Endothelial cells and endothelial progenitor cells in the pathogenesis of systemic sclerosis

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Abstract

Systemic sclerosis (SSc) is a connective tissue disease characterized by excessive fibrosis, microvascularopathy, chronic inflammation, and autoimmunity. Endothelial cell (EC) injury and subsequent endothelial cell dysfunction is believed to be an initial event that eventually leads to a vicious pathogenic cycle. This process is further enhanced by defective angiogenesis and vasculogenesis, as the vascular repair machinery does not work properly. Endothelial progenitor cells (EPCs) are functionally and quantitatively insufficient to recover the endothelium in SSc patients. The dysfunctional ECs and EPCs not only trigger the formation of typical vascular lesions, such as progressive intimal fibrosis in small arteries and the loss of capillaries, but also promote a series of inflammatory and profibrotic processes, such as endothelial-mesenchymal transition and recruitment and accumulation of monocytic EPCs with profibrotic properties. These processes together contribute to the accumulation of extracellular matrix in the affected tissue. This review features current insights into the roles of ECs and EPCs in the pathogenesis of SSc.

Keywords: Scleroderma, systemic sclerosis, endothelial cell, progenitor

Introduction

Systemic sclerosis (SSc) is a connective tissue disease characterized by a combination of excessive fibrosis, microvascularopathy, chronic inflammation, and autoimmunity (1). Vascular involvement in patients with SSc mainly affects small arteries and causes reduced blood flow and tissue ischemia, leading to clinical manifestations, such as digital ulcer and pulmonary arterial hypertension (PAH). The vascular pathologies unique to SSc include progressive intimal proliferation and fibrosis in small arteries, along with the loss of capillaries. The mechanism of SSc vasculopathy is not fully understood, but increasing evidence indicates that endothelial injury and subsequent endothelial dysfunction is a primary event that triggers the subsequent formation of typical vascular lesions (2). In addition, vascular recovery barely occurs in SSc patients, which indicates a defective vascular repair process (3). Postnatal vascular repair is mediated through the collaborative effects of two distinct processes: (1) angiogenesis, i.e., the formation of new blood vessels sprouting from preexisting vessels through proliferation and migration of mature endothelial cells (ECs), and (2) vasculogenesis, i.e., the de novo differentiation of mature ECs through recruitment and differentiation of endothelial progenitor cells (EPCs) (4). This results in the activation of inflammatory and fibrotic processes in perivascular lesions, leading to complex vascular remodeling and irreversible structural changes. This review focuses on the roles of two major cell types that contribute to the homeostasis of the vascular system, ECs and EPCs, in the pathogenic processes of vasculopathy and excessive fibrosis in SSc patients.

Role of ECs in the pathogenesis of SSc

EC apoptosis as a trigger

It is believed that endothelial injury is an initial event that eventually leads to EC dysfunction in SSc patients, and can be triggered via a number of different mechanisms, including infection, ischemia-reperfusion reaction caused by the vasospasm resulting from Raynaud’s phenomenon, oxidative stress through abnormal regulation of reactive oxygen species, turbulent blood flow and shear stress, and the imbalance between coagulation and fibrinolysis (5, 6). In this regard, an infection with human cytomegalovirus induces antibodies to recognize an amino acid sequence on the human cytomegalovirus-derived protein UL94, which is homologous to NAG-2, a surface molecule highly expressed on ECs. Antibodies against UL94 peptide have been shown to induce apoptosis of ECs upon engagement with the NAG-2-integrin complex (7). Another potential contributor is anti-endothelial cell antibody (AECA), which is a heterogeneous antibody family...
that reacts with various cell surface antigens on ECs (8). The mechanisms of AECA-mediated cytotoxicity against ECs include antibody-dependent cell-mediated cytotoxicity (9, 10) and have direct effects through an interaction between the Fas and Fas ligands (11, 12).

Defective angiogenesis

SSc patients represent clinical features that are consistent with insufficient vascular repair, but demonstrate up-regulation of a series of pro-angiogenic factors, including vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), platelet-derived growth factor (PDGF), hepatocyte growth factor, placental growth factor, and CXCL12 (13). Increased levels of circulating VEGF have been reported in SSc patients (14, 15), but it has been shown that increased VEGF isoform is actually anti-angiogenic to VEGF165-b, rather than pro-angiogenic for VEGF165 (16). In contrast, a soluble VEGF receptor (VEGFR)-1 in circulation works as a decoy receptor for VEGF, and is decreased in SSc patients (16). Interestingly, all three forms of VEGF were upregulated in the skin biopsies of SSc patients (17, 18), suggesting that VEGF system, which plays a central role in the development and maintenance of the blood and lymphatic vascular systems, is totally disrupted in SSc patients. In addition, reduced levels of pro-angiogenic angiopoietin-1 along with increased levels of angiopoietin-2, an antagonist of angiopoietin-1, were also observed in SSc patients. The expression of kallikreins 1, 9, 11, and 12, which are powerful modulators of angiogenesis, was down-regulated in ECs as compared to healthy controls (13, 21-23). Endostatin was increased in all phases of the disease, while angiostatin levels were elevated only in the late phase and were correlated with the severity of interstitial lung disease (ILD) (24).

Defective responses of ECs to pro-angiogenic factors in SSc patients can be explained, in part, by the down-regulated expression of their receptors and/or impaired intracellular signaling. In fact, the reduced expression of CXCR4, a receptor of CXCL12, has been found in the affected skin of SSc patients, especially in the late stage of the disease (25). The angiogenic transcription factors implicated in the pathogenesis of SSc include the Friend leukemia integration-1 (FlI1) and Fos-related antigen 2 (Fra-2). FlI1 belongs to the Ets family of transcription factors, and acts as a repressor of collagen transcription in the human skin. A sustained down-regulation of FlI1 in the SSc fibroblasts has been correlated with abnormal matrix deposition in SSc-affected skin (26). Although the FlI1 deficiency in ECs promotes migration, proliferation, and survival, it also suppresses tube formation, which suggests that FlI1 deficiency is potentially attributable to the development of both proliferative obliterative vasculopathy and the loss of vessels, which are characteristic of SSc vasculopathy (27). Fra-2 is a member of the multifunctional activator protein 1 family, and mice overexpressing Fra-2 replicate SSc phenotype, including proliferative obliterative vasculopathy (28, 29). Fra-2 expression was upregulated in SSc fibroblasts, and has been potentially correlated with an increase in the profibrotic effects of transforming growth factor-β (TGF-β) and PDGF (30). Fra-2 appears to contribute to the development of microvasculopathy by inducing EC apoptosis and reducing the migration of ECs (31).

Roles of EC interaction with other cell types in promoting fibrosis

Dysfunctional ECs are also involved in promoting tissue fibrosis in SSc patients by cellular interactions with other cell types, including resident cells within the vascular wall and inflammatory cells infiltrated into the affected tissues. Specifically, ECs in the affected tissue recruit and activate skin fibroblasts by inducing mesenchymal-to-mesenchymal transition through the secretion of a connective tissue growth factor, TGF-β (32). On the other hand, dermal fibroblasts derived from SSc patients are known to overexpress matrix metalloproteinase (MMP)-12, which cleaves the urokinase-type plasminogen activator receptor of microvascular ECs, resulting in the failure of ECs to induce an efficient angiogenic program (33). This bi-directional interaction of ECs and fibroblasts synergistically promotes fibrosis and inhibition of angiogenesis. The altered function of microvascular pericytes has also been reported in patients with early SSc, which resulted from interactions with dysregulated ECs (34, 35). In SSc-affected skin, pericytes expressed activation markers, including PDGF receptor β, high molecular weight melanoma-associated antigen, a regulator of G protein signaling 5 (36), and secreted PDGF-BB that recruits and induces proliferation of pericyte progenitors (37). Interestingly, the co-culture of SSc-derived pericytes with microvascular ECs from healthy controls resulted in an increased production of collagen (38).

Endothelial-mesenchymal transition (Endo-MT)

Given the crucial role of myofibroblasts in the pathogenesis of systemic and organ-specific fibrotic diseases, such as SSc and idiopathic pulmonary fibrosis (IPF), extensive research has previously aimed at precisely identifying their cellular origin. Tissue myofibroblasts can originate from various sources, including quiescent resident tissue fibroblasts, pericytes, adipocytes, macrophages, and epithelial cells (39). Moreover, Karasek et al. (40) have demonstrated that ECs are capable of trans-differentiating toward myofibroblasts in a process called endothelial-to-mesenchymal transition (Endo-MT), by which ECs change their morphological features and acquire a myofibroblast-like phenotype. In SSc patients, evidence has shown that Endo-MT plays a role in vascular remodeling and tissue fibrosis (41). This process is mediated primarily through TGF-β, but the detailed intracellular pathways activated by TGF-β have not been entirely elucidated. TGF-β induces Endo-MT through both Smad-dependent and independent pathways, such as c-Abl kinase, protein kinase c-δ, and β-catenin (42). Moreover, various transcriptional regulators, such as Snail1 and Snail2, Twist, and some members of the Zeb family of proteins, are associated with the regulation of TGF-β-induced Endo-MT (43-47). In addition, many other mediators collaboratively promote Endo-MT, as shown below:

Caveolin-1 (CAV1)

CAV1, a major protein component of caveolae, plays an important role in the pathogenesis of tissue fibrosis and various fibrotic diseases by regulating the internalization, transport, and degradation of TGF-β receptors, thereby modulating TGF-β signaling (48, 49). Indeed, the gene and protein expression of CAV1 was
decreased in the affected tissues of patients with SSc and SSc-associated ILD and in the lung tissue of patients with IPF (50-52). Del Galdo et al. (50) demonstrated that Cav11 mice readily developed pulmonary and skin fibrosis. Restoration of Cav1 function in vitro by supplementing Cav1 with scaffolding domain peptide or overexpression of Cav1 using adenovirus helped to normalize the phenotypes of SSc fibroblasts and suppress TGF-β-induced extracellular matrix production, by inhibiting Smad3 activation and regulation of the c-Jun N-terminal kinase pathway in vitro (50-52). It has also been demonstrated that in vivo restoration of Cav1 by transfer via adenovirus or administration of a cell-permeable Cav1 peptide prevented bleomycin-induced pulmonary fibrosis and monocrotaline-induced PAH through inhibition of the STAT3 signaling cascade (52, 53). In cultures of pulmonary ECs derived from Cav11 mice, Endo-MT occurred spontaneously, as evidenced by the constitutive expression of α-SMA, the high levels of production of type I collagen, and the high expression of Snail1 and Snail2. These observations suggest that Cav1 deficiency may participate in the development of progressive tissue fibrosis and proliferative vasculopathy through the promotion of Endo-MT.

**Endothelin-1 (ET-1)**

Besides its crucial role in the development of PAH, ET-1 has been implicated in the development of organ fibrosis and is an important trigger of the fibrotic process in SSc (54-56). Recent studies have examined whether ET-1 may also play a role in the development of tissue fibrosis by inducing Endo-MT. For example, EC-derived ET-1 promotes cardiac fibrosis and heart failure in diabetic hearts through the induction of Endo-MT (56). However, ET-1 alone was unable to induce Endo-MT in murine lung EC cultures, but did enhance TGF-β-induced Endo-MT (57). The subsequent study confirmed this finding and showed that cultured human ECs induced Endo-MT in vitro when treated with ET-1 in the presence of TGF-β (57), indicating a potent synergistic effect of TGF-β and ET-1 on Endo-MT. Endo-MT induced by TGF-β and ET-1 primarily involved the Smad pathway, and was blocked by an ET-1 receptor antagonist, macitentan (58).

**Notch pathway**

Recent studies indicate that Notch pathways contribute to the pathogenesis of SSc and other fibrotic diseases (59-61), and may also be involved in the regulation of Endo-MT (62). The canonical Notch signaling can act in conjunction with TGF-β to induce Endo-MT by activating the expression of Snail and upregulating a subset of genes through recruiting Smad3 to Smad binding sites (63). However, it should be noted that Kaposi’s sarcoma-associated herpesvirus was found to induce Endo-MT via Notch signaling, which was independent of the TGF-β pathway (64).

**Wnt pathway**

Wnt contains a multigene family of secreted glycoproteins that play important roles during embryogenesis through canonical and non-canonical pathways (65, 66). Recent studies using cultured ECs have demonstrated that canonical Wnt signaling activates Endo-MT pathways (67, 68). On the other hand, the Wnt/β-catenin pathway is involved in the activation of multiple profibrotic steps in SSc pathogenesis (69-72). In fact, increased Wnt activation has been found in skin biopsies from patients with SSc, and Wnt3a-induced myofibroblast differentiation via Smad-dependent autocrine TGF-β signal has also been observed (70). In addition, the nuclear accumulation of β-catenin in activated fibroblasts was detected in fibroblastic foci in the lungs of patients with SSc-associated ILD (73).

**Hypoxia-inducible factor-1α (HIF-1α)**

The transcription factor HIF-1α is a key regulator responsible for inducing a number of cellular and molecular responses to hypoxia and is dysregulated in various pathologic conditions, including SSc (74-76). The mechanisms involved in HIF-1α-induced fibrosis are very complex and may affect numerous gene expression changes, interaction with profibrotic factors (such as TGF-β and VEGF), and the induction of Endo-MT (77-79). One study has shown that the important downstream effects of HIF-1α on Endo-MT induction involve a potent activation of Snail that may ultimately lead to the development of cardiac fibrosis (80).

**Roles of EPCs in pathogenesis of SSc**

**Defective vasculogenesis by aberrant EPCs**

Since EPCs are defined as circulating primitive cells that contribute to postnatal vasculogenesis (81), many studies have been conducted to clarify the contribution of EPCs to the pathogenesis of various vascular and connective tissues diseases (82). In patients with SSc, we first reported a reduced number of circulating EPCs, compared with age- and sex-matched rheumatoid arthritis patients or healthy individuals (83). The subsequent analyses done by other groups confirmed our finding (84-86), but some showed a comparable or even increased count of EPCs in SSc patients (87-91). It is now known that these contradictory results resulted from differences in experimental protocols used for quantifying EPCs. Circulating EPCs are identified as cells expressing CD34 in combination with CD133 and/or CD309/VEGFR2 by multi-color flow cytometry, but accurate quantification is technically difficult due to the extreme rarity of this population in circulation. To overcome this limitation, flow cytometry was combined with procedures that enrich EPCs, such as sorting of CD34+ cells and lineage-negative cells, in some studies (83, 90). In these circumstances, the European League Against Rheumatism Scleroderma Trials and Research (EUSTAR) proposed recommendations for the standardization of EPC research (92). We have directly compared several different protocols for quantifying circulating EPCs, and confirmed that the EUSTAR recommendations are valid when combined with an accurate quantification technique, which substantially improved the reproducibility of the results (93). Using standardized protocols, circulating EPCs were shown to be reduced in SSc patients in comparison with healthy controls. Recently, circulating lymphatic EPCs, identified by CD34+CD133+VEGFR3+ cells, were also decreased in SSc patients, and the lower counts were associated with the current digital ulcer (94).

In terms of functional properties of EPCs, we previously reported an impaired potential of SSc-derived EPCs to differentiate into mature ECs using in vitro cultures with multiple pro-angiogenic factors (83). Another study utilizing cultured EPCs showed an impaired differentiation potential to ECs in SSc-derived EPCs, as compared to EPCs derived from healthy controls (16). We recently developed a system to evaluate the in vivo differentiation potential of EPCs, using a murine tumor neo-vascularization model, in which freshly isolated human CD133+ cells are transplanted into the skin of mice in conjunction with syngeneic mouse tumor cells (95). Using this system, the neovascularization capacity of circulating EPCs, identified by CD34+CD133+VEGFR3+ cells, were also decreased in SSc patients, and the lower counts were associated with the current digital ulcer (96). In these circumstances, the European League Against Rheumatism Scleroderma Trials and Research (EUSTAR) proposed recommendations for the standardization of EPC research (92). We have directly compared several different protocols for quantifying circulating EPCs, and confirmed that the EUSTAR recommendations are valid when combined with an accurate quantification technique, which substantially improved the reproducibility of the results (93). Using standardized protocols, circulating EPCs were shown to be reduced in SSc patients in comparison with healthy controls. Recently, circulating lymphatic EPCs, identified by CD34+CD133+VEGFR3+ cells, were also decreased in SSc patients, and the lower counts were associated with the current digital ulcer (94).
patients, the upregulated expression of MMP-10 in EPC-derived ECs was associated with PAH, and the histologic findings of pulmonary arterial remodeling was suppressed by the blockade of MMP-10 in Fra-2-transgenic mice, a model mimicking the vascular and fibrotic aspects of SSC. These findings together suggest that defective EPC leads to the formation of digital ulcers and other vascular manifestations of SSC.

Currently, little is known about the mechanisms behind decreased numeric and functional aberrations in EPCs in SSC patients. In this regard, Del Papa and colleagues reported an interesting finding, i.e., EPCs in the bone marrow from SSC patients were defective in their ability to proliferate in long-term culture with pro-angiogenic factors, suggesting that EPC precursors were functionally altered before their release into the bloodstream (88). The bone marrow of patients with diffuse cutaneous SSC showed markedly reduced microvascular density and increased fibrosis (98), indicating that the dysregulated microenvironment within the bone marrow may alter the EPC differentiation process. In this regard, we recently found that EPC counts are inversely correlated with the level of circulating pentraxin 3, a multifunctional pattern recognition protein with a capacity to inhibit angiogenesis through suppression of FGF-2 (23). Pentraxin-3 is capable of inhibiting the differentiation of bone marrow stem cells into EPCs in in vitro cultures with FGF-2, indicating that exposure to a high concentration of pentraxin-3 would suppress the FGF2-mediated EPC differentiation in the bone marrow. Finally, EPCs in circulation may be attacked though autocrine mechanisms. In this regard, Zhu and colleagues found that the sera from SSC patients were able to induce apoptosis of EPCs, which was mediated through the Akt-FOXO3a-Bim pathway (84).

Heterogeneity of EPC subsets
There is a great deal of controversy about the definitions and roles of EPCs in postnatal vascular formation (99). This is primarily because of the technical difficulty in identifying those cells due to their extreme rarity in circulation (100). The utilization of a variety of experimental procedures has resulted in a number of definitions of EPCs in the literature. Nevertheless, it is currently accepted that there are at least two EPC subsets that can be discriminated between, based on their surface antigen expression, proliferation potential, and time of emergence in the cell culture system (101). Endothelial colony-forming cells detected in cultures are lineage-restricted progenitor cells that only give rise to endothelium with a clonogenic expansion potential (101), although their circulating origin has not been identified yet. On the other hand, the cells originally identified as “EPCs” are in fact hematopoietic lineage cells that display pro-angiogenic properties, and are now termed pro-angiogenic hematopoietic cells (101). Pro-angiogenic hematopoietic cells are also heterogeneous cell population, including CD14+ monocytic origin (monocytic EPCs) and CD14+ cells positive for CD34, CD133, and CD309 (narrowly defined or conventional EPCs) (102), which were initially termed circulating endothelial progenitors (103). Currently, it is generally accepted that pro-angiogenic hematopoietic cells do not give rise to mature ECs efficiently, rather they work as vascular regenerating and supporting cells (104). Monocytic EPCs especially lack the capacity to proliferate or form tubular structures in the absence of mature ECs. On the other hand, conventional EPCs have typical features of progenitors, including the capacity to proliferate and to differentiate into ECs; however, their efficiency is much lower in comparison with endothelial colony-forming cells. Nevertheless, pro-angiogenic hematopoietic cells, either in a monocytic or conventional subset, are capable of promoting blood vessel formation through multiple mechanisms, including the secretion of a series of pro-angiogenic factors, including VEGF, granulocyte colony-stimulating factor (G-CSF), and stromal cell-derived factor-1 (SDF-1) (105, 106), and differentiation into other elements of the vasculature, such as pericytes and smooth muscle cells. Theoretically, pro-angiogenic hematopoietic cells play a major role in the very early phase of vascular repair by attaching to the denuded vascular endothelium immediately after injury and taking advantage of the large number of ECs in circulation (102). In the following vascular processes, endothelial colony-forming cells and pro-angiogenic hematopoietic cells work in conjunction with platelets and residential ECs to form new blood vessels.

Potential roles of monocytic EPCs in tissue fibrosis
When the number of circulating monocytic EPCs was examined in SSC patients using a culture system developed to enrich this cell population, circulating monocytic EPCs were found to be paradoxically increased in SSC patients as compared to age- and sex-matched healthy controls (107). Intriguingly, monocytic EPCs derived from SSC patients showed enhanced proliferation in vitro tubular structure formation compared with the structures seen in healthy controls. Furthermore, in a murine tumor neovascularization model, the transplantation of SSC-derived monocytic EPCs dramatically promoted tumor growth and tumor vessel formation in vivo, indicating that monocytic EPCs derived from SSC patients have enhanced angiogenic activity. The increased number and enhanced pro-angiogenic potency of monocytic EPCs is likely to be a compensatory response to impaired vasculogenesis due to the malfunction of conventional EPCs. Circulating monocytic EPCs are mobilized from the bone marrow and recruited to affected lesions of SSC in response to chemokines such as monocyte chemotactant protein-1/CCL2 and SDF-1, which are upregulated in the affected skin of SSC patients (108, 109). In addition, the hypoxic condition of the affected tissues of SSC patients are known to stimulate the differentiation of monocytic EPCs through activation of HIF-1α (110). These local stimuli promote the accumulation of functionally altered monocytic EPCs into the affected lesions of SSC. Since monocytic EPCs are capable of differentiating into cells that produce extracellular matrix proteins (111-115), they might participate in the fibrotic process in the affected organs in an CCL2/CCRF2-dependent amplification loop (114, 115). In this regard, the fibrotic clinical features in SSC patients were correlated with an increased proportion of CXCR4+ circulating cells with monocytic and endothelial markers, which correspond to monocytic EPCs (116). Interestingly, monocytic EPCs have common phenotypic features of alternatively activated or M2 macrophages, which are appreciated increasingly as important cells that contribute to the pathogenic process of SSC (117). Recent studies have shown that circulating monocytes with combined classically and alternatively activated features are increased in SSC patients (118, 119). Furthermore, in a phase II clinical trial of tocilizumab in early, active patients with diffuse cutaneous SSC, tocilizumab treatment resulted in down-regulation of M2-associated genes in the skin and a sustained reduction of circulating CCL18, a chemokine associated with M2, in association with the improvement of skin sclerosis (120). Therefore, the pathogenic process of SSC is likely triggered by recruitment and accumulation of circulating monocytic EPCs with M2 features into the affected sites, where they acquire profibrotic properties, i.e., the production of a variety of profibrotic growth factors, cytokines, and chemokines to stimulate resident mesenchymal cells, and their own trans-differentiation into extracellular matrix-producing cells.

Summary: A potential link between aberrant EPC/EC and the pathogenic processes of SSC
Current insights raise the intriguing hypothesis that ECs and EPCs are directly involved in the pathogenesis of SSC by virtue of participating in two major pathologic aspects of the disease: vascular remodeling and excessive...
fibrosis (Figure 1). Specifically, in early phase of SSc, a variety of triggers damage the endothelium, leading to subsequent expression of a series of pro-angiogenic factors, growth factors, and chemokines. Increased levels of these mediators promote recruitment of conventional EPCs from bone marrow, however, the vascular repair machinery is intrinsically impaired, which results in altered EC functions that induce the activation of fibroblasts by a direct interaction and their trans-differentiation into myofibroblasts via Endo-MT. Additionally, in compensation for the insufficient vascular repair process, monocytic EPCs with M2 features are recruited into circulation and are made to accumulate at the affected sites, thereby promoting ECM deposition and tissue fibrosis.

ECs: endothelial cells; vSMCs: vascular smooth muscle cells; EPCs: endothelial progenitor cells; VEGF: vascular endothelial growth factor; FGF-2: basic fibroblast growth factor-2; MCP-1: monocyte chemoattractant protein-1; SDF-1: stromal-derived factor-1; MMP12: matrix metalloproteinase 12; TGF-β: transforming growth factor β; ECM: extracellular matrix.

Figure 1. Roles of ECs and EPCs in pathogenesis of SSc.

A variety of triggers damage the endothelium, leading to subsequent expression of a series of angiogenic factors, growth factors, and chemokines. Increased levels of these mediators promote recruitment of conventional EPCs from bone marrow, however, the vascular repair machinery is intrinsically impaired, which results in altered EC functions that induce the activation of fibroblasts by a direct interaction and their trans-differentiation into myofibroblasts via Endo-MT. Additionally, in compensation for the insufficient vascular repair process, monocytic EPCs with M2 features are recruited into circulation and are made to accumulate at the affected sites, thereby promoting ECM deposition and tissue fibrosis.

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References
1. Varga J, Trojanowska M, Kuwana M. Pathogenesis of systemic sclerosis: recent insights of molecular and cellular mechanisms and therapeutic opportunities. J Scleroderma Rel Disord 2017; 2: 137-52. [Crossref]
2. Guiducci S, Giacomelli R, Cerinic MM. Vascular complications of scleroderma. Autoimmun Rev 2007; 6: 520-3. [Crossref]
3. Liakouli V, Cipriani P, Marrelli A, Alvaro S, Ruscitti P, Giacomelli R. Angiogenic cytokines and
growth factors in systemic sclerosis. Autoimmun Rev 2011; 10: 590-4. [Crossref]

4. Fischer C, Schneider M, Carmeliet P. Principles and therapeutic implications of angiogenesis, vasculogenesis and arteriogenesis. Handb Exp Pharmacol 2006: 157-212. [Crossref]

5. Abraham D, Distler O. How does endothelial cell injury start? The role of endothelin in systemic sclerosis. Arthritis Res Ther 2007; 9(Suppl 2): S2. [Crossref]

6. Pattanaik D, Brown M, Postlethwaite BA, Postlethwaite AE. Pathogenesis of systemic sclerosis. Front Immunol 2015; 6: 272. [Crossref]

7. Lunardi C, Bason C, Navone R, Millo E, Damonte G, Crocco R, et al. Systemic sclerosis immune-noglobulin G autoantibodies bind the human cytomegalovirus late protein UL94 and induce apoptosis in human endothelial cells. Nat Med 2000; 6: 1183-6. [Crossref]

8. Del Papa N, Conforti G, Gambini D, La Rosa L, Tincani A, D’Cruz D, et al. Characterization of the endothelial surface proteins recognized by anti-endothelial antibodies in primary and secondary autoimmune vasculitis. Clin Immunol Immunopathol 1994; 70: 211-6. [Crossref]

9. Marks RM, Czerniecki M, Andrews BS, Penny R. The effects of scleroderma serum on human microvascular endothelial cells. Induction of antibody-dependent cellular cytotoxicity. Arthritis Rheum 1988; 31: 1524-34 [Crossref]

10. Holt CM, Lindsey N, Moul J, Malia RG, Greaves M, Hume A, et al. Antibody-dependent cellular cytotoxicity of vascular endothelium: characterization and pathogenic associations in systemic sclerosis. Curr Exp Immunol 1989; 78: 359-65. [Crossref]

11. Sgonc R, Gruschwitz MS, Boeck G, Sepp N, Mackiewicz Z, Sukura A, Povilenaite D, Ceponis A, Shulman HM, Connolly MK, et al. Capillary regeneration in scleroderma: stem cell therapy reverses skin manifestations and defective vasculogenesis. Arthritis Rheumatol 2015; 67: 498-507. [Crossref]

12. Almeida I, Oliveira Gomes A, Lima M, Silva I, Vasconcelos C. Different contributions of angiostatin and endostatin in angiogenesis impairment in systemic sclerosis: a cohort study. Clin Exp Rheumatol 2016; 34(Suppl 100): 37-42. [Crossref]

13. Mackiewicz Z, Sukura A, Pavleniata D, Ceponis A, Viritanen I, Hakunen M, et al. Increased but imbalanced expression of VEGF and its receptors has no positive effect on angiogenesis in systemic sclerosis skin. Clin Exp Rheumatol 2002; 20: 641-6. [Crossref]

14. Wang Y, Fan PS, Kahaleh B. Association between enhanced type I collagen expression and epigenetic repression of the FUL1 gene in scleroderma fibroblasts. Arthritis Rheum 2006; 54: 2271-9. [Crossref]

15. Toyama T, Asano Y, Miyagawa T, Nakamura K, Hirabayashi M, Yamashita T, et al. The impact of transcription factor Fli1 deficiency on the regulation of angiogenesis. Exp Dermatol 2017; 26: 912-8. [Crossref]

16. Maurer B, Distler O, Distler JH, Schedt A, Acker T, Hirth A, Rethage J, et al. Uncontrolled expression of vascular endothelial growth factor and its receptors leads to insufficient skin angiogenesis in patients with systemic sclerosis. Circ Res 2004; 95: 109-16. [Crossref]

17. Higashi-Kuwata N, Makino T, Inoue Y, Ihn H. Expression pattern of VEGF-R, -2, -3 and D2-40 protein in the skin of patients with systemic sclerosis. Eur J Dermatol 2011; 21: 490-4. [Crossref]

18. Giusti B, Serrati S, Margheri F, Papucci L, Rossi L, Poggi F, et al. The antiangiogenic tissue kallikrein pattern of endothelial cells in systemic sclerosis. Arthritis Rheum 2005; 52: 3618-28. [Crossref]

19. Michalska-Jakubus M, Kowal-Bielecka O, Chodorska W, Krasowska D. Angiopoietins-1 and -2 are differentially expressed in the sera of patients with systemic sclerosis: high angiopoietin-2 levels are associated with higher severity and higher activity of the disease. Rheumatology (Oxford) 2011; 50: 746-55. [Crossref]

20. Rabquer BJ, Koch AE. Angiogenesis and vasculopathy in systemic sclerosis: evolving concepts. Curr Rheum Rep 2012; 14: 56-63. [Crossref]

21. Distler O, Del Rosso A, Giacomelli R, Cipriani P, Conforti ML, Guiducci S, et al. Angiogenic and angiostatic factors in systemic sclerosis: increased levels of vascular endothelial growth factor are a feature of the earliest disease stages and are associated with the absence of fingertip ulcers. Arthritis Res Ther 2002; 4: R11. [Crossref]

22. Shirai Y, Okazaki Y, Inoue Y, Tamura Y, Yasuoka H, Takeuchi T, et al. Elevated levels of pentraxin 3 in systemic sclerosis: associations with vascular manifestations and defective vasculogenesis. Arthritis Rheumatol 2015; 67: 498-507 [Crossref]

23. Almeida I, Oliveira Gomes A, Lima M, Silva I, Vasconcelos C. Different contributions of angiostatin and endostatin in angiogenesis impairment in systemic sclerosis: a cohort study. Clin Exp Rheumatol 2016; 34(Suppl 100): 37-42. [Crossref]

24. Mackiewicz Z, Sukura A, Pavleniata D, Ceponis A, Viritanen I, Hakunen M, et al. Increased but imbalanced expression of VEGF and its receptors has no positive effect on angiogenesis in systemic sclerosis skin. Clin Exp Rheumatol 2002; 20: 641-6. [Crossref]

25. Wang Y, Fan PS, Kahaleh B. Association between enhanced type I collagen expression and epigenetic repression of the FUL1 gene in scleroderma fibroblasts. Arthritis Rheum 2006; 54: 2271-9. [Crossref]

26. Toyama T, Asano Y, Miyagawa T, Nakamura K, Hirabayashi M, Yamashita T, et al. The impact of transcription factor Fli1 deficiency on the regulation of angiogenesis. Exp Dermatol 2017; 26: 912-8. [Crossref]

27. Maurer B, Reich N, Juegeli A, Kriegsmann J, Gay RE, Schett G, et al. Fra-2 transgenic mice as a novel model of pulmonary hypertension associated with systemic sclerosis. Ann Rheum Dis 2012; 71: 1382-7. [Crossref]

28. Maurer B, Distler O, Distler JH. The Fra-2 transgenic mouse model of systemic sclerosis. Vascul Pharmacol 2013; 58: 194-201. [Crossref]
Novel Probiotic Effect of ET-1. PLoS One 2016; 11:e0161988. [Crossref]
58. Cipriani P, Di Benedetto P, Ruscitti P, Cacrepe D, Zaffaroni F, Liakoulis V, et al. The endotheli- 
amel- mesenchymal transition in systemic sclerosis is induced by endothelin-1 and transforming 
expression factor-beta and may be blocked by mac- 
tenin, a dual endothelin-1 receptor antagonist. J 
Rheumatol 2015; 42: 1808-16. [Crossref]
59. Beyrer C, Distler JH. Morphogen pathways in 
systemic sclerosis. Curr Rheumatol Rep 2013; 15: 299. [Crossref]
60. Beyrer C, Dees C, Distler JH. Morphogen path- 
ways as molecular targets for the treatment of 
fibrosis in systemic sclerosis. Arch Dermatol Res 2013; 305: 1-8. [Crossref]
61. Distler A, Lang V, Del Vecchio T, Huang J, Zhang Y, Beyrer C, et al. Combined inhibition of mor- 
phogen pathways demonstrates additive anti-
fibrotic effects and improved tolerability. Ann 
Rheum Dis 2014; 73: 1264-8. [Crossref]
62. Noseda M, McLean G, Niessen K, Chang L, Pollet I, Montpetit R, et al. Notch activation results in 
phenotypic and functional changes consistent 
with endothelial-to-mesenchymal transforma-
tion. Circ Res 2004; 94: 910-7. [Crossref]
63. Fu Y, Chang A, Chang I, Niessen K, Eapen S, Setiadi A, et al. Differential regulation of trans-
forming growth factor beta signaling pathways by Notch in human endothelial cells. J Biol 
Chem 2009; 284: 19452-62. [Crossref]
64. Gasperini P, Espigol-Frigole G, McCormick PJ, Salucci O, Maric D, Uldrick TS, et al. Ka-
posi sarcoma herpesvirus promotes endo-
thelial-to-mesenchymal transition through Notch-dependent signaling. Cancer Res 2012; 
72: 1157-69. [Crossref]
65. Cleverson H, Nusse R. Wnt/beta-catenin signaling 
and disease. Cell 2012; 149: 1192-205. [Crossref]
66. Niehrs C. The complex world of Wnt receptor 
signalling. Nat Rev Mol Cell Biol 2012; 13: 767-
79. [Crossref]
67. Asagbonhin O, Rao M, Ryzhov S, Atria N, Feokti-
Stov I, Hatzopoulos AK. Experimental myocard-
ial infarction triggers canonical Wnt signalling 
and endothelial-to-mesenchymal transition. Dis Model Mech 2011; 4: 469-83. [Crossref]
68. Li L, Chen L, Zang J, Tang X, Liu Y, Zhang J, et 
al. C3a and C5a receptor antagonists amelio-
rate endothelial-myofibroblast transition via the Wnt/beta-catenin signaling pathway in 
diabetic kidney disease. Metabolism 2015; 64: 
597-610. [Crossref]
69. Lafyatis R. Connective tissue disease: SSc-fibro-
sis takes flight with Wingless inhibition. Nat Rev 
Rheumatol 2012; 8: 441-2. [Crossref]
70. Wei J, Fang F, Lam AP, Sargent JL, Hamburger E, 
Hinchcliffe ME, et al. Wnt/beta-catenin signaling is 
hyperactivated in systemic sclerosis and 
induces Smad-dependent fibrotic responses in 
mesenchymal cells. Arthritis Rheum 2012; 67: 
234-45. [Crossref]
71. Beyrer C, Schramm A, Akhmetshina A, Dees C, Kire-
va T, Gelse K, et al. beta-catenin is a central medi-
ator of pro-fibrotic Wnt signaling in systemic 
sclerosis. Ann Rheum Dis 2012; 71: 761-7. [Crossref]
72. Dees C, Schlottmann I, Funke R, Distler A, Pa-
lumbo-Zerr K, Zerr P, et al. The Wnt antagonists 
Dkk1 and Sfrp1 are downregulated by pro-
moter hypermethylation in systemic sclerosis. Ann Rheum Dis 2014; 73: 1322-9. [Crossref]
73. Lam AP, Flozak AS, Russell S, Wei J, Jain M, Mutku 
GM, et al. Nuclear beta-catenin is increased in sys-
temic sclerosis pulmonary fibrosis and promotes 
lung fibroblast migration and proliferation. Am J 
Respir Cell Mol Biol 2011; 45: 915-22. [Crossref]
74. Semenza GL. Hypoxia-inducible factors in phys-
iology and medicine. Cell 2012; 148: 399-408. [Crossref]
75. Lokmic Z, Musyoka J, Hewitson TD, Darby IA. Hypox-
ia and hypoxia signaling in tissue repair and fibrosis. Int Rev Cell Mol Biol 2012; 296: 139-85. [Crossref]
76. Haase VH. Pathophysiological consequences of 
HIF activation: HIF as a modulator of fibrosis. Ann N Y Acad Sci 2009; 1177: 57-65. [Crossref]
77. Sanchez-Elsner T, Botella LM, Velasco B, Corbi A, 
Attisano L, Bernabeu C. Synergistic cooperation 
between hypoxia and transforming growth 
factor-beta pathways on human vascular endo-
thelial growth factor gene expression. J Biol 
Chem 2001; 276: 38527-35. [Crossref]
78. Sun S, Ning X, Zhang Y, Lu Y, Nie Y, Han S, et al. 
Hypoxia-inducible factor-1alpha induces Twist 
expression in tubular epithelial cells subjected to 
hypoxia, leading to epithelial-to-mesenchymal 
transition. Kidney Int 2009; 75: 1278-87. [Crossref]
79. Higgins DF, Kimura K, Bernhardt WM, Shrimank-
er N, Akai Y, Hohenstein B, et al. Hypoxia pro-
motes fibrogenesis in vivo via HIF-1 stimulation of 
ethodelial-to-mesenchymal transition. J Clin 
Invest 2007; 117: 3810-20. [Crossref]
80. Xu X, Tan X, Tampe B, Sanchez E, Zeisberg M, 
Zeisberg EM. Snail is a direct target of hypox-
ia-inducible factor-1alpha (HIF-1alpha) in hypox-
ia-induced endothelial to mesenchymal transi-
tion of human coronary endothelial cells. J Biol 
Chem 2015; 290: 16653-64. [Crossref]
81. Asahara T, Murohara T, Sullivan A, Silver M, van 
der Zee R, Li T, et al. Isolation of putative pro-
genitor endothelial cells for angiogenesis. Sci-
ence 1997; 275: 964-7. [Crossref]
82. Ferrante A, Guggino G, Di Liberto D, Ciccia F, Ci-
priani P, Balistreri CR, et al. Endothelial progen-
itor cells: Are they displaying a function in au-
toimmune disorders? Mech Ageing Dev 2016; 
159: 44-8. [Crossref]
83. Kuwana M, Okazaki Y, Yaukoua H, Kawakami Y, 
Ikedo Y. Defective vasculogenesis in systemic 
sclerosis. Lancet 2004; 364: 603-10. [Crossref]
84. Zhu S, Evans S, Yan B, Povsic TI, Tappson V, Gold-
smith-Clement PJ, et al. Transcriptional regu-
lation of Bim by FoxO3a and Akt mediates 
sclerosis serum-induced apoptosis in eto-
helial progenitor cells. Circulation 2008; 118: 
2156-65. [Crossref]
85. Mok MY, Yu KH, Wong CY, Quivoxi J, Lai 
WH, Wong WS, et al. Low circulating level of 
CDD33+KDR+ cells in patients with systemic 
sclerosis. Clin Exp Rheumatol 2010; 28: 519-25.
86. Andrigueti FV, Arismendi MI, Ebbing PC, Kayser 
C. Decreased numbers of endothelial progenitor 
cells in patients in the early stages of systemic 
sclerosis. Microvasc Res 2015; 98: 82-7. [Crossref]
87. Del Papa N, Colombo G, Fraccioni A, Moneti L, Ingegnoli F, Magione W, et al. Circulating endothelial cells as a marker of ongoing vascular disease in systemic sclerosis. Arthritis Rheum 2004; 50: 1296-304. [Crossref]
88. Del Papa N, Quinci N, Soligo D, Scavullo C, Cortana M, Borsotti C, et al. Bone marrow endothelial progenitors are defective in systemic sclerosis. Arthritis Rheum 2006; 54: 2605-15. [Crossref]
89. Allano J, Batteux F, Avouac J, Assous N, Weill B, Kahan A. Levels of circulating endothelial progenitor cells in systemic sclerosis. Clin Exp Rheumatol 2007; 25: 60-6. [Crossref]
90. Avouac J, Juin F, Wipff J, Couraud P, Chiocchia G, Kahan A, et al. Circulating endothelial progenitor cells in systemic sclerosis: association with disease severity. Ann Rheum Dis 2008; 67: 1455-60. [Crossref]
91. Nevskaya T, Bykovskaia S, Lyssuk E, Shakhov I, Zaprjagaeva M, Mach E, et al. Circulating endothelial progenitor cells in systemic sclerosis: relation to impaired angiogenesis and cardiovascular manifestations. Clin Exp Rheumatol 2008; 26: 421-9. [Crossref]
92. Distler JH, Allano J, Avouac J, Giacomelli R, Guiducci S, Moritz F, et al. EULAR Scleroderma Trials and Research group statement and recommendations on endothelial progenitor cells. Ann Rheum Dis 2009; 68: 163-8. [Crossref]
93. Kuwana M, Okazaki Y. Quantification of circulating endothelial progenitor cells in systemic sclerosis: a direct comparison of protocols. Ann Rheum Dis 2012; 71: 617-20. [Crossref]
94. Manetti M, Pratesi S, Romano E, Rosa I, Bruni Ota and Kuwana. EC/EPC in SSC pathogenesis histochemistry and multiparametric computer. Arthritis Res Ther 2013; 15: R55. [Crossref]
95. Distler O, Pap T, Kowal-Bielecka O, Meyringer R, Zakhartchev S, Alarcón-Gómez R, et al. Soluble factors released by endothelial progenitor cells promote migration of endothelial cells and cardiac resident progenitor cells. J Mol Cell Cardiol 2011; 50: 266-72. [Crossref]
96. Rehm an J, Li J, Orschell CM, March KL. Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors. Circulation 2003; 107: 1164-9. [Crossref]
97. Urbbich C, Aicher A, Heeschen C, Dernbach E, Hofmann WK, Zeiher AM, et al. Soluble factors released by endothelial progenitor cells promote migration of endothelial cells and cardiac resident progenitor cells. J Mol Cell Cardiol 2005; 39: 733-42. [Crossref]
98. Yamaguchi Y, Okazaki Y, Setan T, Sato H. Macrophage polarisation state of M-CSF stimulated monocytes in patients with systemic sclerosis. Ann Rheum Dis 2013; 72: 394-9. [Crossref]
99. Turco A, Balleere A, Jouneau S, Fardel O, Vernhet L, Jego P, et al. M1/M2 polarisation state of M-CSF stimulated cells coexpressing M1 and M2 macrophage surface markers in patients with systemic sclerosis. Ann Rheum Dis 2018; 77: 1842-5. [Crossref]
100. Leocato A, Pareti M, Kimura T, Nomura K, Sato H, Aikawa Y, et al. CXCR4+ circulating progenitor cells expressing CXCR4+CD45+CD133+CD34+ cells in patients with systemic sclerosis. J Pathol 2008; 214: 388-96. [Crossref]
101. Distler O, Pap T, Kowal-Bielecka O, Meyringer R, Zakhartchev S, Alarcón-Gómez R, et al. Soluble factors released by endothelial progenitor cells promote migration of endothelial cells and cardiac resident progenitor cells. J Mol Cell Cardiol 2011; 50: 266-72. [Crossref]
102. Yamaguchi Y, Okazaki Y, Setan T, Sato H. Macrophage polarisation state of M-CSF stimulated monocytes in patients with systemic sclerosis. Ann Rheum Dis 2013; 72: 394-9. [Crossref]
103. Peichev M, Nayer Aj, Pereira D, Zhu Z, Lane WJ, Williams M, et al. Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. Blood 2000; 95: 952-8. [Crossref]
104. Kuwana M, Okazaki Y. Proangiogenic hematoipoietic cells of monocytic origin: roles in vascular regeneration and pathogenic processes of systemic sclerosis. Histol Histopathol 2013; 28: 175-83. [Crossref]
105. Prater DN, Case J, Ingram DA, Yoder MC. Working hypothesis to redefine endothelial progenitor cells. Leukemia 2007; 21: 1141-9. [Crossref]
106. Urbich C, Aicher A, Heeschen C, Dernbach E, Hofmann WK, Zeiher AM, et al. Soluble factors released by endothelial progenitor cells promote migration of endothelial cells and cardiac resident progenitor cells. J Mol Cell Cardiol 2005; 39: 733-42. [Crossref]
107. Yamaguchi Y, Okazaki Y, Setan T, Sato H. Macrophage polarisation state of M-CSF stimulated monocytes in patients with systemic sclerosis. Ann Rheum Dis 2013; 72: 394-9. [Crossref]
108. Distler O, Pap T, Kowal-Bielecka O, Meyringer R, Zakhartchev S, Alarcón-Gómez R, et al. Soluble factors released by endothelial progenitor cells promote migration of endothelial cells and cardiac resident progenitor cells. J Mol Cell Cardiol 2011; 50: 266-72. [Crossref]
109. Peichev M, Nayer Aj, Pereira D, Zhu Z, Lane WJ, Williams M, et al. Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. Blood 2000; 95: 952-8. [Crossref]
110. Kuwana M, Okazaki Y, Kodama H, Izumi K, Yasuoka H, Ogawa Y, et al. Human circulating CD14+ monocytes as a source of progenitors that exhibit mesenchymal cell differentiation. J Leukoc Biol 2003; 74: 833-45. [Crossref]
111. Badorff C, Brandes RP, Popp R, Rupp S, Urbich C, Aicher A, et al. Transdifferentiation of blood-derived human adult endothelial progenitor cells into functionally active cardiomyocytes. Circulation 2003; 107: 1024-32. [Crossref]
112. Kuwana M, Okazaki Y, Kodama H, Izumi K, Yasuoka H, Ogawa Y, et al. Human circulating CD14+ monocytes as a source of progenitors that exhibit mesenchymal cell differentiation. J Leukoc Biol 2003; 74: 833-45. [Crossref]
113. Komagami N, Annunziato F, Liotta F, Lazzari E, Mazzinghi B, Frosali F, et al. CD14+CD45low cells with stem cell phenotypic and functional features are the major source of circulating endothelial progenitors. Circ Res 2005; 97: 314-22. [Crossref]
114. Sakai N, Wada T, Fujuchi K, Shimizu K, Kobuko S, Hara A, et al. MCP-1/CCR2-dependent loop for fibrogenesis in human peripheral CD14-positive monocytes. J Leukoc Biol 2006; 79: 555-63. [Crossref]
115. Masuda A, Yasuoka H, Satoh T, Okazaki Y, Yamaguchi Y, Kuwana M. Versican is upregulated in circulating monocytes in patients with systemic sclerosis and amplifies a CC1L2-mediated pathogenic loop. Arthritis Res Ther 2013; 15: R74. [Crossref]
116. Campioni D, Lo Monaco A, Lanza F, Moretti S, Ferrari L, Cotoni M, et al. CXCR4+CD45+CD133+ peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors. Circulation 2003; 107: 1164-9. [Crossref]
117. Stifano G, Christmann RB. Macrophage Involvement in Systemic Sclerosis: Do We Need More Evidence? Curr Rheumatol Rep 2016, 18: 2. [Crossref]
118. Soldano SP, Trombetta AC, Contini PP, Tornatis VM, Ruaro BM, Brizzolara RP, et al. Increase in circulating cells coexpressing M1 and M2 macrophage surface markers in patients with systemic sclerosis. Ann Rheum Dis 2018; 77: 1842-5. [Crossref]
119. Lescoat A, Ballerie A, Jouneau S, Fardel O, Vernhet L, Jego P, et al. M1/M2 polarisation state of M-CSF blood-derived macrophages in systemic sclerosis. Ann Rheum Dis 2018; 77: e127. [Crossref]
120. Khanna D, Denton CP, Jahreis A, van Laar JM, Feigh TM, Anderson ME, et al. Safety and efficacy of subcutaneous tocilizumab in adults with systemic sclerosis (faSScinate): A phase 2, randomised, controlled trial. Lancet 2016; 387: 2630-40. [Crossref]