSc, and differential VEGF-A splice isoform expression may provide a tool for personalized medicine approaches to disease management.

Key words

Systemic sclerosis (scleroderma)
Pathogenesis
Vascular endothelial growth factor
VEGF-A
VEGF-A_{165b}

Fibrosis
Vasculopathy
Anti-angiogenic
Pro-fibrotic

Abbreviations:

ACA anti-centromere autoantibody, Ang angiopoietin, BM basement membrane, Cav caveolin, CCH, chronic continuous hypoxia, CIH chronic intermittent hypoxia, CTGF connective tissue growth factor, dcSSc diffuse cutaneous SSc, DU digital ulceration, ECM extracellular matrix, EndoMT endothelial-to-mesenchymal transition, ERA endothelin-1 receptor antagonists, HIF hypoxia inducible factor, HRE hypoxia response elements, HSP heparin sulphate proteoglycan, HSC haemopoietic stem cells, Id1 Inhibitor of DNA binding protein-1, IL-1 β Interleukin 1 β , IL6 interleukin-6, ILD interstitial lung disease, IPF idiopathic pulmonary fibrosis, lcSSc limited cutaneous SSc, mRSS modified Rodnan skin score, MSC mesenchymal stem cell, MVEC microvascular endothelial cell, NC nailfold capillaroscopy, NO nitric oxide, NP neuropilin, PAH pulmonary arterial hypertension, PDE5 phosphodiesterase-5, PDGF platelet derived growth factor, PDGFR platelet derived growth factor receptor, PIGF placental growth factor, RNAPIII anti-RNA polymerase III autoantibody, RP Raynaud's phenomenon, Scl-70 anti-topoisomerase autoantibody, SSc Systemic sclerosis, TK tyrosine kinase, TGF β transforming growth factor- β , TNF α tumour necrosis factor- α , VEGF vascular endothelial growth factor, VEGFR vascular endothelial growth factor receptor.

Introduction

Systemic sclerosis (SSc) is a rare multisystem autoimmune disease whose pathological hallmarks constitute a triad of vasculopathy, autoimmunity and aberrant tissue remodeling, manifesting as Raynaud's phenomenon (RP), circulating autoantibodies and cutaneous fibrosis (scleroderma) respectively. SSc is a heterogeneous disease whose clinical phenotype and major subgroup classifications are largely defined by the relative presence and extent of tissue fibrosis and vasculopathy. Autoantibody expression has strong associations with particular clinical phenotypes[1] but these associations are not absolute and the presence and severity of clinical features vary widely between individuals. Despite this, management approaches are relatively uniform focusing predominantly on immunosuppression and vasodilation[2]. LeRoy was the first to propose an important inter-relationship between the pathological hallmarks of the disease, suggesting vasculopathy as an important driver of tissue remodeling and autoimmunity[3]. Many signaling pathways have been implicated in SSc pathogenesis with a significant focus on the pro-fibrotic potential of transforming growth factor- β (TGF β), platelet derived growth factor (PDGF), connective tissue growth factor (CTGF), serotonin and interleukins[4], although therapeutic trials targeting specific pro-fibrotic molecular targets to date have been disappointing[5-7].

Therapeutic approaches targeting the molecular pathways relating to vasculopathy and tissue hypoxia have been less extensively studied in SSc but emerging evidence suggests an important role of proteins including hypoxia inducible factor (HIF)[8] and vascular endothelial growth factor (VEGF)[9-14] in SSc pathogenesis. Indeed, recent clinical trials demonstrating the efficacy of small molecule tyrosine kinase inhibitors targeting VEGF receptor signaling[15] has led to renewed interest in the potential role of VEGF in SSc. This review examines the available evidence of VEGF signaling in SSc and explores the potential contribution to SSc pathogenesis. We shall describe emerging evidence concerning the competing influence of differential VEGF splice isoform expression and the potential implications for therapeutic approaches targeting VEGF in SSc.

Vasculopathy in Systemic sclerosis

Endothelial injury is an important initiating event in SSc and vasculopathy occurs early in the disease process. Endothelial dysfunction and apoptosis, increased vascular permeability, vessel wall remodeling, platelet aggregation, and a perivascular inflammatory cell infiltrate pre-date the development of established tissue fibrosis[16-18]. Clinical manifestations of RP and morphological capillary changes at the nailfold (Figure 1) precede the onset of overt cutaneous fibrosis by an average of four years[19, 20]. A 20-year prospective study following the disease course of patients with RP, noted transition on nailfold capillaroscopy (NC) from giant capillaries to capillary loss occurring in close temporal relationship to the emergence of clinical features that led to a diagnosis of definite SSc (usually defined by the emergence of cutaneous fibrosis)[20]. A number of studies have also identified a positive and progressive association between the severity of microangiopathy on NC and the extent of skin fibrosis[21-25]. Indeed, 'early' changes are more frequently identified in limited cutaneous SSc (lcSSc)[23, 26] whereas 'late' changes are more prevalent in diffuse cutaneous SSc (dcSSc)[19, 25]. The rate of progression of NC changes (and skin fibrosis) varies according to autoantibody specificities. For example, SSc patients carrying anti-RNA polymerase III autoantibodies (RNAPIII) have been noted to develop enlarged capillaries and capillary loss earlier in the disease course (4 vs. 15 years) than patients with anti-centromere autoantibody (ACA)[20].

Unsurprisingly, the progression of NC changes, predict the severity of peripheral vascular manifestations. For example, in lcSSc and dcSSc, capillary loss that accompanies 'late' NC pattern carries an increased risk of developing new digital ulcers (DU) compared with preserved capillary number seen in 'normal' or 'early' patterns[23, 27]. In that vein, DU are more common in dcSSc than lcSSc[28]. However, whilst DU are significantly less common in SSc sine scleroderma (ssSSc), no association with NC pattern has been identified in this subset, perhaps reflecting low study numbers[28]. Larger capillary loop diameter and greater numbers of giant capillaries have also been used to predict the future development of DU in SSc, demonstrating the association between disorganized neoangiogenesis and inadequate tissue perfusion[29].

The principle pathological effect of vasculopathy in SSc is tissue hypoxia. Indeed, oxygen saturations in fibrotic skin of SSc patients are notably low (pO₂ 23.7 \pm 2.1mmHg compared to 33.6 \pm 4.1mmHg in healthy controls (p<0.05))[11]. Tissue hypoxia in SSc is further exacerbated by the oxygen demands of

the inflammatory milieu and impaired oxygen diffusion secondary to aberrant tissue remodeling and accumulation of extracellular matrix proteins.

Tissue hypoxia leads to over-expression of cell signaling molecules such as HIF and VEGF that protect cells from oxidative stress, promote wound healing and enhance tissue perfusion through neoangiogenesis[30].

Hypoxia inducible factor (HIF)

HIF is a family of transcription factors within the PER ARNT SIM transcription group[31]. HIF is a heterodimer consisting of an α subunit (HIF1 α , 2 α , 3 α ; whose expression is dependent on local tissue oxygenation[32]) and a β subunit (a constitutively expressed nuclear protein)[31]. Under normoxic conditions, HIF α is rapidly degraded by proteasomes and is therefore only detected at significant levels under hypoxic conditions[31]. As HIF α accumulates, the HIF α / β dimer (hereafter referred to as HIF1, 2, 3) binds to hypoxia response elements (HRE) to up-regulate gene transcription[32]. HIF1 α is expressed widely throughout almost all tissues, whereas HIF2 α paralog is differentially expressed in endothelium, renal, hepatic, pulmonary and brain tissue[33]. HIF1 has been implicated in a number of diseases characterized by altered angiogenesis, inflammation and fibrosis [32, 34] including pulmonary arterial hypertension (PAH)[35], which can occur as a manifestation of SSc related vasculopathy.

Vascular endothelial growth factor (VEGF) family

The VEGF family comprises placental growth factor (PlGF) and four mammalian VEGF subgroups (VEGF A-D). Figure 2 illustrates the biological actions of VEGF receptors (VEGFR) and the associated co-receptors.

VEGF-A was initially described as a vascular permeability factor[36] and subsequently shown to exhibit both mitogenic and angiogenic properties[37, 38]. Levels are precisely controlled such that a single allele deletion results in embryonic failure[39] and VEGFR1 gene mutation causes disorganized endothelial cell lining and failed angiogenesis[40]. However, its biological actions now appear wider, including neutrophil chemo-attraction[41] and fibrosis[42]. Hypoxia is a major up-regulator of VEGF-A both via up-regulation of HIF[32] and via hypoxic VEGF-A mRNA stabilization[43].

Variable splicing of the 6th and 7th exons of VEGF-A results in different isoforms (hereafter referred to as VEGF-A_{xxx}a); named according to their respective number of amino acids (e.g. VEGF-A_{165a})[44]. VEGF-A_{165a} is the dominant pro-angiogenic factor amongst the VEGF-A family acting through the principle receptor (VEGFR2)[45], to stimulate neoangiogenesis through proliferation and migration of endothelial cells to form new tubular vessel structures[46].

Until recently, all VEGF isoforms were considered pro-angiogenic factors. However, since 2002 a number of alternative splice variants of VEGF-A (VEGF-A_{xxx}b and VEGF-A_x) have been identified, some of which inhibit angiogenesis through competitive binding of VEGFR2[47-49] and absence of neuropilin-1 (NP-1) co-receptor binding (Figure 2)[50]. The latter also directs alternative intracellular trafficking in favour of VEGFR2 degradation[51]. However, VEGF-A_{xxx}b isoforms may have physiologically beneficial roles in placental neoangiogenesis and pre-eclampsia during pregnancy[52] and the inhibition of tumour growth and metastatic progression in many cancers[47].

Other members of the VEGF family (VEGF-B-D and PlGF) also promote angiogenesis through co-binding of VEGFR1 and NP-1[53]. VEGF-B also plays a role in fatty acid transport and may provide a therapeutic target for insulin resistance and type 2 diabetes[53]. VEGF-C and VEGF-D signal through an alternative VEGFR3, promoting lymphangiogenesis in embryonic and postnatal periods [53].

The potential role of VEGF in SSc related vasculopathy

In view of the characteristic microvascular manifestations of SSc, VEGF-A pathways have attracted interest as potentially important drivers of disease pathogenesis. In view of the pronounced capillary drop out found in SSc, the authors of early studies were surprised to identify high levels of circulating serum VEGF-A in both early[9] and established SSc [22, 54], although serum VEGF-A was noted to be comparatively lower in SSc patients with DU[9, 55]. VEGF associations with systemic organ manifestations of SSc have been less extensively studied and are notably varied. Circulating VEGF-A

levels in SSc pulmonary vasculopathy are contradictory[56, 57]. Limited data shows no correlation between elevated serum VEGF-A with ultrasound parameters of renal vasculopathy[58]. One study has reviewed VEGF-A through non-invasive sampling of tears of SSc patients and found levels to be surprisingly low possibly explained by reduced tear secretion associated with dry eye syndrome[59].

The elevated VEGF-A levels initially appeared at odds with the obliterative microangiopathy associated with progressive capillary loss[9, 60, 61]. A proposed explanation for these apparently conflicting findings is cellular compartmentalization of VEGF-A and its receptors; a biological concept that might be important in healthy lung homeostasis[62]. However, the identification of VEGF-A splice variants with opposing angiogenic function provides a deeper and more compelling explanation. VEGF-A_{165a} and VEGF-A_{165b} isoforms differ by only six amino acids at exon 8. Commercially available VEGF-A ELISAs are unable to differentiate between these isoforms. Thus, aforementioned studies[9, 22, 54, 60, 61] likely detected pan-VEGF-A (representing co-detection of VEGF-A_{xxx}a and VEGF-A_{xxx}b soluble isoforms). Subsequent studies used isoform VEGF-A_{165b} specific detection methods to confirm an association between VEGF-A_{165b} and the 'late' avascular patterns on NC[14]. Furthermore, those with 'early' nailfold changes (i.e. few microvascular changes) have similar VEGF-A_{165b} levels to healthy controls[14], suggesting that anti-angiogenic isoform expression evolves with disease progression, although longitudinal studies have yet to confirm this.

VEGF receptor status in SSc skin, serum and cell culture is mixed and inconclusive[11, 63-66]. However, higher levels of circulating soluble VEGFR2 appear to be associated with telangiectasia[64]. Urokinase-type plasminogen activator receptor (uPAR), which is required for VEGFR2 internalization, is reduced in SSc skin[50]. Additionally, NP-1 is reduced in skin and serum[50, 65] and associates with DU and more advanced (active/late) NC patterns in SSc[65]. Interestingly, despite evidence of microvasculopathy at the nailfold, reduced serum NP-1 does not appear to associate with specific NC patterns in those with pre-SSc[67]. However, exposure of MVEC to patient sera attenuates NP-1 expression; a phenomenon demonstrated even by sera from pre-SSc donors[67]. In combination, the functional status of VEGFR2 appears to be impaired from multiple co-factors, potentially reducing the pro-angiogenic potential of VEGF-A_{xxx}a and potentiating VEGF-A_{xxx}b inhibitory action.

Given the relationship between NC pattern and VEGF-A_{165b}, and known correlations between capillary density and both gas transfer[22] and the presence of SSc-related pulmonary disease (both interstitial lung disease (ILD) and PAH)[68], the relationship between inhibitory VEGF-A_{xxx}b isoforms and pulmonary vasculopathy is of interest but has not been investigated to date. Interestingly, transgenic mice over-express anti-angiogenic pulmonary VEGF-A_{165b} do not develop vascular abnormalities[42] whereas overproduction of VEGF-A_{164a} (the murine equivalent of VEGF-A_{165a}) results in increased vessel number and wall thickness[69] and dilated and disorganized vasculature[70] suggesting it is the relative rather than absolute level of the anti-angiogenic isoforms that dictates vascular morphology.

The roles of VEGF-B-D have been less extensively investigated in SSc. VEGF-C/D regulate lymphangiogenesis and lymphatic endothelium (Figure 2). SSc lesional skin displays a progressive reduction in lymphatic number[71, 72] despite the fact that circulating VEGF-C and cutaneous VEGF-D[73] and its receptor (VEGFR3)[72] are increased. This might indicate impaired downstream signaling of VEGFR3 or the presence of splice variants of VEGF-C/D with opposing functions; akin to the aforementioned VEGF-A_{xxx}b isoforms. In one study, plasma VEGF-D levels were shown to increase at the time of PAH diagnosis[74].

The potential role of VEGF-A in fibrosing disease

HIF and VEGF-A have been implicated in a number of fibrosing diseases including graft versus host disease, hepatic fibrosis and idiopathic pulmonary fibrosis (IPF) [75-82].

A pro-fibrotic role for VEGF-A_{165a} in SSc is supported by the demonstration of increased collagen induction in both healthy and SSc dermal fibroblasts in response to VEGF-A_{165a} with more pronounced effects observed in SSc fibroblasts[83]. Serum panVEGF-A levels in SSc correlate with skin scores[60] and increased levels are associated with dcSSc[9, 60] and anti-topoisomerase autoantibodies (anti-Scl-70)[9, 84]. PanVEGF-A and HIF1 α are increased in SSc hypoxic lesional skin[8, 11] and over-expressed by dermal fibroblasts cultured under hypoxic conditions[85]. Furthermore, panVEGF-A is overexpressed in non-lesional skin predating the onset of fibrosis[11] implicating VEGF-A as an early signaling protein

in fibrosis. However, whilst lesional skin is notably hypoxic, the pO₂ of non-lesional skin is normal[11] suggesting that VEGF-A expression may be stimulated by factors beyond hypoxia.

To date, the focus of VEGF-A_{165b} investigation in SSc has been with regard to its anti-angiogenic function and there are few observations reported with regard to fibrosis in SSc. Studies have identified increased VEGF-A_{165b} in the skin (mRNA) and plasma of SSc patients[12, 14], which may account for the majority of panVEGF-A overexpression[12]. VEGF-A_{165b} appears to be particularly elevated in certain autoantibody profiles (anti-centromere and anti-Scl-70)[86, 87] although, in contrast to panVEGF-A, VEGF-A_{165b} levels (in skin and plasma) do not apparently correlate with extent of skin involvement[12, 14]. Recent data from murine models of an alternative fibrotic disease (IPF), suggest that VEGF-A_{xxx}a isoforms act as pro-fibrotic drivers of IPF whilst the VEGF-A_{165b} isoform has opposing anti-fibrotic properties[42]. In this model, the balance between VEGF-A_{165a} and VEGF-A_{165b} expression may also be important in IPF pathogenesis[42]. Considering this parallel fibrotic disease, it may be hypothesized that VEGF-A_{165b} could detrimentally contribute to the progressive vasculopathy in SSc whilst encouraging regression of skin fibrosis later in disease. Interestingly, VEGF-A_{165b} appears to be higher in the skin of early SSc (despite comparable circulating plasma levels)[12]. This may suggest the occurrence of early isoform switching.

There is conflicting data regarding the association of circulating panVEGF-A levels with the degree of pulmonary fibrosis on computerized tomography[22, 57, 60, 88]. As discussed for SSc-PAH, VEGF-A_{xxx}b isoform expression has not been specifically studied and conflicting reports in SSc-ILD may be a consequence of panVEGF-A detection.

What is the potential cellular source of VEGF-A isoforms in SSc?

If VEGF-A is at the forefront of disease initiation then identifying its cell origin is paramount to understanding and modifying its signaling network. In SSc, panVEGF-A and VEGF-A_{165b} is expressed in fibroblasts, endothelial and perivascular inflammatory cells[11, 12] with additional expression of VEGF-A_{165b} in vascular smooth muscle cells in *ex vivo* lesional skin[12]. Circulating mononuclear cells[89] and skin keratinocytes[8, 11] also produce increased panVEGF-A levels.

In vitro, cultured microvascular endothelial cells (MVEC) express higher VEGF-A_{165b} (co-localized with increased VEGFR2 but with impaired signaling function) than controls[12] (Figure 3). Additionally, when MVEC, from non-lesional SSc skin, are co-cultured in vitro with activated fibroblasts from lesional skin, panVEGF-A and CD31 expression in the former are reduced whilst VEGF-A_{165b} is increased[90]. This is associated with reduced microtubule formation and increased endothelial-to-mesenchymal transition (EndoMT)[90], demonstrating the potential paracrine activity of SSc fibroblasts on the vasculature and potential to perpetuate the cycle posed by the vascular hypothesis[90].

Platelets are an important source of circulating panVEGF-A in SSc[91] and recent investigation has also proven them to be an important source of VEGF-A_{165b}[92]. Furthermore, tubule formation by dermal MVEC in vitro is impaired when incubated with SSc platelet releasate[92] potentially due to the anti-angiogenic action of VEGF-A_{165b}. It is not known whether the platelet load of VEGF-A_{xxx}a/_{xxx}b isoforms remains consistently elevated in SSc or whether isoform switching occurs at some stage in the disease course.

Additional mediators implicated in enhanced VEGF-A signaling in Systemic sclerosis

Whilst hypoxia is the major driver of VEGF-A expression, other cytokines and growth factors can potentiate VEGF signaling, or are themselves potentiated by VEGF-A expression, which could have important implications for SSc pathogenesis. For example, angiopoietins (Ang-1 and -2) are additional regulators of angiogenesis. Under normoxic conditions, Ang-1 aims to maintain vessel stability through Tie2 signaling, whilst Ang-2 is released under hypoxic stress and acts differentially to either facilitate angiogenesis or angio-regression depending on the presence or absence of VEGF-A respectively[93]. Reported circulating levels of angiopoietins and Tie2 are variable in the literature[84, 88, 93-96]. However, noting the results of a recent study, there is a reduction in Ang-1/-2 ratio in serum of both pre-SSc and SSc with particular association with DU history[93]. Furthermore, increased vascular expression of Ang-2, reduced Tie2 and comparable Ang-1 in SSc skin versus controls[93] potentially represents a shift towards an anti-angiogenic environment. Progressive study regarding the association of Ang-2 with

VEGF-A_{xxx}b isoforms may help map the divergent nature of Ang-2 with VEGF-A expression and the implications of VEGF-A isoforms on angiopoietin function.

Inhibitor of DNA binding protein 1 (Id-1) is a transcription factor and chemokine required for endothelial cell migration and is reduced in SSc endothelial cells, resulting in impaired endothelial cell response to VEGF-A stimulation[97]. The influence of Id-1 expression on responses to specific VEGF-A isoforms has not been investigated.

Increased plasma 8-isoprostane reflects increased oxidative stress in SSc[98] and contributes to impaired angiogenesis[98] via increased TXAR/RhoA/ROCK expression and signaling[98] and subsequent inhibition of VEGF-A induced endothelial cell migration[98]. Interestingly, increased plasma 8-isoprostane appears specific to dcSSc and SSc-ILD and not present in lcSSc and SSc-PAH[98]. Once again, further investigation of these pathways with respect to specific VEGF-A isoforms and correlation with SSc subtype is of interest.

Elevated levels of TGF β are evident in skin and lung tissue[99] and peripheral B cells[100] in SSc alongside increased TGF β receptor expression by cultured SSc fibroblasts[101]. HIF1 increases TGF β transcription, which in turn stabilizes HIF1 α [85, 102](Figure 3). This provides potential for TGF β mediated indirect VEGF-A stimulation, but it also directly stimulates VEGF-A production in SSc dermal fibroblasts[103]. Furthermore, the effects of HIF and TGF β on VEGF-A are synergistic in human MVEC *in vitro* via complimentary action at the HRE on the VEGF promoter region[104]. Moreover, and relevant to SSc pathogenesis, TGF β encourages a switch from proximal to distal splicing of VEGF-A exon 8 via p38 MAPK signaling[105] favouring VEGF-A_{165b} production in cultured SSc-MVEC[12]. This could ameliorate VEGF-A mediated fibrosis and offer an explanation for the late improvements in skin thickening that characterizes the natural history of SSc. Increased VEGF-A_{165b} in SSc may therefore in part be directed by TGF β , potentially as part of a negative feedback loop and resulting in mRSS plateau and late improvement.

Treatment of cultured retinal epithelial cells with tumour necrosis factor- α [TNF α], meanwhile, induces a switch from dominant VEGF-A_{165b} at rest to VEGF-A_{165a}[105]. To our knowledge this relationship has not been investigated in SSc specifically. Whilst a previous trial of anti-TNF α agents failed to demonstrate definite improvement in scleroderma[106], the theoretical effects of TNF α inhibition on VEGF-A isoform switching in arresting scleroderma progression is of interest.

PDGF is known to stimulate VEGF-A via phosphatidylinositol 3 kinase[107]. PDGF and its receptors (PDGFR) are increased in SSc and *in vitro*, PDGF can attenuate panVEGF-A production by SSc fibroblasts[11]. Furthermore, there is PDGFR up-regulation (in skin and lung fibroblasts) in response to TGF β stimulation[108]. Thus, it is possible that PDGF may compliment TGF β directed VEGF-A activation in SSc fibroblasts.

Hypoxia induces increased synthesis of CTGF mRNA in both healthy and SSc dermal fibroblasts via HIF1 α dependent pathways[109]. Circulating CTGF is increased in SSc and associations have been found with diffuse skin disease, pulmonary fibrosis and disease duration[110]. *In vitro*, CTGF levels are over-expressed in SSc mesenchymal stem cells (MSC) and increased further by VEGF-A stimulation[111].

Caveolins (Cav) are the principle protein constituent of caveolae (cell membrane invaginations that act as ‘gate keeper’ organelles for a range of cell signaling tasks)[112]. Cav-1 and -2 are the principle caveolins in EC, fibroblasts and adipocytes. Cav-1 acts to down-regulate TGF β [113, 114] and VEGF-A signaling[111] through receptor internalization providing protection against fibrosis such that Cav-1 knockout mice develop SSc-like features[115]. Accordingly Cav-1 levels and therefore VEGFR2 degradation are reduced in SSc[111, 114] with resultant increase in CTGF expression[111]. Impaired expression of Cav-1 in SSc may therefore contribute to increased VEGF-A_{xxx}a_{xxx}b signaling via VEGFR2.

Cytokines such as interleukin-1 β (IL-1 β) and Interleukin-6 (IL-6) are pro-inflammatory and pro-fibrotic mediators that induce HIF and VEGF-A through NF κ B[53] and signal transducer and activator (Stat3)[116] respectively. Circulating IL-1 β is increased in SSc[117] and up-regulates panVEGF-A production *in vitro* SSc fibroblasts[11]. IL-6 is raised in sera[100] and cultured fibroblasts (with further TNF α driven attenuation)[118] and peripheral B cells[100] of patients with SSc, with a correlation between B cell derived IL-6 and mRSS[100]. Accordingly, the potential for anti-IL-6 receptor antibody (Tocilizumab) to treat skin disease in SSc is currently under investigation [119].

Effects of hypoxic design on HIF paralog expression and vascular pathology

In rodent models, differential HIF1 α and HIF2 α paralog expression occur in chronic intermittent hypoxia ((CIH); as is found in obstructive sleep apnoea) as opposed to chronic continuous hypoxia ((CCH); as occurs in chronic lung disease) and important differences in vascular sequelae occur under these varying hypoxic conditions[30]. Specifically, CIH exposure *induces* HIF1 α and *inhibits* HIF2 α in mice resulting in systemic hypertension[120, 121] compared to protective effects of heterozygous HIF1 α ^{+/-} and HIF2 α ^{+/-} on pulmonary vascular remodeling and PAH in transgenic rodents under CCH[122, 123]. SSc uniquely demonstrates both patterns of intermittent tissue hypoxia with distinct attacks of RP early in disease course, as well as more continuous tissue ischaemia as structural vascular changes progress. We hypothesize, the transition from early disease where vasculopathy and fibrosis are developing to established RP and scleroderma may both be precipitated by and feed forward to influence differential HIF paralog function and downstream VEGF-A_{xxx}^a/_{xxx}^b signaling. Notionally, this may explain the heterogeneity of vascular manifestations in lcSSc versus dcSSc. Indeed, an association between a HIF1A (gene encoding HIF1 α) polymorphism and ACA lcSSc suggests further evaluation of HIF signaling in SSc is warranted [124].

Implications of VEGF-A signaling in the management of Systemic sclerosis

Phosphodiesterase-5 (PDE5) inhibitors and dual endothelin-1 receptor antagonists (ERA) form an integral part of current pharmaceutical therapy for SSc related digital and pulmonary vasculopathy. PDE5 inhibitors effectively improve SSc-RP and digital blood flow through inhibition of cyclic guanosine monophosphate degradation and attenuation of nitric oxide (NO) driven vasodilation[125]. VEGF-A and NO are known reciprocal activators[43], however, PDE5 inhibition in SSc related RP does not appear to alter circulating panVEGF-A levels in sera[125], which may suggest that either NO/VEGF-A potentiation occurs locally in tissues or that by the time PDE5 inhibitors are initiated other factors influencing VEGF-A dominate.

In the previously described *in vitro* model, Corallo et al., [90] demonstrated the ability of ERA to reduce EndoMT and reverse the ratio of panVEGF-A:VEGF-A_{165b} in favour of angiogenesis. Indeed, in some studies NC patterns demonstrate devolution after ERA therapy[126]. Furthermore, Corrado et al., [127] suggested that ERA may have anti-fibrotic potential in SSc-ILD. Further study to examine the potential of ERA to ameliorate progression of both vasculopathy and fibrosis is warranted.

Drugs directly targeting VEGF signaling are now used in a variety of clinical settings including malignancy[53], retinopathy[53] and IPF[15]. The latter, Nintedanib (a blanket tyrosine kinase inhibitor including VEGFR1-3, PDGFR and fibroblast growth factor receptor,) has been shown to slow disease progression in IPF[15] and is currently being evaluated in SSc-ILD (NCT02597933) following encouraging work using pre-clinical murine models of lung fibrosis, skin fibrosis and PAH[4]. The effect of Nintedanib on VEGF-A_{165b} signaling specifically is however unknown.

Concluding remarks

The evidence presented suggests VEGF-A in particular is an important signaling factor contributing to SSc pathogenesis even at the earliest clinically detectable stages of disease. More precisely, the anti-angiogenic isoform VEGF-A_{165b} contributes to progressive capillary loss and tissue ischaemia. Herein, we have discussed multiple mediators of VEGF signaling and potential implications in SSc, including but not exclusive to: HIF as a major up-regulator of VEGF-A, the divergent angiogenic potential of Ang-1/-2, pro-inflammatory cytokine IL-6 and pro-fibrotic TGF β with the ability to ‘flip the switch’ to proximal VEGF-A_{165b} splicing. Of equal importance in the complex SSc story, are the potential cellular sources of VEGF-A isoforms. We have considered with particular interest inflammatory cells, platelets, endothelial cells and fibroblasts; all of which have been demonstrated to produce anti-angiogenic VEGF-

A_{165b} and have been repeatedly implicated in SSc pathology. Whilst SSc is primarily a disease of vascular pathology, fibroblasts undeniably play a role in bolstering the vicious cycle through paracrine action, altering endothelial function and phenotype and encouraging a switch in favour of VEGF-A_{165b} production.

With increasing interest in VEGF-A in SSc, a deeper understanding of the isoform specific responsibilities is required. In particular the role of VEGF-A_{165b} in fibrosis is yet to be elucidated. In collating the literature presented here, we have postulated on our own hypotheses. HIF α paralog expression, determined by the nature of tissue hypoxia and local cytokine expression in SSc may contribute to differential VEGF-A isoform expression and is currently the focus of further investigation. Extrapolating from knowledge in parallel diseases, it may be hypothesized that VEGF-A_{165b} is inhibitory of both angiogenesis and fibrosis and may therefore account for progressive microvascular destruction and the natural regression in skin fibrosis that accompanies established SSc. Furthermore, the relative ratio of VEGF-A_{xxx}a:-A_{xxx}b may be important in determining the burden of these clinical features and thus variance in clinical phenotype. This raises the question, whether targeted inhibition of VEGF-A_{165b} may then have beneficial effects on vascular abnormalities but worsen tissue fibrosis? Alternatively, if tissue oxygenation is improved through inhibition of VEGF-A_{165b}, would this break the cycle of the Vascular hypothesis and ultimately abrogate both pathologies? Ultimately, identifying a single molecular target in this multifaceted disease continues to be a challenge. However, VEGF-A and its specific isoforms remain in the spotlight as both potential future biomarkers and therapeutic targets.

Figure legends:

Figure 1 illustrates the evolution of SSc specific nailfold capillaroscopy (NC) changes, from normal through early, active and late patterns[129]. Normal NC pattern as seen in healthy individuals, is recognized by 7-9 regular hairpin shaped capillaries per millimeter. Early pattern maintains capillary number but enlarged* (>20µm limb diameter) and occasionally giant** (>50 µm) capillaries are present. Active pattern shows frequent giant capillaries, microhaemorrhages^o and some reduction in capillary number. Late pattern is classified primarily by severe capillary loss and evidence of neoangiogenesis[⌘] with few/absent giant capillaries/microhaemorrhages.

Figure 2 adapted from collective reports from[43, 45, 48, 53, 130], illustrates vasculogenic actions of VEGF family through their respective signaling receptors including three tyrosine kinase receptors (VEGF receptor-1 (VEGFR1/flt1), VEGF receptor-2 (VEGFR2/KDR/flk1), VEGF receptor-3 (VEGFR3/flt4)), supported by co-receptors (neuropilin-1 (NP-1), neuropilin-2 (NP-2) and heparin sulphate proteoglycan (HSP)). VEGFR2 is the principal receptor for VEGF-A signaling including VEGF-A_{xxx}b isoforms with additional low affinity binding for VEGF-C and VEGF-D following proteolysis. VEGFR1 and VEGFR3 impose regulatory function on VEGFR2. VEGF-A binding to NP-1 and HSP is isoform specific, dependent upon exon splicing[43, 49, 53]. Lack of VEGF-A_{xxx}b affinity for NP-1 contributes to its anti-angiogenic action. Data reporting the affinity of VEGF-A_{121a} for NP1 is mixed and therefore inconclusive[48]. Abbreviations: BM basement membrane, EC endothelial cell, ECM extracellular matrix, HSC haemopoietic stem cells, PIGF placental growth factor, TK tyrosine kinase.

Figure 3 demonstrates the pathway of hypoxia induced VEGF-A induction via HIF and hypoxia mRNA stabilization[32, 43, 53, 104]. Known cellular sources of VEGF-A in SSc are illustrated based on available evidence (panVEGF-A = (a), VEGF-A₁₆₅b = (b))[11, 12, 89, 91, 92, 103, 111, 114, 131]. *TGFβ and HIF1α synergistically increase VEGF-A in endothelial cells[104]. Whilst TGFβ has been shown to favour VEGF-A₁₆₅b production in SSc-MVEC, similar evidence is not available in other cell lines, where only results for panVEGF-A have been reported. Abbreviations: HRE hypoxia response elements (found in the VEGF-A gene promoter region).

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