Endothelin receptor antagonists: effects on extracellular matrix synthesis in primary cultures of skin fibroblasts from systemic sclerosis patients

S. Soldano¹, P. Montagna¹, R. Brizzolara¹, C. Ferrone¹, A. Parodi², A. Sulli³, B. Seriolo¹, B. Villaggio³, M. Cutolo¹

¹Research Laboratory and Academic Unit of Clinical Rheumatology, Department of Internal Medicine, University of Genova, Italy; ²Department of Health Science, Unit of Dermatology, University of Genova, Italy; ³Research Laboratory of Nephrology, Department of Internal Medicine, University of Genova, Italy

INTRODUCTION

In systemic sclerosis (SSc), the fibrosis of skin and internal organs is an important pathological feature and it is primarily determined by the transition of quiescent to activated fibroblasts, called myofibroblasts, that become effector cells by enhancing the synthesis and deposition of collagens and other extracellular matrix (ECM) macromolecules (1-4). Fibrosis is regulated by a complex interaction between cytokines and growth factors, such as transforming growth factor-β (TGF-β) and endothelin-1 (ET-1) which act on different cell types involved in the fibrotic process (pericytes, mesenchymal stem cells, and fibroblasts, in particular) (5,6). In a recent study, TGF-β was shown to induce ET-1 expression in human dermal fibroblasts and promoted their differentiation to profibrotic and contractile myofibroblasts, confirming the crucial role of the TGF-β/ET-1 axis in the development of skin fibrosis.
Endothelin receptor antagonists

of fibrosis, primarily in the skin (5). ET-1 is one of the major over-expressed soluble mediators in both early and late stage of SSc fibrosis. This molecule, besides being a potent vasoconstrictor and a direct downstream target of TGF-β1, acts like a pro-fibrotic cytokine stimulating fibroblast chemotaxis and proliferation, as well as inducing procollagen and fibronectin (FN) synthesis (2-7). Since ET-1 mediates its biological effects via endothelin (ET) A and B receptors on fibroblasts, there is growing evidence to suggest that antagonizing ET receptors might represent a possible strategy to block the effects of this molecule, at least in SSc skin fibrosis (8, 9).

The aim of the present in vitro study was to evaluate the contrasting effects of single ETA and dual ET A/B receptor antagonist (ET ARA and ETA/BRA) on type I collagen (COL-1), FN and fibrillin-1 (FBL-1) in primary cultures of SSc skin fibroblasts.

## MATERIALS AND METHODS

### Cell cultures and treatments

Skin biopsies were obtained during diagnostic procedures (Dermatology Clinic, University of Genoa, Italy) from 6 female SSc patients (mean age 64±8 years) after informed consent and Ethical Committee approval were obtained. In order to reduce variability, study participants showed the same level of skin/disease involvement (limited SSc), and average disease duration (4.6±4 years). Ongoing treatments limited to vasodilator drugs (loftyl, iloprost) were stopped at least one month before the diagnostic procedure and subsequently restarted. Skin biopsies were cut, incubated with collagenase 0.1 µg/mL (Sigma-Aldrich, Milan, Italy) and finally plated in cell culture dishes in the presence of culture medium made up of RPMI 1640 at 10% of fetal bovine serum (FBS, Lonza, South America), and 1% penicillin-streptomycin and 1% of L-glutamine (Lonza) at 37°C 5% of CO₂ to obtain skin fibroblasts. The cells were grown in culture medium until the 4th passage. To detect the optimal concentration able to antagonize the mitogenic effects of ET-1, a concentration range (0.1 µM -100 µM) for ET ARA (sitaxentan) and ETA/BRA (bosentan) was tested before starting with the experiments. The optimal concentration found to reduce the ET-1 effects was 2 µM for ET ARA and 10 µM for ETA/BRA (data not shown), in agreement with other in vitro studies (2, 5). SSc fibroblasts at the 4th passage were conditioned with or without pre-treatment for 1 h with ET ARA (2 µM) or ETA/BRA (10 µM) and then treated with ET-1 (100 nM) for 24 and 48 hrs in order to test the effects of the ET receptor antagonists. SSc fibroblasts were also treated with ET ARA (2 µM) and ETA/BRA (10 µM) alone for 24 and 48 hrs. Primary cultures of human SSc skin fibroblasts not treated with ET-1 or ET receptor antagonists (ET ARA and ETA/BRA) were used as controls. Normal fibroblast cultures have been previously tested in our laboratory and were found to be responsive to ET-1 treatment in the same way as SSc fibroblasts (6). For ethical reasons, in this in vitro study, new biopsies from healthy subjects were not performed.

### Immunocytochemistry

SSc fibroblasts at the 4th passage were cultured in Flexi PERM chamber slides (1x10⁴ cells/spot) and treated according to the experimental design. At the end of treatment, the cells were incubated with primary antibodies to human FN (dilution 1:100, Sigma-Aldrich), COL-1 (dilution 1:100, Abcam, Cambridge, UK) and FBL-1 (dilution 1:100, Millipore, Billerica, MA, USA). Linked antibodies were detected by biotinylated universal secondary antibody and subsequently with horseradish-peroxydase-streptavidine complex (Vector Laboratories, Burlingame, CA, USA). The slide evaluation was performed on 30 light power fields for each condition by light microscopy (magnification 40X) and computerized image analysis with Leica Q500MC Image Analysis System (Leica, Cambridge, UK).

### Western blot analysis

SSc fibroblasts at the 4th passage, cultured up
to 80% of confluence, were treated according to the experimental design. At the end of treatment, the cells were lysed with NucleoSpin RNA/protein (Macherey-Nagel, Duren, Germany) and protein quantification was performed by the Bradford method. For every condition, 15 µg of protein were separated by electrophoresis on a 10% sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) gel and transferred into Hybond-C-nitrocellulose membrane (GE Healthcare, Milan, Italy). Membranes were incubated in a blocking solution (PB-S1x, 0.1% triton-X and 5% non-fat powdered milk) and subsequently with primary antibodies to human FN (dilution 1:1000, Sigma-Aldrich), COL-1 (dilution 1:4000, Abcam), FBL-1 (dilution 1:500, Millipore) and actin (dilution 1:2000, DB Biotech, Kosice, Slovakia) at 4°C overnight.

Figure 1 - Immunocytochemistry of type I collagen (COL-1) synthesis at 24 and 48 hours. (A) *In vitro* evaluation by immunocytochemistry and relative image analysis of COL-1 synthesis in primary cultures of SSc skin fibroblasts at 24 hrs and (B) 48 hrs. ET-1, endothelin-1 [100 nM]; ET<sub>A</sub>RA, endothelin-A receptor antagonist [2 µM]; ET<sub>A/B</sub>RA, endothelin-A/B receptor antagonist [10 µM].
Membranes were subsequently incubated with secondary antibodies: anti-mouse Ig for FN and FBL-1 (dilution 1:10000, GE-Healthcare), and anti-rabbit Ig for COL-1 and actin (dilution 1:10000, Santa Cruz Biotechnology, California, USA). Protein synthesis was detected using the enhanced chemiluminescence system (Immobilon-P, Millipore, USA). Densitometric analysis of Western blotting was performed by computerized image analysis with Leica Q500MC Image Analysis System (Leica). Protein synthesis was evaluated by the quantification of pixel intensity and indicated as positive area.

**Statistical analysis**

Statistical analysis was carried out by non-parametric test. Friedman’s test was performed to compare the paired treatments. A P value <0.05 was considered statistically significant.

**Figure 2** - Immunocytochemistry of fibronectin (FN) synthesis at 24 and 48 hours. (A) In vitro evaluation by immunocytochemistry and relative image analysis of FN synthesis in primary cultures of SSc skin fibroblasts at 24 hrs and (B) 48 hrs. ET-1, endothelin-1 [100 nM]; ET~A~RA, endothelin-A receptor antagonist [2 µM]; ET~A~B~RA, endothelin-A/B receptor antagonist [10 µM].
RESULTS

Effects of ET-1 on ECM protein synthesis

Results showed that ET-1 significantly increased COL-1 and FN synthesis both at 24 and 48 hrs (P<0.001 for every protein) vs untreated fibroblasts (Fig. 1 and 2). ET-1 was able to increase the FBL-1 synthesis after 48 hrs of treatment (P<0.001), whereas no difference was observed at 24 hrs vs untreated fibroblasts (Fig. 3). These data were obtained by immunocytochemistry and relative image analysis. COL-1, FN and FBL-1 synthesis was also investigated by Western blotting and relative densitometric analysis. Results showed that ET-1 was able to induce a statistically significant increase in COL-1 and FN synthesis both at 24 hrs (P<0.01 for both) and 48 hrs (P<0.05 for both) vs untreated fibroblasts (Fig. 4). Finally, FBL-1 production was found significantly increased only at 48 hrs.

Figure 3 - Immunocytochemistry of fibrillin-1 (FBL-1) synthesis at 24 and 48 hours. (A) In vitro evaluation by immunocytochemistry and relative image analysis of FBL-1 synthesis in primary cultures of SSc skin fibroblasts at 24 hrs and (B) 48 hrs. ET-1, endothelin-1 [100 nM]; ET,RA, endothelin-A receptor antagonist [2 µM]; ET,RA, endothelin-A/B receptor antagonist [10 µM].
by ET-1 treatment vs untreated fibroblasts (P<0.05) (Fig. 4B).

**Effects of ET receptor antagonists on ECM protein synthesis**

Immunocytochemistry and Western blotting analysis were performed to detect the *in vitro* effects of ET receptor antagonists on ECM protein synthesis. At 24 hrs, both ET<sub>A</sub>RA and ET<sub>A/B</sub>RA significantly antagonized the ET-1-mediated increase in COL-1 (P<0.001 for both) and FN (P<0.001 for

![Figure 4](image)

**Figure 4** - Western blotting analysis of ECM protein synthesis at 24 and 48 hours. (A) *In vitro* evaluation by Western blotting and relative densitometric analysis of type I collagen (COL-1), fibronectin (FN), fibrillin-1 (FBL-1) and actin synthesis in primary cultures of SSc skin fibroblasts at 24 hrs and (B) 48 hrs. ET-1, endothelin-1 [100 nM]; ET<sub>A</sub>RA, endothelin-A receptor antagonist [2 µM]; ET<sub>A/B</sub>RA, endothelin-A/B receptor antagonist [10 µM].
both) synthesis, whereas no significant difference was observed in FBL-1 synthesis vs ET-1-treated fibroblasts (Fig. 1A, 2A and 3A). At 48 hrs, ET_ARA still significantly contrasted the increase in COL-1 and FBL-1 synthesis induced by ET-1 (P<0.01 and P<0.001, respectively), but it lost any effect on FN synthesis vs ET-1-treated cells (Fig. 1B, 2B and 3B). On the contrary, at 48 hrs ET_A/BRA still significantly antagonized the ET-1-mediated increase in all the investigated ECM protein synthesis (P<0.05 for COL-1; P<0.01 for FN; P<0.001 for FBL-1 vs ET-1-treated fibroblasts) (Fig. 1B, 2B and 3B). The effect of ET_A/BRA treatment on FN was found sustained also at 72 hrs (data not shown). These data were obtained by immunocytochemistry and relative image analysis.

Western blotting analysis confirmed the results observed by immunocytochemistry showing that both ET_A and ET_A/BRA significantly antagonized COL-1 (P<0.01 and P<0.05, respectively) and FN synthesis induced by ET-1 (P<0.001 and P<0.05, respectively) at 24 hrs vs ET-1-treated fibroblasts (Figure 4A). At 48 hrs, ET_A contrasted the ET-1-mediated increase in both COL-1 and FBL-1 synthesis (P<0.05 and P<0.01, respectively) whereas no difference was observed for FN vs ET-1-treated fibroblasts (Figure 4B). Conversely, ET_A/BRA maintained the ability to antagonize the increase in synthesis of all the investigated ECM proteins induced by ET-1 also at 48 hrs of treatment vs ET-1-treated cells (P<0.05 for COL-1 and FN; P<0.01 for FBL-1) (Figure 4B). The effect of ET_A/BRA treatment on FN was found sustained also at 72 hrs (data not shown). The SSc skin fibroblasts were also treated with ET_A or ET_A/BRA alone and it was shown that both ET receptor antagonists reduced COL-1 and FN synthesis, especially after 24 hrs, and FBL-1 synthesis at 48 hrs, but this was not statistically significant (data not shown).

### DISCUSSION

The present *in vitro* study investigated the effects of the single and dual ET-1 receptor antagonists (ET_A and ET_A/BRA) on the ET-1-induced increase in ECM protein synthesis (COL-1, FN and FBL-1) in primary cultures of SSc skin fibroblasts. Firstly, results confirm that ET-1 is able to induce a significant increase in the synthesis of all the evaluated ECM proteins, supporting a possible involvement of this potent soluble vasoconstrictor also in the development of SSc skin fibrosis, as proposed in other conditions (10).

In particular, endogenous ET-1 signalling was previously found to contribute to COL-1 and connective tissue growth factor (CTGF) overexpression in fibrotic fibroblasts (11). However, recent reports support the profibrotic role of ET-1 *in vitro* by showing that TGF-β1 and ET-1 may act in cooperation to induce the acquisition of pro-fibrotic phenotype in human normal skin fibroblasts and suggesting a possible synergistic role of the TGF-β1/ET-1 axis in the development of fibrosis (2, 5).

Therefore, since ET-1 via ET_A and ET_B receptors contributes to the ability of TGF-β to promote a pro-fibrotic phenotype, this effect is consistent with the notion that ET-1 receptor antagonism may be beneficial in potentially controlling the fibrogenic responses in lung fibroblasts and might represent an important strategy to contrast the profibrotic effects of ET-1 on SSc progression (2, 5, 10, 12, 13).

The results showed that the single ET_A was able to down-regulate the ET-1-mediated increase in COL-1 and FN synthesis in the short term, whereas the dual ET_A/BRA induced a more prolonged and significant effect in reducing especially the ET-1-mediated increase of FN synthesis, together with COL-1.

Interestingly, since COL-1-containing fibrils do not form in the absence of increased FN and FN-binding and collagen-binding integrins, the best target to control the progression of the fibrotic process seems to be the increased FN (and integrins as organizers) (14). The capability of ET_A and ET_A/BRA to antagonize the ET-1-induced ECM increase in cultured SSc fibroblasts and, in particular, the more
sustained inhibitory effects exerted by the ET$_{A/B}$-RA on FN synthesis, seems to support some of the clinical aspects. For example, recent clinical studies showed that the recognized effect of ET$_{A/B}$-RA antagonist in preventing digital ulcers may also be effective in reducing skin fibrosis in patients with SSc (4, 15, 16).

Finally, the analysis of FBL-1 showed that ET-1 increased the synthesis of this protein and this increase was temporarily secondary to the COL-1 and FN. As we know, FBL-1 is the main component of ECM microfibrils and plays an important role on the profibrotic phenotype of SSc fibroblasts (17). As observed for COL-1 and FN, the ET$_{A}$-RA and ET$_{A/B}$-RA contrasted significantly with the increase in FBL-1 synthesis induced by ET-1, an effect that might be the consequence of a general downregulation of the ECM synthesis exerted by these antagonists.

In agreement with these results, a recent study showed that in SSc, dermal fibroblasts the microfibril abnormalities might be a secondary event of matrix deregulation and in ECM remodeling, probably due to the alteration of proteolitic process induced by growth factors (i.e., TGF-$eta$) that characterize the SSc fibrotic process (18).

**CONCLUSIONS**

In conclusion, *in vitro* interference of the ET receptor antagonism with the ECM protein synthesis and the resultant possible therapeutic effect on progressive fibrosis, suggest that ET antagonism agents could act as potential disease modifying molecules in combination therapies for SSc (18).

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**REFERENCES**