Effects of bosentan on collagen type I synthesis on *in vitro* culture of scleroderma skin fibroblasts

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Abstract

The present study evaluated the effects of a non-selective endothelin ($ET_{A/B}$) receptors antagonist, on collagen type I (COL1) synthesis on in vitro culture of scleroderma (SSc) skin fibroblasts (Fb). Fb were obtained from skin biopsies of 6 female SSc patients (mean age 64.1±6 years), after informed consent and Ethical Committee Approval. Cells were treated with endothelin-1 [ET-1, 100nM] for 24 and 48 hrs, pre-treated for 1 hr with $ET_{A/B}$ receptors antagonist [10mM] alone or followed by ET-1 for 24 and 48 hrs. Untreated Fb were used as controls. Immunocytochemistry and western blot analysis were performed to evaluate COL1 synthesis. ET-1 increased COL1 synthesis both at 24 and 48 hrs when compared to controls. $ET_{A/B}$ receptor antagonist blocks the increased COL1 synthesis ET-1-mediated both at 24 and 48 hrs vs. ET-1. Results showed that ET-1 receptors blockage by $ET_{A/B}$ receptors antagonist might prevent the excessive synthesis of COL1, supporting its positive action in the management of skin fibrosis.

Introduction

Systemic sclerosis (SSc) is a progressive autoimmune disease characterized by vascular injury, inflammation and fibrosis [1]. In SSc patients, fibrosis of the skin and internal organs arises from excessive synthesis and deposition of collagen and other extracellular matrix (ECM) molecules by activated fibroblasts (Fb), which represent the key effectors of this pathological process [2, 3].
Among the many potential mediators of the fibrotic process, three molecules appear important: transforming growth factor beta, connective tissue growth factor and endothelin-1 (ET-1) [2].
ET-1 is known to be the most potent vasoconstrictor and in addition to its important role in regulating vascular tone, this peptide showed strong proliferative and mitogenic properties [4]. ET-1 was found increased in plasma levels of SSc patients and it can stimulate the down-regulation of metalloproteinase-1 (MMP-1 or collagenase), the induction of fibronectin as well as the mitogenesis and chemotaxis of Fb [3, 4]. The vasoconstrictor, inflammatory, proliferative and fibrotic effects of ET-1 are mediated by two specific cell surface receptors, the ET-A and ET-B ($ET_{A/B}$) receptors, which have been detected in human cell types, including endothelial cells and Fb. ET_A is selective for ET-1 and ET_B can bind all three ET isoforms with similar affinity. Moreover ET_B was found to be increased in SSc and ET_A decreased in both fibrotic lung and skin derived Fb [5].
The administration of an oral non-selective $ET_{A/B}$ receptors antagonist was approved for the treatment of pulmonary arterial hypertension associated with systemic rheumatic diseases [6]. It has been shown to decrease inflammatory reactions, to prevent the increase in permeability of pulmonary vessels, and to prevent the development of fibrosis in animal with pulmonary inflammation [7].
Based on these concepts, the aim of the present preliminary study was to evaluate the effects of ET-1 receptor blockage on collagen type I (COL1) synthesis on *in vitro* culture of human SSc skin Fb by $ET_{A/B}$ receptors antagonist as a possible strategy to reduce the fibrotic process in SSc.

Materials and methods

Cell cultures and treatments

Skin biopsies were obtained during diagnostic procedures (Academic Dermatological Clinic, University of Genova) from 6 female SSc patients (mean age 64.1±6 years) after informed consent and Ethical Committee Approval. In order to reduce the variability, the SSc patients enrolled into the study showed same skin involvement (limited SSc), average disease duration (8.6±4 years) and ongoing treatments limited to vasodilator drugs (loftily, iloprost).

The skin biopsies were cut, incubated with collagenase 0.1 mgr/ml (Sigma-Aldrich, Milan, Italy), and finally plated in cell culture dish with RPMI 1640 at 10% of fetal bovine serum (South America, Lonza, Milan, Italy), 1% penicillin-streptomycin (Sigma-Aldrich) at 37°C 5% of CO_2 to obtain skin Fb. At 4th passage SSc Fb were treated with ET-1 (100 nM, Vinci-Biochem, Vinci, Italy) for 24 and 48 hours, pre-treated for 1 hour with $ET_{A/B}$ receptors antagonist (10 mM) alone or followed by ET-1 for 24 and 48 hours. Untreated SSc Fb were used as controls (CNT).

Immunocytochemistry (ICC)

SSc Fb at 4th passage were cultured in Flexi PERM chamber slides (10×10^3 cells/spot) (Sartorius, Germany) and treated according to the experimental design. At the end of treatment, the cells were incubated with primary antibody to human COL1 (dilution 1:100, Abcam, Cambrige, UK) for 45 minutes. Linked antibody was detected by biotinylated universal secondary antibody and subsequently with horseradish-peroxydase-streptavidine complex (Vector Laboratories, California, 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). Experiments were done in triplicate.

Western blot analysis (WB)

SSc Fb at 4th passage, culture 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 Bradford methods. For every condition, 15 mgr of protein were separated by electrophoresis on a 10% SDS-PAGE gel and transferred into Hybond-C-nitrocellulose membrane (GeHealthcare, Milan, Italy). Membranes were incubated for 1 hour in a solution of PBS, 0.1% triton-X, 5% powered milk and

subsequently with primary antibody rabbit anti-COL1 (dilution 1:4.000, Abcam) at 4°C over night. To perform the normalization step, actin was used as housekeeping protein and detected by a rabbit anti actin primary antibody (dilution 1:2.000, DB Biotech, Kosice, Slovakia). Membranes were finally incubated for 1 hour with secondary antibody anti-rabbit Ig for COL1 and actin (dilution 1:10.000, Santa-Cruz Biotechnology, California, USA). Protein synthesis was detected using the enhanced chemiluminescence system (Immobilon-P, Millipore). Densitometric analysis of WB 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. Experiments were done in triplicate.

Statistical analysis

The statistical analysis was carried out by non-parametric test. Friedman test was performed to compare the paired treatments. Probability values (P-values) less than 0.05 were considered statistically significant.

Results

To understand the effects of $ET_{A/B}$ receptors antagonist on COL1 synthesis, ICC and WB analysis were performed on human SSc skin Fb cultures treated as described in the Material and Methods section. The results showed that ET-1 induced a significant increase in COL1 synthesis both at 24 and 48 hours vs. CNT ($p<0.001; p<0.001$, respectively) (fig. 1A and B). COL1 production was significantly reduced in $ET_{A/B}$ receptors antagonist pre-treated SSc Fb before ET-1 both at 24 and 48 hours vs. ET-1-treated cells ($p<0.01; p<0.05$, respectively) (fig. 1A and B). The data were obtained by ICC and relative image analysis. COL1 synthesis was also investigated by WB and evaluated by relative densitometric analysis. ET-1 was able to induce
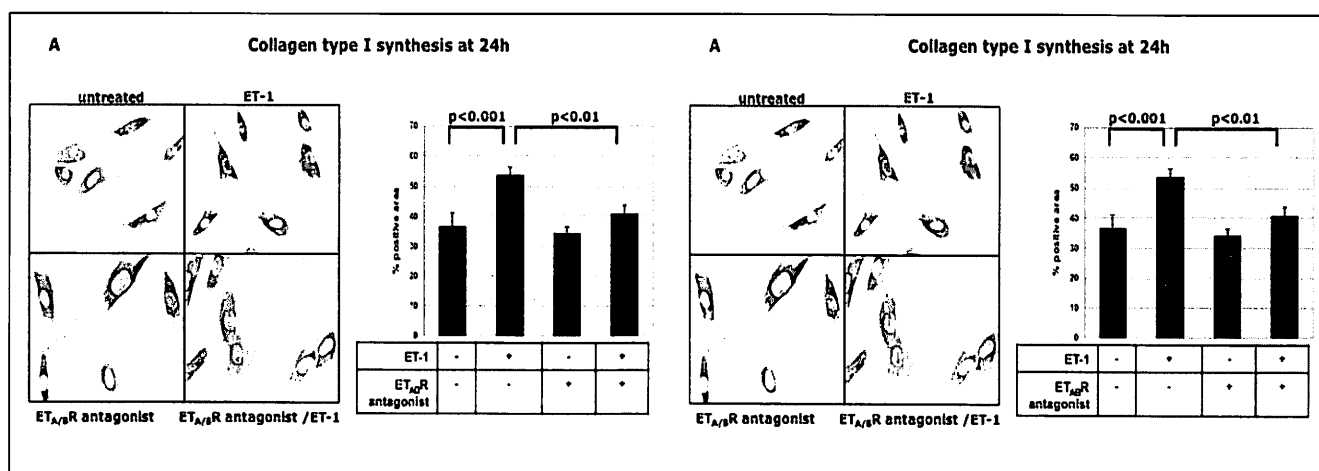

Figure 1. (A) Evaluation of collagen type I synthesis in culture of human scleroderma skin fibroblasts untreated, treated with endothelin-1 (ET-1) for 24 hrs, pre-treated for 1 hr with $ET_{A/B}$ receptors antagonist alone ($ET_{A/B}R$ antagonist) or followed by ET-1 for 24 hrs ($ET_{A/B}R$ antagonist/ET-1) by immunocytochemistry and relative image analysis. (B) Evaluation of collagen type I synthesis in culture of human scleroderma skin fibroblasts untreated, treated with endothelin-1 (ET-1) for 48 hrs, pre-treated for 1 hr with $ET_{A/B}$ receptors antagonist alone ($ET_{A/B}R$ antagonist) or followed by ET-1 for 48 hrs ($ET_{A/B}$ R antagonist/ET-1) by immunocytochemistry and relative image analysis.
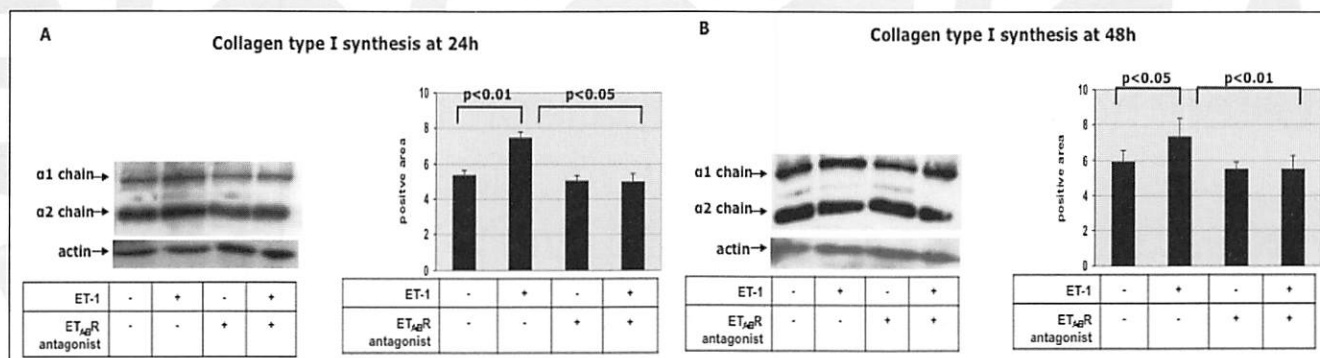
Figure 2. (A) Evaluation of collagen type I synthesis in culture of human scleroderma skin fibroblasts untreated, treated with endothelin-1 (ET-1) for 24 hrs, pre-treated for 1 hr with $ET_{A/B}$ receptors antagonist alone ($ET_{A/B}R$ antagonist) or followed by ET-1 for 24 hrs ($ET_{A/B}R$ antagonist/ET-1) by western blot and relative densitometric analysis. (B) Evaluation of collagen type I synthesis in culture of human scleroderma skin fibroblasts untreated, treated with endothelin-1 (ET-1) for 48 hrs, pre-treated for 1 hr with $ET_{A/B}$ receptors antagonist alone ($ET_{A/B}R$ antagonist) or followed by ET-1 for 48 hrs ($ET_{A/B}R$ antagonist/ET-1) by western blot and relative densitometric analysis.

a statistical significant increase of COL1 vs. CNT both at 24 and 48 hours ($p<0.01$; $p<0.05$ respectively) (fig. 2A and B respectively). Pre-treatment with $ET_{A/B}$ receptors antagonist before ET-1 was found to reduce COL1 synthesis vs. ET-1-treated Fb at 24 and 48 hours ($p<0.05$; $p<0.01$ respectively) (fig. 2A and B, respectively). All these data confirmed the results observed by ICC.

Discussion

Present results confirm that ET-1 is able to induce an increase of COL1 synthesis and this effect is mediated *via* $ET_{A/B}$ receptors expressed on Fb.

As known the biological effects of ET-1 can be divided into two types of functional response: the first "early" response involves the cell signalling and calcium metabolism whereas the second "phenotypic" response culminates in the genes activation which codifies the ECM proteins as collagen, MMPs and tissue inhibitor of MMPs resulting in the tissue architecture alteration [5, 8].

COL1 is the most important molecule in the fibrotic process of SSc, and it is largely responsible for the functional failure of the affected organs [9, 10]. The effects of ET-1 in increasing the COL1 synthesis might support the major role of this soluble mediator as target for therapeutic intervention in the fibrotic process as recently discussed in several studies [3, 5, 11].

These findings enhance the recent evidences that antagonizing the ET-1 receptors might be an important strategy in the pathogenesis of SSc [12].

In this study the block of $ET_{A/B}$ receptors with a non-selective receptors antagonist was found to antagonize the ET-1 effects on increased synthesis of ECM, in particular COL1. The action of $ET_{A/B}$ receptors antagonist showed the importance of ET_A/ET_B receptors to mediate ET-1 effects in SSc skin Fb indicating that a blockage of these receptors might represent a possible tool in the management of SSc fibrosis.

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