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# Pathogenesis of the Endothelial Damage and Related Factors

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## 1. Introduction

Systemic sclerosis (SSc) is an autoimmune connective tissue disorder characterized by a widespread microangiopathy, autoimmunity and fibrosis of the skin and of various internal organs. Vascular damage occurs early in the course of the disease as showed by the presence of Raynaud's phenomenon (RP) that can precede the fibrotic process of months or years. A complex interaction between endothelial cells (ECs), smooth muscle cells (SMCs), pericytes, extracellular matrix (ECM), and intravascular circulating factors is now recognized to contribute to the vascular reactivity, remodeling, and occlusive disease of SSc (Gabrielli et al., 2009). Chronic platelet activation and enhanced coagulation with reduced fibrinolysis, secondary to EC activation that leads to fibrin deposits and contribute to the intimal proliferation and luminal narrowing are also found. The identity of the initial trigger of EC damage remains unknown. Current hypotheses suggest a possible infectious or chemical trigger(s) that activates both cellular and humoral immunity. Products of immune activation may lead to vascular injury possibly through the production of autoantibodies and the release of products of activated T cells that can directly damage the endothelium (Kahaleh, 2008). Microangiopathy is characterized by a reduced capillary density and an irregular chaotic architecture that leads to chronic tissue hypoxia and organ dysfunction with eventual organ failure. Vascular complications, including pulmonary arterial hypertension (PAH) and scleroderma renal crisis (SRC) have emerged as leading causes of disability and mortality in SSc (Guiducci et al., 2007). Despite the hypoxic conditions, there is no evidence for a sufficient compensative angiogenesis in SSc (Distler O et al., 2002). Furthermore, vasculogenesis, the *de novo* formation of blood vessels, is also impaired. An imbalance between angiogenic and angiostatic factors as well as functional alterations of the cellular players, involved in the angiogenic and vasculogenic program, might explain the pathogenetic mechanisms of SSc vasculopathy (Liakouli, 2011; Cipriani, 2011). Either angiogenic or vasculogenic mechanisms may potentially become in the future the target of novel therapeutic strategies to promote vascular regeneration in SSc.

## 2. Vascular endothelial cell damage

Microvascular endothelial cell (MVEC) injury and apoptosis is an early and central event in the pathogenesis of SSc vasculopathy that leads to microcirculatory dysfunction and loss of

capillaries with consequent vascular desertification, tissue chronic ischemia and eventual organ failure. The initiating factors or cause of the vascular insult in scleroderma remain unknown. Current hypotheses in SSc vascular disease pathogenesis suggest a possible infectious or chemical trigger(s) that activates both cellular and humoral immunity. Products of immune activation may lead to vascular injury possibly through the production of autoantibodies and the release of products of activated T cells that can directly damage the endothelium. In particular, primary activation of ECs in SSc include autoantibodies showing cross-reactivity between Cytomegalovirus (CMV) epitopes and specific surface molecules of ECs, inducing apoptosis. However, this is unlikely to be the only aetiological factor, since CMV is ubiquitous in the normal population (Lunardi et al., 2000). Anti-endothelial cells antibodies (AECAs) present in the SSc sera are reported to activate EC and, induce EC apoptosis *in vivo* independent of the Fas-Fas ligand pathway. This is clearly shown in the chicken model of SSc (UCD-200), where serum transfer into normal chicken embryos results in binding of antibodies to the microvasculature in the chorioallantoic membrane in association with endothelial apoptosis (Worda et al., 2003). The exact identity of the endothelial antigen is not known; (Worda M et al., 2003). Moreover, SSc sera containing distinct AECA subsets (ACAs for limited cutaneous SSc or anti-topoisomerase I antibodies for diffuse cutaneous SSc) can induce EC apoptosis in association with increased gene expression of caspase 3 and the reexpression of EC SSc autoantigen fibrillin 1 (Ahmed et al., 2006). Anti-endothelial cells antibodies (AECAs) are present in 40–50% of the SSc sera and are mostly of the IgG1 isotype. The antibody titres correlate negatively with pulmonary diffusion capacity and positively with pulmonary hypertension and with digital ischemic ulcers, suggesting a pathological role in the development of the vascular disease. The only published proteomic analysis of endothelial antigen(s) recognized by AECA identified 53 proteins consisting of cytoskeleton proteins, proteins involved in cellular mobility, regulation of apoptosis and senescence as well as proteins implicated in clotting and antigen presentation (Bordron et al., 1998). Thus, vascular cell apoptosis could, in turn, expose autoantigens to immune surveillance, evoking an autoimmune response and perpetuating autoimmunity to blood vessels in SSc (Ahmed et al., 2006). However, AECAs are detected in a variety of vascular diseases and specific epitopes and mechanisms have not been clarified in SSc. High levels of reactive oxygen species and oxidative stress have been directly or indirectly implicated in scleroderma (Sambo et al., 2001, 1999; Servettazet al., 2007). The source of reactive oxygen species is the membrane NADPH oxidase system, which is stimulated in all cell types within or surrounding the vessel wall in response to injury (Lassegue, 2001; Sturroch, 2005; Holland, 1998). In scleroderma, the high levels of reactive oxygen species in mesenchymal cells (MSCs) are relatively independent of the inflammatory status; they persist *in vitro* in the absence of growth factors and cytokines, render cells sensitive to stress, and induce DNA damage (Svegliati et al., 2006). MSCs become progressively hypersensitive to cytokines induced by local reactive oxygen species (Sullivan et al., 2008). Cytokines activate mesenchymal precursor cells and lead to the transformation of fibroblasts to myofibroblasts with consequent abnormal collagen synthesis. Furthermore, free radicals contribute to the release of mediators implicated in fibrosis (Bellocq et al., 1999; Barcellos-Hoff et al., 1996). EC apoptosis may also activate the immune-inflammatory system by dendritic cells and macrophage presentation of self-antigen present in the apoptotic debris to CD8<sup>+</sup> T cells, and by the direct activation of the alternate complement

and coagulation cascades leading to microvascular thrombosis and further vessel compromise. MVEC apoptosis can result from their interaction with cytotoxic T cells either by Fas or granzymes/perforin-related mechanisms. For example, CD4<sup>+</sup> T cells can mediate MVEC apoptosis by a Fas-related mechanism as seen in cytolytic T cells killing of vascular endothelium in the rejection reaction, whereas the granzyme/perforin system mediates apoptosis by the major cytotoxic cells, the CD8<sup>+</sup> T cells, NK and LAK cells. Involvement of cytotoxic T cells in SSc is suggested by the presence of a 60 kDa protein in SSc sera that was described as an endothelial cytotoxic factor. This factor was characterized as the granular enzyme, and was detected in the perivascular spaces in SSc skin biopsies. Cytotoxic T lymphocytes (in particular CD8<sup>+</sup> T cells, NK cells, LAK cells) granule-specific products such as granzyme B and perforin are able to induce apoptosis in cultured ECs. Granzymes gain access to the cells following cellular membrane damage by perforin (Kahaleh, 1997). The majority of autoantigens targeted in SSc can be cleaved by granzyme B and are recognized preferentially by patients antibodies (Schachna et al., 2002). Furthermore, the activation of cytotoxic cell-mediated pathways is plausible and may be involved in early vascular injury thus initiating and propagating the autoimmune response in SSc. Antibody-dependent cellular cytotoxicity of vascular endothelium is reported in up to 40% of the SSc patients. The effector cells express Fc receptors and are both non-T cells and non adherent T lymphocytes, while the antibody is an IgG with MVEC specificity that mediate MVEC cytotoxicity via the Fas pathway (Sgonc et al., 2000). EC apoptosis may also confer a state of resistance to apoptosis by the surrounding fibroblasts that may lead to myofibroblast differentiation and tissue fibrotic changes that follow (Laplante et al., 2005). However, EC damage and apoptosis is an early event in the course of the disease with progressive loss of capillaries, responsible on one hand of the typical clinical manifestations of vasculopathy and on the other hand the chronic tissue ischemia that leads to organ dysfunction and eventual organ failure.

### **3. Vascular endothelial cell alterations and fibroproliferative vasculopathy**

Vascular disease in scleroderma patients is both functional and structural with reversible vasospasm as well as a reduction in the capillary density followed by obliterative vasculopathy. These vascular changes involving capillaries, arterioles and small arteries may be observed by nail-fold capillaroscopy. Important features of the tissue lesions in various stages of scleroderma are early microvascular damage, mononuclear-cell infiltrates, and slowly developing fibrosis. In later stages of scleroderma, the main findings are very densely packed collagen in the dermis, loss of cells, and atrophy. In particular, in the early phase of the disease, endothelial damage is characterized by collapse of vimentin's filaments in the perinuclear region, vacuolization, granular degeneration of the nucleus, cellular necrosis, gaps between endothelial cells, reduplication of basal membranes, followed by vascular lumen obstruction, altered permeability of vessel wall that induce increased passage of both plasma and mononuclear cells with perivascular infiltrates formation in which T lymphocytes and monocytes bearing macrophage markers predominate (Fleischmajer, 1980, 1977; Ishikawa, 1992) with more CD4<sup>+</sup> than CD8<sup>+</sup> cells (Roumm, 1984). In fact, T cells in skin lesions are predominantly CD4<sup>+</sup> cells, display markers of activation, exhibit oligoclonal expansion (Sakkas et al., 2002) and are predominantly type 2 helper T (Th2) cells (Mavalia et al., 1997). Moreover, an

increase in the Vdelta1 + T cells subset that express both adhesion molecules and activation markers suggests a selective V gene subset expansion (Giacomelli et al., 1998). In advanced phases, intimal thickening, delamination, vessel narrowing or obliteration and perivascular fibrosis are present (Rodnan et al., 1980).

At the cellular level, the changes that characterized the early lesions are: loss of endothelial cells, proliferating pericytes and vascular smooth muscle cells, and immune cells in the perivascular space. Endothelial cells are the only mesenchymal cell type that undergo apoptosis in the early phase of scleroderma, whereas vascular smooth-muscle cells and pericytes proliferate vigorously thus leading to the characteristic fibroproliferative SSc vasculopathy. The activation of vascular smooth muscle leads to migration of these cells into the intimal layer of the vessel where they differentiate into a myofibroblast. Fibroblasts and pericytes may also transform into myofibroblasts in scleroderma disease (Rajkumar et al., 2005). It is also suggested that, following vascular injury, bone marrow-derived circulating mesenchymal progenitor cells (e.g., fibrocytes), and epithelial cells via epithelial to mesenchymal transition (EMT) can become myofibroblasts. Recently, a study provided evidence that abnormal fibrillin-1 expression and chronic oxidative stress mediate endothelial-mesenchymal transition (EndoMT) in the tight skin murine model of SSc (Xu et al., 2010) and more recently evidence indicates that the c-Abl tyrosine kinase and the protein kinase C  $\delta$  (PKC- $\delta$ ), are crucial for TGF $\beta$  induction of EndoMT *in vitro*, and that imatinib mesylate and rottlerin, or similar kinase inhibitor molecules, may be effective therapeutic agents for SSc and other fibroproliferative vasculopathies in which EndoMT is involved (Li & Jimenez, 2011). The exact mediator of cell activation in scleroderma is unknown, but speculation includes the release of mediators from the activated endothelium (e.g., endothelin-1 (ET-1)) and platelets (e.g., thromboxane or platelet derived growth factor). In fact, endothelial damage cause an imbalance in endothelial vascular signals with increased endothelin production and impaired nitric oxide and prostacyclin release that mediates the vasospasm and contribute to intimal proliferation and vascular fibrosis and stiffness of the vessel wall. Recently, Interleukin 33 (IL-33), a novel member of the IL1 family that promotes Th2 responses and inflammation through the ST2 receptor, was found to be abnormally expressed in the SSc tissue and sera. In particular, in the early phase of the disease, upon EC activation/damage IL-33 may be mobilised from ECs into the circulation to signal through ST2 in key profibrotic players such as inflammatory/immune cells and fibroblasts/myofibroblasts. (Manetti, 2010, 2011). This step probably corresponds to the first symptom of scleroderma. Recurrent Raynaud's phenomenon could be the direct consequence of the structural changes of the vessel and the perturbed control of vascular tone due to an imbalance between vasodilatory and vasoconstrictive mediators. At this stage, the patient may have early signs of skin and visceral fibrosis. Platelet activation and enhanced coagulation with reduced fibrinolysis lead to fibrin deposits and contribute to the intimal proliferation and luminal narrowing.

Besides the skin, fibroproliferative vasculopathy is present in the lungs, kidneys and other organs (Dorfmueller et al., 2007; Nagai et al., 2007; Guiducci et al., 2007). However, the mechanisms underlying the pathological vascular changes in SSc still remain unclear.

#### 4. Impaired angiogenesis and vasculogenesis in SSc

In the adult mammalian organism, the vasculature is normally quiescent and the ECs have an extremely low turn-over rate with the exception of the reproductive cycle (ovulation, implantation, pregnancy) and wound healing or tissue regeneration (Carmeliet, 2003).

However, after endothelial cell injury, and in response to appropriate stimuli, mature and progenitor ECs they can form new blood vessels through a combination of two separate processes: angiogenesis and vasculogenesis. The term angiogenesis describes the formation of new capillaries and larger vessels by sprouting of differentiated EC from pre-existing vessels. Angiogenesis is a highly complex and requires a dynamic, temporally and spatially interaction among ECs, ECM molecules, adhesion molecules, proteolytic enzymes and the subtle balance between proangiogenic and angiostatic factors (Distler et al., 2003). In particular, proangiogenic stimuli activate EC, which degrade the basal membrane and the perivascular extracellular matrix, proliferate and migrate into the site of new vessel formation. Stabilisation of vessel wall by pericytes is the final process of sprouting angiogenesis and leads to a functional network of new capillary. In contrast to angiogenesis, vasculogenesis describes the formation of new vessels by circulating EPC, independent from pre-existing vessels. Vasculogenesis was regarded to be restricted to embryogenesis but the discovery of EPC in adult bone marrow and peripheral blood has challenged this theory (Asahara et al., 1997; Shi et al., 1998). After birth, postnatal vasculogenesis contributes to vascular healing in response to endothelial injury through the processes of rapid reendothelialization of denuded vessels and collateral vessel formation in ischemic tissues. In particular, following tissue ischemia, EPC are mobilized from their bone marrow niches into the circulation in response to stress- and/or damage related signals, migrate through the bloodstream and home to the sites of vascular injury, where they contribute to the formation as well of neovessels as to the repair of damaged vessels, collaborating with pre-existing mature EC (Urbich et al., 2004). Several studies showed that EPCs promotes structural and functional repair in several organs such as the heart, liver, kidney or brain. However, as mentioned above, progenitor cells can migrate to sites of vascular injury and differentiate not only into an endothelial phenotype (vascular repair), but also into vascular smooth muscle cells contributing to neointimal hyperplasia and eventually fibroproliferative vasculopathy.

Both angiogenic and vasculogenic processes are impaired in SSc. The progressive loss of capillaries on one hand, and the vascular remodeling of arteriolar vessels on the other result in insufficient blood flow, causing severe and chronic hypoxia. Tissue hypoxia usually initiates the formation of new blood vessels from the pre-existing microvasculature leading to the expression of pro-angiogenic molecules, mainly of Vascular Endothelial Growth Factor (VEGF), which triggers the angiogenic process. Despite the hypoxic conditions and the increased levels of VEGF in skin and serum of SSc patients, there is, paradoxically, no evidence for a sufficient angiogenesis, thus perpetuating the vicious circle leading to tissue ischemia (Distler JH et al., 2006; Distler O et al., 2004, 2002). Vasculogenesis, is also impaired in SSc patients with a decreased number and several functional defects of endothelial progenitor cells (EPCs) (Kuwana et al., 2004; Del Papa et al., 2006) and mesenchymal stem cells (MSCs), the latter deriving from the bone marrow population and the tissue resident cells, was observed in SSc patients (Cipriani et al., 2007).

## 5. Endothelial cells

The aetiological factors involved in the pathogenesis of SSc-associated vascular defects determine a complicate network of EC alterations which account for the lost ability of these cells to perform *in vitro* angiogenesis.

Angiogenic process is an invasive event in which proteolytic activities by EC are required. Specific proteases are needed for the degradation of the membrane basement, for cell

migration and for creating space in the matrix, to allow the formation of new tubules. Besides their substrate specific properties, proteases exert more complex pro- or anti-angiogenic activities, including the activation and modification of growth factors, cytokines and receptors and the generation of matrix fragments which inhibit angiogenesis (Van Hinsberg et al., 2008). Evidence for a mechanism of dysregulated angiogenesis involving these proteases in SSc has emerged from recent experimental studies. A decreased urokinase plasminogen activator (uPA) dependent invasion, proliferation, and capillary morphogenesis, was showed in SSc EC. Urokinase plasminogen activator receptor (uPAR) undergoes truncation between domains 1 and 2, and this modification prevents EC from entering in an angiogenic program (D'Alessio et al., 2004). Furthermore, SSc MVECs produce large amounts of antiangiogenic molecules such as matrix metalloproteinase 12 (MMP-12), involved in the cleavage of the domain of the uPAR, and pentraxin 3 (PTX3). Silencing these two molecules in SSc-MVEC, restore their ability to produce capillaries in vitro (Margheri et al., 2010). Recent work has provided the evidence for the association between a uPAR gene variant, *UPAR* rs344781, and vascular complications, such as digital ulcerations, suggesting a role of this gene in the vascular pathophysiology of SSc (Manetti et al., 2011).

Endothelial cells are directly involved also in vasculogenic process, through the expression of SDF-1. In fact, SDF-1 is a pivotal molecule in the recruitment and retention of CXCR4+ EPC into neo-angiogenic niches (Petit et al., 2007). This molecule, expressed and presented by EC at the site of injury triggers cell arrest and emigration of circulating cells, facilitating the formation of stable vasculature and supporting organ repair (Yao et al., 2003). Additionally, SDF-1 has an angiogenic effect on endothelial cells by inducing cell proliferation, differentiation, sprouting and tube formation in vitro (Salvucci et al., 2002; Yamaguchi et al., 2011). It has been reported that SDF-1 and CXCR4 are clearly up-regulated in the skin and in microvascular endothelial cells during the early edematous phases of SSc. The production of these two molecules progressively decreased, with the lowest levels in the latest phases of the disease. These data strongly suggest that an impairment of EC ability to promote a sustained response to chronic ischemic stress through SDF-1 expression could compromise an adaptive angiogenesis and vasculogenesis in the disease, contributing to the disappearance of the vessels (Cipriani et al., 2006).

Finally, it has been demonstrated that in SSc skin, along with the loss of capillaries, there is a dramatic change in the endothelial phenotype of residual microvessels, characterized by loss of vascular endothelial cadherin (VEcadherin), supposed to be an universal endothelial marker required for tube formation, as well as the over-expression of the anti-angiogenic interferon- $\alpha$  (IFN- $\alpha$ ) and over-expression of RGS5, a signaling molecule whose expression coincides with the end of new vessel formation during embryo development and tumour angiogenesis (Fleming et al., 2008).

## 5.1 Endothelial progenitors

### 5.1.1 Hematopoietic endothelial progenitor cells

Endothelial progenitor cells are known to be a key cellular effectors of vascular regeneration. Growing evidence shows that EPC play an important role in the homeostasis of physiologic vascular network and are involved both in new vessel formation after ischemic insult and in the repair mechanisms of existing vessels (Shi et al., 1998; Urbich et al., 2004; Zammaretti & Zisch, 2005; Adams et al., 2004). Endothelial progenitor cells have emerged as crucial regulators of cardiovascular integrity. Reduced numbers and altered

functions of these cells have been found to be involved in the pathogenesis of cardiovascular disease (Adams et al., 2004, Dimmeler et al., 2001; Hill et al., 2003).

At least two different types of circulating progenitors appear able to become mature endothelium (Smadja et al., 2007). One type of progenitor cells displays the markers CD133, CD34, and vascular endothelial growth factor receptor 2 (VEGFR2) (Hristov et al., 2004). These are harvested from late-outgrowth cultures, possess a high proliferation capacity and are sometimes referred as "true EPC"; most of studies focus on this population. A second type of progenitor population is a subset of CD14<sup>+</sup> monocytes distinguishable from the conventional endothelial progenitor cells by the fact that they are CD34<sup>-</sup> (Hristov et al., 2004; Zhao et al., 2003), arise from short-term cultures and show little proliferative capacity. Both circulating progenitor cell types can differentiate into mature endothelium in culture. It has been precisely characterized using, genome-wide transcriptional study, the molecular fingerprint of two distinct EPCs, showing that early-outhgrowth EPC are haematopoietic cells with a molecular phenotype linked to monocytes; whereas late-outgrowth EPC exhibit commitment to the endothelial lineage (Medina et al., 2010). Interestingly both populations can form capillary tubes *in vitro*, mediate reendothelialization after injury and improve neovascularization (Urbich et al., 2004). It has been previously demonstrated that both subsets contribute to angiogenesis, but through different mechanisms, CD14<sup>+</sup> EPC supporting vasculogenic process by paracrine production of growth factors, while late-outgrowth CD14<sup>-</sup> EPC directly incorporating into vessel wall (Sieveking et al., 2008; Mukai et al., 2008). Neovascularization and reendothelialization event are complex multistep processes, requiring EPC chemoattraction, adhesion, and finally differentiation into mature EC. Although the signaling cascades that regulate these steps are still incompletely understood, it is well known that VEGF and SDF-1, induced by hypoxia, play a pivotal role in the EPC mobilization from bone marrow, differentiation and attraction to site of ischemia. Recently several studies have demonstrated a role of EPC in the pathogenesis of SSc, suggesting that alteration in the vasculogenic process might contribute to the vasculopathy, distinctive features of the disease. Apparently conflicting results have raised on quantitative and functional characteristics of EPC from SSc patients, probably due to unclear distinctive markers of the several cell subsets belonging to the EPC population.

A lower number of circulating EPC, defined as CD34<sup>+</sup>CD133<sup>+</sup>VEGFR-2<sup>+</sup> mononuclear cells in patients with SSc than in patients with RA or in healthy subjects was seen (Kuwana et al., 2006). In SSc patients, EPC counts did not correlate with the disease subset, the disease duration, or the modified Rodnan skin thickness score. However, the numbers of these cells were lower in SSc patients with pitting scars and active fingertip ulcers. Furthermore, EPC, obtained from the peripheral blood of SSc patients demonstrated an impaired differentiation capacity into mature EC, as shown by a reduced expression of the EC marker von Willebrand factor.

In contrast to these findings, in another study was found a significantly increase in the number of circulating EPC, identified via the same cell surface markers CD34<sup>+</sup>CD133<sup>+</sup>VEGFR-2<sup>+</sup>, in SSc patients. Further subgroup analysis revealed a negative correlation between EPC count and disease duration (Del Papa et al., 2008). Based on this finding, the authors suggested that differences in disease duration might account for the discrepancy between their results and the findings reported by the other authors. Apart from disease duration, no correlations between EPC counts and clinical parameters, including digital ulcers, were observed. In this study bone marrow EPC were also evaluated. The number of bone marrow CD133<sup>+</sup> cells was significantly decreased in SSc patients compared to healthy controls. Their ability to differentiate into EC *in vitro* was

found reduced in SSc patients. Finally, the number and the size of colonies were reduced, and cells from patients showed morphologic signs of cellular senescence.

Another study assessed EPC counts in the whole blood of patients with SSc, osteoarthritis (OA), and RA (Allanore et al., 2007). Circulating CD34+CD133+ cells were increased in patients with SSc as compared with patients with OA, but the same cells were lower than those in RA patients. The analysis of potential correlations with clinical parameters showed that CD34+CD133+ counts increased in parallel with the European League Against Rheumatism Scleroderma Trial and Research (EUSTAR) group disease activity score. Of note, the authors did not analyse the expression of VEGFR-2.

In another study, the same authors (Avouac et al., 2008) assessed EPC, evaluating VEGFR-2 and lineage (Lin) markers as additional markers, and using 7-aminoactinomycin D (7-AAD) as viability marker. Again, patients with SSc displayed higher numbers of circulating Lin-7AAD-CD34+CD133+VEGFR-2+ EPC than did healthy subjects. Lower EPC counts in SSc patients were associated with higher Medsger severity scores for SSc and with digital ulcers. A decreased CD34+CD133+VEGFR-2+ EPC counts was found in both limited and diffuse subsets of recent-onset, and late-stage SSc, as compared with healthy individuals. The authors showed an increased rate of apoptosis in freshly isolated EPC from SSc patients. Addition of sera from the same patients to cultured EPC from healthy volunteers was able to induce apoptosis of EPC. The proapoptotic effects of SSc sera were abolished by depletion of the IgG fraction, suggesting the presence of anti-EPC autoantibodies in the SSc patient sera (Zhu et al., 2008).

It has been also found a raise of circulating EPC in early stage SSc, in response to tissue ischemia, but they dropped with disease progression. EPC reduction was linked with endothelial dysfunction and capillary loss, as well as the development of severe cardiac involvement and pulmonary arterial hypertension (Nevsakaya et al., 2008).

Whether EPC counts are altered in the peripheral blood of SSc patients is still a matter of controversy. Apparent contradiction in EPC counts between the different studies might be explained by use of different combinations of surface markers, resulting in the analysis of different cell subsets, or by differences in the mean disease duration and severity. Nevertheless, functional defects of EPC in the peripheral blood as well as in the bone marrow have consistently been reported (Del papa et al., 2006; Kuwana et al., 2004) indicating a critical role of EPC in the pathogenesis of SSc. Further studies on the molecular mechanisms underlying these defects is needed in order to develop specific treatment options and restore functional vasculogenesis in patients with SSc.

Several lines of evidence indicate that EPC obtained by short term culture of peripheral blood mononuclear cells (PBMCs) in media favouring endothelial differentiation, are composed predominantly of endothelial-like cells derived from circulating monocytes (Rehman et al., 2003; Urbich et al., 2003). Moreover, it has been identified a monocyte-derived multipotent cells positive for CD14, CD45, CD34, which contain progenitors able to differentiate into several distinct mesenchymal cell types, including bone, cartilage, fat, and skeletal and cardiac muscle cells, as well as neurons (Kuwana et al., 2003; Kodama et al., 2005, 2006). It has been recently shown that this population of multipotent cells, is able to differentiate into endothelium of a mature phenotype with typical morphologic and functional characteristics (Kuwana et al., 2006). These findings indicate a potential developmental relationship between monocytes and endothelial cells and suggest that the monocyte population could be recruited for vasculogenesis and represent an endothelial precursor population.

A recent study demonstrated that circulating monocytic EPCs were increased in the peripheral blood of SSc patients. *In vitro* and *in vivo* functional analyses revealed that monocytic EPCs derived from SSc patients had an enhanced ability to promote blood vessel formation, when co-cultured with HUVEC. In contrast, the EPC ability to be incorporated into vessels and differentiate into mature endothelial cells was rather impaired in SSc patients. This characteristic was primarily attributable to an enhanced angiogenic property through production of angiogenic factors (Yamaguchi et al., 2010). Because EPCs may critically contribute to the homeostasis of the physiological vascular network, these progenitor cells might be considered interesting candidates for novel cell therapies for the treatment of various ischemic diseases.

### 5.1.2 Mesenchymal stem cells (MSCs)

Endothelial cells could also originate from non-hematopoietic stem cells of the bone marrow (Drake et al., 2003). Mesenchymal stem cells are multipotent cells that are present in the bone marrow and in some tissues as resident stem cells. They retain the capacity to differentiate into several cell lineages of mesenchymal tissues, i.e. bone, cartilage, muscle, tendon or adipose tissue and serve for the preservation and repair of tissues and organs (Tuan et al., 2003; Arnhold et al., 2007). Multipotent mesenchymal progenitor cells have been isolated from bone marrow. Under exposition to angiogenic factors, these cells differentiated to angioblasts *in vitro* (CD34-positive, flk-1-positive, vascular endothelial cadherin-positive) and finally to mature endothelial cells, expressing specific endothelial markers, showing characteristics endothelial functional features and participating in neovascularization of tumours or wound healing *in vitro* (Reyes et al., 2001, 2002). Transplanted MSCs were able to enhance angiogenesis and contribute to remodelling of the vasculature *in vitro* (Ladage et al., 2007; Choi et al., 2008; Copland et al., 2008). Taken together, these data suggest that human MSCs may be considered an alternative source of EPCs (Oswald et al., 2004).

There are several evidences that in SSc there is a complex impairment in the BM microenvironment, involving not only the endothelial compartment but also the MSC, thus hypothesizing that alteration in this cell population may also contribute to the defective vasculogenesis in SSc (Del Papa et al., 2006; Cipriani et al., 2007). In particular, it has been reported that the number of colonies formed by MSC obtained from bone marrow of SSc patients were reduced in comparison with healthy controls. The colonies were small and did not expand, and the cells rapidly showed aging and signs of stress (Del Papa et al., 2006). Furthermore, no other study provided evidence of a reduced differentiative ability *in vitro* toward an endothelial phenotype of bone marrow MSC from SSc patients. These cells displayed both an early senescence and decreased capacity to perform specific endothelial activities, such as capillary morphogenesis and chemoinvasion, after VEGF and SDF-1 stimulation (Cipriani et al., 2007). All above suggests that a functional impairment of this population of endothelial progenitors cells may affect endothelial repair machinery, contributing to the defective angiogenesis and vasculogenesis in the disease.

In the last few years bone marrow-derived MSCs have shown great promise for tissue repair. In experimental models of acute myocardial infarction, intramyocardial injection of mesenchymal stem cells restored cardiac function through the formation of a new vascular network and arteriogenesis (Torensma et al., 2004). Autologous implantation of bone marrow-derived mesenchymal stem cells into chronic nonhealing ulcers has been shown to accelerate the healing process and significantly improve clinical parameters (Martens et al.,

2006). Recently, it has been showed that in one patient with systemic sclerosis who have severe peripheral ischemia, intravenous infusion of expanded autologous mesenchymal stem cells may promote the recovery of the vascular network, restore blood flow, and reduce skin necrosis (Guiducci et al., 2010). Further studies on a larger number of patients with systemic sclerosis are needed to confirm the short- and long-term efficacy and safety of mesenchymal stem-cell infusion as treatment of severe digital ulcers and gangrene of the extremities that are resistant to conventional therapies.

## **6. Angiogenic factors**

### **6.1 Vascular endothelial growth factor (VEGF)**

VEGF, one of the strongest angiogenic factors known in biology, is involved in several steps of physiological and pathological angiogenesis. VEGF increases the vascular permeability, stimulates the migration and proliferation of ECs and induces tube formation. The biological effects of VEGF are extremely dose dependent. Loss of even a single allele results in lethal vascular defects in the embryo, and postnatal inhibition of VEGF leads to impaired organ development and growth arrest in mice. Application of VEGF as a recombinant protein or by gene transfer augmented perfusion and development of collateral vessels in animal models of hindlimb ischemia, thereby making VEGF an interesting target for therapeutic angiogenesis (Takeshita et al., 1994, 1996). During SSc, VEGF is strongly overexpressed in the skin and sera (Distler O et al., 2002) of these patients. VEGF exerts its biological functions by binding to 2 different tyrosine kinase receptors: VEGFR-1 and VEGFR-2, which are both upregulated in the affected skin of SSc patients (Distler O et al., 2004), although non compensative new vessel formation is observed. The serum levels of VEGF significantly correlate with the development of fingertip ulcers in these patients. Although elevated levels of VEGF are consistent with active angiogenesis, an uncontrolled chronic over-expression, as seen in SSc patients, throughout various disease stages, might contribute to disturbed vessel morphology and endothelial disturbances rather than to promote new vessel formation. On the other hand, a brief upregulation of VEGF results in instability of newly formed vessels ((Dor et al., 2002). The mechanisms that lead to an over-expression of VEGF in SSc are unclear. Isolated microvascular endothelial cells (MVECs) from SSc patients show an impaired response to VEGF and other growth factors in the Matrigel capillary morphogenesis assay, suggesting that VEGF receptor signaling might be impaired in these cells (D'Alessio et al., 2004). Hypoxia-induced expression of hypoxia inducible factor-1 $\alpha$  (HIF1- $\alpha$ ) does not appear to play a major role in the induction of VEGF in SSc (Distler O et al., 2004), whereas induction by cytokines such as platelet derived growth factor (PDGF) and transforming growth factor beta (TGF- $\beta$ ) appear to be more important. The function of TGF- $\beta$  in angiogenesis is strongly context dependent. A weak TGF- $\beta$  stimulation may cause induction of several angiogenic regulators such as VEGF and matrix proteins to promote angiogenesis, whereas at high TGF- $\beta$  concentrations, growth-inhibitory effect dominate (Bobik et al., 2006). Functionally important gene polymorphisms that lead to an impairment in biological properties of VEGF have not still been shown in SSc patients (Allanore et al., 2007).

### **6.2 Transforming growth factor beta (TGF- $\beta$ ) and Endoglin (ENG)**

The vascular effect of TGF- $\beta$  in angiogenesis results in activation of ECs and vascular smooth muscle cells (VSMCs). It regulates the activation state of ECs, via 2 different types of I receptors, ALK-5, and ALK-1. The TGF- $\beta$ /ALK-1 pathway stimulates ECs proliferation and migration, whereas the TGF- $\beta$ /ALK-5 pathway inhibits these processes. ALK-5

deficiency not only impairs TGF- $\beta$ /ALK-5 signaling but also reduces TGF- $\beta$ /ALK-1 responses, suggesting that ALK-5 is essential for efficient ALK-1 activation and recruitment into a TGF- $\beta$ /receptor complex (Bobik et al., 2006). These effects are mainly mediated by ENG (CD105), a coreceptor of TGF- $\beta$ , predominantly expressed on cell surfaces of ECs. ENG plays a role in vascular integrity and endothelium functioning, whereas soluble ENG (sENG) acts as an antiangiogenic protein interfering with the binding of TGF- $\beta$  to its receptors. Conflicting results have been reported in the available literature concerning the relationship between ENG and PAH. A previous paper reported an association between a 6-base insertion in intron 7 (6bINS) polymorphism of ENG gene and SSc-related PAH (Wipff et al., 2007). More recently, increased sENG levels were found in SSc patients, both with and without PAH, suggesting a role for ENG in SSc vasculopathy, independent of PAH presence (Coral-Alvarado et al., 2010). Furthermore, ENG might act on fibroblasts to modulate TGF- $\beta$  signaling by acting as a molecular link regulating or reducing the total pool of TGF- $\beta$  available for activating signal-transducing receptors.

### 6.3 Platelet derived growth factor (PDGF)

The PDGF family consists of four different PDGF strands (A-D), establishing functional homodimers (PDGF-AA, PDGF-BB, PDGF-CC, and PDGF-DD) or an heterodimer PDGF-AB. They exert their biological activities by activating 2 structurally related tyrosine kinase receptors, PDGFR $\alpha$  and PDGFR $\beta$ . Ligand-induced receptor homo- or hetero-dimerization leads to autophosphorylation of specific tyrosine residues within the cytoplasmic domain. PDGF-A activates PDGFR $\alpha$  exclusively, while PDGF-B is capable of activating PDGFR $\alpha\alpha$ , PDGFR $\alpha\beta$  and PDGFR $\beta\beta$ . PDGF-AB and PDGF-C activate PDGFR $\alpha\alpha$  and PDGFR $\alpha\beta$ , whereas PDGF-D preferentially activates PDGFR $\beta\beta$ . PDGF-B and the PDGFR $\beta$  are primarily required for the development of the vasculature both under physiological and pathological angiogenic conditions e.g. during myocardial infarction (Zymek et al., 2006) and tumor vascularization (Vrekoussis et al., 2007) while, they are almost undetectable in healthy tissues. PDGFBB/PDGFR $\beta$  signaling-axis is also involved in recruiting pericytes and smooth muscle cells thus contributing to the maturation of the vessel wall (Lindblom et al., 2003). PDGF plays an important role in the pathogenesis of scleroderma and elevated expression of PDGF and its receptors has been found in SSc skin and lung diseases. In particular, expression of PDGF-B was detected in endothelial cell lining of small capillaries and in the infiltrating cells (Gay et al., 1989; Klareskoog et al., 1990). Likewise, elevated levels of PDGF-A and PDGF-B were found in bronchoalveolar lavage (BAL) fluid obtained from SSc patients (Ludwicka et al., 1995). Moreover, elevated plasma levels of PDGF-BB were reported in patients with SSc (Hummers et al., 2009). Indeed, recent studies suggest that the reciprocal induction of pro-angiogenic factors could promote vascularization and improve vessel maturation compared with the release of a single factor, and the interplay between PDGF and VEGF-mediated signalling pathways is just emerging (Bianco et al., 2007; Reinmuth et al., 2007). In fact, activated platelets carry in their alpha granules a set of angiogenesis stimulators such as VEGF, PDGF and SDF-1. Moreover, EPCs trigger EC thus inducing a pro-angiogenic phenotype including the up-regulation of PDGFR $\beta$ , thereby turning the PDGFBB/PDGFR $\beta$  signaling-axis into a critical element of EPC-induced endothelial angiogenesis (Wyler von Ballmoos et al., 2010). This finding may be utilized to enhance EPC-based therapy of ischemic tissue in future. Furthermore, the recent discovery of novel agonistic antibodies targeting the PDGF receptor that represents a pathogenetic link between immune system and fibrosis might lead to perform intense investigations to clarify the possible role that PDGF might exert on the SSc vasculopathy (Baroni et al., 2006). Finally,

PDGF receptor antagonist STI571 (imatinib) reversed advanced pulmonary vascular disease in 2 animal models of pulmonary hypertension (Schermuly et al., 2005).

#### **6.4 Stromal cell-derived factor 1 (SDF-1/CXCL12)**

The CXC chemokine SDF-1, the most important chemokine induced by ischemia, and its receptor, CXCR4, regulates specific steps in new vessel formation (Salcedo et al., 2003). Experimental deficiency in *SDF-1* or *CXCR4* gene in the embryo results in a lethal phenotype characterised by defective development of cardiovascular system (Kucia et al., 2004). As already mentioned above, SDF-1 is a potent chemoattractant for mature ECs, hematopoietic stem cells (HSCs) and EPCs expressing CXCR4 on their surface, thus influencing both angiogenesis and vasculogenesis (De Falco et al., 2004). SDF-1-CXCR4 interaction further amplifies angiogenesis by increasing VEGF release by ECs. VEGF elevated levels, in turn, promote enhanced expression of CXCR4 on endothelial cells, which can then respond to SDF-1. SDF-1, also prevents the apoptosis of EPCs. Also, SDF-1 contributes to the stabilization of neo-vessel formation by recruiting CXCR4+PDGFR+cKit+ smooth muscle progenitor cells during recovery for vascular injury. In SSc, due to the transient nature of SDF-1 expression, its modulation could be considered a future therapeutic target for inducing new vessel formation in this disease (Cipriani et al., 2006). Finally, SDF-1 polymorphism may modulate SSc vascular phenotype, further arguing for a critical role of SDF-1/CXCR4 axis in the vascular component of SSc pathogenesis (Manetti et al., 2009).

#### **6.5 Endothelin 1 (ET-1)**

ET-1, a highly vasoconstrictor molecule produced from endothelial cells and mesodermal cells such as fibroblasts and smooth muscle cells, promotes leukocyte adhesion to the endothelium as well as vascular smooth muscle cell proliferation and fibroblast activation (Abraham & Distler, 2006). ET-1 expression levels are increased in blood vessels, lung, kidney and skin of patients with SSc. Plasma ET-1 levels are also increased in SSc patients in both early and late stage (Kahaleh, 1991; Kadono et al., 1995). ET-1 mediates its biological effects via the ETA and ETB receptors. ETA receptors are expressed by vascular smooth muscle cells and can mediate vasoconstriction, smooth muscle cell proliferation, fibrosis and inflammation. ETB receptors are predominantly expressed on endothelial cells mediating vasodilation via the release of nitric oxide or potassium channel activation, and removing ET-1 from the circulation. In SSc vasculopathy, ETB receptors are down regulated on endothelial cells which may diminish their vasodilatory role while are up-regulated on smooth muscle cells and can contribute to cell proliferation, hypertrophy, inflammation, fibrosis and vasoconstriction (Abraham et al., 1997; Bauer et al., 2002). ETA/B receptor antagonists including bosentan are now commonly used for the treatment of PAH (Channick et al., 2001; Rubin et al., 2002) and of the prevention of new digital ulcers related to SSc (Matucci-Cerinic et al., 2011). ET-1 represents a potent molecular target for intervention in the management of patients with SSc.

#### **6.6 Angiopoietins**

Angiopoietins are known to be involved in the development, remodeling and stability of blood vessels. Recently has been clarified as they might act alongside VEGF (Ashara et al., 1998). However, Ang-1 and -2 have opposing functions. Ang-1 under physiological conditions has vasoprotective and anti-inflammatory actions (Kim et al., 2001), mediates

vessel maturation and maintains vessel integrity by the recruitment of periendothelial cells. On the contrary, Ang-2 acts as a vessel-destabilizing cytokine, playing an essential role in vascular remodeling. Recently, has been demonstrated that Ang-1 and -2 are differentially expressed in the sera of patients with SSc. Ang-1, was significantly decreased in SSc patients contributing to the development of SSc-related vasculopathy through promoting activation and apoptosis of ECs and destabilization of blood vessels. On the other hand, Ang-2, was significantly increased in SSc patients (Michalska et al., 2010)). Moreover, high Ang-2 levels are associated with greater severity and higher activity of the disease. Thus, the Ang-1/Ang-2 imbalance might contribute to the development of the disease, and represent a new promising therapeutic target in SSc.

### 6.7 uPAR and kallikreins

As already mentioned above, MVECS can perform angiogenesis only when provided with a proper enzymatic machinery, enabling them to lyse the extracellular matrix and invade the surrounding tissue. In this regard, the serine protease urokinase- type plasminogen activator uPA-uPAR system is known to play a crucial role in angiogenesis by modulating the adhesive properties of ECs in their interactions with the extracellular matrix and in the degradation of matrix components (Van Hinsberg et al., 2008); D'Alessio et al., 2004; Margheri et al., 2006; Manetti et al., 2011).

Another angiogenesis-associated serine protease family, potentially involved in the angiogenic program of SSc are some members of kallikreins. Kallikreins hydrolyze kininogen to kinin. Kinins promote angiogenesis, since they play a role that leads to endothelial cell migration, proliferation and differentiation. Kallikreins 9, 11, and 12, which are associated with proangiogenesis, were downregulated in SSc patients, whereas anti-angiogenic kallikrein 3 was upregulated. Further experiments using healthy MVECs treated with antibodies against the relevant kallikreins revealed that while kallikreins 9, 11, and 12 induced cell growth, only kallikrein 12 regulated invasion and capillary morphogenesis. Buffering of kallikrein 12 with antibodies resulted in the acquisition of an SSc-like pattern by normal cells *in vitro* angiogenesis (Giusti et al., 2005).

### 6.8 Adhesion molecules

Another pro-angiogenic marker of SSc vasculopathy is the presence of adhesion proteins involved in cell-cell interaction and cell-extracellular matrix interactions that are found increased in SSc skin (Gruschwitz et al., 1992). In particular, increased expression of the adhesion proteins such as intercellular adhesion molecule 1 (ICAM-1), endothelial leukocyte adhesion molecule 1 (ELAM-1), vascular adhesion molecule (VCAM-1), E-selectin, P-selectin in endothelial cells in the skin of patients with a rapidly progressive systemic sclerosis was observed (Sollberg et al., 1992; Gruschwitz et al., 1995; Ihn et al., 1997; Kiener et al., 1994; Ihn et al., 1998). To evaluate the relationship between systemic manifestations and immunological markers of endothelial cell activation, soluble VCAM-1 (sVCAM-1), soluble E selectin (sE-selectin), VEGF, and ET-1 were determined. Interestingly, the injury to the pulmonary and renal vascular trees might have distinct pathogenic mechanisms (Stratton et al., 1998). In particular, in patients with SRC, the level of E-selectin, sVCAM-1, and soluble ICAM-1 (sICAM-1) were elevated, but they were not consistently elevated in patients with pulmonary hypertension.

## 7. Angiostatic factors

### 7.1 Endostatin and Angiostatin

Breakdown of the extracellular matrix by granzyme B and other proteases contained in T cell granule content, may contribute to defective wound healing and vascular repair in SSc patients. Among these extracellular matrix derived angiostatic growth factors, endostatin has been characterized as a potent inhibitor of VEGF-induced angiogenesis. Endostatin is a C-terminal, 20 kDa fragment of the basement protein collagen type XVIII that inhibits angiogenesis and tumor growth strongly by reducing endothelial cell proliferation and migration. Circulating endostatin concentrations are significantly increased in patients with SSc and this increase is associated with the presence of more severe clinical involvement (Distler O et al., 2002). Angiostatin is another antiangiogenic factor derived from the cleavage of the plasminogen and proangiogenic plasmin. Recent data suggest that there is a decreased presence and activity of proangiogenic plasmin, and increased production of antiangiogenic angiostatin in SSc plasma. This increase in angiostatin production may account for some of the vascular defects observed in patients with SSc (Mullighan-Kehoe et al., 2007). Finally, circulating thrombospondin-1 (TSP-1), a counteradhesive protein with angiostatic and apoptotic properties was found increased in patients with SSc (Macko et al., 2002).

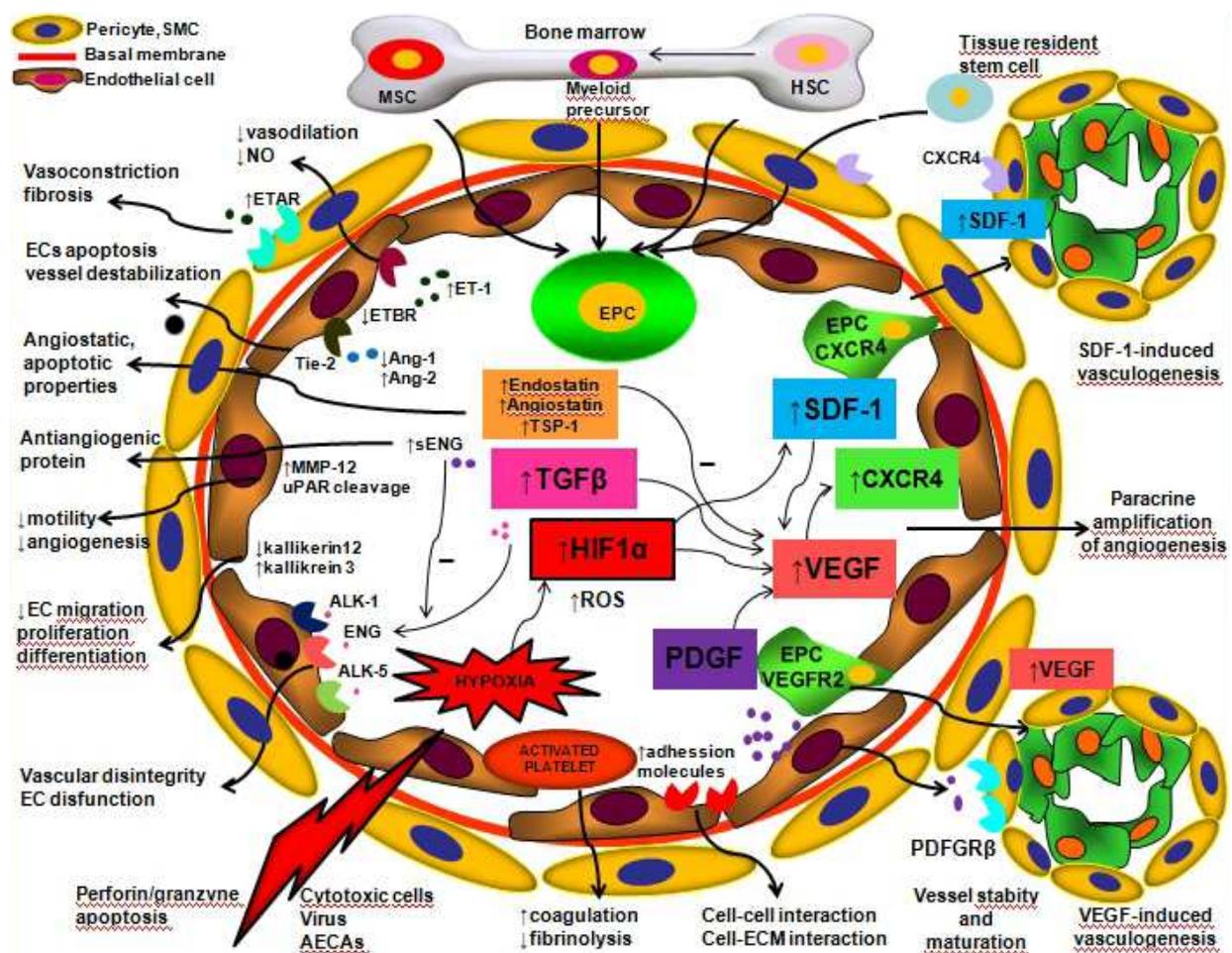


Fig. 1. Angiogenic endothelial response following ischemic injury.

For the specific function of involved molecules and cells see the text. EPC: circulating endothelial progenitors; MSC: mesenchymal stem cells; HSC: hematopoietic stem cells; ROS: reactive oxygen species; TGF $\beta$ : transforming growth factor beta; HIF1 $\alpha$ : hypoxia inducible factor 1 alfa; PDGF: platelet derived factor; PDGFR: platelet derived factor receptor; VEGF: vascular endothelial growth factor; VEGFR: vascular endothelial growth factor receptor; SDF-1: stromal cell-derived factor 1; CXCR4: SDF-1 receptor; ENG: endoglin; sENG: soluble endoglin; ALK-1 and ALK-5: activin receptor-like kinase-1 and -5; Ang-1 and Ang-2: angiopoietin 1 and 2; Tie-2: tyrosine kinase receptor; ET1: endothelin-1; ETAR and ETBR: endothelin receptor A and B; MMP12: matrix metalloproteinase 12; uPAR: urokinase-type plasminogen activator.

## 8. Conclusions

Vascular endothelial cell damage is and early an probably initiating event in the pathogenesis of the SSc resulting in endothelial dysfunction and loss of capillaries. The dysregulation of the angiogenic homeostasis seen in SSc, leads to failure in replacing damaged vessels, thus contributing to the vascular desertification and the chronic ischemia characteristic of the disease. Vasculogenesis is also impaired in SSc. Failure of the angiogenic process in SSc largely depends on alteration in the balance between angiogenic and angiostatic factors. At present, data describing the process of dysregulation in angiogenic homeostasis are incomplete and need further research. On the other hand functional alterations of the cellular players involved in the angiogenic and vasculogenic program are also demonstrated. The possibility of reversing this impairment opens new perspectives for regenerative cellular therapy for the vascular damage of this disease.

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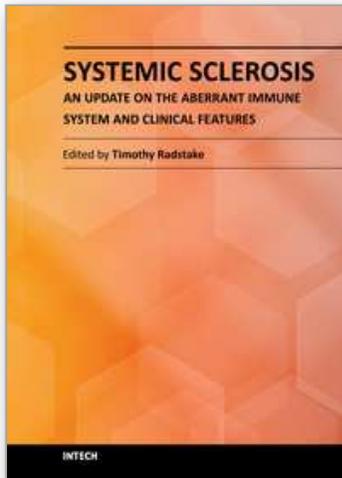
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## **Systemic Sclerosis - An Update on the Aberrant Immune System and Clinical Features**

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Systemic sclerosis (SSc), or often referred to as Scleroderma (tight skin), is characterized by an exaggerated formation of collagen fibers in the skin, which leads to fibrosis. Accumulating evidence now points toward three pathological hallmarks that are implicated in Ssc, the order of which has yet to be determined: endothelial dysfunction, autoantibody formation, and activation of fibroblasts. This current book provides up-to-date information on the pathogenesis and clinical features of this severe syndrome. It is our hope that this book will aid both clinicians and researchers in dealing with patients with this clinical syndrome. In addition, we hope to shed more light on this rare and severely disabling syndrome, ultimately leading to better research and successful therapeutic targeting.

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