Concise report

Rosiglitazone alleviates the persistent fibrotic phenotype of lesional skin scleroderma fibroblasts

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Abstract

Objective. The transcription factor peroxisome proliferator-activated receptor (PPAR)-γ plays an important role in controlling cell differentiation. The aim of the present study was to examine whether PPAR-γ expression was reduced in skin scleroderma fibroblasts and whether PPAR-γ agonists could suppress the persistent fibrotic phenotype of skin scleroderma fibroblasts.

Methods. Dermal fibroblasts were isolated from site-, age- and sex-matched healthy individuals and lesional areas of individuals with dcSSc. Western blot and collagen gel contraction analyses were used to detect protein expression in the presence or absence of the PPAR-γ agonist rosiglitazone.

Results. PPAR-γ expression was reduced in dcSSc fibroblasts. The PPAR-γ agonist rosiglitazone alleviated the persistent fibrotic phenotype of dcSSc fibroblasts.

Conclusion. Rosiglitazone may alleviate the extent of fibrosis in dcSSc.

Key words: α-Smooth muscle actin, Myofibroblast, Rosiglitazone, Connective tissue growth factor, CCN2, Type I collagen.

Introduction

Tissue repair involves the reconstitution of connective tissue [1]. Specialized fibroblasts called myofibroblasts are responsible for the adhesive and tensile forces involved with this process. In normal tissue repair, myofibroblasts disappear from the wound; however, in fibroproliferative diseases such as dcSSc myofibroblasts persist [2, 3].

Peroxisome proliferator-activated receptor (PPAR)-γ is a transcription factor that binds to PPAR response elements in promoters [4]. PPAR-γ agonists inhibit pulmonary myofibroblast differentiation and collagen production [5]. Recently, we showed that mice detected for PPAR-γ in skin possessed increased susceptibility to bleomycin-induced skin fibrosis [6]. However, it is unclear whether PPAR-γ expression is reduced in dcSSc fibroblasts and if PPAR-γ agonists can affect the persistent fibrotic phenotype of these cells.

In this report, we investigate the contribution of PPAR-γ to the dermal sclerosis observed in dcSSc by examining the expression of PPAR-γ in normal and dcSSc fibroblasts and by examining the effect of the PPAR-γ agonist rosiglitazone on the persistent fibrotic phenotype of dcSSc fibroblasts. Our results reveal new insights into the molecular mechanism underlying the fibrosis in dcSSc as well as how to control the fibrosis observed in this disease.

Experimental procedures

Patients

Individuals enrolled were five ScS patients with active dcSSc (all females; aged 28–56 years, average age 44 years) and five normal volunteers (one male, four females; aged 26–58 years, average 43 years). Skin biopsies were taken from the most recently involved ScS skin of patients, all of whom fulfilled the criteria of the ACR for the diagnosis of ScS. Mean duration of ScS from the...
onset of the non-Raynaud’s symptom was 32 months. None of the subjects was taking corticosteroids or had received anti-fibrotic or immunosuppressive therapy prior to skin biopsy. Ethical approval was obtained from the Royal Free Hospital Ethical Practices Sub-Committee, and informed consent was obtained before the patients entered the study.

Cell culture, IF and western analysis

Fibroblasts were grown by explant culture from skin of SSc patients and from healthy individuals, all used between passages 2 and 5 [6, 7]. Cells were lysed in 2% SDS, proteins quantified (Pierce, Rockford, IL, USA) and subjected to western blot analysis as previously described [2]. Antibodies used were: anti-\(\alpha\)-smooth muscle actin (SMA) (Sigma, 1:5000, St Louis, MO, USA), anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1 : 5000, Sigma), anti-connective tissue growth factor (CTGF) (Abcam, 1:500), anti-type I collagen (1 : 1000, Biodesign, Saco, ME, USA) and anti-PPAR-\(\gamma\) (Cell Signaling, 1 : 500). Densitometric analysis was conducted (Biospectrum AC Imaging System Ultraviolet Products, Cambridge, UK) on blots and relative expression of target protein to GAPDH expression was calculated. For some studies, cells were incubated in the presence or absence of rosiglitazone (10 \(\mu\)M; Glaxo Smith Kline, Brentford, UK) for 24 h. Cells were stained with anti-\(\alpha\)-SMA or anti-vinculin (Sigma) antibodies, and with FITC-conjugated secondary antibody (Jackson) before subjecting the cells to IF microscopy (Zeiss, Welwyn Garden City, UK).

Floating and fixed collagen gel cultures and quantitation of gel contraction

Experiments were performed essentially as described [8]. Tissue culture plates were pre-coated with BSA. Trypsinized fibroblasts were suspended in MCDB medium and mixed with collagen solution [one part 0.2 M N-2-hydroxyethylpiperazine-N’-2-ethanesulphonic acid, pH 8.0; four parts collagen (Vitrogen-100, 3 mg/ml, Cohesion Technologies, Palo Alto, CA, USA) and five parts MCDB \(\times 2\)] resulting in a final concentration of 80 000 cells/ml and 1.2 mg/ml collagen. Collagen–cell suspension (1 ml) was added to each well and allowed to polymerize. For floating contraction assays, in which cell migration and myofibroblast differentiation are assessed, cells were detached from wells 1 h post-polymerization, and contraction quantified by loss of gel weight and decrease in gel diameter over a 24-h period. For fixed contraction assays, in which cells differentiate into myofibroblasts due to mechanical loading and the attachment of the cell–collagen mixture to the tissue culture dish, cells were detached from wells 24 h post-polymerization and contraction was quantified by loss of gel weight and decrease in gel diameter over a 1-h period. For inhibition experiments, cells were pre-incubated in the presence of inhibitor for 45 minutes before initiation of the assay. Comparison of collagen gel contraction was performed by using Student’s unpaired \(t\)-test (\(P < 0.05\) was considered as statistically significant).

Fibroblast populated collagen lattices

Measurement of contractile force generated within a 3D, tethered floating fibroblast-populated collagen lattice was performed as described previously [8]. Using \(1 \times 10^6\) cells/ml of collagen gel (First Link, Birmingham, UK), we measured the force generated across the collagen lattice with a culture force monitor. This instrument is capable of measuring the minute forces exerted by cells within a collagen lattice [9], over 24 h as fibroblasts attach, spread, migrate and differentiate into myofibroblasts. In brief, a rectangular fibroblast-seeded collagen gel was cast and floated in medium, tethered to two flotation bars on either side of the long edges, in turn attached to a ground point at one end and a force transducer at the other. Cell-generated tensile forces in the collagen gel are detected by the force transducer and logged into a personal computer. Graphical readings are produced every 15 s providing a continuous output of force (Dynes: \(1 \times 10^{-5}\)) generated [9]. The cells used in these experiments were passage matched; all control and inhibition experiments were run in parallel.

Results

PPAR-\(\gamma\) protein expression is lower in dcSSc fibroblasts

We subjected dermal fibroblasts cultured from explants to western blot analysis using an anti-PPAR-\(\gamma\) antibody (Fig. 1A). Five normal and five dcSSc (lesional) fibroblasts were used. PPAR-\(\gamma\) protein expression was significantly reduced in dcSSc fibroblasts compared with normal healthy fibroblasts. The PPAR-\(\gamma\) agonist rosiglitazone reverses the persistent fibrotic phenotype of dcSSc fibroblasts

Based on these data, we investigated whether a PPAR-\(\gamma\) agonist could reverse the persistent fibrotic phenotype of dcSSc fibroblasts. We found that, as expected, expression of \(\alpha\)-SMA, type I collagen and CTGF protein expression were elevated in dcSSc fibroblasts (Fig. 1B). PPAR-\(\gamma\) protein was lower in dcSSc fibroblasts (Fig. 1B). Rosiglitazone did not appreciably affect protein expression in normal dermal fibroblasts (Fig. 1B). Rosiglitazone treatment suppressed in \(\alpha\)-SMA, type I collagen and CTGF protein expression in dcSSc fibroblasts, but increased PPAR-\(\gamma\) expression over a 24-h period (Fig. 1B). Moreover, rosiglitazone suppressed the enhanced ability of dcSSc fibroblast to contract both ‘floating’ and mechanically loaded ‘fixed’ collagen gel matrices (Fig. 2A and B). Similarly, rosiglitazone alleviated the enhanced ability of dcSSc fibroblasts to generate contractile forces across a fixed collagen gel lattice (Fig. 2C). Consistent with this notion, rosiglitazone suppressed the myofibroblast phenotype of dcSSc fibroblasts as visualized by the presence of \(\alpha\)-SMA stress fibres and the enhanced vinculin staining of so-called ‘supermature’ focal adhesions (Fig. 2D and E). Collectively, these results
Fig. 1 Scleroderma fibroblasts show reduced PPAR-γ expression and the PPAR-γ agonist rosiglitazone inhibits the overexpression of a cohort of pro-fibrotic genes in scleroderma fibroblasts. Dermal fibroblasts from five normal individuals (normal) and five individuals with SSc (SSc) were cultured. (A) Equal amounts of protein extracts were subjected to SDS-PAGE and western blot analysis with anti-PPAR-γ and anti-GAPDH antibodies. Statistical analysis Student’s t-test shows that there is a statistical significance (*P < 0.05) between PPAR-γ expression levels in SSc fibroblasts. (B) Dermal fibroblasts from normal individuals (NF) and individuals with SSc (SScF) were cultured in the presence or absence of rosiglitazone (concentrations as indicated) for 24 h. Cell extracts were then subjected to western blot analyses with anti-CTGF, anti-α-SMA, anti-type I collagen, anti-PPAR-γ and anti-GAPDH antibodies.

Discussion

In this report, we test whether: (i) reduced PPAR-γ protein expression exists in fibroblasts cultured from lesional areas of patients with dcSSc; and (ii) PPAR-γ agonists might be used to reverse the persistent fibrotic phenotype of these cells.

indicate that reduced PPAR-γ promotes fibrogenic responses in skin scleroderma fibroblasts and that PPAR-γ agonists may be used to reverse the persistent fibrotic phenotype of these cells.
Rosiglitazone reduced the ability of lesional SSc dermal fibroblasts to contract a collagen matrix and to possess α-SMA stress fibres and ‘supermature’ focal adhesions. The effect of rosiglitazone on contractility of SSc dermal fibroblasts was investigated using a (A) floating and (B) fixed collagen gel contraction assay. Fibroblasts isolated from four normal (NF) and four SSc (SScF) patients were embedded in collagen gel matrices and detached from a tissue culture dish either after (A) 1 h or (B) 24 h. Cells were cultured in the presence or absence of rosiglitazone. Weights of the contracted collagen gels were measured at the conclusion of the experiments. Values shown are average (±s.d.) for the four sets of contracted collagen gels. A Student’s t-test confirmed that rosiglitazone suppressed the contractile ability of SSc dermal fibroblasts as well as normal fibroblasts differentiated into myofibroblasts in mechanically loaded, fixed collagen gels. treated and the control (P < 0.05). (C) fibroblast populated collagen lattice assay. The effect of rosiglitazone on contractile force generated by both SSc (SScF) and normal (NF) fibroblasts were investigated using a culture force monitor. Note the higher contractile forces generated by cells derived from SSc-involved dermis in comparison with fibroblasts from normal dermis. Note reduction in overall contractile force generated following treatment. (D, E) IF analysis. Fibroblasts were isolated from normal (NF) and SSc (SScF) patients and treated for an additional 24 h with or without rosiglitazone. Cells were fixed with paraformaldehyde and stained to detect (D) α-SMA and (E) vinculin. Cells were counterstained with 4', 6-diamidino-2-phenylindole to detect nuclei.
lesional dcSSc fibroblasts [3, 10, 11]. Moreover, rosiglitazone caused a reduction in the enhanced ability of lesional dcSSc fibroblasts to contact collagen gel matrices, which is a major phenotypic feature distinguishing lesional dcSSc fibroblasts from their healthy counterparts [3]. These results extend recent observations that rosiglitazone can alleviate bleomycin-induced skin fibrosis [12]. In a recent study, we showed that mice deficient in PPAR-γ expression in fibroblasts possess enhanced susceptibility to bleomycin-induced skin fibrosis including elevated Smad3 phosphorylation in response to bleomycin and TGF-β [6]. It is interesting to note, however, that the mere loss of PPAR-γ in mouse skin was insufficient in itself to cause fibrosis [6]. Similarly, small interfering RNA reducing PPAR-γ expression in normal fibroblasts is insufficient to cause either an elevation in Smad3 phosphorylation or in CTGF expression (data not shown). These results suggest that loss of PPAR-γ may not directly result in fibrosis, but may result in an increased sensitivity to certain fibrotic stimuli, such as inflammation. However, it should be pointed out that in our study TGF-β type I (ALK5) receptor/Smad3-dependent activities are not consistently up-regulated in lesional SSc fibroblasts and are not responsible for the overexpression of α-SMA in these cells [13, 14]. Thus, additional mechanisms must mediate the effects of rosiglitazone on SSc fibroblasts. These results collectively suggest that PPAR-γ normally suppresses fibrogenesis in vivo and that the reduction in PPAR-γ expression in SSc fibroblasts contributes to the persistent fibrotic phenotype of these cells.

In summary, our studies examining the involvement in PPAR-γ in the function of dcSSc fibroblasts may have profound implications for fibrotic processes by contributing to our understanding of basic mechanisms regarding fibrogenesis. As a consequence, our results may have future therapeutic implications for the treatment of SSc.

Rheumatology key message

• Rosiglitazone may be considered as a possible new treatment for dcSSc.

Acknowledgements

A.L. is a New Investigator of the Arthritis Society (Scleroderma Society of Ontario), the recipient of an Early Researcher Award and a member of the Canadian Scleroderma Research Group New Emerging Team.

Funding: This work is supported by grants from the Canadian Foundation for Innovation, the Canadian Institutes of Health Research, the Ontario Thoracic Society (to A.L.) and the Arthritis Research Campaign and the Reynaud’s and Scleroderma Association (to D.J.A. and C.P.D.).

Disclosure statement: The authors have declared no conflicts of interest.

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