Concise Report

Dermal tissue and cellular expression of fibrillin-1 in diffuse cutaneous systemic sclerosis

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Abstract

Objective. To assess dermal expression and fibroblast production of fibrillin-1 (FBN-1) in SSc.

Methods. In vivo analysis of microfibrillar network was performed using EM from affected and unaffected skin biopsy specimens of dcSSc patients (n = 5) compared with healthy controls (n = 2). FBN-1 matrix deposition and organization by dermal fibroblast cultures from dcSSc (n = 6), healthy (n = 5) and Marfan (n = 4) controls was analysed in vitro by IF with or without TGF-β1 activation. Finally, production of FBN-1 by cultured dermal fibroblasts was evaluated by western blot (WB) and real-time PCR.

Results. We observed a striking decrease of tissue microfibrillar network in the dermis of SSc patients compared with healthy controls affecting both clinically involved and uninvolved skin. In cultures, SSc dermal fibroblasts displayed no apparent in vitro alteration of synthesis, secretion and organization of microfibril network. The WB and real-time PCR analyses showed similar FBN-1 amounts in matrix and FBN1 gene expression in SSc and healthy controls.

Conclusions. We observed a striking decrease of in vivo microfibrillar network in clinically affected and unaffected skin in early dcSSc patients. This does not relate to an inability of SSc dermal fibroblasts to produce, secrete and organize microfibrils in vitro. Therefore, the disturbances of microfibrils in SSc may be a secondary event to matrix remodelling that occurs in this disease.

Key words: Systemic sclerosis, FBN-1, Fibroblasts, TGF-β.

Introduction

Fibrosis is a hallmark of SSc. The fibrosis corresponds to massive deposits of extracellular matrix (ECM) substances such as collagen [1]. Microfibrillar network displays a structural role in the tissue-specific organization of ECM. Fibrillin-1 (FBN-1) is the main component of the 10–12 nm ECM microfibrils [2].

Numerous data support the role of FBN-1 in SSc: (i) the tight skin 1 (Tsk1/+ mouse results in a partial in-frame duplication of FBN-1 and develops a thickened and abnormally structured dermis and is a widely used model for studying SSc [3]; (ii) FBN-1 auto-antibodies have been described in SSc and are suspected to induce a profibrotic phenotype in fibroblasts [4]; and (iii) FBN-1 microfibril abnormalities have been reported in SSc but with conflicting results [5, 6]. Fleischmajer showed an in vivo increased amount of microfibrils in the lower dermis of SSc patients [5], whereas in vitro metabolic labelling studies have suggested a defect of FBN-1-containing microfibrils due to excessive instability [6].

Moreover, several association studies raise the question of the implication of FBN1 gene polymorphisms in genetic susceptibility to SSc in particular in Choctaw Indians [7]. However, in a recent study [8], we found no significant association between FBN1 gene polymorphisms and the disease in a large cohort of European Caucasian SSc patients.
Besides, it is now accepted that ECM is involved in the regulation of various cytokines and growth factors among which TGF-β is the prototypic profibrotic cytokine [9]. Altogether, these data suggest that FBN-1 defects contribute to SSc but we hypothesized that this potential role could not be through FBN1 gene susceptibility. Thus, we investigated whether expression and production of FBN-1 could be altered in the dermis of SSc patients.

Patients and methods

Patients

SSc patients were recruited in the Rheumatology A Department of Cochin University Hospital. All patients had a disease duration of <5 years since the first non-Raynaud symptom and a cutaneous diffuse form according to LeRoy’s criteria [10]. The skin status (affected and unaffected) was clinically evaluated by two clinicians (J.W. and Y.A.). Ethical approval for this study was obtained from CCPPRB Paris-Cochin. All the patients gave written informed consent for participating in the study.

Electron microscopy

Biopsies for EM were performed from clinically affected and non-affected skin of SSc patients and healthy controls who were matched for age and site of biopsy. Ultrathin sections, enclosed in Epon, had been stained by uranyl acetate and lead citrate. Visualization was performed at 40 000× using an electron microscope (Zeiss Le Pecq, France).

Cell cultures

Dermal fibroblasts were explanted from skin biopsy pairs from six SSc and four Marfan syndrome (MFS) patients and from five healthy controls. All SSc patients had systematically one biopsy each in clinically affected and unaffected areas of skin. The healthy controls were matched for age and site of biopsy (forearm area for lesional skin and upper part of the arm for unaffected skin). Fibroblasts were stored in liquid nitrogen (−196 °C) and were used at passages 2–8.

Immunofluorescence

Fibroblasts (250 000 cells/well) were placed into four-well tissue culture Lab-Tek slides (Permanox; Nunc, Rochester, NY, USA). After 24 h, the medium was replaced by fetal calf serum free DMEM with or without TGF-β1 at 10 ng/ml (R&D Systems Europe, Lille, France). After 5 or 6 days, the samples were incubated with primary antibody to FBN-1 (clone 11C1.3; Lab Vision Corporation, Fremont, CA, USA) and were subsequently exposed for 1 h at 37 °C with a Alexa Fluor 488 F(ab)2 fragment of goat anti-mouse IgG (H+L) (Invitrogen, Paisley, UK). After nuclei counterstaining with propidium iodide, visualization was performed with confocal Zeiss LSM 5 Pascal microscopy.

Western blot

At the confluence of fibroblasts, culture medium was eliminated and serum-free DMEM was added with or without TGF-β1 (10 ng/ml) for 6 days. ECM proteins were extracted from cell lysates and were equally loaded into each lane of an SDS–PAGE gel (Invitrogen, Carlsbad, CA, USA). After transfer, nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, USA) were probed with primary antibody mouse monoclonal anti-FBN-1 (clone 11C1.3), and then with the corresponding secondary antibody (goat anti-mouse; Amersham Biosciences, Buckinghamshire, UK). The membranes were reprobed with actin antibody (Santa Cruz, CA, USA) to correct loading differences and efficiency in electrophoretic transfer.

Gene expression assays

Total mRNA was obtained using the RNeasy Mini Extraction Kit (Qiagen, Courtaboeuf, France). Gene expression assays were performed in triplicate as previously described [11] with primer sequences (Applied Biosystems, Courtaboeuf, France) for the FBN1 (Hs00171191_m1) and the GAPDH gene (Hs99999905_m1), the housekeeping control gene. Results were the average of triplicate assays and were expressed in expression arbitrary units (EAUs) of FBN1 gene normalized with respect to the GAPDH gene.

Statistical analysis

Gene expression assays are presented as EAU (s.e.m.). Comparison between any two groups was by unpaired t-test for normally distributed data. P < 0.05 was considered statistically significant.

Results

Electron microscopy

EM results are shown in Fig. 1. EM analysis (40 000×) focused on the microfibrillar network surrounding the elastic fibres but collagen fibres were also visualized. The ‘bead-on-a-string’ appearance of the microfibrils cannot be visualized because rotary shadowed microscopy was not available and electron microscopic magnification was not sufficient. Thus, analysis was restricted to the aspect of microfibrils excluding abnormalities of morphology. The comparison of EM images between control and SSc patients showed that, in vivo, elastin-associated microfibrillar network appeared to be thicker in controls than in SSc. When comparing the dermal SSc elastic and collagen fibres with controls, no difference was detected. In SSc patients, affected and unaffected skin provided globally similar aspect of microfibrils, both showing a decrease.

Immunofluorescence

Without TGF-β1 activation, the classical IF microscopy aspect is similar between healthy controls and SSc: there are a few fluorescent linear stripes that are absent
**Discussion**

Our results show that (i) there is a striking decrease of *in vivo* elastin-associated microfibrillar network in non-affected and affected SSc skin compared with healthy controls; and (ii) this alteration is not due to deficient production, secretion and organization of FBN-1 and microfibrils by SSc dermal fibroblasts.

**Western blot**

The amounts of FBN-1 were evaluated by western blot (WB) of matrix protein from cell lysates of cultured dermal fibroblasts after scraping cells onto dishes. Without TGF-β, fibroblasts from affected and unaffected SSc skin synthesized and secreted equivalent amounts of FBN-1 compared with controls (Fig. 2). The FBN1-mutated Marfan fibroblasts secreted a similar FBN-1 amount to that of the two others. As expected, TGF-β activation increased FBN-1 amounts in the same proportion for controls and SSc from fibroblast cultures of affected or unaffected SSc skin. Marfan fibroblasts responded in the same way to TGF-β induction.

Semi-quantitative analysis from WB was performed using Image J software. The results confirmed the absence of difference between FBN-1 amounts in SSc fibroblast cultures from unaffected and affected skin; in healthy controls (data not shown) without and with TGF-β; and in Marfan with a density of FBN-1 protein after TGF-β activation 2.44-, 1.77-, 1.54- and 2.11-fold, respectively.

**Gene expression analysis**

We postulated that increased gene expression could explain the activation of the *in vitro* organization of FBN-1-containing microfibrils in matrix by TGF-β in SSc and controls and the concomitant increased matricial amounts of FBN-1. To test this hypothesis, gene expression assays by real-time PCR were performed from fibroblast cultures with or without 48 h of TGF-β activation.

At basal state and without TGF-β activation, amounts of FBN-1 mRNA were similar between healthy controls and SSc samples for unaffected skin and affected skin (data not shown). Furthermore, results showed that there was no significant enhancement of *FBN1* gene expression in controls and SSc patients after TGF-β activation. When comparing potential enhancement between controls and SSc, there was no statistical difference.

**Discussion**

Our results show that (i) there is a striking decrease of *in vivo* elastin-associated microfibrillar network in non-affected and affected SSc skin compared with healthy controls; and (ii) this alteration is not due to deficient production, secretion and organization of FBN-1 and microfibrils by SSc dermal fibroblasts.

EM results demonstrated that, *in vivo*, the microfibrillar network associated with elastin appears to be reduced compared with healthy controls in SSc dermal biopsies. Furthermore, this reduction was also present in unaffected skin of these dcSScs, which are able to develop cutaneous fibrosis. This suggests that the histological alteration of microfibrils in SSc could precede clinical involvement. This *in vivo* result should be put in perspective with the fact that the capacity of SSc dermal fibroblasts to produce and organize a meshwork of microfibrils *in vitro* is intact at basal state i.e. without TGF-β activation. These results could reveal that *in vivo* histological abnormalities of the microfibrillar network occur at an early stage of SSc, precede clinical skin fibrosis and thus may be one of the factors participating in the secondary establishment of fibrosis. The observed microfibrillar abnormalities are probably a secondary event of matrix deregulation inherent in SSc.

Kikuchi et al. [12] have raised the hypothesis of a higher sensitivity of SSc fibroblasts to TGF-β activation in
particular for growth regulation. Our IF results suggest that
the role of TGF-β in microfibrillar network organization
seems unaffected in SSc as compared with healthy con-
trols. Secondly, the WB results suggest that the intrinsic
behaviour of SSc fibroblasts in culture with or without
TGF-β is similar between SSc and controls. Our results
are in accordance with a previous study [13]. To go fur-
ther, real-time PCR results confirmed previous published
northern blot results [13] that demonstrated that the influ-
ence of TGF-β on matrix FBN-1 amounts was not through
activation of FBN1 gene expression. Hypotheses about
FBN-1 matrix accumulation upon TGF-β stimulation are:
(i) post-transcriptional stabilization of FBN-1 precursors
into cytoplasm by TGF-β; (ii) recruitment of soluble
FBN-1 from supernatant [13]; (iii) activation of the fibrillin
matrix assembly; and (iv) abnormal proteolysis.

Another matter of debate relates to the difference
of phenotype between fibroblasts from affected and
non-affected areas of the SSc skin in published results.
In our study, investigations from involved and uninvolved
dermis fibroblasts did not detect any behaviour difference.
Currently, opinions are divided on this topic: some
authors support the hypothesis that fibroblasts from
non-affected areas of dcSSc skin samples have the
same pre-clinical activation phenotype as that of the
involved skin [14, 15]; conversely, others describe differ-
ences [16, 17]. Therefore, it remains undefined whether
fibroblasts from affected or non-affected SSc skin share
an identical phenotype.

Finally, some authors think that the biological relation-
ship between FBN-1 and TGF-β in the fibrilliniopathies, of
which SSc is one, seems to be an abnormal activation of
the TGF-β pathway [18]. The main hypothesis, taking into
account the abnormalities of the microfibrillar network
and TGF-β signalling in SSc, might lie in abnormal proteolysis
of FBN-1-containing microfibrillar network with the follow-
ing sequence of events: normal interaction between
FBN-1 and TGF-β but inadequate proteolysis of microfibrillar
network with excessive secondary activation of TGF-β
and its effectors. This hypothesis might be in accordance
with recent published data showing abnormal MMP1
levels in supernatants of reconstructed dermis with fibro-
blasts from lesional late-stage SSc patients [19].
Investigations of these aspects of the pathogenesis of
fibrosis are ongoing.

Conclusions

The in vivo quantitative decrease of the microfibrillar net-
work in both clinically affected and unaffected SSc skin
appears not to be due to a deficiency of SSc fibroblast
FBN-1 synthesis, secretion and microfibrillar assembly.
Our results observed in SSc suggest that disturbances
of the microfibrillar network are probably a secondary
event to matrix deregulation inherent to SSc, which may
be inadequate proteolysis of the microfibrillar network
leading to excessive release of TGF-β, in turn leading to
fibrosis.
SSc patients show a striking decrease in the in vivo microfibrillar network in affected and unaffected skin.
In vitro production, secretion and organization of microfibrils by SSc fibroblasts are normal.

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