Surface morphology and mechanical properties of fibroblasts from scleroderma patients

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Abstract

Overproduction of extracellular matrix components by fibroblasts plays a key role in the pathogenesis of scleroderma. To investigate whether these functional alterations are accompanied by changes in the mechanical properties and morphology of fibroblast, atomic force microscopy was applied to dermal fibroblasts derived either from scleroderma patients or from healthy donors. No significant morphological differences could be observed among the different cell strains showing long cytoskeleton fibres similar in length and irregularly distributed protrusions on the cell surface. In contrast, significant differences in cellular stiffness of dermal fibroblasts derived from scleroderma lesions were detected. Compared to fibroblasts from healthy donors, diseased cells were characterized by a reduced elastic constant both when the global and local mechanical properties were probed. The altered stiffness of scleroderma fibroblasts may be important in the pathogenesis of the disease as it could lead to the abnormal response of fibroblasts to mechanical stimuli.

Keywords: atomic force microscopy • cytoskeleton • extracellular matrix • fibroblast • morphea • systemic sclerosis

Introduction

Systemic scleroderma (SSc) is characterized by a complex, multifaceted pathology involving immunological activation, microvascular damage and progressive fibrosis of the skin and internal organs, including lungs and the gastrointestinal tract [1–3]. The prominent fibrosis of the skin and viscera in SSc is caused by activated fibroblasts that overproduce collagens (*e.g.* types I, III and VII) and other components of the extracellular matrix (ECM), including glycosaminoglycans, fibronectin and decorin [2–5]. In cell culture, the phenotype of activated fibroblasts can be maintained for several passages [2]. The reversibility of this abnormal phenotype suggests that fibroblast activation is an epigenetic phenomenon [2, 3]. The stimuli leading to fibroblast activation remain unclear. It was proposed that fibroblasts might be activated initially

by blood mononuclear cells, platelets, mast cells or endothelial cells, and later on by other fibroblasts in an autocrine and paracrine fashion leading to sustained production of ECM components [2]. The main signalling molecules involved in ECM overproduction were found to be transforming growth factor β (TGF- β), connective tissue growth factor (CTGF), endothelin, interleukin (IL)-1, IL-4 and IL-6 [2, 6, 7].

ECM–cell interactions not only determine the shape and orientation of cells within the ECM but also regulate important functions, including migration, differentiation, and proliferation [8, 9]. Fibroblasts are sensitive to changes in ECM composition and structure. Thus, compared to cells cultivated on planar surfaces, fibroblasts cultured in three-dimensional collagen matrices show different morphologies [10]. Upon mechanical stimulation dermal fibroblasts showed increased proliferation rate and assume a ‘synthetic’ phenotype characterized by augmented connective tissue synthesis. Simultaneous matrix degradation is inhibited [11, 12]. Fibroblasts are the key effector cells in the pathogenesis of SSc. Therefore, we investigated, whether the functional alterations of SSc fibroblasts are accompanied by changes in cell morphology. Applying atomic force microscopy (AFM) [13] dermal fibroblasts

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Table 1 Clinical characteristics of subjects included in the study

Strain	Gender	Age (years)	Disease duration (years)	Antibody	Organ involvement	Biopsy site
SSc-1	Male	65	1	RNA polymerase	Skin, lungs	Lower arm
SSc-2	Male*	71	0.5	Unclassified	Skin, lungs, kidneys	Upper arm
SSc-3	Female	37	2	Scl-70	Skin, lungs, esophagus	Lower arm
LSc	Female	18	5	Absent	Skin	Thigh
Control-1	Male	6	NA	NA	NA	Lower arm
Control-2	Female	31	NA	NA	NA	Lower arm

*This patient also suffered from hypernephroma, NA – not applicable.

from systemic (SSc) and localized scleroderma (LSc) patients were compared with fibroblasts from healthy donors. In contrast to conventional electron microscopy, AFM allowed to characterize cells in their physiological, aqueous environment at nanoscale resolution [14]. Using AFM tip as an indenter the mechanical properties of the different cell strains were studied. Using both approaches we demonstrate that fibroblasts from scleroderma patients are softer than cells derived from healthy donors, while the surface topography is not altered.

Methods

Patients and controls

Skin biopsies were obtained after informed, written consent from two healthy donors, three patients with SSc and one patient with LSc. Details on the clinical characteristics of the analysed patients are demonstrated in Table 1. Patients with scleroderma were not under treatment at the time of biopsy.

Fibroblast culture

Fibroblasts were obtained by enzymatic digestion of dermal tissue. In brief, biopsies were rinsed three times with antibiotics/antimycotics solution (Invitrogen, Karlsruhe, Germany), cut into small pieces (2–3 mm²) and incubated overnight at 4°C with dispase (2 U/ml in DMEM) (Invitrogen). Following the removal of the epidermis, the dermis was disrupted and cells were liberated by vortexing for 5 min. with 0.05% trypsin/EDTA. Cells were recovered by centrifugation (300 g, 4 min.) and maintained in DMEM supplemented with 10% FCS (Invitrogen), 2 mm glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (both from Invitrogen) at 95% relative humidity and 5% CO₂ at 37°C. For imaging, fibroblasts were seeded on ethanol-cleaned glass coverslips (24 mm diameter) inserted into 35-mm tissue culture dishes (Nunc, Wiesbaden, Germany) and grown for 1–3 days. The cells were used between the 2nd and 7th passage.

Measurements of fibroblast mechanical properties

For stiffness measurements cells were trypsinized, centrifuged at 300 g for 4 min. at RT, resuspended in 0.5 ml of CO₂-independent medium (Invitrogen), placed on glass coverslips (~3–4 × 10⁴ cells/coverslip) and allowed to attach for 10–15 min. A BioCell (JPK Instruments, Berlin, Germany) allowed measurements to be conducted at 37°C. To probe global mechanical properties: bead-modified (Ø 5 µm; Kisker, Steinfurt, Germany) and NP-O cantilevers (Veeco Instruments, Santa Barbara, CA, USA) were used. To probe local mechanical properties of cells, NP-S cantilever (Veeco Instruments) having conical styli (tip angle = 35°) with nominal tip radii of ≈20 nm were employed. Cantilevers were calibrated prior to each measurement [14]. The bead-modified cantilevers had a mean spring constants of 75.4 ± 5.3 mN/m and cantilevers with conical tips had a mean spring constants of 73.7 ± 5.9 mN/m. For stiffness measurements beads were pushed onto cells exerting contact forces of ≤0.6 nN while cantilevers having a conical probe were pushed onto cells at forces of ≤0.35 nN. Force-distance curves were collected from the center of the cell. For each cell line a minimum of 25 cells were analysed in two to four independent experiments. At least three force-distance curves were collected per cell with 15-sec periods between approach/retract cycles. The approach speed of the cantilever was set to 5 µm/s. The vertical range pulling of the piezo was 10 µm.

Force-distance curves were processed using in-house algorithms for Igor Pro 5.0 (Wavemetrics Inc, Lake Oswego, OR). A representative force-distance curve is presented in Figs. 1 and 2A. To extract mechanical properties of cells from force-distance curves, Hook's law as well as a method elaborated by Al-Hassan *et al.* [15] were applied to the data. According to Hook's law the spring constant (k_s) of cells was calculated from the indentation depth of the AFM probe into cell (δ) resulting from the applied force (F) (Eq. 1).

$$k_s = \frac{F}{\delta} \quad (1)$$

According to the Hertz-Sneddon model adapted by Al-Hassan *et al.* [15] the elastic constant (k) of cells is related to the indentation depth of the AFM probe into the cell (δ) and the work (W) done by the cantilever by pushing the probe onto the cell as demonstrated in Eqs. (2) and (3). R is the bead radius (Eq. 2), and α is the tip angle (Eq. 3) [15]. The work done by the cantilever is equal to the area under the curve from the contact point to the defined setpoint (Fig. 1).

Fig. 1 Typical force-distance curve collected during approaching and pushing an AFM cantilever probe onto a cell. The work done by the cantilever is equal to the area under the force-distance curve from the contact point to defined setpoint. The contact point corresponds to the moment of touching the cell with the cantilever probe.

$$k_{\text{Bead}} = \frac{8}{15} \frac{\sqrt{R}}{\pi W} \delta^{5/2} \quad (2)$$

$$k_{\text{Tip}} = \frac{1}{6} \frac{\tan(\alpha)}{W} \delta^3 \quad (3)$$

The relation between the elastic constant (k) and the elastic modulus (E) is presented in Eq. (4), where ν is the Poisson ratio.

$$k = \frac{1-\nu}{\pi E} \quad (4)$$

stained with TRITC-labeled phalloidin (Sigma, Munich, Germany) diluted 1:1000 in PBS for 30 min at RT and visualized with a fluorescence microscope (Zeiss Axiovert 200M microscope, Carl Zeiss, Goettingen, Germany).

Statistical analysis

Statistica 7.0 software (Statsoft, Cracow, Poland) was used for running Kolmogorov–Smirnov one-sample test on data to check the normal distribution of analysed parameters. Univariate analyses of variance and Scheffé *post hoc* test were used to assess differences between compared cell lines. Significance was set at $P < 0.05$.

AFM imaging

For AFM imaging, cells were fixed with 4% formaldehyde/PBS for 10–15 min or alternatively with 2% glutaraldehyde/PBS for 1 min, followed by 4% formaldehyde/PBS for 10–15 min. A NanoWizard® AFM (JPK Instruments, Berlin, Germany), mounted on an inverted optical Zeiss Axiovert 200M microscope (Carl Zeiss, Goettingen, Germany) was used. Coverslips were mounted in a custom-metal holder and cells were imaged in PBS containing 1 mM CaCl_2 and 1 mM MgCl_2 at room temperature (RT). Images were acquired in low force contact mode using 200 μm long V-shaped cantilevers, with nominal spring constants of 0.01 N/m (MLCT-AUMN, Veeco Instruments, Santa Barbara, CA, USA). The force applied to the cantilever was set manually to ≈ 50 pN and the feedback gains were manually adjusted to obtain optimal resolution. Images were collected at line-scan rates ranging from 0.7 to 1.2 Hz [14, 16]. Height and vertical deflection images were collected simultaneously in both, trace and retrace direction. Light microscopy images were collected before AFM imaging. Topographical cell surface features of AFM images were analysed with Image Processing® software (JPK Instruments, Berlin, Germany) using the cross-section function. Light microscopy images were processed using MetaMorph® (Universal Imaging Corporation, Downingtown, PA, USA).

Actin staining with TRITC-phalloidin

Fixed cells were washed twice with PBS and permeabilized for 3 min. with 0.5% Triton X-100®/PBS at RT. Unspecific binding sites were blocked with 0.5% BSA/PBS (bovine serum albumin, Sigma, Munich, Germany) for 15 min.,

Results

Fibroblast stiffness

Using a bead-modified cantilever ($\varnothing$ 5 μm), the global stiffness of cells was assed. The average elastic constants of SSc-1, SSc-2 and SSc-3 cell lines were significantly higher than those measured for both control fibroblasts strains ($P < 0.05$) (Table 2, Fig. 1C). The mean spring constants of all three SSc strains calculated according to Hook's law were significantly reduced in comparison to controls ($P < 0.05$) (Table 2, Fig. 1D).

In order to refine these measurements we probed the cell strains with a cantilever equipped with a conical tip to determine fibroblast elasticity in defined, small areas. These measurements confirmed the global surface stiffness measurements. For SSc-2 and -3 cells, the mean elastic constants were significantly higher than for the two controls, while the mean spring constants were reduced (Table 2, Fig. 1E). The corresponding values for SSc-1 and LSc were intermediate between controls and the other two SSc strains (Table 2), indicating some degree of variation in local stiffness, which do not affect global differences.

Using both types of cantilevers, no significant differences were detected between the two control cell strains with respect

Fig. 2 (A) Overlay of force-distance curves representative for control 1 cell line (blue curve), LSC cell line (red curve) and SSC-1 cell line (green curve). All force-distance curves were collected using a 5- μm spherical bead glass glued onto a cantilever. (B) Distribution of the work done by pushing the bead onto control 1 cell line (filled red bars) and SSC-2 cell line (open black bars). (C) Comparison of elastic constants of analyzed cell lines measured by pushing the bead onto the cell surface. (D) Comparison of the spring constants of analysed cell lines measured by the deflection of the AFM cantilever. (E) Comparison of elastic constants of cell lines indented by AFM cantilevers having a conical tip. (F) Comparison of the spring constants of analysed cell lines measured using cantilevers having a conical tip. Abbreviations Standard error, SE; systemic scleroderma, SSC localized scleroderma, LSc.

to work done by the cantilevers, indentation of the cell, elastic or spring constant. Moreover, these four parameters did not change significantly with passage number over four consecutive passages (data not shown), indicating that they are very

stable. In summary, our measurements of fibroblast stiffness suggest that fibroblasts derived from the skin of scleroderma patients are softer than control fibroblasts from healthy donors.

Table 2 Detailed comparison of elastic properties between fibroblasts isolated from scleroderma patients and healthy donors

	Cell line					
	Control 1	Control 2	LSc	SSc-1	SSc-2	SSc-3
Cantilever with 5 μm bead:						
Number of analyzed cells	104	54	78	77	54	53
Applied force (pN)	600	600	600	600	600	600
Work (fJ)	0.26 $\pm$ 0.09	0.27 $\pm$ 0.09	0.3 $\pm$ 0.12	0.32 $\pm$ 0.11	0.37 $\pm$ 0.12	0.29 $\pm$ 0.08
Indentation (μm)	1.11 $\pm$ 0.35	1.01 $\pm$ 0.3	1.18 $\pm$ 0.41	1.38 $\pm$ 0.42	1.58 $\pm$ 0.53	1.38 $\pm$ 0.41
Elastic constant ($\text{m}^2/\text{N} \times 10^{-3}$) (acc. to Eq. 2)	2.0 $\pm$ 0.97	1.68 $\pm$ 0.81	2.02 $\pm$ 1.13	2.81 $\pm$ 1.34	3.4 $\pm$ 1.81	3.19 $\pm$ 1.92
Spring constant ($\text{N/m} \times 10^{-3}$) (acc. to Eq. 1)	0.6 $\pm$ 0.24	0.61 $\pm$ 0.21	0.56 $\pm$ 0.18	0.48 $\pm$ 0.18	0.43 $\pm$ 0.17	0.47 $\pm$ 0.14
Cantilever with conical tip:						
Number of analyzed cells	79	50	82	105	55	50
Applied force (pN)	350	350	350	350	350	350
Work (fJ)	0.16 $\pm$ 0.04	0.16 $\pm$ 0.05	0.21 $\pm$ 0.07	0.19 $\pm$ 0.05	0.21 $\pm$ 0.05	0.18 $\pm$ 0.05
Indentation of the cell (μm)	1.16 $\pm$ 0.29	1.17 $\pm$ 0.35	1.39 $\pm$ 0.52	1.33 $\pm$ 0.32	1.65 $\pm$ 0.34	1.56 $\pm$ 0.45
Elastic constant ($\text{m}^2/\text{N} \times 10^{-3}$) (acc. to Eq. 3)	0.92 $\pm$ 0.67	0.94 $\pm$ 0.66	1.4 $\pm$ 1.5	1.2 $\pm$ 0.61	1.89 $\pm$ 0.87	1.97 $\pm$ 1.61
Spring constant (N/m) (acc. to Eq. 1)	0.33 $\pm$ 0.09	0.33 $\pm$ 0.09	0.29 $\pm$ 0.12	0.29 $\pm$ 0.1	0.23 $\pm$ 0.07	0.25 $\pm$ 0.09

All results are mean $\pm$ standard deviation. Results significantly different from control cell lines are presented in bold

Abbreviations: fJ, femtojoules; LSc, localized scleroderma; m, meter; μm , micrometer; N, Newton; pN, pico Newton; SSc, systemic scleroderma.

Surface topography of fibroblasts

Phase contrast images of all fibroblast strains tested revealed bipolar or stellate shapes with prominent lamellipodia (Fig. 3). Numerous actin stress fibres were detected by TRITC-phalloidin staining (Fig. 3). Usually, the cells extended over more than 100 μm . Since the maximum x,y scan range of AFM was 100 $\times$ 100 μm^2 , scanning was limited to defined areas of the cells.

The most prominent part of the cells was the nuclear region. The mean height of LSc cells was 2.87 $\pm$ 0.85 μm (n = 20), SSc-1 cells 2.49 $\pm$ 0.58 μm (n = 17), SSc-2 cells 2.1 $\pm$ 0.37 μm (n = 19) and SSc-3 cells 2.35 $\pm$ 0.28 μm (n = 22). For comparison, the mean height of control fibroblasts was 2.98 $\pm$ 0.55 μm for control 1 (n = 22) and 2.14 $\pm$ 0.52 μm for control 2 (n = 25) (Table 3). Although statistical analysis revealed some significant differences in height among the cell lines derived from patients, the difference was not significant (P = 0.54) between the mean heights of fibroblasts from all scleroderma patients (2.46 $\pm$ 0.63 (n = 78)) and healthy donors (2.53 $\pm$ 0.68 μm (n = 47)).

The most prominent fibroblast surface structures observed by AFM were long fibres located submembranously parallel to the cells long axis (Figs. 3 and 4). We detected these fibres both in fibroblasts from scleroderma patients and healthy donors. Comparison of AFM images with fluorescent TRITC-phalloidin staining enabled us to identify these filaments as actin stress

fibres (Fig. 3). Their mean number, calculated by scanning the surfaces of 60 SSc and 20 LSc cells, was 22.1 $\pm$ 6.2 and 20.5 $\pm$ 6.5 fibres/10 μm^2 surface, respectively (for details, see Table 3). These values did not differ significantly in control fibroblasts (21.9 $\pm$ 6.8 fibres/10 μm^2 of cell surface, n = 40) (P = 0.63). The height of these fibres was similar in all cell lines (Table 3).

In addition, small, smooth and irregularly distributed protrusions were observed on cell surfaces. The mean height of these protrusions did not differ significantly between scleroderma and control fibroblasts (136.4 $\pm$ 58.2 nm in LSc (n = 50), 155.0 $\pm$ 59.3 nm in SSc-1 (n = 50), 142.2 $\pm$ 48.0 nm in SSc-2 (n = 50), 147.7 $\pm$ 52.7 nm in SSc-3 (n = 50), 156.1 $\pm$ 66.6 nm in control 1 (n = 50) and 144.5 $\pm$ 57.6 nm in control 2 cell line (n = 50) (Table 3)). No significant changes in surface morphology were detected when cells were inspected at different days after seeding and at different passage numbers (data not shown).

The morphology of fibroblast surfaces as visualized by AFM depended on the method of fixation. Cells fixed with formaldehyde showed smoother and more homogenous surfaces with demarcated fibres and sporadic, small protrusions (Fig. 4A–F). Surfaces of fibroblasts fixed with glutaraldehyde followed by formaldehyde were rougher, showed more protrusions and less-pronounced fibres. These cells also featured well-demarcated nuclear regions with distinct nucleoli (Fig. 4G–L). Moreover, they were $\approx$ 10–15% less high (2.35 $\pm$ 0.63 μm) compared to cells just fixed with formaldehyde (2.68 $\pm$ 0.63 μm , P < 0.01).

Fig. 3 Morphology of fibroblasts derived from healthy donor (A) to (D) and localized scleroderma patient (E) to (H). (A) and (E) represent phase contrast images, (B) and (F) fluorescence images recorded after FITC-phalloidin staining, (C) and (G) AFM deflection and (D) and (H) corresponding AFM height images of fibroblasts. Fibrous structures detected by AFM corresponded to FITC-phalloidin stained actin filaments. The vertical full scales of the AFM topographs correspond to 2 μm (D) and 2.5 μm (H). All AFM images were recorded in trace scan direction.

Discussion

AFM can image biological surfaces at nanometre resolution in their natural aqueous environment [16–18]. AFM was also applied to visualize surface morphology of fibroblasts [19–28]. In this study, cultured fibroblasts obtained from patients with localized or SSc were studied by AFM analysing their mechanical properties, surface topology and submembranous actin fibres. The skin of scleroderma patients is characterized by excessive accumulation and dense packing of ECM, in particular collagen bundles. Being embedded in and interacting with this environment, resident fibroblasts are subjected to altered mechanical forces contributing to the characteristic changes in gene expression, *e.g.* enhanced production of ECM proteins, protease inhibitors and fibrogenic mediators [11]. The aim of this study was to assess whether the difference in mechanical loading will be reflected in fibroblast stiffness and surface topology. Scleroderma fibroblasts imaged by AFM revealed stellate or elongated body shapes with prominent fibrous structures on the cell surfaces. Identical structures were also identified on control cells. Parameters such as cell height, and height of protrusions on fibroblasts surfaces did not differ from those on control fibroblasts. Interestingly, the surface morphology of scleroderma fibroblasts and controls alike depended markedly on the fixation method. Cells fixed with glutaraldehyde showed rougher surface and had more protrusions but less clearly visible fibres compared to cells fixed with formaldehyde. These differences in cell surface morphology were most probably caused by an increase in osmolality when adding glutaraldehyde to PBS and making the solution hypertonic for cells (SW Schneider, personal communication). Increased osmolality may lead to partial

dehydration of cells and contraction of the cell body during fixation. Fixation may also influence the rigidity or stiffness of cells, resulting in a surface topology different from that of living cells [19, 26, 29]. A sharp increase in the elastic modulus was observed even after mild glutaraldehyde fixation (0.5% for 60 sec.), accompanied by a dramatic improvement of imaging reproducibility [26]. Comparing our results with previously published data [19, 21], we observed that AFM images of cells fixed with formaldehyde resembled AFM pictures of living fibroblasts whereas images of cells fixed with glutaraldehyde were rather similar to fibroblast images obtained by electron microscopy [19]. Braet *et al.* [19] suggested that imaging living cells reveals submembranous structures, while scanning of fixed cells results in images dominated by surface details such as microvilli, microspikes and membrane ruffles.

Cell mechanical properties extracted from force-distance curves show that fibroblasts derived from scleroderma lesions seem to be softer compared to control cells. It could be suggested that softer cells may be more sensitive to activation by mechanical stimuli leading to enhanced production of ECM components and less sensitive to inhibitory signals. Nho *et al.* [30] observed decreased integrin-linked kinase activity in response to collagen matrix contraction correlating with serine/threonine kinase Akt dephosphorylation and induction of fibroblast apoptosis. *In vivo*, this mechanism seemed to be involved in fibroblast removal during scar resolution after wounding [30]. We speculate that soft fibroblasts from scleroderma skin might be less sensitive to this mechanism and might therefore be protected from apoptosis [10]. This response would lead to cell survival and further production of ECM by activated fibroblasts despite high extracellular collagen content and could thereby contribute to the pathological skin sclerosis in scleroderma patients.

Fig. 4 The surface morphology of fibroblasts derived from the skin of systemic scleroderma patients. Cells were fixed with 4% paraformaldehyde/PBS (A)–(F) and with 2% glutaraldehyde/PBS followed by 4% paraformaldehyde/PBS (G)–(L). (A) to (C) and (G) to (I) represent AFM deflection images with increasing magnification. The corresponding AFM height images are represented in (D) to (F) and (J) to (L). The vertical full scales the AFM topographs correspond to 2.5 μm (D) 2.2 μm (E), 0.4 μm (F), 2 μm (J) and (K) and 0.4 μm (L). All AFM images were recorded in trace scan direction.

Mechanical properties of fibroblasts strongly depend on the intracellular cytoskeleton network [20, 21, 24, 31]. However, although significant differences in stiffness between scleroderma and control fibroblasts were measured, we were unable to detect even subtle changes in the submembranous fibre distribution between scleroderma and control fibroblast. We cannot exclude the possibility that the lack of detection of difference in fibroblast surface morphology was due to artificial cellular conditions, as it was clearly shown that fibroblasts in three-dimensional collagen matrices show different morphologies compared to those cultivated on planar surfaces [10].

The cytoskeletal network in fibroblasts consists of actin microfilaments, microtubules and vimentin intermediate filaments. According to the tensegrity model of cellular architecture [32, 33], the three filament networks are interconnected to physically couple the ECM *via* integrin receptors to the nucleus providing a structure for mechanosensation and mechanosignalling. This model postulates that the cytoskeletal elements are arranged such that actin microfilaments and vimentin intermediate filaments are intracellular tension elements, connected to compression-resistant microtubules. For scleroderma cells with unaltered number and thickness of actin stress fibres, this may mean that

Table 3 Comparison of surface morphology between dermal fibroblasts isolated from healthy controls and patients suffering from scleroderma (all results are mean $\pm$ standard deviation)

	Cell line					
	Control 1	Control 2	LSc	SSc-1	SSc-2	SSc-3
Number of analysed cells	22	25	20	17	19	22
Cell height (μm)	2.98 ± 0.55	2.14 ± 0.52	2.87 ± 0.85	2.49 ± 0.58	2.1 ± 0.37	2.35 ± 0.28
Number of fibres/ $10 \mu\text{m}^2$ of cell surface ($n = 20$)	20.1 ± 4.5	23.7 ± 8.2	20.5 ± 6.5	24.1 ± 7.0	22.9 ± 6.4	19.3 ± 4.3
Height of fibres ($n = 50$) (nm)	53.8 ± 25.6	53.6 ± 30.7	49.6 ± 22.7	52.2 ± 26.0	53.5 ± 30.2	55.1 ± 29.5
Height of protrusions ($n = 50$) (nm)	156.1 ± 66.6	144.5 ± 57.6	136.4 ± 58.2	155.0 ± 59.3	142.2 ± 48.0	147.7 ± 52.7

increased flexibility could result from differences in microtubuli structure or dynamics.

However, viscoelastic properties of cells also depend on factors not related to the cytoskeleton, including nuclear stiffness, cell shape and aging [24, 28, 34]. Cell shape was similar in both control and scleroderma fibroblasts. Cells were round-shaped at the onset of measurement, and all measurements were performed at the largest vertical diameter above the nucleus. For this particular cellular region, we applied the Hertz-Sneddon model, which provides reliable data for thicker parts of the cell [23]. Furthermore, all cells were in early passages. Thus, cell shape and age were probably negligible parameters. By contrast, we cannot rule out that differences in nuclear elasticity contributed to reduced stiffness in scleroderma fibroblasts. Important roles for nuclear stability have recently been implied for cell division [35] and for multiple, as yet unrelated pathological disorders [36]. For scleroderma fibroblasts, the biological relevance for either nuclear elasticity or microtubule dynamics remains to be shown.

The results presented here should be viewed with caution in the context of cells not being homogeneous structures. Thus, the true mechanical properties of different cellular compartments or tissues may differ significantly. Therefore, our data should be considered as an approximation of elastic properties and not as defined constants.

Abbreviations

AFM, atomic force microscopy; BSA, bovine serum albumin; CTGF, connective tissue growth factor; DMEM, Dulbecco's modified Eagle medium; EDTA, ethylenediaminetetraacetic acid; ECM, extracellular matrix; FCS, foetal calf serum; IFN- γ , interferon γ ; IL, interleukin; LSc, localized scleroderma; PBS, phosphate buffer saline; RT, room temperature; SE, standard error; SD, standard deviation; SSc, systemic scleroderma; TGF- β , transforming growth factor β ; TNF- α , tumour necrosis factor α ; TRITC, tetramethylrhodamine isothiocyanate.

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