Hypoxia induced interstitial transformation of microvascular endothelial cells by mediating HIF-1 α /VEGF signaling in systemic scleroderma

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Abstract

Background: Endothelial mesenchymal transition (EndMT) is a key pathological event for vasculopathy, and is one of the early features and hallmarks of systemic sclerosis (SSc). It has been well-established that hypoxia contributes to EndMT. However, little is known about the effects of EndMT induced by hypoxia on the skin microvascular remodeling of SSc, as well as the underlying mechanism.

Methods: Skin biopsy was performed for SSc patients and healthy controls, and skin tissues were collected for isobaric tags for the relative and absolute quantification (iTRAQ)-based proteomics and immunohistochemical test. Human microvascular endothelial cell line-1 (HMEC-1) cultured in hypoxic or normal conditions was treated by tamoxifen or bevacizumab. The expression of hypoxia inducible factor-1 α (HIF-1 α), vascular endothelial growth factor (VEGF)-a, CD31, α -smooth muscle actin (α -SMA), VE-cadherin, and fibronectin were detected at both the protein and mRNA level.

Results: The iTRAQ-based proteomics indicated the significantly upregulated HIF-1 signal in the skin tissues of SSc patients. The immunohistochemical results demonstrated the significant downregulation of endothelial cell (EC) marker CD31 and the distinct positive staining of interstitial cell (IC) marker α -SMA at sites that lined the vessel lumens in skin tissues of SSc. Meanwhile, the positive staining of HIF-1 α , which is a key transcription factor in response to chronic hypoxia, and VEGF-a were found to be diffusely distributed in SSc skin tissues. Consistent with these observations, HMEC-1 cells cultured under hypoxic conditions exhibited a significant decrease in CD31 and VE-cadherin expression, alongside the marked increase in the expression of α -SMA and fibronectin, as well as the distinct upregulation of HIF-1 α and VEGF-a, when compared with those under normal conditions. It is noteworthy that the inhibition of HIF-1 α by tamoxifen effectively downregulated the hypoxic induction of VEGF-a and α -SMA, while rescuing the hypoxic suppression of CD31. In addition, the VEGF-a inhibitor bevacizumab treatment had the same effect on the hypoxic expression of α -SMA and CD31, as a tamoxifen intervention, but did not reduce HIF-1 α .

Conclusion: These results suggest that the HIF-1 α /VEGF signaling pathway has a critical role in mediating the effect of hypoxia-induced EndMT on the skin microvascular remodeling of SSc.

Background

Systemic sclerosis (SSc) is a rare connective tissue disease with high disability and mortality rate, featured by autoimmunity, a broad microvasculopathy, and the fibrosis of the skin and visceral organs [1]. Endothelial-to-mesenchymal cell transition (EndMT) has emerged as a key player in the pathogenesis of tissue fibrosis and fibro-proliferative vasculopathy in various diseases, including cardiac and intestinal fibrosis, diabetic nephropathy, portal hypertension, and pulmonary arterial hypertension (PAH) [1,2].

Furthermore, experimental evidence supports the role of EndMT in the lung tissues of patients with interstitial lung disease associated with SSc [3,4]. In addition, cells in the intermediate stages of EndMT were identified in the dermal microvessels of both patients with SSc, and bleomycin-induced and urokinase-type plasminogen activator receptor deficient mouse models [5]. That is, EndMT causally connects the two hallmarks of SSc: the persistent endothelial injury and the aberrant fibrogenesis.

In the pathogenesis of SSc, reduced capillary blood flow coupled with deficient angiogenesis leads to chronic hypoxia, enforcing a positive feed-forward loop for sustaining vascular remodeling, which further promotes the irreversible extensive tissue fibrosis [2]. This indicates that chronic hypoxia is a prominent trait that contributes to vasculopathy and tissue fibrosis in SSc. In recent years, a large number of studies have confirmed that hypoxia could induce EndMT in vitro and in vivo [6,7,8]. However, the process of EndMT induced by hypoxia in the pathogenesis of SSc remains unclear.

Hypoxia inducible factor-1 α (HIF-1 α) is a key transcription factor that responds to chronic hypoxia, and is presently considered as the "main regulatory factor" of the hypoxia environment [9]. It has been reported that HIF-1 α promotes fibrotic disease, and its implicated function includes the stimulation of excessive extracellular matrix (ECM), vascular remodeling and futile angiogenesis, with further exacerbation of the chronic hypoxia, the deterioration of the pathofibrogenesis, and EndMT [10,11]. The excessive expression of HIF-1 α has been detected in tissues of systemic sclerosis [12,13]. In addition, vascular endothelial growth factor (VEGF) is the predominant proangiogenic factor regulated by HIF-1 α in hypoxia-related diseases, and the remarkable upregulation of VEGF has been observed in SSc specimens [10,13]. Recently, the inhibition of the HIF-1 α /VEGF signaling pathway has been reported to reverse the anti-angiogenesis effects of chrysophanol [14]. Nevertheless, it needs to be further verified whether hypoxia induces the EndMT in the pathogenesis of SSc dependent on HIF-1 α /VEGF signaling.

The present study provides immunohistological evidence for verifying the EndMT in the dermal specimens of patients with SSc, and the excessive increase in expression of HIF-1 α and VEGF-a. The investigators further presented that human microvascular endothelial cell line-1 (HMEC-1) cells cultured under hypoxic conditions exhibited the characteristics of EndMT, and the activation of both HIF-1 α and VEGF-a. Finally, the inhibition of HIF-1 α /VEGF signaling by tamoxifen and bevacizumab effectively inhibited EndMT. These observations provide strong support to the hypothesis that HIF-1 α /VEGF signaling has a critical role in mediating the effect of hypoxia-induced EndMT on the skin microvascular remodeling of SSc. This novel mechanism may represent an important and novel therapeutic target for the complications of SSc-associated fibro-proliferative vasculopathy and fibrosis.

Materials And Methods

Patients and tissue samples

Skin tissue samples obtained from eight patients with SSc and eight age and gender-matched healthy controls were investigated (Table 1). The tissue samples of patients and controls were obtained by skin biopsy, which was performed at the Affiliated Hospital of Southwest Medical University. The

investigators only included patients who met the American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) 2013 classification criteria for SSc [15]. The eligibility criteria excluded the following key conditions: (1) aged <18 or >75 years old; (2) body mass index (BMI) >25; (3) patients with severe primary diseases, such as liver, kidney, hematopoietic system damage, and other skin and connective diseases; (4) patients with recent infection; (5) tumor patients. The control samples were obtained from the normal skin surrounding the nevus, when it was removed. All specimens were taken from the fingers, the back of the hand, and the forearm. All surgical biopsies were performed after the patient's provision of informed consent, and according to the Institutional Review Board–approved protocols of the Affiliated Hospital of Southwest Medical University.

Reagents and antibodies

For the immunohistochemistry and western blot, the following primary antibodies were used: anti-CD31, anti- α -smooth muscle actin (α -SMA), anti-HIF-1 α , anti-VEGF-a, anti-fibronectin, and anti-VE-cadherin. These were all purchased from Abcam (UK). The mouse anti-rabbit IgG (1:5,000 dilution) was purchased from Santa Cruz (USA). The anti- β -actin and goat anti-mouse IgG (1:3,000 dilution) were obtained from Bioss (China). The specific HIF-1 α inhibitor tamoxifen was purchased from Solarbio (China). The specific VEGF-a inhibitor bevacizumab was purchased from MedChemExpress (USA).

Histopathology and immunohistochemistry

The qualified skin tissue was fixed with 10% formalin, embedded in paraffin and sliced into 4- μ m sections. Slides that contained the paraffin-embedded skin tissue sections were histopathologically examined by staining with hematoxylin and eosin, and by immunohistochemical staining with the specific EC and mesenchymal (myofibroblast) cell markers. The procedure for the immunohistochemical staining was, as follows: After dewaxing and hydration, the specimens were washed with phosphate buffer saline (PBS) and repaired with antigen in citric acid buffer. Then, catalase blockers were added, and the antibodies were added. Next, the slides were initially incubated overnight with one of the following antibodies: anti-CD31 (1:50 dilution), anti- α -SMA (1:200 dilution), anti-HIF-1 α (1:100 dilution), or anti-VEGF-a (1:100 dilution). The IgG binding was revealed after the incubation with the secondary antibody for one hour. Then, the slides were colored with the diaminobenzidine (DAB) chromogenic agent. Finally, the positive signals of each index were semi-quantitatively counted using ImageJ, and the analysis was carried out.

Extraction of total proteins from skin tissues

The skin tissues were taken from four patients and matched controls. These were immediately soaked in liquid nitrogen before storing at -80°C . Each tissue was grounded in lysis buffer using Tissue Lyser II at a frequency of 25 Hz for a period of 30 minutes. The skin tissues were chopped up with scissors, and an additional 30 minutes of sonication performed using the intelligent ultrasonic processor DH99-IIIN (Lawson, China) to lyse the tissues until total grinding. Then, these tissues were lysed in lysis buffer (4% sodium dodecyl sulfate, 0.1 M of dithiothreitol in 0.1 M of Tris-HCl, pH 7.6) at a ratio of 100 mg of tissue per 1 mL of lysis buffer. Afterwards, the tissues were vortexed and incubated for five minutes at 95°C before being lysed for 20 seconds with one second OFF one second ON at 20% amplitude using the ultrasonic processor probe. Subsequently, the lysate was centrifuged at $14,000\times g$ for 20 minutes at 4°C to remove the cell debris. Then, the supernatant was recovered in a new tube, and the amount of proteins was measured using the bicinchoninic acid (BCA) protein assay kit, according to manufacturer's instruction. The protein assay through the BCA method was used for the quantification of total proteins in the sample.

Absolute quantitation (iTRAQ)-based proteomics

An amount of 1 mg of protein from each sample was deposited in a filtration centricon Microcon YM-30 (Millipore). The washing steps were performed with 8 M of urea, 0.1 M of Tris-HCl, and pH 8.5 buffer. Then, the cysteine residues were blocked with 12 mM of methyl methanethiosulfonate for 30 minutes at room temperature. Next, the proteins were digested by trypsin with enzyme to a substrate ratio of 1:50 (w/w) at 37°C for 15 hours. Furthermore, the digested peptides were labeled with the iTRAQ Reagent Kit (Applied Biosystems, Foster city, CA, USA). The iTRAQ-labeled samples were analyzed using the NanoAquity UPLC system connected to the Q Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, USA). All protein analyses were performed by Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China). The raw data files were analyzed using the Proteome Discoverer (Thermo Scientific, Version 2.1) against the Homosapiens database (<https://www.uniprot.org/taxonomy/9606>). The MS/MS search criteria were, as follows: mass tolerance of 10 ppm for MS and 0.02 Da for MS/MS tolerance, trypsin was the enzyme with two missed cleavage allowed, the carbamido methylation of cysteine and the iTRAQ of the N-terminus and lysine side chains of peptides were the fixed modification, and methionine oxidation was the dynamic modification. The false discovery rate (FDR) of peptide identification was set as $\text{FDR} \leq 0.01$. A minimum of one unique peptide identification was used to support the protein identification. The functional enrichment analysis was performed for the identified proteins based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<http://www.genome.jp/kegg/>).

Cell culture

The HMEC-1 cell line was obtained from Zhongqiao Xinzhou (Shanghai, China). The cells were cultured in endothelial cell medium (ECM, ScienCell) containing 5% fetal bovine serum (FBS), 1% penicillin/streptomycin double antibody, and 1% ECGS endothelial growth factor. Then, the cells were maintained at 37°C in a humidified atmosphere with 5% CO₂. The anaerobic gas production bag (Hpebio, Qingdao, China) was used to promote the hypoxia environment, and this was placed into the anaerobic culture box. Then, the HMEC-1 cells were treated with hypoxia by culturing in the anaerobic culture box for 72 hours. The phenol blue oxygen indicator was used to monitor the oxygen concentration and hypoxia status.

Tamoxifen was dissolved in dimethyl sulfoxide (DMSO) to construct a solution at 5-mmol/L concentration. Then, this was filtered through a needle sterile filter, and stored at -20°C in the dark. These HMEC-1 cells could also be co-cultured with 5 umol/L of tamoxifen, with DMSO as the negative control. Bevacizumab was dissolved in PBS to construct a solution of 3-mg/ml concentration. Then, this was filtered through a needle sterile filter, and stored at -20°C in the dark. The HMEC-1 cells could also be co-cultured with 0.3 mg/ml of bevacizumab, with PBS as the negative control.

Quantitative reverse transcription–polymerase chain reaction (PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, USA), according to manufacturer's instructions. Then, β -actin was used as the internal control to detect the mRNA expression of transcription factors HIF-1 α , VE-cadherin, fibronectin, CD31, α -SMA, VEGF-a, VEGFR1 and VEGFR2. The EC transcript levels were quantified using the Rever Tra Ace qPCR RT Kit (Code No. FSQ-201; TOYOBO, Japan) on the ABI PRISM 7500 system (Applied Biosystems, USA). The quantification was performed using the $2^{-\Delta\Delta CT}$ method. The primers used in the PCR analyses were, as follows:

HIF-1 α : forward 5'-AGCAACTTGAGGAAGTACCATT-3', Reverse 5'-AGGTGAACTTTGTCTAGTGCTT-3';

VE-cadherin: forward 5'-AAAGAATCCATTGTGCAAGTCC-3', Reverse 5'-CGTGTTATCGTGATTATCCGTG-3';

Fibronectin: forward 5'-AATAGATGCAACGATCAGGACA-3', Reverse 5'-GCAGGTTTCCTCGATTATCCTT-3';

CD31: forward 5'-TCGTGGTCAACATAACAGAACT-3', Reverse 5'-TTGAGTCTGTGACACAATCGTA-3';

α -SMA: forward 5'-CCGGGAGAAAATGACTCAAATT-3', Reverse 5'-CTCAGCAGTAGTAACGAAGGAA-3';

VEGF-a: forward 5'-ATCGAGTACATCTTCAAGCCAT-3', Reverse 5'-GTGAGGTTTGATCCGCATAATC-3';

VEGFR1: forward 5'-CAAGATTTGCAGAACTTGTGGA-3', Reverse 5'-CTGTCAGTATGGCATTGATTGG-3';

VEGFR2: forward 5'-GGAGCTTAAGAATGCATCCTTG-3', Reverse 5'-GATGCTTTCCCCAATACTTGTC-3'.

Western blot

HMEC-1 cells were lysed with RIPA lytic buffer (Beyotime Biotechnology, China). Then, the same amount of protein samples was separated by 10% SDS-PAGE, and transferred onto a NC membrane (Millipore, USA). After blocking with 5% skim milk, the membranes were incubated overnight at 4°C with one of the following primary antibodies: β -actin (1:1,000), HIF-1 α (1:1,000), α -SMA (1:500), CD31 (1:500), VEGF-a (1:500), VE-cadherin (1:500), and fibronectin (1:500). Then, the membranes were washed with PBS, and incubated for one hour with the secondary antibody. Finally, these were visualized using the electrochemiluminescence (ECL) reagent (Millipore, USA). The protein images were captured using ImageJ.

Immunofluorescence

HMEC-1 cells were fixed in 4% paraformaldehyde (Biosharp, China) for 30 minutes at room temperature, and permeabilized with 0.2% Triton X-100 (Sigma, USA) and 2% BSA. After blocking with 2% BSA for one hour, these cells were incubated with the primary antibody overnight at 4°C. Then, these were washed with PBS for three times, and incubated with the secondary antibody for one hour. Finally, the DAPI reagent was added in the dark, and the images were quickly captured using a fluorescence microscope. The following antibodies were used: HIF-1 α (1:50), α -SMA (1:50), CD31 (1:50), VEGF-a (1:50), VE-cadherin (1:50), and fibronectin (1:100).

Statistical analysis

All experiments were repeated for at least three times with similar results. The values were expressed as the mean $\pm$ SD of at least three independent experiments in triplicate. The statistical differences were assessed by one-way analysis of variance (ANOVA) (Kruskal–Wallis) using the SPSS 17.0 software. A *P*-value of <0.05 was considered statistically significant.

Results

Background of participants and iTRAQ-based proteomics

The clinical features of the SSc patients and healthy controls, whose skin tissues were studied, are shown in Table 1. The protein patterns from four patients and four controls were analyzed using the iTRAQ-based quantitative approach. A total of 2,159 proteins were identified. For the amino acid sequences of the identified proteins, the protein dataset was matched to the local database, and 43 proteins were found to be significantly different between SSc patients and controls, with *P* < 0.05, FC > 1.2, or < 0.83, as shown in Additional File 1. The functional enrichment analysis was performed for the

identified proteins based on the KEGG database. The top 15 up and down pathways are shown in Figure 1A. Interestingly, the HIF-1 signal was significantly upregulated in the skin tissues of SSc patients.

Table 1. Clinical and demographic characteristics of SSc patients and controls

Characteristics	SSc (n=8)	Controls (n=8)	P-value
Age (years)	51±8.49	51±8.49	1.00
Female/Male	6/2	6/2	1.00
BMI (Kg/m ²)	21.75±2.63	22.06±1.19	0.77
Duration of disease (years)	1.04±0.82	-	-
Involve organs (n/N)			
Skin	8/ 8		
Raynaud phenomenon	7/ 8	-	-
Lung	4/ 8	-	-
Gastrointestinal tract	3/ 8	-	-
Joint	5/ 8		
Kidney	1/ 8	-	-
Heart	2/ 8	-	-
Biopsy site (n)			
Finger/Back of hand/ Forearm	4/ 2/ 2	4/ 2/ 2	1.00
ANA titier	1:1000 (5/ 8), 1:3200 (2/ 8), 1:320 (1/ 8)	-	-
SCL-70	+++ (8/ 8)	-	-
Pattern of microangiopathy	perivascular inflammation (8/ 8), intimal hyperplasia (6/ 8), obliteration of the lumen (2/ 8), vascular loss (7/ 8), microthrombi (1/ 8)	-	-
Skin fibrosis	6/ 8		

The EndMT and activated HIF-1 α /VEGF signal were detected in patients with systemic sclerosis

Compared with the controls, all eight skin tissue specimens obtained from SSc patients displayed varying degrees of interstitial fibrosis, along with a pattern of infiltration of inflammatory mononuclear cells around the vessels, marked narrowing, and occlusion of the vessel lumen and vascular loss, as illustrated in Figures 1B and 1C. EC marker CD31 and IC marker α -SMA were used in the

immunohistologic analyses to identify the EndMT present in the cutaneous tissue. As expected, CD31 positive cells significantly decreased in SSc tissues, and these were detected at sites that lined the vessel lumens, when compared with those in control tissues, as illustrated in Figures 1F and 1G. Meanwhile, the positive α -SMA staining of α -SMA was perivascularly shown in the specimens of patients, and on the epidermal cells, but was negative in the control specimen, as illustrated in Figures 1H and 1I.

The HIF-1 α /VEGF signal markers were also analyzed by immunohistochemistry. All skin biopsies obtained from SSc patients were both HIF-1 α and VEGF positive. However, HIF-1 α was not present, and VEGF was lowly expressed in the healthy tissue samples (Figs. 1J, 1K, 1L and 1M). The staining of these two markers were observed throughout the keratinocytes of the epidermis, and this was diffusely distributed in the dermis.

Hypoxia induced the interstitial transformation of microvascular endothelial cells in vitro

In order to determine whether hypoxia is able to induce the conversion of ECs into ICs, the EC cell line HMEC-1 was exposed to an anaerobic gas production bag for hypoxia treatment, and the endothelial and fibrotic characteristics were measured. ECs exposed to anaerobic gas exhibited a decrease in mRNA expression for endothelial proteins VE-cadherin and CD31 (Figs. 2A and 2B) at every tested hypoxia time point. Furthermore, consistent with the establishment of the fibrotic process, ECs challenged with anaerobic gas exhibited an increase in mRNA level for fibrotic markers α -SMA and fibronectin (Figs. 2C and 2D) at every tested hypoxia time point. At the protein level, the expression level of CD31 and VE-cadherin was temporarily upregulated after 24 hours of hypoxia, and gradually declined with the extension of hypoxia, as illustrated in Figure 2E, 2F and 2G. The protein of α -SMA and fibronectin exhibited a continuous increase with the prolongation of the hypoxia treatment, as illustrated in Figures 2E, 2H and 2I. The immunofluorescence of cells revealed a similar trend, as illustrated in Additional File 2.

The effect of hypoxia on the cellular morphology and polarity of ECs was also investigated. In the absence of hypoxia, HMEC-1 cells presented as circular adherent cells. When these cells were anoxic for 12 hours, there was a small amount of death, but the shape was still full, and the outline was clear. With the duration of hypoxia, the number of cell death increased, and the morphology changed to fusiform, gradually losing the adhesion connection and polarization. The data is shown in Additional File 3.

Hypoxia activated the HIF-1 α and VEGF signaling in vitro

Considering the postulation of the HIF-1 α /VEGF signaling pathway, which is involved in the hypoxia-mediated EndMT, the investigators were prompted to determine whether hypoxia induced the activation of signal molecules and their ligands at both the mRNA and protein level. There was no change in HIF-1 α mRNA level at the different time points after hypoxia (Fig. 3A). However, ECs in the presence of hypoxia exhibited the increase in VEGF-a mRNA level at each of the tested time point (Fig. 3B). Meanwhile, the

expression of the two ligands of VEGF, Vascular Endothelial Growth Factor Receptor-1 (VEGF-R1) and VEGFR2, were detected at different time points of hypoxia by real-time quantitative polymerase chain reaction (qRT-PCR). Finally, the activation of VEGF-R1 and the inhibition of VEGF-R2 were found, as illustrated in Figures 3C and 3D. At the protein level, the detection of HIF-1 α and VEGF-a were performed by western blot, which indicated the upregulation of both molecules (Figs. 3E, 3F and 3G).

The downregulation of HIF-1 α inhibits the hypoxia-induced EndMT and VEGF signaling

Although tamoxifen has been reported to be the specific inhibitor of HIF-1 α [9], further investigations are needed to determine whether HIF-1 α signaling and its probable downstream VEGF signaling induced by hypoxia could be effectively abolished. To test this, hypoxia-treated ECs were incubated with tamoxifen for 72 hours, and signal molecules HIF-1 α and VEGF-a were detected at the protein level. The results revealed that hypoxia-treated ECs with tamoxifen exhibited the sustained decrease in expression of both HIF-1 α and VEGF-a (Figs. 4A, 4B and 4C).

Taking into account that hypoxia induces EndMT, and the expression of HIF-1 α and VEGF-a, the investigators determined whether the activation of the HIF-1 α /VEGF signaling is necessary for the EndMT-induced by hypoxia. Finally, the investigators used tamoxifen, the specific inhibitor of HIF-1 α /VEGF signaling, and determined the expression of CD31, α -SMA and fibronectin in hypoxia-treated ECs. The results revealed that the decrease in mRNA expression of EC marker CD31 in hypoxia-treated HMEC-1 cells could be inhibited by tamoxifen intervention (Fig. 5A). Furthermore, hypoxia-treated ECs with tamoxifen exhibited a significant decrease in mRNA expression of fibrotic protein α -SMA and fibronectin, which could upregulate in EC induced by hypoxia, without the inhibition of HIF-1 α /VEGF signaling (Figs. 5B and 5C). The protein levels of CD31 and α -SMA were also detected by western blot. It was found that the expression of EC marker CD31 could not decrease, and even IC marker α -SMA was inhibited in hypoxia-treated ECs while co-culturing with tamoxifen (Figs. 5D, 5E and 5F).

Hypoxia-induced EndMT is dependent on the activation of HIF-1 α /VEGF signaling

Although VEGF-a has been verified as the predominant proangiogenic factor regulated by HIF-1 α in hypoxia-related diseases, it remains unknown whether the hypoxia-induced EndMT mediated by the upregulation of HIF-1 α signaling was dependent on the activation of VEGF-a. Bevacizumab, the inhibitor of VEGF-a, was used to treat ECs in hypoxia conditions for 72 hours, and HIF-1 α , VEGF-a, CD31 and α -SMA were detected at the protein level [16]. VEGF-a could be gradually inhibited by its inhibitor, which could not intervene with the activation of HIF- α induced by hypoxia (Figs. 6A, 6B and 6C). The downregulation of CD31 and upregulation of α -SMA induced by hypoxia could be perfectly rescued by the inhibition of VEGF-a (Figs. 6D, 6E and 6F).

Discussion

Therapeutic improvements have been reported to alleviate the relative damage of SSc complications on patient prognosis. However, previous articles did highlight a high mortality rate in SSc patients and SSc vasculopathy, in terms of pulmonary PAH, heart involvement, and scleroderma renal crisis, which still represents the first cause of disease-related mortality [17-20]. Although the mechanism of SSc vasculopathy is not fully understood, increasing evidence indicates that endothelial injury and subsequent endothelial dysfunction is a primary event that triggers the subsequent formation of typical vascular lesions [21]. In clinical practice, Raynaud's phenomenon (RP), the most common symptom and clinical sign of the disease, is just the result from endothelial injury, an important initiating event in SSc [22-24]. In this study, seven of eight SSc patients clinically manifested as RP, but none of them had digital ulceration, even gangrene, which involved 70% of European SSc cases at the end of the 10-year observation period [25]. The short duration of the disease (1.04 ± 0.82) for patients included in this study could partly explain the absence of symptoms of digital ulcer. Meanwhile, the decreased staining of CD31 and increasing expression of α -SMA around dermis vessels in the specimen of these patients suggested the developing EndMT for them. It is known that EndMT, a key player in the remodelling of injured vessels, may be reversible, which further accounts for the clinical results, showing that none of the irreversible ischemic tissue injury was associated to ulceration in this study, and this also possibly offers novel cues for treatment [2].

In addition, it has been proposed that the SSc-related EndMT process have differential pathogenetic roles depending on the type of affected vessels [26, 27]. In arterioles and small arteries, EndMT may lead to an accumulation of profibrotic myofibroblasts in the vessel intima and media, thereby contributing to vessel remodeling, and clinically manifesting as digital ulcers and gangrene of the extremities. When affecting capillary vessels, EndMT may lead to the increase in the number of perivascular myofibroblast, thereby contributing to tissue fibrosis and a parallel loss of endothelial cells, characterized by microvessel rarefaction, which clinically promote the nailfold video-capillaroscopy abnormalities and dermal fibrosis [27]. In this study, most of the patients suffered from clinical RP, high titer of antinuclear antibody (ANA), strongly positive expression of anti scl-70 antibody, and pathological skin fibrosis, along with perivascular inflammation, intimal hyperplasia and vascular loss, while very few cases had pathologically occlusive vasculopathy such as obliteration of the lumen and microthrombi. None of these patients had digital ulcer. Meanwhile, the decrease in staining of CD31 and increased expression of α -SMA were shown to be mainly around the dermis vessels in the specimen of patients with SSc, and the positive HIF- α and VEGF signaling were diffusely distributed in the dermis. These findings support the importance of EndMT in skin microangiopathy during the active progression of SSc, and to which the HIF- α /VEGF signaling may contributed much.

It is common knowledge that EndMT refers to the trans-differentiation process, during which ECs downregulate the expression of its specific EC markers, such as CD31 and VE-cadherin, acquiring a mesenchymal phenotype, which is characterized by the expression of α -SMA, fibronectin, type I collagen, and others [26]. Meanwhile, ECs gain a spindle-shaped fibroblast-like morphology, disaggregate, and lose

polarity during this transition [26], which has been well-observed in HMEC-1 cells intervened by hypoxia in this study. Together, the decreased expression of CD31 and the upregulation of α -SMA could also be detected by both RT-PCR at the mRNA level and western blot and immunofluorescence at the protein level for hypoxia-treated HMEC-1 cells, which was consistent with the findings in scleroderma tissue. These accumulated results testified that HMEC-1 cells are the reliable cell model for hypoxia-induced EndMT, although highly proliferative SV40-transformed cells sometimes do not differentiate into stable phenotypes as primary endothelial cells at the same time.

Previous studies have confirmed that hypoxia could induce EndMT in various diseases, including scleroderma, which contributes to the vicious circle of ongoing pathology, and is considered as an important hallmark in SSc patients [6-8, 28-30]. The Galectin-3, Endothelin-1 (ET-1), HIF-1 α /Twist1, BMP-7/Smads and TGF β 1/SMAD signaling pathways have been proven to be critical mediators in PAH by regulating EndMT, and have a critical role in mediating the effect of hypoxia-induced EndMT in pulmonary arterial and cardiovascular remodeling [6,8, 29,30]. In addition, ET-1 is another important factor for promoting the EndMT process on both endothelial cell lines and primary ECs isolated from SSc patients [31-33]. However, the role of HIF-1 α /VEGF signaling in inducing EndMT was first discussed in the present study throughout literatures.

HIF-1 α is the master transcriptional regulator of the adaptive response to hypoxia, which is the tightly regulated form of HIF-1, and will quickly be hydroxylated and degraded in normoxic conditions by prolyl hydroxylases [34-37]. However, in hypoxia conditions, the expression of HIF-1 α would dramatically upregulate [38,39]. HIF-1 α has been postulated to be dysregulated in various pathologic conditions, which was also detected throughout the keratinocytes of the epidermis in all skin biopsies obtained from scleroderma patients [13, 40, 41]. In the present study, the positive staining of HIF-1 α was not only throughout the keratinocytes of the epidermis, but also diffusely distributed in the dermis, which further hints the important role of hypoxia in the pathogenesis of SSc. In addition, HIF-1 α has no effect on the change of mRNA. Hypoxia inactivates HIF-1 α hydroxylase, which inhibits ubiquitin degradation, and this mainly occurs at the protein level.

VEGF has been reported to be significantly upregulated in all stages of fibrosis and dendritic endothelial cells, and was considered to be one of the main transcriptional targets of HIF-1 α in hypoxia-related diseases [13,42]. Traditionally, VEGF is identified as a key mediator of angiogenesis, which induce the differentiation, proliferation, and migration of endothelial cells, consequently contributing to the formation of vessels through both angiogenesis and vascular remodeling [42]. VEGF exerts its biological functions by binding to its receptors, that is, tyrosine kinase receptors VEGFR1 and VEGFR2. However, unexpectedly, skin tissues of SSc patients with characterized vessel obstruction and loss demonstrate the strong upregulation of VEGF and its two receptors, with a more intense staining for VEGFR-2 than for VEGFR-1 [13,38]. Furthermore, the increase in VEGF and HIF-1 α , accompanied by the characteristics of EndMT, were observed in the skin of SSc patients in both previous articles and in the present study. Meanwhile, HMEC-1 treated by hypoxic exhibited the downregulated level of CD31, VE-cadherin expression, and there was a marked increase in the expression of α -SMA and fibronectin, along with the

increase in expression of HIF-1 α and VEGF-a. It is noteworthy that the hypoxia induced EndMT was effectively reversed by tamoxifen and bevacizumab, the inhibitor of HIF-1 α /VEGF pathway.

The present study has limitations, such as the lack of validation in primary cells. Although highly proliferative SV40-transformed cells could not sometimes differentiate into stable phenotypes as primary endothelial cells did at the same time, the expression of all markers were detected by both RT-PCR at the mRNA level and western blot and immunofluorescence at the protein level, which were consistent with the findings found in scleroderma tissues of patients. These accumulated results confirm the reliability of these results. Another limitation of the present study was that protein inhibitors are directly used, instead of employing gene-level interventions, such as gene silencing or overexpression. However, tamoxifen and bevacizumab have been widely used as effective inhibitors of HIF- α and VEGF, which requires less cost, when compared to genetic intervention.

Conclusion

Taken together, the results from the present study provides evidence that hypoxia is a crucial factor in inducing the conversion of ECs into ICs through an HIF-1 α /VEGF dependent mechanism that consequently promotes skin microvascular remodeling and fibrosis in SSc. This information would be beneficial for designing novel and improved therapeutic strategies against the complications of SSc associated to fibro-proliferative vasculopathy and fibrosis.

Abbreviations

EndMT endothelial mesenchymal transition

SSc systemic scleroderma

iTRAQ isobaric tags for relative and absolute quantification

HMEC-1 Human microvascular endothelial cell line-1

HIF-1 α Hypoxia inducible factor-1 α

VEGF vascular endothelial growth factor

α -SMA α -smooth muscle actin

EC endothelial cell

IC interstitial cell

PAH pulmonary arterial hypertension

ECM extracellular matrix

ACR	American College of Rheumatology
EULAR	European League Against Rheumatism
BMI	Body Mass Index
PBS	phosphate buffer saline
DAB	Diaminobenzidine
BCA	bicinchoninic acid
KEGG	Kyoto Encyclopedia of Genes and Genomes
ECM	Endothelial Cell Medium
FBS	fetal bovine serum
ECGS	endothelial growth factor
DMSO	dimethyl sulfoxide
ECL	electrochemiluminescence
ANOVA	Analysis of Variance
VEGFR	Vascular Endothelial Growth Factor Receptor
qRT-PCR	Real-time Quantitative polymerase chain reaction
RP	Raynaud's phenomenon
ANA	antinuclear antibody
ET-1	Endothelin-1

Declarations

Ethics approval and consent to participate

All the surgical biopsies were performed following the patients' provision of informed consent and according to Institutional Review Board–approved protocols from the Affiliated Hospital of Southwest Medical University.

Consent for publication: Not applicable

Availability of data and materials

The iTRAQ-based proteomics data from this study will be made freely available from the link: <http://cloud.majorbio.com>; The accession number is deng123.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions: J Mao performed the experiments, analyzed the data, and wrote manuscript. JX Liu collected the data, and revised the manuscript. JX Liu, M Zhou and GQ Wang participated in the sample and data collection. Xia Xiong helped optimize the research and proofread the paper. YQ Deng designed and optimized the research, wrote and revised manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

The iTRAQ-based proteomics, and the histopathologic and immunohistologic assessments of skin tissues obtained from patients with SSc. (A) The KEGG pathway enrichment analysis was carried out

based on the protein-concentrated proteins using the independent development process of Majorbio Bio-pharm Technology Co., Ltd. Fisher's exact test was employed. With the adjusted $P < 0.05$, the KEGG pathway was considered to be significantly enriched. The KEGG enrichment diagram revealed the corresponding relationship between the target protein set, the annotation and enrichment of the KEGG pathway. The left side is the protein, and the right is the name of the KEGG pathway that enriched the target protein. Log2FC was used to show the upregulation and downregulation protein. The Z score value was employed to identify the upregulation or downregulation pathways. The Z score for the HIF-1 signaling pathway was 1.0. (B and C) Hematoxylin and eosin staining of skin tissues of SSc patients, indicating a large amount of fibrous tissue in the dermis and infiltration of inflammatory mononuclear cells around vessels, as well as narrowing, occlusion of the vessel lumen and vascular loss. (D and E) Original magnification $\times 100$. Negative controls for immunohistochemistry. (F, G, H and I) Immunohistochemical staining of skin tissues for CD31 and α -SMA. In the tissue from patients similar to that in G and I, the presence of CD31-positive cells decreased and α -SMA-positive signaling around the vessels increased in the dermis, when compared to control tissue, similar to that in F and H. (J, K, L and M) The immunohistochemical staining of skin tissues for the HIF-1 α /VEGF signal. The specimens from SSc from a patient were both HIF-1 α and VEGF positive, similar to that in K and M, and HIF-1 α was not present, and VEGF was lowly expressed in the normal tissue samples, similar to that in J and L.

Figure 2

Hypoxia-induced changes in endothelial and fibrotic markers expression. (A, B, C and D). ECs were exposed to hypoxia for 72 hours, and the mRNA expression was analyzed. (E) Representative images from western blot experiments performed for the detection of endothelial marker CD31, VE-cadherin, and fibrotic marker α-SMA and fibronectin, similar to that in E. (F, G, H and I) Densitometric analyses of the western blot experiments. The statistical differences were assessed by one-way analysis of variance (ANOVA) (Kruskal–Wallis), followed by Dunn’s post hoc test. * $P < 0.05$ against the untreated condition. The graph bars show the mean $\pm$ SD.

Figure 3

The hypoxia-induced changes of HIF-1α, VEGF-a, VEGFR1 and VEGFR2 at the mRNA and protein level. (A, B, C and D) The ECs were exposed to hypoxia for 72 hours, and the mRNA expression of HIF-1α, VEGF-a, VEGFR1 and VEGFR2 was analyzed. (E) The representative images from the western blot experiments performed for the detection of HIF-1α and VEGF-a. (F and G) Densitometric analyses of the western blot experiments. The statistical differences were assessed by one-way analysis of variance (ANOVA) (Kruskal–Wallis), followed by Dunn’s posthoc test. * $P < 0.05$ against the untreated condition. The graph bars show the mean $\pm$ SD.

Figure 4

The hypoxia-treated ECs were incubated with tamoxifen, the HIF-1α inhibitor, and the expression of HIF-1α and VEGF-a were detected. (A) The representative images from the western blot experiments performed for the detection of HIF-1α and VEGF-a, with and without tamoxifen treatment. (B and C) Densitometric analyses of the western blot experiments. The statistical differences were assessed by one-way analysis of variance (ANOVA) (Kruskal–Wallis), followed by Dunn’s posthoc test. *P<0.05 against the untreated condition. The graph bars show the mean ± SD. The asterisk on top of the bars correlate to the 24, 48 and 72 hours, which represent the comparison with the bars at zero hour.

Figure 5

Hypoxia-treated ECs were incubated with tamoxifen, the HIF-1 α inhibitor, and the expression of CD31, fibronectin and α -SMA were detected. (A, B and C) The relative expression of CD31, fibronectin and α -SMA mRNA. (D) Representative images from the western blot experiments performed for the detection of HIF-1 α and VEGF-a, with and without tamoxifen treatment. (E and F) Densitometric analyses of the western blot experiments. The statistical differences were assessed by one-way analysis of variance (ANOVA) (Kruskal–Wallis), followed by Dunn’s posthoc test. * $P < 0.05$ against the untreated condition. The graph bars show the mean $\pm$ SD. The asterisk on top of the bars correlate to the 24, 48 and 72 hours, which represent the comparison with the bars of the two groups at the same time point.

Figure 6

The hypoxia-treated ECs were incubated with bevacizumab, the VEGF-a inhibitor, and the expression of HIF-1α, VEGF-a, CD31 and α-SMA were detected. (A and D) Representative images from the western blot experiments performed for the detection of HIF-1α, VEGF-a, CD31 and α-SMA, with and without bevacizumab treatment. (B, C, E and F) Densitometric analyses of the western blot experiments. The statistical differences were assessed by one-way analysis of variance (ANOVA) (Kruskal–Wallis), followed by Dunn’s posthoc test. *P<0.05 against the untreated condition. The graph bars show the mean ± SD. The asterisk on top of the bars correlate to the 24, 48 and 72 hours, which represent the comparison with the bars at zero hour.

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