Original article

Focal adhesion kinase and reactive oxygen species contribute to the persistent fibrotic phenotype of lesional scleroderma fibroblasts

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Abstract

Objective. Fibrotic diseases such as SSc (systemic sclerosis, scleroderma) are characterized by the abnormal presence of the myofibroblast, a specialized type of fibroblast that overexpresses the highly contractile protein α-smooth muscle actin. Myofibroblasts display excessive adhesive properties and hence exert a potent mechanical force. We aim to identify the precise contribution of adhesive signalling, which requires integrin-mediated activation of focal adhesion kinase (FAK)/src, to fibrogenic gene expression in normal and fibrotic SSc fibroblasts.

Methods. We subject either FAK wild-type and knockout fibroblasts or normal and SSc fibroblasts treated with FAK/src inhibitors to real-time polymerase chain, western blot, cell migration and collagen gel contraction analyses.

Results. FAK operates downstream of both integrin β1 and reactive oxygen species (ROS) to promote the expression of genes involved in matrix production and remodelling, including CCN2, α-smooth muscle actin and type I collagen. Blocking either FAK/src with PP2 or ROS with N-acetyl cysteine alleviates the elevated contractile and migratory capability of lesional SSc dermal fibroblasts.

Conclusions. Excessive adhesive signalling is intimately involved with the fibrotic phenotype of lesional SSc fibroblasts; blocking adhesive signalling or ROS generation may be beneficial in controlling the fibrosis observed in SSc.

Key words: adhesion, integrin, ROS, CCN2, FAK, α-SMA, collagen.

Introduction

It has been estimated that nearly 45% of all deaths in the developed world are caused by fibrotic conditions [1].

There is no therapy for fibrosis. An example of a fibrotic disease is the chronic disease diffuse systemic sclerosis/scleroderma (dSSc) in which both the skin and the internal organs can be affected [2]. Within connective tissue, including in dSSc, the critical cell type considered to be responsible for fibrosis is the myofibroblast, a specialized form of fibroblast characterized by the overexpression of the pro-contractile protein α-smooth muscle actin (α-SMA) [3, 4]. α-SMA is organized into stress fibres, which are attached to the extracellular matrix (ECM) through cell surface structures called focal adhesions (FAs), enabling the myofibroblast to adhere to and remodel the ECM [5]. Indeed, fibroblasts isolated from fibrotic lesions of dSSc patients are characterized by excessive adhesion and contraction of ECM [4].

FA components play direct roles in the fibrotic phenotype. The essential cell surface receptors within FAs responsible for binding ECM are the integrins. Integrins are
overexpressed in lesional SSC fibroblasts [5]; integrin β1 is required for the excessive adhesive and contractile phenotype of these cells [6]. Moreover, mice deleted for β1 integrin in fibroblasts are resistant to bleomycin-induced skin scleroderma [7]. Focal adhesion kinase (FAK), which is phosphorylated post-integrin-mediated cell attachment, is a component of FAs [8]. Elevated adhesive signalling, including FAK activation, is a hallmark of fibrotic cells [4, 9]. Moreover, the ability of the cytokine TGF-β to induce pro-fibrotic mRNA expression in normal fibroblasts involves FAK [10]. However, it remains unknown whether FAK promotes the overexpression of fibrogenic genes in lesional SSC fibroblasts.

In this report we investigate the contribution of adhesive signalling to the phenotype of normal or lesional dSSC fibroblasts by assessing whether FAK contributes to a pro-fibrotic phenotype. Our results uncover new and valuable insights into the complex molecular mechanism underlying fibrosis.

**Methods**

**Cell culture**

Mouse embryonic fibroblasts [deleted or not for PTK2 (the gene coding for FAK); CRL-2644 and CRL-02645; American Type Culture Collection, Manassas, VA, USA], a human dermal fibroblast cell line (CRL-2429; American Type Culture Collection) and mouse integrin β1 wild-type and knockout fibroblasts [7] were grown in DMEM containing 10% fetal calf serum, 2 mM l-glutamine, antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) and 1 mM sodium pyruvate (Invitrogen, Burlington, ON, Canada). Lesional scleroderma fibroblasts were grown in the same medium by explant culture from forearm skin of patients with early-onset dcSSc [11]. The group of SSC patients fulfilled the criteria of the ACR for the diagnosis of SSC. Patients were female; age-, sex- and site-matched fibroblasts from healthy individuals were also similarly isolated and cultured normal fibroblasts (NF). NF and scleroderma fibroblasts (SScF) (each from six different individuals) were used between passages 2 and 5 [12]. Ethical approval was obtained from the Royal Free Hospital Ethical Practices Sub-Committee, and informed written consent was obtained before the patients entered the study.

**Histology**

Punch biopsies of lesional SSC and healthy skin were fixed in 10% neutral buffered formalin, embedded in paraffin and 3 μm serial sections were cut on poly-L-lysine-coated slides. Sections were then stained with anti-phospho-FAK (Y397) antibody (1:200; Abcam, Cambridge, UK), and developed using the Impress reagent detection system (VECTOR Laboratories, Peterborough, UK). For double immunofluorescence studies, frozen sections were fixed in 10% formal saline, washed in PBS (pH 7.4) and blocked in 10% goat serum (VECTOR Laboratories) for 15 min at room temperature.

Phospho-FAK (pFAK; Abcam) was added to the sections and left overnight at 4°C. Slides were subsequently washed in PBS, and a goat anti-rabbit Alexa flour 488 conjugate was applied followed by Cy3-conjugated α-SMA antibody (Sigma, Dorset, UK) for 1 h. Slides were counterstained with 4',6-diamidino-2-phenylindole. Photographs were taken with an axioskope microscope using Axiovision software (Zeiss, Welwyn Garden City, UK).

**Real-time PCR**

Real-time PCR was performed as previously described [13, 14]. Total RNA was isolated using Trizol (Life Technologies, Burlington, ON, Canada). Total RNA (25 ng) was reverse transcribed and amplified using TaqMan Assays on Demand (Life Technologies) in a 15-μl reaction volume with TaqMan One-step Mastermix (Life Technologies) and 6-carboxyfluorescein-labelled TaqMan MGB probe and the Applied Biosystems Prism 7900 HT sequence detector (Life Technologies) according to the manufacturer’s instructions. Triplicate samples were run; transcripts and expression values were standardized to values obtained with control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) RNA primers as previously described using the ΔΔCt method [15]. Statistical analysis was performed by the Student’s paired t-test. Less than 10% variation was seen within replicate samples.

**Western blot analysis**

Cells were serum-starved overnight, lysed in 2% SDS, proteins quantified (Fisher, Nepean, ON, Canada) and subjected to western blot analysis as previously described [6, 10], with anti-pFAK (1:500, Cell Signaling, Hitchin, UK), anti-FAK (1:500, Cell Signaling), anti-GAPDH (1:5000, Sigma, St Louis, MO, USA), anti-CCN2 (Abcam, 1:500), anti-pFAK (1:500, Cell Signaling, Hitchin, UK), anti-β-tubulin (1:2000, Sigma) and anti-type I collagen (1:1000, Meridian, Abingdon, UK) antibodies. Cells were incubated for 24 h in the presence or absence of dimethylsulphoxide (DMSO; Sigma), anti-integrin β1 antibody (20 μg/ml; J10, Millipore, Watford, UK), N-acetyl cysteine (NAC; 40 μM), PP2 (10 μM) (Sigma) or PF-573,228 (5 μM) (Tocris, Minneapolis, MN, USA).

**Collagen gel contraction**

Experiments were performed as described previously [4, 16]. Briefly, 24-well tissue culture plates were pre-coated with BSA. Trypsinized fibroblasts were suspended in MCDB medium (Sigma), mixed with collagen solution [one part 0.2 M N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, pH 8.0; four parts collagen (Nutragen, 3 mg/ml; Advanced Biomatrix, San Diego, CA, USA) and five parts MCDB × 2] to yield 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, after which gels were
detached by adding 1 ml of MCDB medium. Contraction of the gel was quantified by photography after 24 h.

Migration assays
Assays were performed as previously described [17]. Cultured fibroblasts were grown on 12-well plates. Medium was removed; cells were rinsed with serum-free medium + 0.1% BSA and cultured for an additional 24 h in serum-free medium + 0.1% BSA. The monolayer was artificially injured by scratching across the plate with a blue pipette tip (approximately 1.3 mm width). Wells were washed to remove detached cells. The cells were then cultured in serum-free medium in the presence of mitomycin C (Sigma, 10 μg/ml) to prevent cell proliferation. After 24 h, five representative images of the scratched areas under each condition were photographed.

Results
The FAK/src inhibitor reduces the expression of mRNAs encoding pro-fibrotic genes in fibroblasts
Inhibition of FAK and related proteins is considered to represent a novel therapeutic approach for cancers [18]. Previously we showed that mRNA expression of α-SMA, CCN2, P4H (prolyl-4-hydroxylase) and THBS-1 (thrombospondin-1) was found to be significantly reduced in mouse embryonic fibroblasts deleted for FAK compared with wild-type mouse embryonic fibroblasts; the FAK/src inhibitor PP2 resulted in a reduction of α-SMA, CCN2, P4H and THBS-1 mRNAs in ptk2+/− fibroblasts [19]. To begin to assess whether the expression of additional pro-fibrotic genes was dependent on FAK, we first confirmed that, in response to PP2 (10 μM, 24 h), the expression of α-SMA, CCN2 and THBS-1 mRNAs was reduced.

Total RNA was isolated from wild-type (ptk2+/+) mouse embryonic fibroblasts treated with (A) DMSO or PP2 (10 μM, 24 h) or (B) DMSO or PF-573,228 (5 μM, 24 h). (C) wild-type (ptk2+/+) and FAK knockout (ptk2−/−) mouse embryonic fibroblasts or human dermal fibroblasts treated with (D) DMSO or PP2 (10 μM, 24 h) or (E) DMSO or PF-573,228 (5 μM, 24 h). Gene transcripts were examined using real-time RT-PCR and compared with GAPDH expression. α-SMA, COL1A1, P4H, CCN2, THBS-1 and VCL mRNAs were detected. Data presented are the average and s.d of three biological replicates, each with four technical replicates. Expression of mRNAs in the presence of DMSO was taken to represent 1.

Fig. 1 FAK/src inhibition decreases expression of pro-fibrotic mRNAs in fibroblasts.
reduced in wild-type mouse embryonic fibroblasts (Fig. 1A). To extend these data we examined whether expression of collagen type 1 alpha 1 (COL1A1) and vinculin (VCL) mRNAs in mouse embryonic fibroblasts was also suppressed by PP2. These mRNAs were chosen as COL1A1 encoded for the α1 chain of type I collagen, a key constituent of the fibrotic phenotype in SSc [20], and VCL encodes vinculin, a key FA component that is especially abundant in the ‘supermature’ FAs of myofibroblasts [21]. We found that PP2 also reduced COL1A1 and VCL mRNAs (Fig. 1A). Similar data were obtained with the selective FAK inhibitor PF-573,228 (5 μM, 24 h) [19] (Fig. 1B). When FAK wild-type and knockout (i.e. ptk2+/+ and ptk2−/−) fibroblasts were compared, the absence of FAK resulted in decreased expression of α-SMA, CCN2, COL1A1, VCL, P4H and TSP-1 mRNAs (Fig. 1C). Either PP2 or the selective FAK inhibitor PF-573,228 also reduced expression of α-SMA, CCN2, COL1A1, VCL, P4H and TSP-1 mRNAs in a human dermal fibroblast cell line (Fig. 1D and E). Please note that both PP2 and PF-573,228 reduced the expression of pro-fibrotic mRNAs in ptk2−/− fibroblasts (data not shown), consistent with the fact that (i) PP2 inhibits src and (ii) PF-573,228 has other kinase targets than FAK [22]. However, as similar results were obtained when (i) ptk2+/+ and ptk2−/− fibroblasts were compared and (ii) ptk2+/+ fibroblasts were treated with PP2 and PF-573,228 (two selective FAK inhibitors that have different structures and mechanisms of action), these data collectively suggest that FAK contributes to the expression of mRNAs encoding pro-fibrotic genes in fibroblasts.

Fig. 2 FAK/src inhibition decreases expression of pro-fibrotic proteins in normal and SSc dermal fibroblasts.

(A) FAK phosphorylation is lesional SSc skin in vivo. Tissue sections taken from normal and SSc skin were subjected to immunohistochemical analysis with an anti-pFAK antibody. Skin of three healthy individuals and three individuals with SSc were analysed. Representative images are shown. Inserts show double immunofluorescence staining for pFAK (green-Alexafluor 488) and α-SMA (red-Cy3). Note abundant pFAK staining in the dermis of SSc skin (arrow) and increased frequency of α-SMA and double-stained cells in the lower dermis of SSc skin compared with the control.

(B) FAK phosphorylation is elevated in cultured SSc fibroblasts in vitro. Total protein was isolated from fibroblasts isolated from skin of six healthy individuals and six individuals with SSc and subjected to western blot analysis with anti-pFAK or anti-FAK antibodies. (C) PP2 reduces expression of pro-fibrotic proteins in normal and SSc fibroblasts (NF and SScF, respectively). Total protein was isolated from fibroblasts isolated from skin of three healthy individuals and three individuals with SSc that had been treated with DMSO or PP2 (10 μM, 24 h). Proteins were detected using antibodies recognizing α-SMA (SMA), type I collagen (Col-1), CCN2, vinculin and GAPDH proteins. Average (±S.D.) values are shown.
The FAK/src inhibitor PP2 reduces the expression of pro-fibrotic proteins in normal and SSc fibroblasts

Lesional SSc fibroblasts are characterized by an elevated ability to adhere to ECM [4]. To begin to assess whether elevated adhesive signalling operating through FAK could play a role in the persistent fibrotic phenotype of this cell type, we first conducted immunohistochemical analysis to show that, compared with healthy control skin, elevated FAK phosphorylation existed in the dermis of SSc patients (Fig. 2A). We then performed double immunofluorescence staining of control and SSc for pFAK (green colour) and α-SMA (red colour) (Fig. 2A inserts). In control sections, pFAK-positive cells were found largely in the upper dermis (green colour); however, a few pFAK-positive cells also expressed α-SMA (yellow/orange colour) in the lower dermis. In contrast, cells expressing pFAK were abundant in the SSc sections, localized to the deeper dermis, and many of these cells also expressed α-SMA (yellow/orange colour). Next, we assessed whether lesional SSc fibroblasts showed enhanced phosphorylation of FAK. To conduct this analysis, dermal fibroblasts from six different individuals with dcSSc were isolated. As controls, dermal fibroblasts from healthy volunteers were used. Western blot analyses of protein extracts prepared from cultured fibroblasts revealed that, compared with fibroblasts from healthy individuals, fibroblasts from lesions of patients with dcSSc showed elevated phosphorylation of FAK (Fig. 2B). Please note that healthy fibroblasts also displayed FAK phosphorylation (Fig. 2B). Because of the identical results obtained with PP2 and the selective FAK inhibitor PF-573,228 on mouse and human fibroblasts, we focused our studies on PP2. Treatment of dcSSc fibroblasts with PP2 resulted in reduced expression of type I collagen, CCN2, VCL and α-SMA protein (Fig. 2C). Note that, consistent with previously published data [23, 24], CCN2 protein was absent from normal dermal fibroblasts. PP2 reduced expression of type I collagen, VCL and α-SMA in healthy fibroblasts (Fig. 2C), consistent with previous data indicating that PP2 treatment resulted in reduced expression of these mRNAs in normal fibroblasts (Fig. 1).

Integrin β1 is required for elevated FAK phosphorylation seen in dcSSc fibroblasts

Previously it was shown that FAK phosphorylation was elevated in lesional SSc fibroblasts [9, 10]. Lesional SSc fibroblasts overexpress integrin β1 [4]; the elevated adhesive properties of this cell type are significantly reduced by a neutralizing anti-integrin β1 antibody [6]. Thus we reasoned that the elevated FAK phosphorylation observed in SSc fibroblasts might be mediated by integrin β1. To begin to test this hypothesis, we incubated healthy and dcSSc fibroblast cells with an anti-integrin β1 antibody identical to what was previously shown to block adhesion [6]. Protein extracts were prepared and subjected to western blot analysis to detect FAK phosphorylation. Application of a neutralizing anti-integrin β1 antibody caused reduced FAK phosphorylation in lesional dSSc fibroblasts (Fig. 3A). Consistent with this observation, mice dermal fibroblasts deleted for integrin β1 [26] were subjected to western blot analysis to detect FAK phosphorylation. Application of a neutralizing anti-integrin β1 antibody caused reduced FAK phosphorylation in lesional dSSc fibroblasts (Fig. 3A). Consistent with this observation, mice dermal fibroblasts deleted for integrin β1 [27] showed reduced FAK phosphorylation (Fig. 3A). Finally, a neutralizing anti-integrin β1 antibody caused reduced CCN2, type I collagen and α-SMA expression in dSSc fibroblasts (Fig. 3B).

To provide a functional context for our observations, both PP2 and a neutralizing anti-integrin β1 antibody reduced the enhanced ability of lesional dcSSc fibroblasts to contract a collagen gel matrix (Fig. 4).

Moreover, an in vitro ‘scratch wound’ assay was used
to show that (i) dcSSc fibroblasts migrated faster than normal fibroblasts and (ii) PP2 and a neutralizing anti-integrin β1 antibody blocked this ability (Fig. 5). Collectively these data are consistent with the notion that elevated integrin β1 activity in dcSSc fibroblasts results in elevated FAK phosphorylation, resulting in the enhanced fibrotic phenotype of this cell type.

**Fig. 5** FAK/src, integrin β1 and ROS are required for the elevated migration of SSc dermal fibroblasts.

Normal and SSc dermal fibroblasts (Fb) were subjected to the scratch wound assay of cell migration in the presence or absence of PP2, NAC or anti-integrin β1 antibody, as indicated. Relative migration after 24 h [average (± S.D.)] of fibroblasts from three healthy individuals and three individuals with dcSSc (SScF) is shown.

Reactive oxygen species are required for elevated FAK phosphorylation in dcSSc fibroblasts

Intracellular reactive oxygen species (ROS) are generated after integrin engagement through rac1; these oxidant intermediates are necessary for integrin signalling during fibroblast adhesion and spreading and wound repair [28, 29]. To assess whether inhibition of ROS could suppress FAK phosphorylation in dSSc fibroblasts and the persistent fibrotic phenotype of dcSSc fibroblasts, we used the antioxidant NAC. Application of NAC to fibroblasts reduced the elevated FAK phosphorylation (Fig. 3A), type I collagen, α-SMA and CCN2 protein expression (Fig. 6), ECM contraction (Fig. 4) and migration (Fig. 5) observed in lesional dcSSc fibroblasts. Please note that GADPH protein expression was not altered by the treatments used (Fig. 6).

Collectively these data are consistent with the hypothesis that elevated integrin β1 expression in lesional SSc contributes to the profibrotic activity of lesional dcSSc fibroblasts through elevated FAK phosphorylation via the generation of ROS.

**Fig. 4** FAK/src, integrin β1 and ROS are required for the elevated ECM contraction of SSc dermal fibroblasts.

Normal and SSc dermal fibroblasts were subjected to the floating collagen gel model of ECM contraction in the presence or absence of PP2, NAC or anti-integrin β1 antibody, as indicated. Relative contraction after 24 h [average (±S.D.)] of fibroblasts from three normal healthy individuals (NF) and three individuals with dcSSc (SScF) is shown.
FIG. 6 Neutralizing anti-integrin β1 antibody or the antioxidant NAC reduces the overexpression of pro-fibrotic genes in human dermal SSc fibroblasts.

Protein extracts were prepared from normal (left three lanes) and SSc (right three lanes) dermal fibroblasts treated with or without NAC or anti-integrin β1 antibody, as indicated. Relative expression [average (± S.D.)] from fibroblasts from three healthy individuals and three individuals with dcSSc is shown. Antibodies detecting type I collagen, α-SMA, CCN2 and GAPDH were used.

Discussion

Much effort has been expended on the contribution of exogenous growth factors (e.g. TGF-β and ET-1 [30, 31]) in promoting fibrogenesis, and in particular, myofibroblast differentiation. However, substantial evidence has now emerged linking mechanical loading and tension to myofibroblast differentiation [4, 26, 27, 32, 33]. In particular, the involvement of elevated mechanical stress on activation of latent TGF-β has been elucidated [26, 32]. Adhesion is not merely a process in which fibroblasts attach to ECM, but is also a key event in elevating the expression of genes that mediate tissue remodelling and repair [19]. Moreover, the propensity of tissues to scar appears to relate directly to the relative adhesive properties of fibroblasts residing within the underlying connective tissue [4, 32, 33].

In our current study we have provided data that, in dcSSc fibroblasts, FAK is constitutively phosphorylated in a fashion that is dependent on integrin β1. These results extend previous data indicating that FAK is constitutively phosphorylated in cultured Ssc fibroblasts [9], and that integrin β1 is essential not only for tissue repair and fibrogenesis in vivo but also for the elevated ability of dcSSc fibroblasts to adhere to ECM [6, 7, 27]. Expression of fibrogenic genes in fibroblasts was reduced in the absence of FAK or in the presence of FAK inhibitors. Addition of the FAK/src inhibitor PP2 suppressed not only the expression of fibrotic genes in normal and SSc fibroblasts, but also the enhanced contractile and migratory properties of this cell type. It is important to note that selective FAK inhibitors have been proposed to represent novel therapeutic approaches for cancers [18, 34]; they may be suitable for fibrotic diseases as well (this report, [35]). Based on results outlined in this report as well as other published studies [9, 10, 19, 21, 25, 31–33, 35–37], we would expect that the FAK/adhesive pathway would play a key role in fibrosis by mediating myofibroblast differentiation and recruitment.

It is interesting to note that NAC, an antioxidant, has been shown to inhibit TGF-β-induced profibrotic responses in lung fibroblasts [38]. Moreover, NAC ameliorates epithelial–mesenchymal transition in alveolar epithelial cells [39]. In the randomized placebo-controlled European Idiopathic Pulmonary Fibrosis International Group Exploring N-acetylcysteine 1 Annual (IFIGENIA) trial in idiopathic pulmonary fibrosis, high-dose NAC given for 1 year together with prednisone and AZA significantly slowed disease progression as visualized by various aspects of lung function [40]. In addition, one study in SSc patients with mild–moderate pulmonary fibrosis reported that i.v. NAC administration was shown to slow the rate of deterioration [41]. Finally, a recent study suggested that NAC monotherapy might be useful to combat fibrosis in early stage idiopathic pulmonary fibrosis patients [42]. Our data provide a mechanistic insight into how NAC might act in SSc, and support the notion that NAC might be used clinically, in the future, to treat the fibrosis in SSc. In this regard, it is important to note that we have used a therapeutically relevant concentration of NAC for our study [43].

These results emphasize the key contribution of enhanced adhesive signalling to the fibrotic phenotype and are consistent with the notion that autocrine-activated adhesive signalling may be sufficient to result in the phenotype of fibrotic diseases such as scleroderma. Blocking adhesive signalling may be of therapeutic benefit in controlling fibrotic disease.

Rheumatology key messages

- FAK/src contributes to the fibrotic phenotype of SSc fibroblasts in a mechanism involving integrin β1 and ROS.
- Inhibitors of FAK/src, integrin β1 or ROS might be used in the future to treat SSc clinically.

Funding: Our work is funded by the Canadian Institute of Health Research (CIHR) and the Scleroderma Society of Ontario (to A.L. and M.B.), the Scleroderma Society and the Arthritis Research Foundation (to C.P.D. and D.J.A.). K.T. is the recipient of summer research awards from the CIHR Strategic Training Initiative in Health Research/Joint Motion Program and the Canadian Scleroderma Research Group.

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