RESEARCH ARTICLE

Role of endothelin-1 in the skin fibrosis of systemic sclerosis

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ABSTRACT. Endothelin-1 (ET-1) acts as a key regulator of vasoconstriction and fibrosis. Many previous studies have focused on the role of ET-1 in scleroderma (systemic sclerosis, SSc). We investigated the effects of ET-1 on the production of extracellular matrix in SSc and normal skin fibroblasts. Primary cultured dermal fibroblasts from SSc patients and healthy controls were treated with ET-1 (25 ng/mL) for 0 min, 15 min, 1 h, 24 h, 48 h and 72 h, respectively. Our results showed that, in SSc fibroblasts, ET-1 upregulated collagen type I, connective tissue growth factor (CTGF), type I plasminogen activator inhibitor (PAI-1) and pAkt in a time-dependent manner within 72 h; in normal fibroblasts, 25 ng/mL ET-1 stimulation correlated with high levels of CTGF, PAI-1 and pAkt. The secretion of fibronectin (FN), collagen type I, and PAI-1 is markedly increased in the supernatant of both SSc fibroblasts and normal fibroblasts. Furthermore, ET-1 phosphorylates Smad2 and Smad3 in normal fibroblasts, but not in SSc fibroblasts. In conclusion, our results demonstrated that ET-1 may induce fibrosis in dermal fibroblasts through Akt signals.

Key words: endothelin-1, systemic sclerosis, fibroblast, smad, Akt

Scleroderma (systemic sclerosis, SSc) is a chronic, autoimmune, connective tissue disorder characterized by micro- and macro-vasculopathy, inflammation and tissue remodeling that often leads to excessive extracellular matrix deposition and fibrosis in the skin and multiple internal organs [1, 2]. The co-occurrence of endothelial dysfunction and fibrosis indicates that endothelial cell-derived factors, such as endothelin-1 (ET-1), may be vital mediators in the pathogenesis of SSc [3].

ET-1 is a potent vasoconstrictor peptide produced by vascular endothelial cells, which mediates proliferation, fibrosis, and inflammation vascular cells via ETA receptor and ETB receptor [4]. It is thought that ET-1 might activate and re-program functional vascular smooth muscle cells, microvascular pericytes and tissue fibroblasts into profibrogenic cells with myofibroblast-like properties [2]. In the context of wound healing, ET-1 acts with other profibrotic mediators to recruit fibroblasts, and prompts their differentiation into myofibroblasts [5]. Abnormal ET-1 expression and signaling has also been implicated in a variety of other disease states such as cancer, pulmonary hypertension and fibrosis [6, 7]. Increased levels of ET-1 are found in SSc endothelial cells and fibroblasts, which correlates with the severity of the fibrotic phenotype [8]. Plasma ET-1 levels were found to be elevated in SSc patients [9, 10] and this correlated with the number of digital ulcers and scars [11, 12]. Moreover, ET-1 receptor antagonism can improve clinical phenotypes of SSc patients, which also suggests a role for ET-1 in the pathogenesis of scleroderma [2].

Until now, there has been very little information about the role of ET-1 in the recruitment and activation of various downstream molecules in the tissue remodeling and fibrosis seen during the development of scleroderma. Our study was performed to help understand the potential contribution and the underlying mechanisms of ET-1-induced fibrosis.

MATERIALS AND METHODS

Materials and reagents

Dispase, trypsin and fetal bovine serum (FBS) were obtained from Gibco and Invitrogen (Invitrogen, Auckland, USA). Dulbecco's modified Eagle's medium (DMEM) was from HyClone (Thermo Fisher Scientific, MA, USA). Recombinant human endothelin-1 was from PeproTech Inc. (NJ, USA). Rabbit anti-human fibronectin, CTGF and PAI-1 antibody were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). Rabbit anti-human Smad1, phospho-Smad1, Smad2, phospho-Smad2, Smad3, phospho-Smad3, Smad4, AKT, phospho-AKT (Ser473), p44/42 MAP kinase, and phospho-p44/42 MAPK (Thr202/Tyr204) antibody were obtained from Cell Signaling Technology (Beverly, MA, USA). Mouse anti-human β-actin was from Kangcheng Bio-tech (Shanghai, China). Primary antibody was visualized using horseradish

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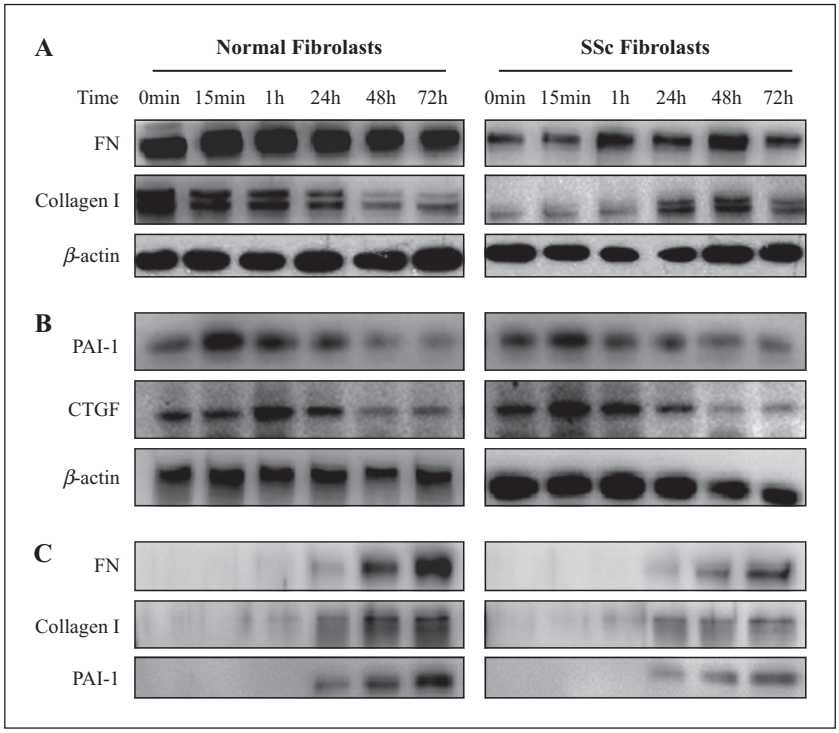

Figure 1
Effect of ET-1 on production of FN, collagen type I, CTGF and PAI-1 in cultured normal and SSc fibroblasts and supernatant. Cells were incubated with 25 ng/mL ET-1 for the indicated times. **A)** Cellular levels of FN and collagen type I; **B)** Cellular level of PAI-1 and CTGF; **C)** secreted FN, collagen I and PAI-1 in the supernatants of cultured normal and SSc fibroblasts. Figures are representative of five independent experiments.

peroxidase–linked anti-mouse IgG or anti-rabbit IgG from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Fibroblast culture

Fibroblasts were obtained from biopsies of lesional areas of the skin of patients with systemic sclerosis (n = 10, 8 females, 2 males, aged 52.6 ± 9.7) who were referred to the Dermatology clinic in the second affiliated hospital of Zhejiang University.

A diagnosis of SSc was given according to the classification criteria of the American College of Rheumatology valid at the time of the study [13, 14]. The patients had not received immunosuppressive medication or corticosteroids for at least one month before biopsy. Punch biopsies were obtained from the dorsal forearm of subjects with informed consent. Normal fibroblasts (n = 10) were taken from healthy donors that were matched with the SSc patients for age and gender. The biopsy tissue was placed in 1 mL of 0.25% collagenase solution (Sigma, St. Louis, MO, USA) and incubated at 37°C, 5% CO2 overnight. Fibroblasts were cultured according to a previous protocol [15], and used at passages 2 to 5.

Western blot analysis

Western blot was performed according to our previous procedures [16-18]. Fibroblasts were serum-starved in DMEM for 24 h, and then stimulated with 25 ng/mL ET-1 for 0 min, 15 min, 1 h, 24 h, 48 h, and 72 h. Supernatant was collected and then stored at -80°C. SSc and normal fibroblasts were washed twice with ice-cold PBS, scraped, and centrifuged. The pellet was incubated for 1 h in modified RIPA lysis buffer. The supernatant was boiled for 10 min in loading buffer. Total protein extracted from each sample was separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose membranes (Millipore, MA, USA). The membranes were blocked for 1h with 7% milk in PBS containing 1% Tween-20, and then probed with the indicated primary antibodies overnight at 4°C in PBS containing 1% Tween-20. The blots were then incubated with a horseradish peroxidase–conjugated secondary antibody (1:5000, Jackson ImmunoResearch, West Grove, PA, USA). Immunoreactivity was detected using an enhanced chemiluminescent (ECL) plus reagent kit (Millipore), and captured by using a chemiluminescence imaging system (BIO-RAD Laboratories, Richmond, CA, USA). A negative control consisted of normal fibroblasts or SSc fibroblasts without ET-1 stimulation at 0, 1 h, 24 h, 48 h, and 72 h.

RESULTS

Regulation of FN, collagen I, PAI-1 and CTGF by ET-1 in normal and SSc fibroblasts

The protein levels of cellular FN in normal fibroblasts and SSc fibroblasts were slightly decreased by ET-1 within 72 h, and reached a minimum level at 72 h (*figure 1A*). However, secreted FN in the supernatants of both normal and SSc fibroblasts was upregulated by ET-1 in a time-dependent manner within 72 h, and reached a maximum level at 72 h (*figure 1C*). Cellular collagen type I in normal fibroblasts was decreased by ET-1 in a time-dependent manner, and reached a minimum at 72 h. Conversely, production of cellular collagen type I in SSc fibroblasts was upregulated by ET-1 in a time-dependent manner, and

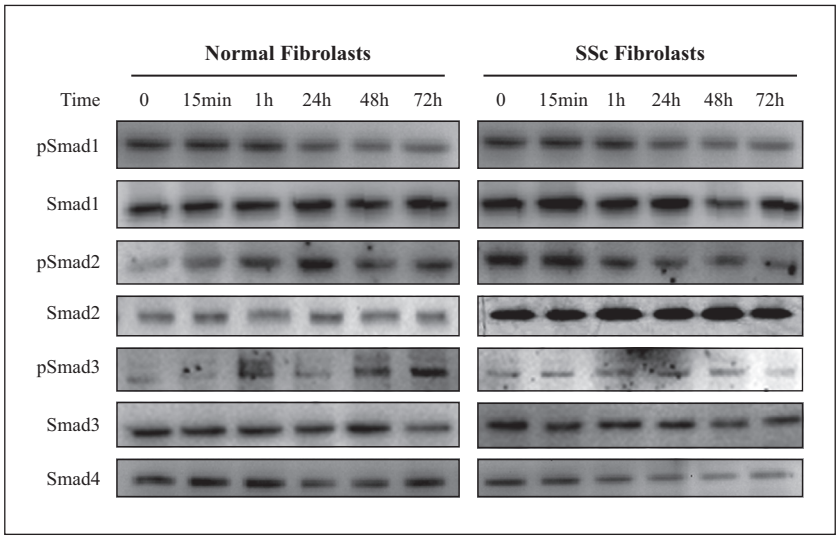

Figure 2
Effect of ET-1 on the phosphorylation of Smads in normal and SSc fibroblasts. Normal and SSc fibroblasts were incubated with ET-1 (25 ng/mL) for the indicated times, and the phosphorylation of Smad1, Smad2 and Smad3 was analyzed by Western blot.

reached a maximum at 72 h (*figure 1A*). Secreted collagen type I increased in a time-dependent manner within 72h in the supernatants of both normal and SSc fibroblasts (*figure 1C*). Cellular PAI-1 was regulated by ET-1, increasing at 15 min, and then decreasing after 1 h in normal and SSc fibroblasts. At 48 h and 72 h however, cellular PAI-1 decreased to below the basal level in both cell types (*figure 1B*). Secreted PAI-1 in the supernatants of normal and SSc fibroblasts was upregulated by ET-1 in a time-dependent manner within 72 h, and reached a maximum at 72h (*figure 1C*). Cellular CTGF in normal fibroblasts was increased by ET-1 at 1h, while being increased at 15min in SSc fibroblasts. At 48h and 72h, production of CTGF was inhibited to below the basal level in both cell types (*figure 1B*). Secreted CTGF in the supernatants of the both cells was not detected by Western blot.

Phosphorylation of Smads by ET-1 in normal and SSc fibroblasts

To determine if the Smads pathway is associated with ET-1-regulated production of FN, collagen type I, CTGF and PAI-1, phospho-Smad1, 2 and 3 levels were determined by Western blot. In normal fibroblasts, Smad2 was phosphorylated by ET-1 at 15min, reached the highest level after 24 h stimulation, and then decreased at 48h and 72h (*figure 2*). Phospho-Smad3 was upregulated by ET-1 in a time-dependent manner and reached a maximum at 72 h (*figure 2*). In both normal and SSc fibroblasts, phospho-Smad1 was not changed by ET-1 at 15min and 1h, but decreased after 24 h, (*figure 2*). Phospho-Smad2 shows a similar tendency to phospho-Smad1 in SSc fibroblasts. ET-1 displayed no effect on the phosphorylation of Smad3 in SSc fibroblasts (*figure 2*). Protein levels of Smad1, 2, 3 and 4 were not altered by ET-1 within 72 h (*figure 2*).

ET-1 promotes phosphorylation of AKT in normal and SSc fibroblasts

To investigate further the role of ET-1 in non-Smad signals, phosphorylation of Akt and ERK1/2 were included. As shown in *figure 3*, phosphorylation of AKT was upregulated by ET-1 in a time-dependent manner, in both normal and SSc fibroblasts. Phospho-Akt reached maximum at

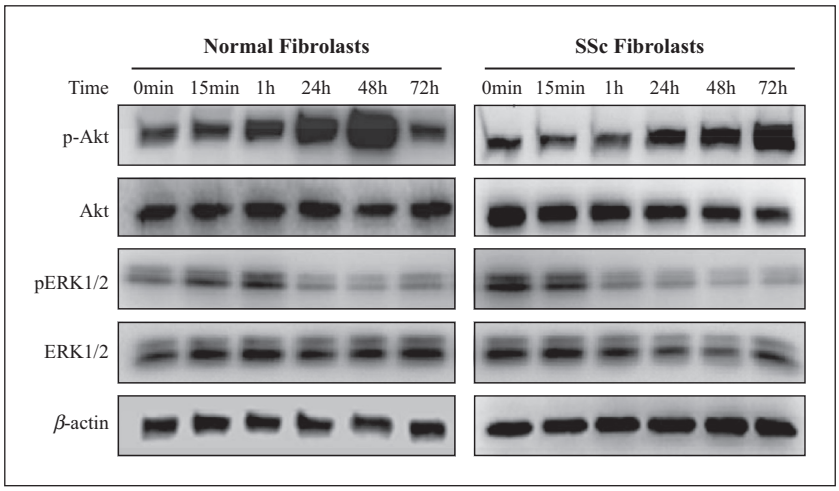

Figure 3
Effect of ET-1 on the phosphorylation of Akt and ERK1/2 in normal and SSc fibroblasts. Normal and SSc fibroblasts were incubated with ET-1 (25 ng/mL) for the indicated times, and the phosphorylation of Akt and ERK1/2 was analyzed by Western blot.

48 h and 72 h respectively, in normal and SSc fibroblasts. ERK1/2 was phophorylated slightly by ET-1 at 1 h, and then regressed to basal level within 72 h in normal fibroblasts, but did not show any stimulatory effect in SSc fibroblasts. The protein levels of Akt and ERK1/2 were not altered by ET-1 within 72 h (*figure 3*).

DISCUSSION

In this study, we have demonstrated that ET-1 contributes to an increased production of cellular collagen-I in SSc fibroblasts, and cellular PAI-1 and CTGF in both normal and SSc fibroblasts. Furthermore, ET-1 accelerates secretion of FN, collagen type I and PAI-1 in the supernatant of cultured normal and SSc fibroblasts.

Previous study has shown that secretion of collagen I and III from normal fibroblasts is increased by incubation with 100 nM ET-1 [3]. ET-1 at 1 nM increased FN production in SSc fibroblasts, while no change was observed in normal fibroblasts [3]. Our results have shown that ET-1, at 25 ng/mL (10 nM), upregulates production of collagen I in SSc fibroblasts, but not in normal fibroblasts. Importantly, 10 nM ET-1 also accelerated secretion of FN, collagen I and PAI-1 in normal and SSc fibroblasts.

These data indicate that different concentrations of ET-1 may have different effects on the production of ECM in fibroblasts.

CTGF establishes an autocrine loop with TGF-β and amplifies the signaling for fibrosis [5, 19]. We have observed that 10 nM ET-1 prompts CTGF production at 15 min in SSc fibroblasts, and at 1 h in normal fibroblasts, which suggests that ET-1 induces a fibrogenic phenotype in normal dermal fibroblasts resembling that seen in SSc fibroblasts. Considering the increased CTGF in SSc fibroblasts, the immediate response of SSc fibroblasts to ET-1 might suggest the pre-activated extracellular CTGF production in SSc [8].

PAI-1, a potent inhibitor of urokinase/tissue-type plasminogen activators, plays a major role in ECM accumulation and degradation [20, 21]. Our observation shows that cellular PAI-1 is upregulated by ET-1 within 15min, and then decreases after 1h in normal and SSc fibroblasts. This decrease may be due to accelerated extracellular secretion of PAI-1.

ET-1, as a downstream regulator or co-factor of the TGF-β signaling pathway, was previously demonstrated to promote fibrotic changes in SSc fibroblasts in concert with TGF-β [7, 8]. TGF-β signaling, the most important pathway contributing to SSc fibrosis, is mediated by both Smads and non-Smads signals. In our study, ET-1 fails to induce phosphorylation of Smad1, but activates Smad2 and Smad3 in normal fibroblasts. No stimulatory effect of ET-1 on the activation of Smad1, 2 and 3 was observed in SSc fibroblasts. Smad2 and Smad3 are transcription factors that are overexpressed in human SSc fibroblasts [22]. These results suggest that the pathways that regulate the balance between ET-1 and Smads are disrupted in SSc fibroblasts. As seen in our study, ET-1 may mediate a profibrotic phenotype of SSc fibroblasts, less dependent on the TGF-β/Smad signaling pathway than normal fibroblasts [3, 19]. These results indicate that a Smads-dependent pathway may not be the principle signaling in ET-1-induced fibrosis in SSc fibroblasts. Therefore, Smads-independent signals, ERK1/2 and Akt were investigated further.

ET-1 activates ERK1/2 slightly in normal fibroblasts, but not in SSc fibroblasts. However, ET-1 activates AKT markedly in a time-dependent manner, in both normal and SSc fibroblasts. ERK was previously demonstrated to mediate a ET-1-induced, matrix-associated genetic program characterized by the expression of collagen isoforms, as well as of contractile proteins involved in enhanced myofibroblast contraction and migration [23]. Phosphorylated AKT has been demonstrated in SSc skin fibroblasts [24]. The elevated levels of ET-1 produced by the SSc fibroblast directly contribute to scar formation by being responsible for the enhanced ability of the SSc fibroblast to contract extracellular matrix via a PI3-kinase/Akt-dependent mechanism [2].

All these data suggest that ET-1 may play its role, at least partly, through Akt signaling to regulate production of FN, collagen type I, CTGF and PAI-1. This is consistent with previous observations that ET-1 produced by the SSc fibroblast directly contributes to scar formation via a PI3-kinase/Akt-dependent mechanism [2, 25]. In addition, although Bosentan, an ET-1 receptor antagonist, does not improve SSc-associated interstitial lung disease [26], it is effective in stabilizing the microcirculation involvement and in improving skin fibrosis irrespective of scleroderma patterns [27]. Therefore, both ET-1 and ET-1 receptor antagonists may be useful in improving skin fibrosis in SSc patients.

In summary, our study adds to the understanding of the pathogenesis of SSc and identifies ET-1 as a candidate therapeutic target for SSc. We found that Akt might be the key signal that mediates ET-1-induced fibrosis: further investigations should be undertaken to clarify the underlying mechanism.

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