The Endothelial-mesenchymal Transition in Systemic Sclerosis Is Induced by Endothelin-1 and Transforming Growth Factor-β and May Be Blocked by Macitentan, a Dual Endothelin-1 Receptor Antagonist

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ABSTRACT. ***Objective.*** High endothelin-1 (ET-1) and transforming growth factor-β (TGF-β) levels may induce in healthy endothelial cells (EC) an endothelial-to-mesenchymal transition (EndMT). The same cytokines are associated with fibrosis development in systemic sclerosis (SSc). Although EndMT has not been definitively shown in SSc, this process, potentially induced by a stimulatory loop involving these 2 cytokines, overexpressed in this disease might contribute to fibroblast accumulation in affected tissues. Macitentan (MAC), an ET-1 receptor antagonist interfering with this loop, might prevent EndMT and fibroblast accumulation.

Methods. EC, isolated from healthy controls (HC) and patients with SSc, were treated with ET-1 and TGF-β and successively analyzed for gene and protein expressions of endothelial and mesenchymal markers, and for Sma- and Mad-related (SMAD) phosphorylation. Further, in the supernatants, we evaluated ET-1 and TGF-β production by ELISA assay. In each assay we evaluated the ability of MAC to inhibit both the TGF-β and ET-1 effects.

Results. We showed that both TGF-β and ET-1 treatments induced an activation of the EndMT process in SSc-EC as reported in HC cells. The ELISA assays showed a mutual TGF-β and ET-1 induction in both SSc-EC and HC-EC. A statistically significant increase of SMAD phosphorylation after treatment was observed in SSc-EC. In each assay, MAC inhibited both TGF-β and ET-1 effects.

Conclusion. Our work is the first demonstration in literature that SSc-EC, under the synergistic effect of TGF-β and ET-1, may transdifferentiate toward myofibroblasts, thus contributing to fibroblast accumulation. MAC, interfering with this process *in vitro*, may offer a new potential therapeutic strategy against fibrosis. (First Release August 15 2015; J Rheumatol 2015;42:1808–16; doi:10.3899/jrheum.150088)

Key Indexing Terms:
SYSTEMIC SCLEROSIS ENDOTHELIAL CELLS MESENCHYMAL TRANSITION
FIBROSIS MACITENTAN

Systemic sclerosis (SSc) is a chronic disease characterized by the activation of the immune system, fibroproliferative vasculopathy, and tissue fibrosis[1]. Vascular abnormalities are involved in many organ dysfunctions, including lung, heart, and kidney[2], and probably drive the first symptom of the disease, the Raynaud phenomenon. Although different mediators have been identified as active players in the vascular remodeling in SSc, such as endothelin-1 (ET-1) and transforming growth factor-β

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(TGF-β)[1,2,3], the mechanisms underlying SSc vasculopathy and how this damage may lead to fibrosis remain poorly understood.

In this setting, myofibroblasts are considered the effector cell in the fibrotic process. The differentiation of quiescent fibroblasts toward cells displaying a proliferating, matrix-producing, contractile phenotype has been shown to be a basic step in this process[4]. Further, the use of *in vivo* animal models in which fibrillar collagen-producing cells have been tracked, helped us to understand that both pericytes and resident fibroblasts are a source of activated myofibroblasts as shown during the evolution of chronic kidney fibrotic diseases[5,6,7]. Several reports[8,9,10] showed that myo-fibroblasts may also originate through the mesenchymal transition of endothelial cells (EC), the so-called endothelial-to-mesenchymal transition (EndMT), first observed in studies on cardiac development[11], and to date, emerging as a possible pathogenetic mechanism in different diseases, including diabetic nephropathy, cardiac fibrosis, intestinal fibrosis, pulmonary hypertension, and SSc[12,13,14,15,16].

During EndMT, resident EC delaminate from the polarized cell layer and invade the underlying tissue. This EC "mesenchymal" phenotype is characterized by the loss of cell-cell junctions and EC markers, such as Von Willebrand factor (vWF), CD31, and vascular endothelial-cadherin (VE-cadherin), as well as the acquisition of invasive properties associated with the gain of mesenchymal markers, such as α-smooth muscle actin (α-SMA), smooth muscle 22 (Sm22), and collagen1 (Col1A1)[11,17,18,19]. Despite evidence suggesting that EndMT is involved not only in pathological[12,13,14,15,16,17,18,19] but also in physiological conditions[20], the underlying molecular mechanisms involved in this process are largely unknown. Evidence has shown the crucial role of TGF-β signaling in the initiation of EndMT[17]. Of note, the same molecule is also considered a pivotal player in many fibrotic diseases, including SSc[21,22,23]. TGF-β exerts its profibrotic role by binding with specific receptors, and the signal is transduced to the nucleus by members of the Sma- and Mad-related (SMAD) family. It has been shown that TGF-β may induce phosphorylation of both SMAD1 and SMAD5, together with phosphorylation of SMAD2/3, in different cell lines such as EC, epithelial cells, fibroblasts, and cancer-derived cell lines[24,25,26]. The activation of intracellular transcription factors supported the production of other fibrotic molecules, such as ET-1[27]. Further, TGF-β–mediated ET-1 release has been associated to the fibrotic response observed in scleroderma fibroblasts in the context of skin and lung fibrosis[28].

ET-1, a 21–amino acid peptide, is known to be one of the most potent vasoconstrictors. In addition to its role in regulating vascular tone through its interaction with 2 specific receptors, endothelin receptor A (ETRA) and B (ETRB), this peptide further displays some fibrotic activity. *In vitro* studies showed that ET-1 promotes myofibroblast switch in fibroblasts, including the SSc fibroblasts[19].

A better understanding of the mechanisms responsible for EndMT may be of primary importance in recognizing clinically useful biomarkers, predicting fibrotic remodeling, and/or developing effective antifibrotic therapies in different fibrotic conditions. Considering that EndMT may induce a profibrotic phenotype and contribute significantly to the vessel's instability and capillary rarefaction during SSc, we investigated the ability of ET-1 in inducing EndMT in SSc-EC and the possible role of macitentan (MAC) in blocking this process. MAC is a novel ETRA/ETRB antagonist, showing in a longterm event-driven Phase III trial (SERAPHIN) the ability to reduce the risk of morbidity and mortality in patients with pulmonary arterial hypertension[29].

To our knowledge, ours is the first report showing that SSc-EC may undergo EndMT and that ET-1 strongly modulates this process. Further, EndMT may be partially blocked by MAC. Our data suggest that this new ET-1 antagonist, interfering *in vivo* with this process, might offer a new therapeutic opportunity for fibrosis in SSc, a condition still lacking an appropriate therapy.

MATERIALS AND METHODS

EC isolation and culture. After obtaining approval from the San Salvatore University Hospital ethics committee and written informed consent from patients, microvascular EC were acquired from 10 patients with SSc with the diffuse cutaneous form of recent onset (disease duration < 3 yrs calculated since the first non-Raynaud symptom of SSc)[30,31] by skin biopsies. Demographic and clinical characteristics of the patients are shown in Table 1.

Patients discontinued corticosteroids, oral vasodilators, intravenous prostanoids, or other potentially disease-modifying drugs at least 1 month before biopsies. None took immunosuppressants.

Ten frozen healthy control (HC) EC samples obtained from age-matched women donors (skin samples for research purposes) were used as controls.

Biopsy samples (1 × 0.5 cm) of the involved forearm skin (skin score 1/2 at the biopsy site) were washed with phosphate buffered saline (PBS; Life Technologies), and 4 explants were placed into a 50-ml tube containing 15 ml of trypsin (Sigma-Aldrich) and then to digest for 45 min at 37°C. Cells were cultured in EGM2-MV (Lonza) at 37°C in a humidified atmosphere of 5% CO_2.

Before the cells reached confluence, after about 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). The beads rapidly targeted and partially coated the EC expressing the CD31 receptor.

After incubation, the cells were placed in a magnet (Dyna MPC-S; Invitrogen, Life Technologies) for 2 min, following the manufacturer's recommended protocol for washings and final extraction. The CD31-negative cells were removed during the successive washings. The positive-selected cells were 99% EC with specific phenotype (CD-31, CD-34, CD-144; Figure 1A). The cells were used at third passages (P3).

EC treatment with TGF-β, ET-1, and MAC. To establish the optimal concentrations of TGF-β (R&D), ET-1 (Sigma-Aldrich), and MAC in our system, a dose/response curve was performed on α-SMA expression (data not shown) using P3 EC obtained from both 1 control and 1 patient.

Each experiment was performed in triplicate and the optimal stimulation dose for TGF-β was assessed to be 10 ng/ml, for ET-1, it was assessed to be 200 nM, and for MAC, it was 1 μm.

For EndMT gene and protein expressions, EC were treated in the following conditions: (1) untreated (UT) EC, (2) EC + TGF-β (10 ng/ml), (3) EC pretreated (1 h) with MAC (1 μm) before being treated with TGF-β, (4) EC + ET-1 (200 nM), and (5) EC pretreated (1 h) with MAC (1 μm)

Table 1. Clinical and demographic features of the 10 patients with diffuse SSc.

Sex/Age, Yrs	Yr of SSc Onset/Disease Duration at Skin Biopsy, Yrs	mRSS/Score at Skin Biopsy	Autoantibodies	Lung Involvement from HRCT/PFT	Heart Involvement/ Scleroderma Renal Crisis	Raynaud Phenomenon/ Digital Ulcers
F/46	2010/2	12/2	ANA/Scl-70	Normal/Normal	Normal/No	Yes/No
F/21	2009/3	13/1	ANA/Scl-70	Normal/Normal	Normal/No	Yes/Yes
F/31	2011/1	13/2	ANA/Scl-70	Normal/Normal	Normal/No	Yes/Yes
F/36	2010/2	11/2	ANA/Scl-70	Normal/Normal	PAH/No	Yes/Yes
M/20	2010/2	11/1	ANA/Scl-70	Normal/Normal	Normal/No	Yes/No
F/41	2010/2	15/2	ANA/Scl-70	Normal/Normal	Normal/No	No/No
F/30	2010/2	10/1	ANA/Scl-70	Normal/Normal	Normal/No	Yes/No
F/21	2010/2	09/1	ANA/Scl-70	Normal/Normal	Normal/No	Yes/No
F/31	2009/3	14/1	ANA/Scl-70	Normal/Normal	Normal/No	Yes/No
F/42	2009/3	16/2	ANA/Scl-70	Fibrosis/Normal	Normal/No	Yes/No

SSc: systemic sclerosis; mRSS: modified Rodnan skin score, maximum possible score 51; HRCT: high-resolution computed tomography; PFT: pulmonary function test; ANA: antinuclear antibodies; Scl-70: antitopoisomerase; PAH: pulmonary arterial hypertension.

before being treated with ET-1. EC were cultured for 6 days in accordance with published studies[19,32].

To assess SMAD 1/5 and SMAD 2/3 phosphorylation, EC were treated in the following conditions: (1) UT EC, (2) EC + TGF-β (10 ng/ml), (3) EC pretreated (1 h) with MAC (1 μm) before being treated with TGF-β, (4) EC + ET-1 (200 nM), and (5) EC pretreated (1 h) with MAC (1 μm) before being treated with ET-1. The experimental conditions were applied for 24 h.

Quantitative real-time (qRT)-PCR analysis. Total RNA was extracted from EC using NucleoSpin RNAXS (Macherey Nagel) according to manufacturer's instructions and reverse transcribed into cDNA with the ThermoScript reverse transcription–PCR system (Invitrogen, Life Technologies).

The qRT-PCR was performed using SYBR green kits (Applied Biosystems). 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 National Center for Biotechnology Information: β-actin: 5′-CCT GGC ACC CAG CAC AAT-3′ (forward) and 5′-AGT ACT CCG TGT GGA TCG GC-3′ (reverse); vWF: 5′-AGC CTT GTG AAA CTG AAG CAT-3′ (forward) and 5′-GGC CAT CCC AGT CCA TCT G-3′ (reverse); CD31: 5′-CCA AGG TGG GAT CGT GAG G-3′ (forward) and 5′-TCG GAA GGA TAA AAC GCG GTC-3′ (reverse); VE-cadherin: 5′-GTT CAC GCA TCG GTT GTT CAA-3′ (forward) and 5′-CGC TTC CAC CAC GAT CTC ATA-3′ (reverse); Sm22: 5′-CCG GTT AGG CCA AGG CTC-3′ (forward) and 5′-GCG GCT CAT GCC ATA GGA-3′ (reverse); α-SMA: 5′-CGG TGC TGT CTC TCT ATG CC-3′ (forward) and 5′-CGC TCA GTC AGG ATC TTCA-3′ (reverse); Col1A1: 5′-AGG GCC AAG ACG AAG ACA GT-3′ (forward) and 5′-AGA TCA CGT CAT CGC ACA ACA-3′ (reverse)]. Each gene was normalized to those for β-actin.

Western blot. To perform Western blot assays, EC cells were pelleted, washed twice with PBS, and lysed in sodium dodecyl sulfate (SDS) sample buffer. The protein concentration was calculated by Bradford protein assay reagent (Bio-Rad). There were 50 µg of proteins separated by SDS-polyacrylamide gel and transferred to nitrocellulose membranes. After 1 h at room temperature in blocking buffer [5% nonfat milk in Tris-buffered saline/1% tween 20 (TBS/T)], the membranes were washed 3 times for 5 min each in TBS/T and incubated overnight at 4°C with the primary antibodies: CD31, VE-cadherin (R&D Systems), Sm22 (Abcam), vWF, α-SMA, Col1A1 (Santa Cruz), Phospho-SMAD1/5, and Phospho-SMAD2/3 (Cell Signaling), diluted in 5% bovine serum albumin in TBS/T. Following 3 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 3 times with TBS/T. The detection was done with enhanced chemiluminescence detection electrochemiluminescent reaction (Amersham Pharmacia Biotechnology). All the results were normalized to the levels of proteins of UT HC-EC and normalized to the actin signal (Sigma-Aldrich). Immunoreactive bands were quantified with densitometry using ImageJ software (National Institutes of Health).

ELISA. The concentrations of TGF-β and ET-1 released in EC supernatants were determined by ELISA using Quantikine Human Immunoassay kits (all by R&D Systems) according to the manufacturer's protocol.

Statistical analysis. GraphPad Prism 5.0 software was used for statistical analyses. Results are expressed as median (range). Because of the nonparametric 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

Effect of MAC on endothelial marker expression in SSc-EC. As shown in Figure 1 (B, C, D), after TGF-β or ET-1 treatment, the mRNA expression of endothelial markers markedly decreased in both HC-EC and SSc-EC when compared with UT cells. Of note, MAC significantly reverted both the TGF-β and the ET-1 effects on HC-EC and SSc-EC. As shown in Figure 1E, these results were confirmed at the protein level by Western blotting analyses.

Effect of MAC on mesenchymal markers expression in SSc-EC. In both HC-EC and SSc-EC, TGF-β or ET-1 treatment induced a significant increase of mRNA expression of the myofibroblast markers when compared with UT cells (Figure 2A, B, C). MAC reverted both the TGF-β and the ET-1 effects on HC-EC and SSc-EC, mirroring the results obtained in the endothelial markers analyses. The Western blot in Figure 2D showed that at protein levels, the results mirror the changes observed in gene expression levels.

The synergic ET-1/TGF-β production in SSc-EC. ET-1 production was assessed using a specific ELISA assay before and after cells treatment with TGF-β for 24 h. The results showed that ET-1 was significantly increased in UT SSc-EC when compared with UT HC-EC [UT HC-EC: 5.35 pg/ml (4.00–6.40) vs UT SSc-EC: 11.20 pg/ml (9.60–12.70), p = 0.0002]. Further, TGF-β significantly induced ET-1

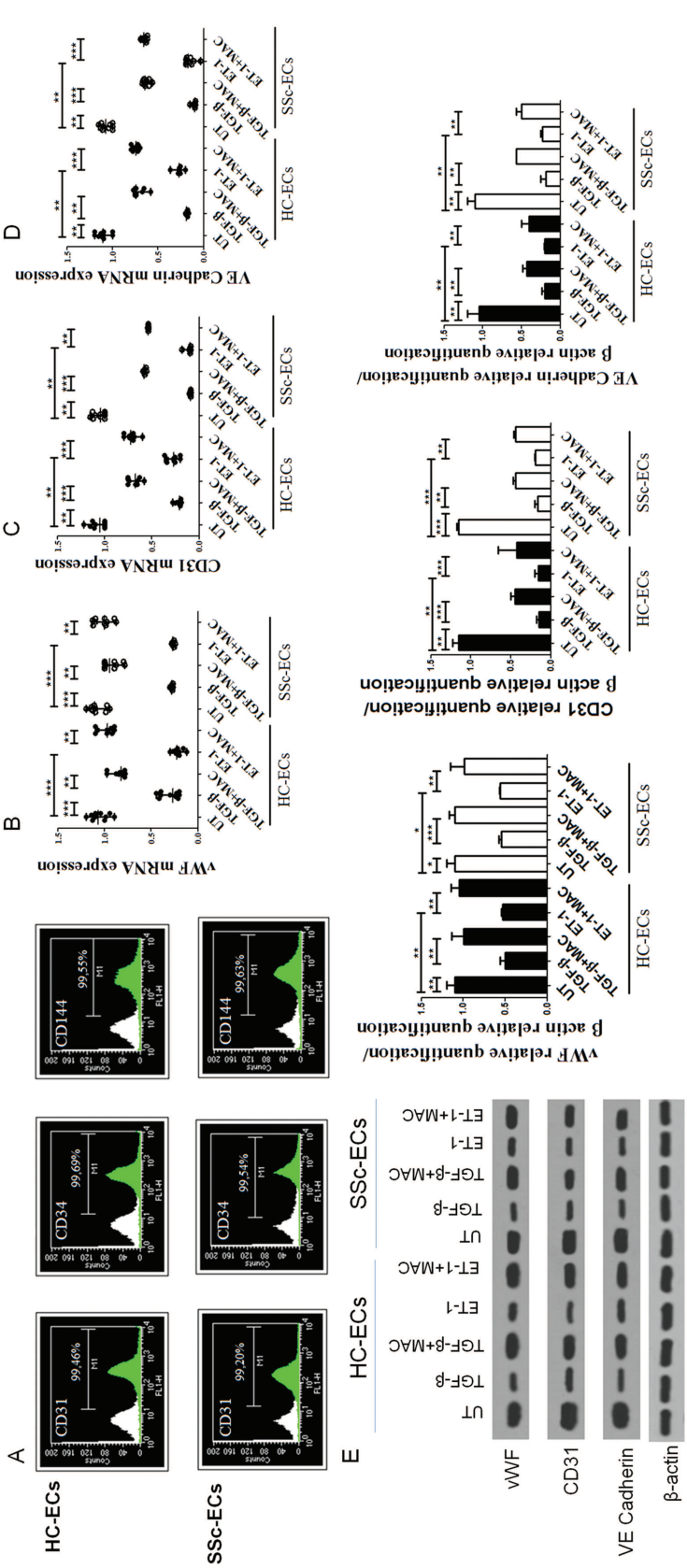

Figure 1. Decrease of endothelial marker in SSc-EC. (A) Expression of EC markers CD31, CD34, and CD144 in both HC-EC and SSc-EC by flow cytometric analysis. The histograms in each graph show the increase in fluorescence for each EC marker (green line) compared with the expression of an isotype-matched irrelevant mAb used as control (white line). (B–D) Quantification by qRT-PCR of vWF (B), CD31 (C), and VE-cadherin (D) mRNA levels in HC-EC and SSc-EC. In both HC-EC and SSc-EC, the treatment with TGF-β and ET-1 induced a significant reduction of endothelial markers when compared with UT cells. MAC administration inhibited the TGF-β and ET-1 effects on endothelial markers expression in both HC-EC and SSc-EC. Any single dot in the figures represents the median of triplicate experiments for each patient. (E) Western blot analyses confirmed the results observed by qRT-PCR analyses. Pictures are representative of all experiments. Protein bands were quantified by densitometry and the values were expressed as protein relative quantification/β actin relative quantification. * p = 0.02. ** p = 0.0002. *** p = 0.0001. SSc: systemic sclerosis; EC: endothelial cells; HC: healthy controls; qRT-PCR: quantitative real-time PCR; vWF: Von Willebrand factor; VE-cadherin: vascular endothelial-cadherin; TGF-β: transforming growth factor-β; ET-1: endothelin-1; UT: untreated; MAC: macitentan; mAb: monoclonal antibody.

Figure 2. Increase of mesenchymal markers in SSc-EC. (A–C) Quantification by qRT-PCR of Sm22 (A), α-SMA (B), and Col1A1 (C) mRNA levels in HC-EC and SSc-EC. In both HC-EC and SSc-EC, the treatment with TGF-β and ET-1 induced a significant increase in all the mesenchymal markers when compared with UT cells. MAC administration inhibited the TGF-β and ET-1 effects on mesenchymal markers expression in both HC-EC and SSc-EC. Any single dot in the figures represents the median of triplicate experiments for each patient. (D) Western blot analyses confirmed the results observed by qRT-PCR analyses. Pictures are representative of all experiments. Protein bands were quantified by densitometry and the values were expressed as protein relative quantification/β actin relative quantification. ** p = 0.0002. *** p = 0.0001. SSc: systemic sclerosis; EC: endothelial cells; qRT-PCR: quantitative real-time PCR; Sm22: smooth muscle 22; α-SMA: α-smooth muscle actin; Col1A1: collagen1; HC: healthy controls; TGF-β: transforming growth factor-β; ET-1: endothelin-1; UT: untreated; MAC: macitentan.

production with significantly higher levels in SSc-EC when compared with HC-EC [HC-EC + TGF-β: 10.90 pg/ml (9.50–12.40) vs SSc-EC + TGF-β: 21.65 pg/ml (19.70–23.50), p = 0.0002; Figure 3A].

We further evaluated the TGF-β production after ET-1 stimulation. Already in UT condition, SSc-EC produced significantly higher levels of TGF-β when compared with HC cells [UT HC-EC: 30.90 pg/ml (28.20–35.20) vs UT SSc-EC: 62.85 pg/ml (60.90–66.10), p = 0.0002]. ET-1 significantly induced TGF-β production, and this increase was significantly higher in SSc-EC when compared with HC-EC [HC-EC + ET-1: 60.10 pg/ml (57.90–63.90) vs SSc-EC + ET-1: 92.75 pg/ml (88.90–95.70), p = 0.0002; Figure 3B].

SMAD phosphorylation is inhibited by MAC treatment in SSc-EC. The Western blot in Figure 3C shows that in UT SSc-EC, the levels of SMAD phosphorylation were higher than in HC-EC. After TGF-β treatment, we observed a significant increase of SMAD phosphorylation in both HC-EC and SSc-EC, the latter significantly higher than HC-EC. After ET-1 treatment, in both HC-EC and SSc-EC, the SMAD phosphorylation was significantly increased when compared with UT EC. Further, after ET-1 treatment, the levels of SMAD phosphorylation in SSc-EC were higher than in HC-EC. MAC significantly blocked the ET-1 effect on SMAD activation in both HC-EC and SSc-EC. Further, we observed that MAC significantly inhibited the SMAD phosphorylation induced by TGF-β.

DISCUSSION

To our knowledge, our work is the first to demonstrate that ET-1 induces EndMT in SSc-EC and that this pathologic process may be inhibited by MAC, a new ET-1 dual receptors blocker. It is well known that ET-1 is strongly upregulated in patients with SSc and an endothelial dysfunction is involved in SSc pathogenesis. In addition to its well-known vasoconstrictive action, ET-1 displays a wide range of biological effects such as proliferation, fibrosis, and inflammation, and involving different cell types. ET-1 induces a profibrotic phenotype in fibroblasts through the increased expression of extracellular matrix proteins, such as Type I and Type III collagen and fibronectin, as well as decreasing the expression of matrix metalloproteinase 1[33,34]. On the other hand, much evidence supports the hypothesis that ET-1 may induce the α-SMA expression in human EC, as well as an increased collagen production, thus modulating their transdifferentiation toward myofibroblasts[8,9,10]. The recruitment of myofibroblasts in affected tissues, associated with the persistence of their elevated biosynthetic functions, may be considered as pivotal determinants for the extent and the progression rate of the fibrosis in SSc[10].

Available literature suggests that myofibroblasts may derive from several sources, including the expansion of tissue resident fibroblasts, the migration of bone marrow-derived circulating fibrocytes, pericytes, and the epithelial cells that underwent epithelial-mesenchymal transition[5,7]. A more recent study showed that EC, under specific stimuli, can also be considered a source of myofibroblasts undergoing EndMT, and this phenotypic transition could mainly be of interest in SSc pathogenesis by providing a cellular link between vasculopathy and fibrosis[10].

We showed that SSc-EC acquired a myofibroblast-like phenotype after treatment with ET-1, as previously observed for normal EC after treatment with ET-1 and TGF-β[9,27]. Further, to assess the ability of SSc-EC to mirror the behavior of normal EC, we stimulated these cells with TGF-β, also showing that this cytokine may modulate EndMT in SSc-EC. In fact, SSc-EC displayed a reduction of endothelial markers (CD31, vWF), as well as an increase of profibrotic markers, such as stress fibers (Sm22, α-SMA) and collagen, after ET-1 and TGF-β stimulation. It has been shown that the endothelial damage in SSc skin is characterized by a progressive loss of the endothelial-specific marker VE-cadherin[35], a strictly endothelial-specific adhesion molecule located at junctions between EC[36]. It has been recently shown that during EndMT, VE-cadherin expression is reduced in EC undergoing transdifferentiation[37]. Of note, in our experiments, we showed that after ET-1 and TGF-β stimulation, SSc-EC displayed a significant decrease of VE-cadherin expression, suggesting the induction of the EndMT program in our cells. This evidence allows us to speculate that this profibrotic switch may be considered a normal response of EC in any condition characterized by an overexpression of TGF-β and ET-1, such as the physiologic wound healing or the pathologic response to an unknown trigger, as occurs during SSc.

We recently reported the pathologic role that perivascular mesenchymal cells may play in the fibrotic evolution during SSc[38,39,40] and the results of our present work suggest that in the process leading to SSc fibrosis, not only perivascular mesenchymal cells but also EC may contribute to myofibroblast generation through the EndMT program.

In this setting, considering the role of ET-1 in triggering EndMT[41], ET-1 blockade may represent an important and still unexplored target to preserve EC integrity, thus decreasing the fibroblast accumulation in SSc.

In our experimental model, MAC treatment significantly reverted the ET-1 effect, as expected, but interestingly MAC was also able to inhibit the TGF-β–mediated EndMT on both HC-EC and SSc-EC. The inhibitory effect of MAC on the EndMT induced by TGF-β suggests that ET-1 may represent the ultimate mediator of the TGF-β actions. In line with this evidence, it has been reported[42,43] that in both EC and fibroblast, TGF-β induces the ET-1 gene promoter activity by SMAD activation. To clarify this aspect, we further assessed the ET-1 production after TGF-β stimulation by ELISA assays. Our results confirmed that TGF-β stimulation increased the ET-1 production by EC. Of note, SSc-EC displayed higher ET-1 production, already at basal levels, when compared with HC-EC. Further, we observed that ET-1

Figure 3. ET-1 and TGF-β production and pSMAD expression in SSc-EC. (A) ET-1 and (B) TGF-β ELISA assays. ET-1 and TGF-β were quantified in the supernatants of both HC-EC and SSc-EC cultures. (A) The release of ET-1 in UT cells was significantly increased in SSc-EC. The production of TGF-β after ET-1 treatment was significantly higher in SSc-EC when compared with HC-EC. (B) Spontaneous TGF-β production after ET-1 stimulus was significantly increased in SSc-EC. Any single dot in the figures represents the median of triplicate experiments for each patient. (C) Western blot showed that pSMAD 1/5 and pSMAD 2/3 were significantly increased in the UT SSc-EC when compared with HC-EC. TGF-β and ET-1 induced a significant increase of both SMAD 1/5 and SMAD 2/3 phosphorylation in both HC-EC and SSc-EC, but the levels of phosphorylated proteins were always significantly increased in SSc-EC when compared with HC-EC. MAC inhibited the TGF-β and ET-1 effects. Pictures are representative of all experiments. Protein bands were quantified by densitometry and the values were expressed as protein relative quantification/β actin relative quantification. * p = 0.02. ** p = 0.0002. *** p = 0.0001. ET-1: endothelin-1; TGF-β: transforming growth factor-β; pSMAD: phospho Sma and Mad; SSc: systemic sclerosis; EC: endothelial cells; HC: healthy controls; UT: untreated; SMAD: Sma- and Mad-related; MAC: macitentan.

stimulation was able to increase the TGF-β production in both HC-EC and SSc-EC, the latter showing significantly higher values of TGF-β before treatment. Under our experimental conditions, UT HC-EC and UT SSc-EC did not show any change in their phenotypic features, although SSc-EC constitutively produced higher levels of ET-1 and TGF-β than HC-EC. We showed that HC-EC and SSc-EC *in vitro* begin their morphological changes after 6 days of strong stimulation by ET-1 and TGF-β, a setting mirroring the cytokines milieu in which these cells may be exposed *in vivo*, considering the number of these molecules produced by activated fibroblasts and inflammatory-recruited cells.

An alternative hypothesis to explain the inhibitory effect of MAC on TGF-β–induced EndMT could be the cooperation between TGF-β receptors and ETR in inducing this program. It has been shown that G-protein–coupled receptors such as ET-1 receptors may cross over and interact with the pathways of serine/threonine kinase receptors, such as the TGF-β Type I receptor (TβRI). Therefore, agonists of ETR may also activate TβRI signaling and their downstream products, such as phospho Sma and Mad (pSMAD)[44,45]. In this setting, the effects of ET-1 may be due to crosstalk between these 2 receptors induced by the hijack of TβRI signaling machinery by ETR[46]. Our results showed that, similarly to TGF-β, ET-1 induced the phosphorylation of SMAD in HC-EC and SSc-EC, confirming the hypothesis of a possible TGF-β/ET-1 receptor cooperation. Of note, in UT SSc-EC, the levels of pSMAD were significantly increased when compared with UT HC-EC. We may speculate that in our experiments at basal condition, the TGF-β signaling pathway was already activated as a result of the overexpression of TGF-β and ET-1 during SSc, thus conditioning the higher levels of pSMAD observed in SSc-EC. In this scenario, MAC, interacting with the ETR, might also inhibit the TβRI transactivation and consequent SMAD phosphorylation induced after both TGF-β and ET-1 stimulation[46]. A further speculative and unexplored aspect may be the block of TβRI. However, to date, unlike ET-1, we still do not have any licensed drug able to interfere with TβRI[47], and from a clinical point of view, ET-1 receptor antagonist now represents the only possibility we have to modulate this functional complex.

We showed for the first time, to our knowledge, the finding that ET-1 induces a phenotypic switch toward mesenchymal cells in SSc-EC, characterized by a downregulation of endothelial markers and an increased expression of profibrotic genes and proteins. Taken together, our results point out the possible contribution of damaged endothelia in the generation of profibrotic cells, and support the hypothesis of a pathologic link between vascular and fibrotic alterations in the pathogenesis of SSc.

Focusing on the involvement of TGF-β and ET-1 as early mediators of both vascular and fibrotic components of SSc, we suggest that these molecules may be considered key therapeutic targets in the early phases of the disease. MAC, the new dual ETR antagonist (as shown in our *in vitro* study), in inhibiting the EndMT, may offer new potential therapeutic strategies that target the TGF-β/ET-1 loop, and may prevent the early pathways leading to fibrosis in SSc.

Further studies are needed to translate these preclinical findings into the clinical setting. In fact, although many *in vitro* studies suggested the antifibrotic effect of another ET-1 receptor antagonist, bosentan[42,48], no clinical benefits were observed when this drug was used in patients with SSc with lung fibrosis[49]. It must be taken into account that the failure of the clinical trials evaluating its efficacy on interstitial lung disease may be biased by the choice of a non-sensitive primary endpoint, as well as the heterogeneity of the enrolled patients and the lack of histological classifications. On this basis, any further study of the ET-1 receptor antagonist in patients with fibrosis might need more stringent inclusion criteria and perhaps primary and secondary endpoints that might be more responsive to change[50].

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REFERENCES

1. Trojanowska M. Cellular and molecular aspects of vascular dysfunction in systemic sclerosis. Nat Rev Rheumatol 2010; 6:453–60.
2. Pattanaik D, Brown M, Postlethwaite AE. Vascular involvement in systemic sclerosis (scleroderma). J Inflamm Res 2011;4:105–25.
3. Piera-Velazquez S, Li Z, Jimenez SA. Role of endothelial-mesenchymal transition (EndoMT) in the pathogenesis of fibrotic disorders. Am J Pathol 2011;179:1074–80.
4. Wynn TA. Cellular and molecular mechanisms of fibrosis. J Pathol 2008;214:199-210.
5. Kida Y, Duffield JS. Pivotal role of pericytes in kidney fibrosis. Clin Exp Pharmacol Physiol 2011;38:467-73.
6. Humphreys BD, Lin SL, Kobayashi A, Hudson TE, Nowlin BT, Bonventre JV, et al. Fate tracing reveals the pericyte and not epithelial origin of myofibroblasts in kidney fibrosis. Am J Pathol 2010;176:85-97.
7. Duffield JS. The elusive source of myofibroblasts: problem solved? Nat Med 2012;18:1178-80.
8. Frid MG, Kale VA, Stenmark KR. Mature vascular endothelium can give rise to smooth muscle cells via endothelial-mesenchymal transdifferentiation: in vitro analysis. Circ Res 2002;90:1189–96.
9. Arciniegas E, Sutton AB, Allen TD, Schor AM. Transforming growth factor beta 1 promotes the differentiation of endothelial cells into smooth muscle-like cells in vitro. J Cell Sci 1992;103:521–9.
10. Jimenez SA. Role of endothelial to mesenchymal transition in the pathogenesis of the vascular alterations in systemic sclerosis. ISRN Rheumatol 2013;23:835948.
11. Zeisberg EM, Tarnavski O, Zeisberg M, Dorfman AL, McMullen JR, Gustafsson E, et al. Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. Nat Med 2007;13:952–61.
12. Arciniegas E, Frid MG, Douglas IS, Stenmark KR. Perspectives on endothelial-to-mesenchymal transition: potential contribution to vascular remodeling in chronic pulmonary hypertension. Am J Physiol Lung Cell Mol Physiol 2007;293:L1-8.
13. Li J, Bertram JF. Review: Endothelial-myofibroblast transition, a new player in diabetic renal fibrosis. Nephrology 2010;15:507-12.
14. Xu H, Zaidi M, Struve J, Jones DW, Krolikowski JG, Nandedkar S, et al. Abnormal fibrillin-1 expression and chronic oxidative stress

mediate endothelial mesenchymal transition in a murine model of systemic sclerosis. Am J Physiol Cell Physiol 2011;300:C550-6.
15. Kizu A, Medici D, Kalluri R. Endothelial-mesenchymal transition as a novel mechanism for generating myofibroblasts during diabetic nephropathy. Am J Pathol 2009;175:1371-3.
16. Potts JD, Runyan RB. Epithelial-mesenchymal cell transformation in the embryonic heart can be mediated, in part, by transforming growth factor beta. Dev Biol 1989;134:392–401.
17. Zeisberg EM, Potenta S, Xie L, Zeisberg M, Kalluri R. Discovery of endothelial to mesenchymal transition as a source for carcinoma-associated fibroblasts. Cancer Res 2007;67:10123–8.
18. Lin F, Wang N, Zhang TC. The role of endothelial-mesenchymal transition in development and pathological process. IUBMB Life 2012;64:717-23.
19. Widyantoro B, Emoto N, Nakayama K, Anggrahini DW, Adiarto S, Iwasa N, et al. Endothelial cell-derived endothelin-1 promotes cardiac fibrosis in diabetic hearts through stimulation of endothelial-to-mesenchymal transition. Circulation 2010; 121:2407–18.
20. Fu Y, Chang A, Chang L, Niessen K, Eapen S, Setiadi A, et al. Differential regulation of transforming growth factor beta signaling pathways by Notch in human endothelial cells. J Biol Chem 2009;284:19452-62.
21. Lafyatis R. Transforming growth factor β—at the centre of systemic sclerosis. Nat Rev Rheumatol 2014;10:706-19.
22. Yan Z, Kui Z, Ping Z. Reviews and prospectives of signaling pathway analysis in idiopathic pulmonary fibrosis. Autoimmun Rev 2014;13:1020-5.
23. Lee SY, Kim SI, Choi ME. Therapeutic targets for treating fibrotic kidney diseases. Transl Res 2015;165:512-30.
24. Goumans MJ, Valdimarsdottir G, Itoh S, Lebrin F, Larsson J, Mummery C, et al. Activin receptor-like kinase (ALK)1 is an antagonistic mediator of lateral TGFbeta/ALK5 signaling. Mol Cell 2003;12:817–28.
25. Goumans MJ, Valdimarsdottir G, Itoh S, Rosendahl A, Sideras P, ten Dijke P. Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. EMBO J 2002;21:1743–53.
26. Daly AC, Randall RA, Hill CS. Transforming growth factor beta-induced Smad1/5 phosphorylation in epithelial cells is mediated by novel receptor complexes and is essential for anchorage-independent growth. Mol Cell Biol 2008;28:6889-902.
27. Rodríguez-Pascual F, Busnadiego O, González-Santamaría J. The profibrotic role of endothelin-1: is the door still open for the treatment of fibrotic diseases? Life Sci 2013;18:156-64.
28. Ahmedat AS, Warnken M, Seemann WK, Mohr K, Kostenis E, Juergens UR, et al. Pro-fibrotic processes in human lung fibroblasts are driven by an autocrine/paracrine endothelinergic system. Br J Pharmacol 2013;168:471-87.
29. Pulido T, Adzerikho I, Channick RN, Delcroix M, Galiè N, Ghofrani HA, et al; SERAPHIN Investigators. Macitentan and morbidity and mortality in pulmonary arterial hypertension. N Engl J Med 2013;369:809–18.
30. van den Hoogen F, Khanna D, Fransen J, Johnson SR, Baron M, Tyndall A, et al. 2013 classification criteria for systemic sclerosis: an American College of Rheumatology/European League against Rheumatism collaborative initiative. Arthritis Rheum 2013; 65:2737-47.
31. van den Hoogen F, Khanna D, Fransen J, Johnson SR, Baron M, Tyndall A, et al. 2013 classification criteria for systemic sclerosis: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Ann Rheum Dis 2013; 72:1747-55.
32. Kitao A, Sato Y, Sawada-Kitamura S, Harada K, Sasaki M, Morikawa H, et al. Endothelial to mesenchymal transition via transforming growth factor-beta1/Smad activation is associated with portal venous stenosis in idiopathic portal hypertension. Am J Pathol 2009;175:616-26.
33. Shi-Wen X, Denton CP, Dashwood MR, Holmes AM, Bou-Gharios G, Pearson JD, et al. Fibroblast matrix gene expression and connective tissue remodeling: role of endothelin-1. J Invest Dermatol 2001;116:417–25.
34. Soldano S, Montagna P, Villaggio B, Parodi A, Gianotti G, Sulli A, et al. Endothelin and sex hormones modulate the fibronectin synthesis by cultured human skin scleroderma fibroblasts. Ann Rheum Dis 2009;68:599–602.
35. Fleming JN, Nash RA, McLeod DO, Fiorentino DF, Shulman HM, Connolly MK, et al. Capillary regeneration in scleroderma: stem cell therapy reverses phenotype? PLoS One 2008;16:e1452.
36. Vestweber D. VE-cadherin: the major endothelial adhesion molecule controlling cellular junctions and blood vessel formation. Arterioscler Thromb Vasc Biol 2008;28:223-32.
37. Liu J, Dong F, Jeong J, Masuda T, Lobe CG. Constitutively active Notch1 signaling promotes endothelial mesenchymal transition in a conditional transgenic mouse model. Int J Mol Med 2014;34:669-76.
38. Cipriani P, Di Benedetto P, Ruscitti P, Campese AF, Liakouli V, Carubbi F, et al. Impaired endothelium-mesenchymal stem cells cross-talk in systemic sclerosis: a link between vascular and fibrotic features. Arthritis Res Ther 2014;16:442.
39. Cipriani P, Di Benedetto P, Capece D, Zazzeroni F, Liakouli V, Ruscitti P, et al. Impaired Cav-1 expression in SSc mesenchymal cells upregulates VEGF signaling: a link between vascular involvement and fibrosis. Fibrogenesis Tissue Repair 2014;7:13.
40. Cipriani P, Marrelli A, Benedetto PD, Liakouli V, Carubbi F, Ruscitti P, et al. Scleroderma mesenchymal stem cells display a different phenotype from healthy controls; implications for regenerative medicine. Angiogenesis 2013;16:595-607.
41. Horstmeyer A, Licht C, Scherr G, Eckes B, Krieg T. Signalling and regulation of collagen I synthesis by ET-1 and TGF-beta1. FEBS J 2005;272:6297–309.
42. Shi-Wen X, Rodríguez-Pascual F, Lamas S, Holmes A, Howat S, Pearson JD, et al. Constitutive ALK5-independent c-Jun N-terminal kinase activation contributes to endothelin-1 overexpression in pulmonary fibrosis: evidence of an autocrine endothelin loop operating through the endothelin A and B receptors. Mol Cell Biol 2006;26:5518-27.
43. Rodríguez-Pascual F, Redondo-Horcajo M, Lamas S. Functional cooperation between Smad proteins and activator protein-1 regulates transforming growth factor-beta-mediated induction of endothelin-1 expression. Circ Res 2003;92:1288–95.
44. Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature 2003;425:577–84.
45. Massagué J, Seoane J, Wotton D. Smad transcription factors. Genes Dev 2005;19:2783–810.
46. Kamato D, Burch ML, Osman N, Zheng W, Little PJ. Therapeutic implications of endothelin and thrombin G-protein-coupled receptor transactivation of tyrosine and serine/threonine kinase cell surface receptors. J Pharm Pharmacol 2013;65:465-73.
47. Badea I, Taylor M, Rosenberg A, Foldvari M. Pathogenesis and therapeutic approaches for improved topical treatment in localized scleroderma and systemic sclerosis. Rheumatology 2009;48:213-21.
48. Akamata K, Asano Y, Aozasa N, Noda S, Taniguchi T, Takahashi T, et al. Bosentan reverses the pro-fibrotic phenotype of systemic sclerosis dermal fibroblasts via increasing DNA binding ability of transcription factor Fli1. Arthritis Res Ther 2014;16:R86.
49. King TE Jr, Brown KK, Raghu G, du Bois RM, Lynch DA, Martinez F, et al. BUILD-3: a randomized, controlled trial of bosentan in idiopathic pulmonary fibrosis. Am J Respir Crit Care Med 2011;184:92–9.
50. Silver RM. Endothelin and scleroderma lung disease. Rheumatology 2008;47 Suppl 5:v25-6.