Decreased Expression of Angiopoietin 1 on Perivascular Mesenchymal Stem Cells from Ssc Patients Induces an Anti Angiogenetic Effect, when Co-cultured with Endothelial Cells

Paola Di Benedetto1*, Vasiliki Liakouli1, Francesco Carubbi1, Piero Ruscitti1, Onorina Berardicurti1, Ilenia Pantano1, Antonio Francesco Campese2, Edoardo Alesse1, Isabella Screpanti1, Roberto Giacomelli1 and Paola Cipriani1

1Department of Applied Clinical Sciences and Biotechnology, Rheumatology Unit, School of Medicine, University of L’Aquila, Italy
2Department of Molecular Medicine, School of Medicine ‘Sapienza’ University of Rome, Italy

Abstract

Introduction: The Angiopoietin (Ang)/Tie2 system plays crucial roles in vascular functions, regulating endothelial-pericyte interaction and promoting vascular stabilization. We assessed if an impaired cross-talk, in Systemic Sclerosis (SSc), between endothelial cells (ECs) and perivascular mesenchymal stem cells (MSCs) may affect the normal interaction among Ang1, Ang2 and Tie2 thus contributing of the impaired angiogenesis in SSc.

Methods: We investigated Ang1, Ang2 and their receptor performing co-cultures with ECs and bone marrow MSCs obtained from patients and healthy controls (HC). After 48 hours, cells were sorted and analysed for molecular assays. Furthermore, we investigated, by ELISA assay, the proteins released in the supernatants. Finally, we silenced Ang-1 expression in HC-MSCs by siRNA-Ang1.

Results: At molecular level, SSc-MSCs, cultured alone, expressed lower amount of Ang1 when compared to HC-MSCs. After co-culture, a significant decreased of Ang1 mRNA levels was observed in the SSc-MSCs/SSc-ECs. On the contrary, SSc-ECs expressed higher levels of Ang2 and Tie2 in each co-culture condition, when compared to the expressions of cells cultured alone. The WB and ELISA assays mirrored the results observed in gene expression. HC-MSCs transfected with siRNA-Ang1 lacked the ability to support the formation of tube like structure.

Conclusions: In this work we provided evidence that an imbalance of Ang1/Ang2 molecules and a decreased expression of their receptor, Tie2, during ECs-perivascular MSCs interplay, may modulate vessel stability, and vascular tube formation, thus contributing to the angiogenic alteration observed during SSc.

Keywords: Systemic sclerosis; Mesenchymal stem cells; Angiogenesis; Angiopoietins

Abbreviations: Ang1 and 2: Angiopoietin1 and 2; Tie2: Tyrosine kinase with Immunoglobulin-like and EGF-like domains-2; EC: Endothelial Cell; MSC: Mesenchymal Stem Cell; HC: Healthy Control; SSc: Systemic Sclerosis; VEGF: Vascular Endothelial Growth Factor; PDGF: Platelet-Derived Growth Factor; TGF: Transforming Growth Factor; ALK: Activin receptor-like Kinase; MMP: Matrix Metalloproteinase

Introduction

SSc is a multisystem autoimmune disease characterized by initial vascular injuries and resultant fibrosis of the skin and internal organs. Although fibrosis is the final consequence of SSc, the evidence of autoimmunity and vascular injuries, prior to the onset of the fibrotic response, appear to play central roles in the pathogenesis of this disorder [1]. In fact, the progressive losses of capillaries on one hand, and the vascular remodelling of arteriolar vessels on the other, result in insufficient blood flow, causing severe and chronic peripheral hypoxia. Despite of elevated levels of vascular endothelial growth factor (VEGF), in skin and serum of SSc patients throughout different disease stages [2], adaptive angiogenic is largely defective [3].

An impaired production of angiogenic molecule produced by ECs and pericytes seems to be involved in this dysfunctional angiogenesis and a deeper knowledge of this early pathogenic alteration, may suggest potentially important molecular and cellular target for the treatment of the disease.

Of note, pericytes are critical for vascular morphogenesis, and, until now an impaired production of angiogenic molecule produced by ECs and pericytes seems to be involved in this dysfunctional angiogenesis and a deeper knowledge of this early pathogenic alteration, may suggest potentially important molecular and cellular target for the treatment of the disease.

The mechanisms governing pericytes migration and differentiation have not been fully established. It is well known that pericytes may play two different functions, as perivascular cells, as well as mesenchymal progenitor cells, the latter involved in the reparative fibrotic response [4], after differentiation toward myofibroblast cells. On this light, to understand the mechanisms leading to the pericyte detachment, from the vessels, might also provide insight on the contribute of these cells in the fibrotic process, during SSc.

*Corresponding author: Dr. Paola Di Benedetto, Department of Biotechnology and Applied Clinical Science, Rheumatology Unit Via Veteto, University of L’Aquila, Coppito, 67100 L’Aquila, Italy, Tel: +390862434775; Fax: +390862434958; E-mail: paola_di_benedetto@libero.it

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The tyrosine kinase with immunoglobulin-like and EGF-like domains-2 and-1 (Tie2 and Tie1) are trans-membrane receptor tyrosine kinases, exclusively expressed on the surface of EC in adult tissues [5]. Tie2 exerts a dual effects on the vascular tree, being able to modulate vessels quiescence or alternatively inducing angiogenesis in a context-dependent manner, via the interaction with its ligands, Ang1 and Ang2 [6,7]. Of note, activation of endothelial Tie2 signalling, by Ang1 which is produces by perivascular cells, enhances ECs barrier integrity and endothelial-pericyte interaction, thereby promoting vascular stabilization [8] and endothelial quiescent status. On the contrary, Ang2, produced and stored in Weibel-Palade bodies in ECs, normally acts as an Ang1 antagonist. Ang2 destabilizes the quiescent vasculature and activates ECs to respond to angiogenic stimuli. Overexpression of Ang2 in the mouse endothelium attenuates physiological Tie2 signalling, thus increasing vascular permeability and counteracting the Ang1 action [9].

Many papers, in last year, explored the role of Ang/Tie2 axis in the pathogenesis of SSc. In 2011, Michalska-Jakubus et al. [10] demonstrated different expression of angiopoietins in sera of patients with SSc, showing increased amount of Ang2 and decreased Ang1 levels. Lately, [11] in SSc higher circulating levels of Ang1, Ang2 and Tie2 were shown. Successively [12], decreased serum Tie2 levels in SSc patients, correlated with clinical symptoms associated with proliferative vasculopathy, such as digital ulcers, scleroderma renal crisis and elevated right ventricular systolic pressure were reported. Recently, [13] it has been supposed that a molecular imbalance among Tie2, Ang1 and Ang2 may reflect a disorder in Tie signal transmission, as well as in its activity, on ECs.

This is the first work, in our knowledge, exploring the Ang/Tie2 axis, before and after EC-MSCs cognate interaction.

Aim of the present study was to assess the modulation of Ang/Tie2 signaling system, during the ECs-pericytes cross-talk, in diffuse SSc patients, in whom the occurrence of fibrosis is generally earlier and rapidly progressive, when compared to the limited form. In this work, bone marrow derived MSCs were used as pericytes surrogate, and rapidly progressive, when compared to the limited form. In this work, bone marrow derived MSCs were used as pericytes surrogate, and this is the first work, in our knowledge, exploring the Ang/Tie2 axis, before and after EC-MSCs cognate interaction.

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Before the cells reached confluence, after approximately 1 week, the heterogeneous pool of cells was exposed to a CD31-positive selection, performed with the Dynabeads magnetic CD31 MicroBeads cell sorting system (Invitrogen, Life Technologies, CA, USA). The heterogeneous cell population was washed twice with PBS and incubated on a rocking platform (25 revolution/min) during 20 min with PBS/0.1% BSA (1.5 × 10⁷ cells/ml) containing pre-washed and re-suspended Dynabeads coated with CD31 antibody (PECAM-1). The beads rapidly target and partially coat the endothelial cells expressing the CD31 receptor. After the incubation, the cells were placed in a magnet (DynaM Magnetic Separation System, Dynal, Oslo, Norway) for 2 min, following the manufacturer’s recommended protocol for washings and final extraction. The CD31-negative cells (with no beads attached) were removed during the successive washings. The positive selected ECs were re-suspended in endothelial cell growth medium and maintained at 37°C and 5% CO₂. The cells obtained with this procedure were 99% ECs with typical cobblestone morphology. The ECs were analyzed for the surface expression of specific antigen (CD-31, CD-34, CD-144) by available literature [19,21,22].

Cell sorting of co-cultured cells

ECs and MSCs were seeded in a 3:1 ratio co-cultures, as suggested by available literature [19,21,22]. The following co-cultures were performed: SSc-ECs/SSc-MSCs, SSc-ECs/HC-MSCs, HC-ECs/HC-MSCs, HC-ECs/SSc-MSCs. As appropriate controls, we used HC-MSCs, SSc-ECs, HC-ECs and SSc-ECs cultured alone, in the same experimental conditions of the co-cultures.

After 48 hrs the total tube length of each well was measured as branching index=(master junction/area)×1000 and photographed. Images were acquired using an Olympus BX53 fluorescence microscope.

Cell sorting of co-cultured cells

After 48 hrs of co-culture, as suggested by available literature [23,24], tube formation was observed. At this point, MSCs and ECs were gently removed from 3D cultures, using Dispase (BD Biosciences, CA, USA). The recovered MSCs and ECs were washed several times with PBS and re-suspended in 0.1% FBS in PBS for cell-sorting. The cells were sorted (purity>95%) by a FACSAria cell sorter (BD Biosciences, CA, USA), The cells were used at third passages.

In vitro angiogenesis assay

Tube formation ability was evaluated using a Matrigel assay. Matrigel (BD Biosciences, CA, USA) (8.6 mg/ml) was used at 1:1 dilution with basal EGM2-MV, without any supplement. ECs and MSCs were labeled, before co-culture in matrigel, using the green fluorescent dye PKH67 and red fluorescent dye PKH26 (Sigma, USA), respectively, according to the manufacturer’s instructions.

After 48 hrs the total tube length of each well was measured as branching index=(master junction/area)×1000 and photographed. Images were acquired using an Olympus BX53 fluorescence microscope.

Transfection of MSC with Ang1 siRNA

MSC were transfected with siRNAs using siPORTTM NeoFXTM (Ambion, USA) to silence Ang1 expression according to the manufacturer’s instructions. Cells were first washed with PBS, trypsinized, and re-suspended in OptiMEM I reduced-serum medium (Invitrogen, USA). Cells were then plated with the transfection medium for 24 h. Cultures were incubated for 24 h with Silencer Select siRNA-Ang1 (Life Technologies, USA) or with Silencer Select Negative Control non-targeting siRNA (NT) (Life Technologies, USA) and after incubation, plates were washed and cells were allowed to recover in normal growth conditions (10% DMEM) for 24 h post-transfection. After, the transfected MSCs were tested for Ang1 expression and, paralleling, the cells were co-cultured with both HC- and SSc-ECs in matrigel.

qRT-PCR analysis

Total RNA was extracted from sorted BM-MSCs and ECs using NucleoSpin RNA XS (Machery Nagel, Dueren, Germany) according to manufacturing instructions and reverse transcribed into complementary DNA (cDNA) with the ThermoScript reverse transcription-PCR system (Invitrogen, Life Technologies, CA, USA).

The qRT-PCR was performed by using SYBR green kits and Taqman gene expression assay (Applied Biosystems, Netherlands). Results were analyzed after 45 cycles of amplification using the ABI 7500 Fast Real Time PCR System. Primers were designed on the basis of the reported sequences (Primer bank NCBI). β-actin: 5'-CCTGGGCACCCAGCAACAT-3' (forward) and 5'-AGTACTCGTGTTGGATGTCG-3' (reverse); Ang1: 5'-CCAGTACACAAACAGCTCT-3' (forward) and 5'-TCTCCGACTCATGTTTCTCAC-3' (reverse); Ang2: 5'-AACCCTTCGGAGAGCATCGGAC-3' (forward) and 5'-CAGATCATTGTCTACGGCGG-3' (reverse). The expression was assessed by applied Taqman gene expression assay (Hs01032443_m1; Hs00176096_m1, respectively).

Western blot

In order to perform western blot assays, sorted BM-MSCs and ECs cells were pelleted, washed twice with PBS, lysed on ice in lysis buffer (1% Triton X-100, 0.5% NP-40, 50 mMTris–Cl, pH 7.5, 150 mMNaCl, 1 mM EDTA, supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 mMNaN3, 5 μg/ml aprotinin, 5 μg/ml leupeptin) for 30 min and cleared by centrifugation. The protein concentration was calculated by Bradford protein assay reagent (Bio-Rad). 50 µg of proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. After 1 hr at room temperature in blocking buffer [5% not fat milk in Tris-buffered saline/1% tween 20 (TBS/T)] the membranes were washed three times for 5 minutes each in TBS/T, and incubated overnight at 4°C with the primary antibodies: Ang1, Ang2 and Tie2 (Abcam, MA, USA), diluted in 5% bovine serum albumin in TBS/T. Following three washes with TBS/T, horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, Biotechnology) diluted in blocking buffer was added for 30 min at room temperature and washed three times with TBS/T. The detection was performed by enhanced chemiluminescence detection ECL reaction (Amersham Pharmacia Biotechnology). All the signals were quantified by normalizing to the tubulin signal (CP06 Anti-a-Tubulin Mouse mAb -DM1A). Immunoreactive bands were quantified with densitometry using ImageJ software (NIH, Bethesda, Maryland, USA).

Enzyme-linked immunosorbent assay

The concentration of Ang1, Ang2 released in single cultures and in co-cultures supernatants were determined by enzyme-linked immunosorbent assay (ELISA) using Quantikine Human Immunoassay kits (all by R&D Systems, Minneapolis, MN), according to the manufacturer’s protocol. Media samples were collected assuming homogeneous distribution of growth factors within the co-culture or monoculture.

Statistical analysis

GraphPad Prism 5.0 software was used for statistical analyses.
Results are expressed as Median (range). Due to the non-parametric distribution of our data the Mann-Whitney U test was used as appropriate for analyses. Statistical significance was expressed by a p value<0.05.

**Results**

**SSc-ECs affect the MSCs skill to support tubulogenesis.**

In our vitro matrigel assay HC-ECs cultured alone formed organized tube-like structure. When these cells were co-cultured with HC- and SSc-MSCs, we observed a significant improvement in the tube formation ability. As shown in Figure 1a and 1b, using SSc-ECs a significant impairment in tube formation was observed.

**Ang1 expression**

The expression of Ang1 was investigated in SSc-MSCs before and after co-culture with both HC- and SSc-ECs. At basal condition the Ang1 mRNA expression in SSc-MSCs was significantly lower compared with HC-MSCs [0.29 (0.09-0.45) vs. 1.25 (0.60-1.60), respectively, p<0.0001]. After co-culture with HC-ECs, the Ang1 mRNA expression was significantly increased in both HC- and SSc-MSCs, when compared to the levels observed when the MSCs were cultured alone (p<0.0001 and p=0.0002, respectively); when SSc-MSCs were co-cultured with SSc-ECs, a lower increase of Ang1 levels was observed (p<0.0002) (Figure 2a).

As shown in Figure 2b the results were confirmed, at the protein level, by western blotting analyses. Ang1 supernatant protein levels were further quantified by ELISA. The results concerning protein secretion confirmed the higher production of Ang1 (Figure 2c) protein in ECs/MSCs co-culture.

**Ang2 expression**

The expression of Ang2 was analyzed in SSc-ECs. Before co-culture, the mRNA levels in SSc-ECs was significantly increased when compared with HC-ECs [2.12 (1.92-2.40) vs. 1.09 (0.97-1.40), respectively, p=0.0002]. When the SSc-ECs were cultured with MSCs, independent of their origin, we observed the highest statistically significant levels of Ang2 transcript, as shown in Figure 3a. These results were confirmed at the protein level by western blotting analyses (Figure 3b). Furthermore, the supernatant Ang2 level assessed by ELISA, and concerning the protein secretion of each culture, mirrored the results observed by qPCR and western blot analysis (Figure 3c).

**Tie2 expression**

The Tie2 expression was analyzed in ECs. Before co-culture, the mRNA levels in SSc-ECs was significantly decreased when compared with HC-ECs [0.46 (0.30-0.54) vs. 1.09 (1.00-1.40), respectively, p=0.0002].

After co-culture with HC-MSCs, both HC- and SSc-EC showed a significant increased expression of Tie2 levels when compared with their...
Figure 2: Production of Ang1 in MSCs co-cultured with HC- and SSc-ECs. The pictures show the quantification by Real-time polymerase chain reaction of Ang1 (a) mRNA levels in HCs- and SSc-MSCs, sorted after co-culture with ECs. SSc-MSCs, cultured alone, expressed lower amount of Ang1 when compared to HC-MSCs. In each co-culture condition a significantly increase of the Ang1 mRNA levels was observed, compared to the expression when cells were cultured alone, a lower but still significant increase of Ang1 mRNA levels was observed in the SSc-MSCs/SSc-ECs coculture (\( p=0.008 \); \( \cdot\cdot\cdot p=0.0002 \); \( \cdot\cdot\cdot\cdot p<0.0001 \)). (b) Western blot analyses: these results confirm, at protein level, those observed by qRT-PCR analyses. Pictures are representative of all experiments. Tubulin was measured as a loading control for normalization. (\( \cdot= p=0.008 \), \( \cdot\cdot\cdot= p=0.0002 \), \( \cdot\cdot\cdot\cdot= p<0.0001 \)). (c) Ang1 ELISA assays. Ang1 were quantified in the supernatants of both single cultures and co-culture conditions. The results mirrored those observed by qRT-PCR and western blot analysis (\( \cdot\cdot\cdot p=0.0002 \); \( \cdot\cdot\cdot\cdot p<0.0001 \)).

Figure 3: Production of Ang2 and Tie2 in ECs co-cultured with HC- and SSc-MSCs. (a) Ang2 mRNA expression in ECs before and after co-culture with MSCs. SSc-ECs expressed higher levels of Ang2 in each co-culture condition, compared to those observed, when cultured alone. (\( \cdot\cdot\cdot\cdot p=0.0002 \); \( \cdot\cdot\cdot\cdot\cdot p=0.0001 \)). (b) Tie2 mRNA expression in ECs before and after co-culture with MSCs. SSc-ECs expressed lower levels of Tie2 in each co-culture condition, compared to those observed, when cultured alone. (\( \cdot\cdot\cdot\cdot p=0.0002 \); \( \cdot\cdot\cdot\cdot\cdot p=0.0001 \)). (c) Ang2 ELISA assays. The Ang2 protein production in the supernatants mirrored the results observed by qRT-PCR and western blot analysis (\( \cdot\cdot\cdot p=0.0002 \); \( \cdot\cdot\cdot\cdot p<0.0001 \)). (d) Western blot analysis of the Ang2 and Tie2 proteins: these results confirmed at protein level the results of qRT-PCR analyses. Pictures are representative of all experiments. Tubulin was measured as a loading control for normalization. (\( \cdot= p=0.008 \), \( \cdot\cdot\cdot= p=0.0002 \), \( \cdot\cdot\cdot\cdot= p=0.0001 \)).
levels, before co-cultures. Intriguingly, as shown in Figure 2b, the Tie2 levels in SSc-ECs were significantly lower than HC-ECs, independent of the MSCs used (SSc or HC) for the co-cultures. These results were confirmed at the protein level by western blotting analyses (Figure 3d).

**HC-MSCs transfected with siRNA-Ang1 lack the ability to support tubulogenesis**

To investigate the functional role of Ang-1 loss in HC-MSCs, we employed a specific si-RNA. In Figure 4a, we showed that in siRNA-Ang1 treated HC-MSCs; a transient silencing of Ang-1 gene expression was observed, when compared to cells treated by negative non-targeting siRNA (NT). Mirroring the data obtained with not-transfected HC-MSCs, siRNA-NT transfected HC-MSCs, when co-cultured with HC-ECs, formed organized tube-like structure. On the contrary, using SSc-ECs, a significant impairment in tube formation was observed (Figure 4b). After Ang-1 silencing, HC-MSCs lacked the ability to support the tubulogenesis of both HC- and SSc-ECs.

**Discussion**

Our study shows a strongly impairment of Ang/Tie2 axis during the cross talk between ECs and perivascular MSCs, during SSc. It is well known that perivascular MSCs, are mesenchymal-derived cells, displaying pericyte specific markers, including α-SMA, NG2, RGS5, desmin and platelet derived growth factor receptor (PDGFR), as well as pericytes functions [19] and are positioned around micro-vessels, covering gaps between ECs. These mural cells are critical for vascular morphogenesis and the mechanisms governing pericyte migration and differentiation are not fully established. In addition to their function as vascular cells, pericytes may contribute to local fibrosis [4]. Thus, a better knowledge of pericytes activities may provide new insight about the behavior of these cells in normal and pathological conditions.

ECs have an intimate contact with mural cells, and their tubular organization and maturation, in structurally stable capillary networks, is required for the development of a functional vascular tree. It is well known that tissue ischemia may lead to activation of both ECs and pericytes, thus promoting neo-angiogenesis. Despite the decreased oxygen tension and the vascular damage in SSc, paradoxically, no increase in effective angiogenesis and vascular repair may be observed [25-27]. To understand this lack of reparative angiogenesis, we recently [19,20] studied the molecular cross-talk between ECs and pericytes, during tubular morphogenesis and reported an altered expression of many molecules and related receptors, which are involved in the vessels formation.

![Figure 4](image-url)

**Figure 4:** Tubular-like structures formation in HC-MSCs transfected with siRNA-Ang1. (a) HC-MSCs was transfected with specific siRNA-Ang1 or non-targeting siRNA (NT), and Ang1 expression was evaluated by qRT-PCR. The cells transfected with siRNA-Ang1 showed a decreased gene expression of Ang1 when compared with cells transfected with siRNA-NT. (b) Phase contrast pictures: HC-MSCs-siRNA-NT co-cultured with HC-ECs (left panel) and SSc-ECs (right panel). HC-MSCs-siRNA-NT, when co-cultured with HC-ECs, formed organized tube-like structure, on the contrary when co-cultured with SSc-ECs, the capacity to improve the tubular formation was decreased. (c) Phase contrast pictures: HC-MSCs-siRNA-Ang1 co-cultured with HC-ECs (left panel) and SSc-ECs (right panel): HC-MSCs-siRNA-Ang1 lack the ability to support the tubulogenesis of both HC- and SSc-ECs. Pictures are representative of all experiments. Original magnifications 20X. (d) The total tube length of each well was measured as branching index= (master junction/area)*1000 and photographed. Results are expressed as Median (range) of triplicate experiments (*p=0.008, **p=0.0002, ***p=0.0001).
Under physiological conditions, pericytes recruitment, around vascular ECs, is controlled by multiple pathways, such as VEGF/VEGFRs, PDGF/PDGFR-β, transforming growth factor β (TGFβ)/activin receptor-like kinase 1 and 5 (ALK1 and ALK5) and matrix metalloprotease (MMP) activity [20,28].

In this work we analyzed the role of Ang1/Tie2 and Ang2/Tie2 axes, which display opposite roles in angiogenesis. Using a threedimensional matrigel system for ECs/MSCs co-cultures, followed by cells sorting, after 48 hrs of co-culture, to generate purified cells populations, we analyzed the changes of Ang1, Ang2 and Tie2 expressions, after ECs/MSCs cognate interactions.

In our experimental system, we showed that SSC-MSCs alone displayed a significantly lower expression of Ang1, when compared with HC-MSCs. This down-regulation may be completely reverted when SSC-MSCs were cultured together with HC-EC. In fact, it is well known that during angiogenesis, ECs are the main stimulus to induce Ang1 production, by pericytes. Ang1 binds Tie2 receptors, expressed by ECs, and this binding stabilizes mature vessels, facilitating EC/MSC interaction, although this mechanism is still not fully understood [29]. Two main functions are described for Ang1, related to the specific target cells: i. the inhibition of endothelial permeability and induction of EC-dependent cytokines release, including TGFβ and PDGF, which results in differentiation of resident perivascular stem cells toward pericytes and recruitment of stem precursors, to stabilize the nascent vessel [30]; ii. blocking endothelial responsiveness to tissue derived pro-angiogenic molecules, e.g. VEGF-A [31,32], to block the new vessels proliferation, directly inhibiting ECs. In our experiments, the Ang1 down-regulation was still evident after co-culture with SSC-ECs, suggesting, on one hand, that HC-ECs are able to induce a normal physiological response in SSC-MSCs, thus reverting the down-regulation of Ang1, and on the other hand, confirming the our previous results, concerning the pathologic role played by Ssc-ECs on the MSCs function, thus inducing the angiogenesis failure, observed during SSc.

We may speculate that, the decreased Ang-1 levels observed in SSC-MSCs, which seems to be modulated by the contact with SSC-ECs, interfering with vessel stabilization and inducing a pericytes/ECs detachment, may contribute to the angiogenic alteration observed, in vivo, during SSc.

The anti-angiogenic state of our patients may be further supported by the higher levels of Ang2 on SSC-ECs, when cultured alone in matrigel. After co-cultures, the contact with MSCs induced a significantly increase of Ang2, in both HC- and SSC-ECs, although the Ang2 levels in SSC-ECs were always higher than those observed in HC-ECs. It is well known that Ang1 and Ang2 show opposite roles in angiogenesis [8,9], Ang1, which is produced by pericytes, promote vascular stabilization [33], Ang2, produced by ECs, negatively modulate the activities of pericytes, competing with Ang1 in Tie2 binding on ECs surface, thus inhibiting pericytes recruitment and supporting the detachment of preexisting mural cells [34,35], interfering with the vessel stability. Our results, after cognate interaction between ECs and MSCs, confirm the pivotal role that SSC-ECs play in the angiogenic failure during SSc. In fact, in any experimental condition, SSC-ECs produced higher levels of Ang2, independent on the MSCs used. The over-expression of Ang2 induces the formation of tenuous and poorly differentiated vessels [35] and, in fact, our results, concerning tube formation, showed a significant decrease of new vessels, when SSC-ECs were cultured with HC-MSCs, as well as with SSC-MSCs. It may be suggested that this constitutive dysregulation of Ang2 in SSC-ECs, unbalancing the normal Ang1/Ang2 equilibrium, may contribute to the progressive vessels desertification, which is one of the main features of the disease.

Tie1 and Tie2 receptors are selectively expressed by ECs, although different cell types, including early hematopoietic cells and some monocytes subsets, may express Tie2. Despite their quite total structural homology, these 2 receptors play different roles in angiogenesis [36]. Functionally, Tie2 binds directly to angiopoietins and displays a strong kinase activity. In contrast, Tie1 does not bind to angiopoietins, showing a weaker kinase activity, and these data were confirmed in Tie1 knockdown model [37]. The lack of angiopoietins binding by Tie1, prompted us to focus our study on the possible role played by Tie2, in our experimental setting. Before co-cultural, SSC-ECs showed a significantly lower Tie2 expression when compared with HC-ECs. After co-cultural, the cognate interaction with MSCs, independent of the MSCs used, induced a significantly increase of Tie2 expression on ECs, although this level was always significantly lower when compared to the levels in HC-ECs, confirming the pathologic behavior of SSC-ECs in the modulation of angiogenesis, as showed by the failure of tube formation in our model.

During angiogenesis, ECs and perivascular-MSCs established a delicate balance, with exchange of many molecules. An altered expression of these molecules could be responsible of the lack of tubulogenesis. Our data showed that, in SSC cells, an altered expression of Ang1, Ang2 and their receptor may be linked to the failure of tube formation.

To confirm the mechanistic contribution of Ang1 during angiogenesis, we silenced the Ang1 expression by siRNA-Ang1 transfection. The silenced HC-MSCs, clearly lack the ability to support normal tube formation, which is considered an experimental surrogate of physiological angiogenesis, mirroring the functional impairment observed using SSC-MSCs, thus confirmed that the loss of Ang1 expression may be associated to the vessel destabilization and the pericytes/ECs detachment, observed in scleroderma patients.

Conclusion

We herein showed an impaired expression, in the cells obtained from SSc patients, of the Ang/Tie2 axis, during ECs-perivascular MSCs cross talk, and this result is strongly associated with the failure of tube formation in our experimental model. These results, in vitro, may explain what observed in vivo, where this alteration, interfering with the vessel stability may contribute to the failure of reparative angiogenesis during SSc. Further studies, targeting the affected molecules, involved in the ECs/perivascular MSCs cross talk during SSc, may open new future possibilities in the treatment of vascular complication of Scleroderma.

Competing Interests

The authors disclose no conflict of interest.

Authors’ Contributions

PDB study conception and design, data interpretation, literature search, figure creation, writing, paper revision and acceptance; VL data collection, literature search, paper revision and acceptance; FC data collection, data interpretation, literature search, paper revision and acceptance; PR data collection, data interpretation, literature search, paper revision and acceptance; OB data collection, literature search, paper revision and acceptance; IP data collection, literature search, paper revision and acceptance; AE data collection, literature search, paper revision and acceptance; IB data collection, literature search, paper revision and acceptance; RG: study conception and design, data interpretation, literature search, figure creation, writing, paper revision and acceptance; PC study conception and design, data interpretation, literature search, figure creation, writing, paper revision and acceptance; All authors gave final approval for submitting the manuscript for review and agree to be accountable for all aspects of the work.
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