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Cells from the skin of patients with systemic sclerosis secrete chitinase 3-like protein 1

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ABSTRACT

Background: The chitinase-like protein, Chi3L1, is associated with increased fibrotic activity as well as inflammatory processes. The capacity of skin cells from systemic sclerosis (SSc) patients to produce Chi3L1, and the stimulation of its synthesis by cytokines or growth factors known to be associated with SSc, was investigated.
Methods: Cells were isolated from forearm and/or abdomen skin biopsies taken from SSc patients and normal individuals and stimulated with cytokines and growth factors to assess Chi3L1 expression. Chi3L1-expressing cells were characterized by immunohistochemical staining.
Results: Chi3L1 was not secreted by skin cells from normal individuals nor was its synthesis induced by any of the cytokines or growth factors investigated. In contrast, Chi3L1 secretion was induced by OSM or IL-1 in cells from all forearm biopsies of SSc patients, and endogenous secretion in the absence of cytokines was detected in several specimens. Patients with Chi3L1-producing cells at both the arm and abdomen had a disease duration of less than 3 years. Endogenous Chi3L1 production was not a property of the major fibroblast population nor of myofibroblasts, but rather was related to the presence of stem-like cells not present in normal skin. Other cells, however, contributed to the upregulation of Chi3L1 by OSM.
Conclusions: The emergence of cells primed to respond to OSM with increased Chi3L1 production appears to be associated with pathological processes active in SSc.
General significance: The presence of progenitor cells expressing the chilectin Chi3L1 in SSc skin appears to play a role in the initiation of the disease process.

1. Introduction

Systemic sclerosis (scleroderma, SSc) is a complex autoimmune disease with a highly variable array of clinical features, the most characteristic being an overproduction and excessive deposition of collagen in the skin and internal organs, with a progressive course and often fatal outcome. It is relatively rare, affecting between 50,000 and 100,000 North Americans, and up to 250,000 Europeans [1,2]. Although less common than other rheumatic diseases, it has one of the highest mortality rates [3].

Disturbances of both the immune and vascular systems are thought to contribute to the development of SSc. Endothelial alterations often occur early in the disease, followed by vascular damage that leads to a cascade of stimulatory changes culminating in tissue fibrosis [4]. This process involves T lymphocytes [5], monocytes, macrophages [6] and mast cells [7] as well as fibroblasts [8]. The activated cells secrete a variety of products, including growth factors, cytokines and their antagonists [9]. These substances cause inflammation and increased deposition of extracellular matrix (ECM) components, leading to progressive and widespread tissue fibrosis. The heterogeneity of various forms of SSc and the difficulty in discriminating between disease activity (aspects of the disease that vary over time and are potentially reversible spontaneously or with drug treatment) and disease damage (irreversible tissue injury that results from the disease) [10] complicate studies of SSc. At present there are no validated biomarkers which can be used to monitor disease progression. There is an extensive literature, however, investigating the relationship between many of the cytokines and effector molecules implicated in the various pathological processes associated with the development and progression of SSc [11–15].

The chitinase-like protein, Chi3L1 (YKL40, HCgp39), has been shown to be associated with increased fibrotic activity as well as inflammatory processes. Chi3L1 is upregulated in many pathological conditions [16–23].

Abbreviations: Chi3L1, chitinase 3-like protein 1; DAPI, 4′,6-diamidino-2-phenylindole; ECM, extracellular matrix; IL, interleukin; mRSS, modified Rodnan skin score; OSM, oncostatin M; PDGF, platelet-derived growth factor; αSMA, α-smooth muscle actin; SBTI, soybean trypsin inhibitor; SSc, systemic sclerosis (scleroderma); TIE2, tyrosine kinase with Ig and EGF homology domains-2; TGFβ, transforming growth factor-β

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Elevated serum levels of Chi3L1 are associated with poor prognosis, shorter recurrence-free interval and low overall survival [16,17] in patients with a broad range of cancers, including breast [16] and colorectal cancers [17]. Patients with diseases characterized by inflammation and tissue fibrosis, including rheumatoid arthritis [18], osteoarthritis [19], pneumonia [20], liver cirrhosis [21] and systemic sclerosis (SSc) [22–24] are also reported to have elevated serum Chi3L1 levels. Chi3L1 belongs to the family of mammalian chitinase-like proteins, which share primary sequence homology and three-dimensional structure with the family 18 glycohydrolases [25] but lack chitinolytic activity. The lack of catalytic activity is due to two amino acid substitutions in the active site region of the protein, the most critical one being substitution of the catalytic glutamate residue with leucine [26].

Chi3L1 is produced by macrophages, synovial cells and chondrocytes from arthritic joints [27], and neutrophils [28]. It has been shown to act synergistically with insulin-like growth factor (IGF-1) in fibroblasts to stimulate cell growth [29]. The protein also has mitogenic effects on chondrocytes and synovial cells and promotes proteoglycan synthesis in these cells [30]. Furthermore, Chi3L1 has been shown to be a migration and adhesion factor for vascular smooth muscle cells, which suggests a role in angiogenesis [31]. Previous work has demonstrated that Chi3L1 can modulate the response of connective tissue cells to inflammatory cytokines, such as IL-1 or TNF-α [32]. Chi3L1$^{-/-}$ mice display an exaggerated inflammatory response in a lung injury model [33], supporting this observation. The detailed molecular mechanisms by which Chi3L1 exerts its biological effects are not known.

In SSc, dermal fibroblasts are one of the main effector cells involved in the development of fibrotic lesions, and their biological activity is regulated by a variety of inflammatory cytokines and growth factors. Chi3L1 had been implicated in other pathologies leading to excessive fibrosis [34], and thus its production might be upregulated in affected tissues in patients with SSc. The goals of the present work were to investigate the capacity of skin cells from SSc patients and healthy individuals to synthesize Chi3L1 and to assess the regulation of this process by growth factors and cytokines shown to be associated with this disease.

2. Materials and methods

2.1. Patients, controls, and skin biopsies

Full-thickness biopsies were obtained by an experienced rheumatologist (MB) from the skin of the distal forearm and abdomen of 41 SSc patients recruited from the Canadian Scleroderma Research Group (CSRG) Registry. Similar skin biopsies were also obtained from 10 healthy control individuals. To be eligible for the Registry, patients must have a diagnosis of SSc made by the referring rheumatologist, be age ≥18 years, and be fluent in English or French. Registry patients undergo an extensive clinical history, physical evaluation, and laboratory investigations, and complete a series of self-report questionnaires. Clinical sclerodermatous involvement of skin from biopsy sites was determined by an experienced rheumatologist (MB). Patients and individuals for control biopsies provided written informed consent, and the sample collection and analysis protocols were approved by the McGill University Institutional Review Board. Patients were classified as having limited cutaneous SSc (lcSSc) or diffuse cutaneous SSc (dcSSc) according to the classification by LeRoy et al. [35]. Disease duration was calculated from the date of appearance of the first non-Raynaud's symptom of SSc. This is based on both cutaneous and systemic symptoms, including respiratory symptoms, finger ulcers, inflammatory arthritis, telangiectasia, skin tightening anywhere, fatigue, puffy extremities (hands or feet), weight loss, heartburn or dysphagia and erectile dysfunction.

2.2. SSc clinical outcomes

Using standardized definitions, the recruiting rheumatologist (MB) reported whether or not the patients had active or healed digital ulcers, interstitial lung disease and pulmonary hypertension. Disease activity was measured using the Valentini Scleroderma Disease Activity Index (SDAI) [36,37], consisting of 10 variables with weights ranging from 0.5 to 2.0 and resulting in a total score ranging from 0 to 10. Variables being measured with the SDAI include modified Rodnan skin score (>14), scleredema, change in skin symptoms in the last month, digital necrosis, change in vascular symptoms in the last month, arthritis, lung diffusion capacity <80% predicted, change in cardiopulmonary symptoms, erythrocyte sedimentation rate >30 mm/h and hypocomplementemia [36,37]. Disease severity was measured using physician global assessments of disease severity (scales ranging from 0 to 10) [38]. Predictors of disease severity have been shown to include skin involvement, severity of Raynaud's phenomenon, shortness of breath, gastrointestinal symptoms and pain, number of fingertip ulcers, tender and swollen joints, creatinine, and fatigue [38].

2.3. Cell isolation and culture

Full thickness skin biopsies were collected at bedside and placed into Falcon tubes containing 10 ml Dulbecco's modified Eagle medium (DMEM) (Gibco BRL, Grand Island, NY, USA). To separate epidermis from dermis 0.5% dispase (Invitrogen, Burlington, Canada) was added and the skin biopsies were incubated at 37 °C, in 5% CO_2 for 2 h. Cells were isolated by incubation of dermal samples overnight at 37 °C under gentle mixing in a solution of 0.2% collagenase H (Sigma, St Louis, MO, USA) in DMEM, and 100 U/ml penicillin, and 100 μg/ml streptomycin (Schering Inc., Pointe Claire, Canada). DMEM with 5% fetal calf serum (FCS) was added to neutralize the activity of collagenase. The mixture was centrifuged for 5 min at 500 *g*. The cell pellet was resuspended with 1 ml DMEM, and cell viability was assessed by Trypan Blue exclusion. Cells were then plated in T-75 culture flasks (Becton Dickinson, Franklin Lakes, NJ, USA) and maintained in 12 ml DMEM supplemented with 5% FCS and antibiotics at 37 °C in a 5% CO_2/95% air atmosphere. Culture media were changed every 3 days until cells reached 90% confluency. At this point the cells were sub-cultured at a ratio of 1:3 by trypsinization. It is expected that these cell preparations would be enriched in fibroblasts, but they could also contain any other cell types capable of adherence to tissue culture plastic.

2.4. Stimulation of skin cells by cytokines and growth factors

Skin cells were plated in T-25 culture flasks at 4×10^5 cells/flask and were cultured to near confluence in DMEM supplemented with 5% FCS and antibiotics, followed by 24 h of incubation in serum-free conditions before stimulation with growth factors or cytokines. Cells were either left untreated or treated with 10 ng/ml recombinant interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-17 (IL-17), oncostatin M (OSM), transforming growth factor-β (TGFβ) or platelet-derived growth factor (PDGF) (R&D Systems, Minneapolis, MN, USA) in 3.5 ml DMEM for 48 h. Conditioned media were collected and secreted proteins were analyzed by SDS-PAGE followed by western blotting.

2.5. Soybean trypsin inhibitor (SBTI) biotinylation

Soybean trypsin inhibitor (SBTI, MW 24 kDa) was used as a control for the efficiency of acetone precipitation of cell culture media prior to SDS-PAGE. The protein was biotinylated with sulfosuccinimidyl-6-(biotin-amido) hexanoate (Sulfo-NHS-LC-Biotin; Pierce, Rockford, IL, USA), following the manufacturer's instructions, to allow detection in western blots with the streptavidin–biotin system used for detection of Chi3L1. Excess Sulfo-NHS-LC-Biotin was removed by overnight dialysis (Nominal MWCO: 12,000–14,000; Fisher Scientific, Ottawa, Canada) into 10 l of 100 mM Tris, pH 7.6 at 4 °C. Ten ng biotinylated SBTI was sufficient for use as a control for detection by western blotting.

2.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

Cell conditioned media (300 μl) supplemented with 10 ng biotinylated SBTI were precipitated with 600 μl cold acetone overnight at −20 °C. The pellet was washed twice with 1 ml cold acetone, dried and resuspended in 24 μl H_2O. Sample buffer containing reducing agent was added to the reconstituted pellet sample, and incubated at 70 °C for 10 min. Proteins were then separated on NuPAGE Bis–Tris 4–12%, 1 mm gels, at 180 V for 1 h. The proteins were then transferred to nitrocellulose membrane (0.45 μm) (Bio-Rad, Hercules, CA, USA) at 30 V for 90 min. After transfer, membranes were blocked for 1 h with 5% dried skimmed milk in Tris-buffered saline–Tween 20 (TBS-T). The membranes were probed overnight at 4 °C with primary antibody: rabbit anti-human Chi3L1 polyclonal antibody raised against the C-terminal peptide CGTNAIFDALAAT conjugated to ovalbumin. The membranes were washed four times, with TBS-T followed by incubation with donkey anti-rabbit, biotinylated species-specific antibody (1:5000 dilution; GE Health Care, Little Chalfont, UK) for 60 min. The membranes were again washed with TBS-T, then incubated with a streptavidin–biotinylated horseradish peroxidase complex (1:750 dilution; GE Health Care, Little Chalfont, UK) for 60 min. After washing the membranes with TBS-T, the protein bands were visualized using ECL reagents (GE Health Care, Pittsburgh, PA, USA). Purified Chi3L1, used as a standard, was isolated from human chondrocyte cultures [39].

2.7. Immunofluorescence analysis of cell phenotype

Fibroblasts were subcultured in 6-well plates on microscope coverslips at a density of 2×10^5 cells per well. Cells were cultured to near confluence in DMEM supplemented with 5% FCS and antibiotics, followed by 24 h in serum-free conditions before stimulation with growth factors or cytokines. Cells were either left untreated or treated with 10 ng/ml OSM or TGFβ in 3.5 ml DMEM for 48 h, then rinsed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde at room temperature for 15 min. After three PBS washes, the cells were permeabilized with PBT (PBS plus 1% Triton X-100) for 30 min prior to blocking with 0.1% bovine serum albumin (BSA), 5% goat serum in PBS for 1 h at room temperature. Primary antibodies diluted in blocking solution were then added and incubated overnight at 4 °C. Primary antibodies: rabbit anti-human Chi3L1 polyclonal antibody at 1:200 dilution, mouse anti-human smooth muscle actin (αSMA) monoclonal antibody at 1:1000 dilution (Invitrogen, Camarillo, CA, USA), mouse anti-human nestin monoclonal antibody at 1:200 dilution, mouse anti-human CD73 monoclonal antibody at 1:200 dilution, mouse anti-human STRO-1 monoclonal antibody at 1:200 dilution, mouse anti-human TIE-2 monoclonal antibody at 1:200 dilution, mouse anti-human CD45 monoclonal antibody at 1:200 dilution, mouse anti-human CD34 monoclonal antibody at 1:200 dilution (Abcam, Cambridge, MA, USA), mouse anti-human LSP-1 antibody at 1:200 dilution (Abnova Cooperation, Taipei, Taiwan), and rabbit NG2 chondroitin sulfate proteoglycan polyclonal antibody at 1:200 dilution (Millipore, Temecula, CA, USA). Mouse IgG1 at 1:200 dilution (Abcam, Cambridge, MA, USA) or normal rabbit IgG at 10 μg/ml were used as negative controls. The cells were then washed three times in PBS before incubation for 1 h at room temperature with secondary antibodies: goat anti-rabbit Alexa 488 and goat anti-mouse Alexa 594. Following three PBS washes, the coverslips were mounted inverted on glass slides with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA) and sealed with nail polish. The labeled cells were evaluated by fluorescence microscopy, and quantified by cell counting using 20 representative fields.

2.8. Statistical analysis

Data were analyzed using the Mann–Whitney U test.

3. Results

3.1. Patient characteristics

Sixty-one skin cell cultures from 41 SSc patients (35 women, 6 men; mean age 57 years, range 39–77 years) were used in this work. The mean disease duration was 10.2 years, ranging from 1.3 to 48.9 years. The features of the patients studied are summarized in Table 1. For 20 patients, skin cells derived from biopsies taken from both the arm and abdomen were available and analyzed with respect to Chi3L1 production, allowing comparison of the behavior of cells from the same patient, isolated from these two different sites (Table 2); for 9 patients, only cells derived from the arm were available (Table 3) and for 12 patients, only cells derived from the abdomen were available (Table 3). Modified Rodnan scores and skin scores from the sites where the biopsies were taken are indicated in Table 4. The control group (Table 5) consisted of 9 females and 1 male, with a mean age of 53 years, range 44–61 years. Biopsies from both the arm and abdomen were taken from 5 members of this group, the remainder provided arm biopsies only.

3.2. Chi3L1 secretion by SSc cells

Chi3L1 production and its regulation by cytokines or growth factors thought to be associated with SSc disease pathology were analyzed in 15 cell lines from the skin of 10 control individuals and 61 cell lines from skin biopsies of 41 SSc patients. Cells were stimulated with IL-1β, IL-6, IL-17, OSM, TGFβ or PDGF and Chi3L1 levels in the culture media were analyzed by SDS-PAGE and western blotting. Chi3L1 secretion was not detectable in any of the 15 unstimulated healthy control skin cell lines analyzed, nor was it induced by any of the cytokines or growth factors tested in this study (Fig. 1A). In contrast, endogenous Chi3L1 secretion was observed in 22 of the 61 SSc skin cell lines analyzed. The level of secreted Chi3L1 was increased when these 22 SSc skin cell lines were stimulated with IL-1β or OSM, with OSM having the more prominent effect (Fig. 1B), while IL-6, IL-17, TGFβ and PDGF had no effect. All cell preparations from SSc patients which produced Chi3L1 always responded to both IL-1β and OSM, and there was no synergism between these cytokines. For those 39 SSc cell lines which did not show endogenous Chi3L1 secretion, the protein was detected in 19 of the cell lines after stimulation with IL-1 or OSM. None of the other cytokines and growth factors tested affected Chi3L1 secretion. Since the greatest stimulation of Chi3L1 production was induced by OSM, this cytokine was used in further studies.

SSc skin fibroblasts have been reported to possess unstable phenotypes with respect to ECM production [40]. This could be due to the sampling site of the biopsy or to variations in the stability of the fibroblast phenotype from different individuals. To ascertain that differences in the secretion of Chi3L1 between the different SSc cell lines were not due to instability of the fibroblast phenotype, 3 cell lines that endogenously secreted Chi3L1 were continued in culture from passage 7 to passage 10. Endogenous Chi3L1 secretion was maintained and remained inducible by OSM at all passages though absolute levels did vary (Fig. 2). Moreover, cell lines that did not express Chi3L1 never attained

Table 1
Summary features of patients studied (n = 41).

Variables	Value	% or range
Age (years) [mean]	57	39–77
Female (n)	35	85%
Disease duration (years) [mean]	10.2	1.3–48.9
Type of systemic sclerosis		
Limited (n)	23	56%
Early limited (n)	9	22%
Diffuse (n)	18	44%
Early diffuse (n)	2	5%

Table 2
Chi3L1 secretion pattern of skin cells isolated from paired biopsy sites of SSc patients.

Patient characteristics					Biopsy site					
Patient ID	Gender	Age (years)	Disease duration (years)	SSc subtype	Forearm			Abdomen		
					Clinically involved	Endogenous Chi3L1 secretion	Induction by OSM	Clinically involved	Endogenous Chi3L1 secretion	Induction by OSM
1	Female	53	2.5	Limited	No	+	+	No	+	+
2	Male	69	1.7	Limited	No	+	+	No	+	+
3	Female	58	1.5	Diffuse	Yes	+	+	Yes	+	+
4	Female	52	1.3	Limited	No	+	+	No	+	+
S	Female	55	2.0	Limited	No	+	+	No	+	+
6	Female	63	1.3	Diffuse	No	+	+	No	+	+
7	Female	60	23.5	Limited	No	+	+	No	−	−
8	Female	46	15.6	Diffuse	Yes	+	+	No	−	−
9	Female	78	11.0	Diffuse	No	+	+	No	−	−
10	Male	63	8.8	Diffuse	No	+	+	No	−	−
11	Female	49	2.9	Limited	No	+	+	No	−	−
12	Female	64	14.8	Limited	No	−	+	No	−	+
13	Female	66	5.6	Limited	No	−	+	No	−	+
14	Female	51	13.6	Diffuse	Yes	−	+	Yes	−	+
15	Female	70	28.5	Limited	Yes	−	+	Yes	−	+
16	Female	61	10.3	Limited	No	−	+	No	−	−
17	Female	69	48.9	Limited	No	−	+	No	−	−
18	Male	77	14.5	Limited	No	−	+	No	−	−
19	Female	46	6.9	Diffuse	Yes	−	+	No	−	−
20	Female	55	12.6	Diffuse	Yes		+	Yes	−	−

Cells were isolated from skin biopsies as indicated and cultured in the absence or presence of OSM. Chi3L1 secretion was determined by western blotting. Chi3L1 secretion was induced by OSM in cells isolated from forearm biopsies in all 20 patients. + indicates detection of endogenous Chi3L1 or an increase over the endogenous level induced by OSM, but does not reflect absolute levels.

this property in culture (data not shown). These observations indicate that Chi3L1 is not secreted by normal skin cells nor is its production induced by inflammatory or fibrogenic cytokines in these cells. In contrast it is produced in an inducible and stable fashion by a majority of SSc skin cell preparations and is likely to reflect an in vivo situation.

Three different patterns of Chi3L1 secretion and regulation were observed in the SSc skin cell preparations used for this study (Fig. 3). Group 1 represents the skin cells that constitutively secreted Chi3L1 and this process was stimulated by OSM. Group 2 represents the skin cells that did not endogenously secrete Chi3L1, but for which Chi3L1 secretion could be induced to various levels by OSM. Group 3 skin cells behaved similarly to normal controls, as they did not endogenously secrete Chi3L1 and Chi3L1 secretion was not inducible with OSM. Of the SSc skin cell lines studied, 22 (36%) belonged to Group 1, 19 (31%) to Group 2, and 20 (33%) to Group 3. Absolute levels of endogenous and/or inducible Chi3L1 varied considerably within each group (Fig. 3) ranging from barely detectable to high levels.

3.3. Comparison of response patterns of peripheral and abdominal skin cells

From the data summarized in Tables 2 and 3 it can be seen that Chi3L1 secretion was inducible by OSM in all 29 cell cultures prepared from the peripheral skin (arm biopsies) of SSc patients. Endogenous secretion was observed in 16 (55%) of these preparations. In contrast,

Table 3
Clinical parameters and Chi3L1 secretion from cells of SSc patients obtained from either the forearm or the abdomen.

Patient characteristics							Chi3L1 secretion	
Patient ID	Gender	Age	Disease duration (years)	SSc sub-type	Biopsy site	Biopsy clinically involved	Endogenous	Induced by OSM
25	Female	70	17.5	Diffuse	Arm	No	+	+
27	Female	62	4.7	Limited	Arm	Yes	+	+
28	Female	46	7.1	Diffuse	Arm	No	−	+
30	Female	63	28.9	Limited	Arm	No	−	+
31	Male	53	6.3	Limited	Arm	Yes	+	+
33	Female	63	5.8	Limited	Arm	No	−	+
36	Female	45	5.6	Diffuse	Arm	Yes	−	+
37	Female	57	5.5	Diffuse	Arm	No	+	+
38	Male	43	4.9	Limited	Arm	No	+	+
21	Female	49	10.1	Diffuse	Abdomen	Yes	−	−
22	Female	59	12.3	Diffuse	Abdomen	No	−	−
23	Female	55	6.9	Limited	Abdomen	No	−	−
24	Male	70	10.8	Diffuse	Abdomen	No	−	−
26	Female	43	18.7	Limited	Abdomen	No	−	−
29	Female	56	5.5	Diffuse	Abdomen	No	−	−
32	Female	39	8.8	Diffuse	Abdomen	No	−	−
34	Female	56	9.7	Limited	Abdomen	No	−	−
35	Female	59	7.9	Diffuse	Abdomen	No	−	−
39	Female	62	1.3	Limited	Abdomen	No	+	+
40	Female	67	12.1	Limited	Abdomen	No	−	−
41	Female	63	2.6	Limited	Abdomen	No	+	+

Cells were isolated from skin biopsies obtained from the indicated sites and cultured in the absence or presence of OSM. Chi3L1 secretion was determined by western blotting of culture media. Chi3L1 secretion was induced by OSM in cells isolated from all forearm biopsies. + indicates detection of endogenous Chi3L1 or an increase over the endogenous level induced by OSM, but does not reflect absolute levels.

Table 4
Patient skin scores.

Patient ID	Gender	Age (years)	mRSS	Skin score (arm)	Skin score (abdomen)
1	Female	53	2	0	0
2	Male	69	4	0	0
3	Female	58	33	4	2
4	Female	52	2	0	0
5	Female	55	9	0	0
6	Female	63	13	0	0
7	Female	60	1	0	0
8	Female	46	17	4	0
9	Female	78	10	0	0
10	Male	63	10	0	0
11	Female	49	1	0	0
12	Female	64	2	0	0
13	Female	66	4	0	0
14	Female	51	31	4	4
15	Female	70	3	0	0
16	Female	61	2	0	0
17	Female	69	2	0	0
18	Male	77	2	0	0
19	Female	46	0	0	0
20	Female	55	29	2	2
25	Female	70	10	2	
27	Female	62	10	1	
28	Female	46	10	0	
30	Female	63	36	5	
31	Male	53	7	0	
33	Female	63	7	1	
36	Female	45	9	0	
37	Female	57	39	4	
38	Male	43	6	0	
21	Female	49	2		0
22	Female	59	2		0
23	Female	55	29		1
24	Male	70	2		0
26	Female	43	2		0
29	Female	56	30		0
32	Female	39	7		0
34	Female	56	29		1
35	Female	59	12		0
39	Female	62	4		0
40	Female	67	12		0
41	Female	63	11		0

abdominal biopsies from 32 patients yielded less responsive cell preparations; induction of Chi3L1 secretion by OSM was observed in 12 samples (38%), and 8 of these produced the protein endogenously, while the remaining 20 samples behaved like cells from healthy individuals, i.e. no Chi3L1 production was detectable under any of the experimental conditions. It should be noted that all cell lines where endogenous secretion of Chi3L1 was observed responded to OSM, independent of the biopsy site (arm or abdomen).

Table 5
Chi3L1 secretion from skin cells obtained from healthy control individuals.

Control ID	Gender	Age	Biopsy site	Chi3L1 secretion	
				Basal	Induced by OSM
1	Male	60	Arm	–	–
2	Female	58	Arm	–	–
			Abdomen	–	–
3	Female	50	Arm	–	–
			Abdomen	–	–
4	Female	47	Arm	–	–
5	Female	51	Arm	–	–
			Abdomen	–	–
6	Female	57	Arm	–	–
7	Female	44	Arm	–	–
8	Female	50	Arm	–	–
			Abdomen	–	–
9	Female	49	Arm	–	–
10	Female	61	Arm	–	–
			Abdomen	–	–

Cells were isolated from skin biopsies obtained from healthy individuals and cultured in the absence or presence of OSM. Culture media were analyzed for the presence of Chi3L1 by western blotting. Paired biopsies were obtained from 5 individuals. No Chi3L1 was detectable in any of the 15 samples either in unstimulated cultures or following exposure to OSM.

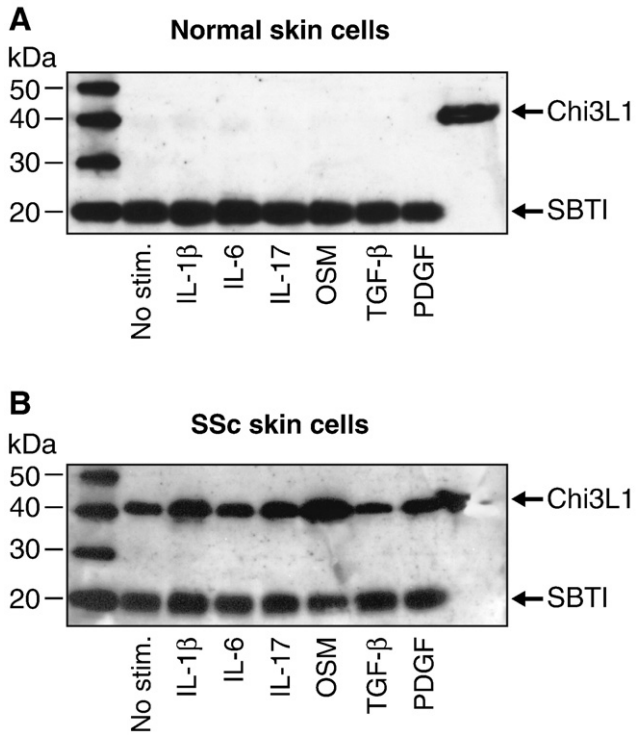

Fig. 1. Comparison of Chi3L1 secretion by normal (A) and SSc (B) skin cells. Results are shown for representative samples from 15 normal skin cells and 22 SSc skin cell lines. Skin cells were grown in monolayer culture and stimulated for 48 h with cytokines or growth factors (IL-1β, IL-6, IL-17, OSM, TGF-β and PDGF). Chi3L1 levels in culture media were measured by SDS-PAGE and immunoblotting. Skin cell lines used in this experiment were in passage 5. The left-hand lane of the gel shows the migration of molecular weight markers, and the right-hand lane shows the migration of purified Chi3L1. Each medium sample was spiked with equal amounts of biotinylated SBTI to monitor acetone precipitation efficiency.

Matching biopsies taken from both the arm and abdomen were available from 20 of the SSc patients used in this study, allowing comparison of the capacity to secrete Chi3L1 relative to the biopsy site in individual patients (Table 2). In 6 patients, unstimulated cells from both sites endogenously produced Chi3L1 and its secretion was increased by OSM, and in a further 4 patients Chi3L1 production was not detectable in unstimulated cells from both sites, but was induced following addition of OSM. No Chi3L1 production either in the absence

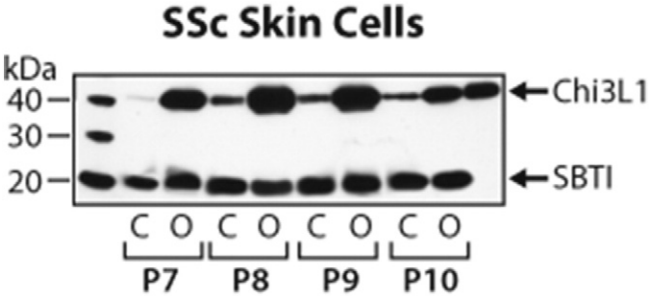

Fig. 2. Comparison of Chi3L1 secretion in response to OSM at different cell passages. One representative sample of 3 SSc skin cell lines that endogenously secreted Chi3L1 and were up-regulated upon stimulation by OSM is shown. Skin cells were grown in monolayer culture with or without OSM stimulation for 48 h. Chi3L1 secretion into the culture medium was measured by SDS-PAGE and immunoblotting. P7–P10: cell passage number; C: unstimulated cells; O: OSM-induced cells. The left-hand lane of the gel shows the migration of molecular weight markers, and the right-hand lane shows the migration of purified Chi3L1. Biotinylated SBTI was added to each medium sample to monitor acetone precipitation efficiency.

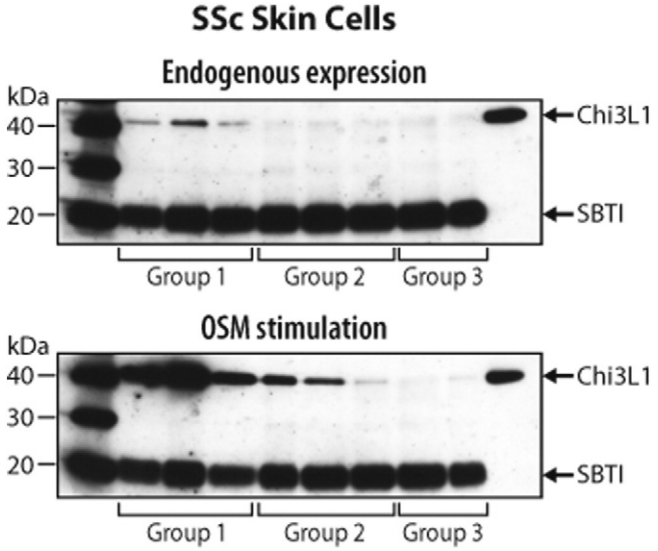

Fig. 3. Classification of SSc skin cell phenotype based on endogenous and OSM-inducible Chi3L1 secretion. Skin cells were grown in monolayer culture with or without OSM stimulation for 48 h. Chi3L1 secretion into the culture medium was then measured by SDS-PAGE and immunoblotting. Skin cell lines used in this experiment were in passage 5. Eight representative samples of 61 cell preparations from 41 SSc patients are shown. Group 1: SSc cells that endogenously secrete Chi3L1 and for which OSM stimulation upregulates Chi3L1 secretion. Group 2: SSc cells that do not endogenously secrete Chi3L1, but the protein is inducible by OSM. Group 3: SSc cells that do not endogenously secrete Chi3L1 and its secretion is not inducible with OSM. The left-hand lane of each gel shows the migration of molecular weight markers, and the right-hand lane shows the migration of purified Chi3L1. Biotinylated SBTI was added to each medium sample to monitor acetone precipitation efficiency.

of stimulation or following addition of OSM was observed in abdominal cell preparations from 10 patients, while peripheral cells from these individuals always produced Chi3L1. This is illustrated in Fig. 4, which shows the response of cells from arm and abdominal skin biopsies taken from two representative SSc patients at the same time and analyzed at the same cell passage level. Skin cells from both the arm and abdomen of patient 14 (Fig. 4A) did not endogenously secrete Chi3L1, but Chi3L1 secretion was inducible by IL-1 and OSM in the cells from both sites. Skin cells from the arm of patient 8 (Fig. 4B) endogenously secreted Chi3L1 and Chi3L1 secretion was upregulated by IL-1 and OSM, but abdominal skin cells from the same patient did not endogenously secrete Chi3L1 nor was the protein inducible by IL-1 or OSM. Thus while peripheral skin cells from SSc patients always contain a cell population which is primed to allow upregulation of Chi3L1 by inflammatory cytokines, this is not always the case for abdominal skin cells.

3.4. Variation in Chi3L1 secretion pattern with disease duration

Chi3L1 secretion patterns did not appear to correlate with any of the typical features associated with SSc. Endogenous secretion and/or inducibility by OSM was observed in patients diagnosed with either limited or diffuse SSc, and in biopsies taken from both clinically involved (fibrotic lesions) and non-involved sites (Tables 2 and 3). Also, Chi3L1 secretion by skin cells of SSc patients was not correlated with skin scores (Table 4) or with clinical outcomes, such as digital ulcers, interstitial lung disease and pulmonary hypertension, disease activity and disease severity (data not shown). However, it was noted that patients exhibiting endogenous secretion of Chi3L1 in cells from both biopsy

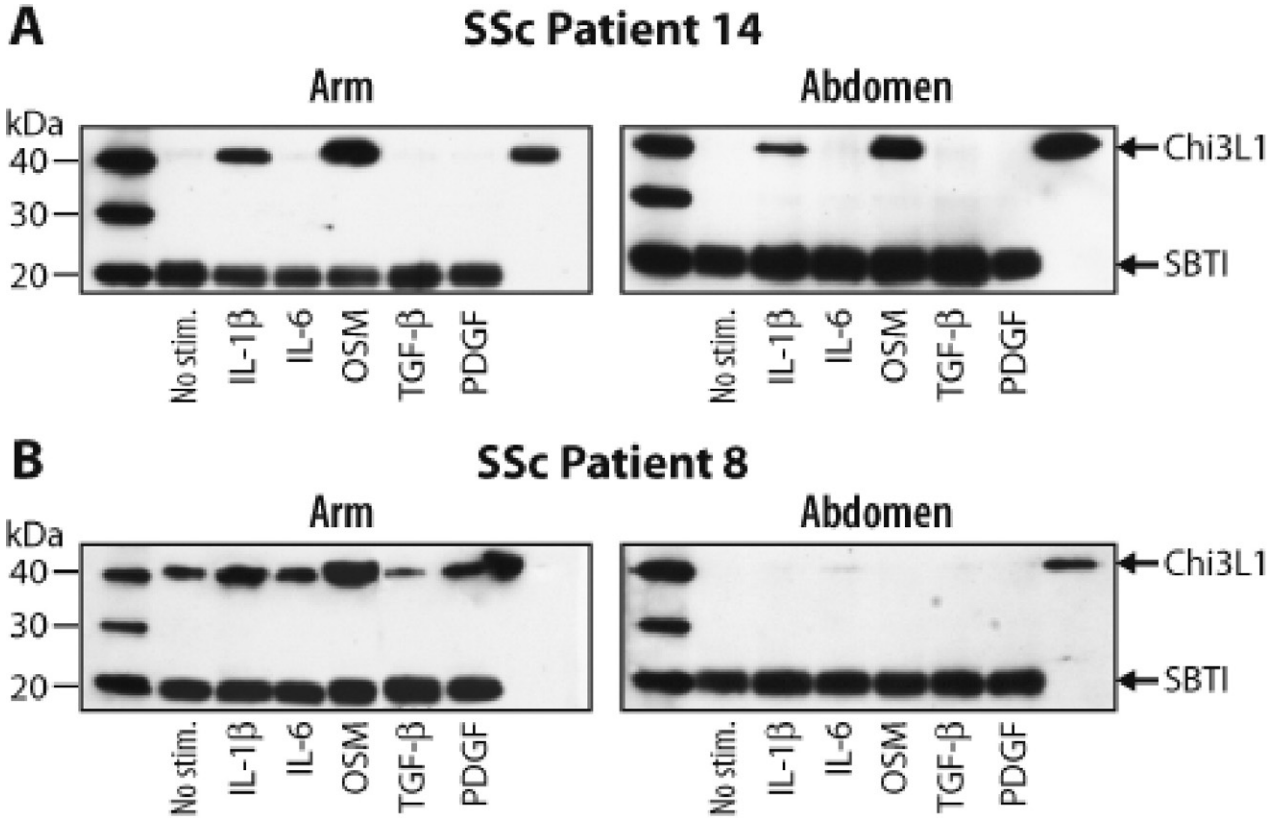

Fig. 4. Comparison of Chi3L1 secretion by skin cells from arm and abdominal biopsies. Results from two SSc patients are shown. Skin cells were grown in monolayer culture and stimulated for 48 h with a variety of cytokines and growth factors (IL-1β, IL-6, OSM, TGF-β and PDGF). Skin cell lines used in this experiment were in passage 5. Chi3L1 secretion into the culture medium was measured by SDS-PAGE and immunoblotting. The left-hand lane of the gel shows the migration of molecular weight markers, and the right-hand lane shows the migration of purified Chi3L1. Biotinylated SBTI was added to each medium sample to monitor acetone precipitation efficiency. Phenotype details of patients 14 (A) and 8 (B) are presented in Table 2.

sites were in the early phase of disease, with a disease duration of <2.5 years (Table 2). The shortest disease duration for patients exhibiting other secretion patterns was 2.9 years with a median of 12.6 years. Mean disease duration was significantly reduced for patients showing Chi3L1 expression in cells from both sites ($P = 0.0005$) and at only the forearm site ($P = 0.026$). This suggests that the capacity for endogenous production of Chi3L1 may be related to pathological processes active at early disease stages.

3.5. Characterization of Chi3L1-expressing cells

Six dermal cell lines from SSc patients previously shown to endogenously produce Chi3L1 in cells derived from both arm and abdomen were analyzed in this study. A heterogeneous cell population was observed, with Chi3L1 endogenous expression only being detected in 5.4–10.7% of the cells, which were often seen in clusters (Fig. 5A). Thus Chi3L1 production is not a feature of the general fibroblast

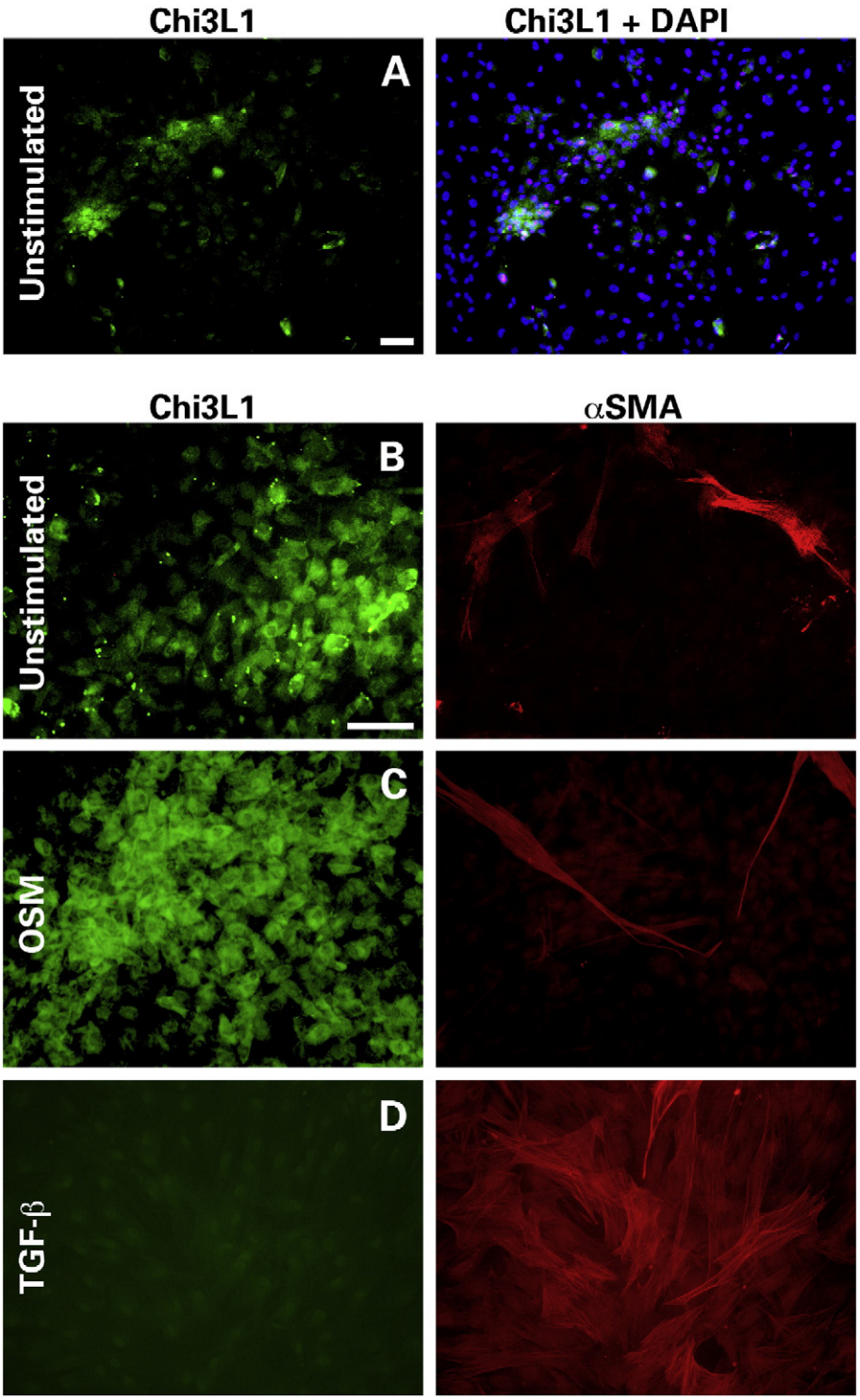

Fig. 5. Identification of cells in SSc dermal cell culture that endogenously express Chi3L1. Cells were identified for Chi3L1 and αSMA, a marker of mature myofibroblasts, using immunofluorescence microscopy. One representative of 6 culture lines is shown for cells in passage 6. (A) The Chi3L1 expressing cells are present in clusters and represent approximately 5–10% of the cell population. (B) Unstimulated Chi3L1-expressing cells do not co-localize with myofibroblasts. (C) OSM stimulation nearly doubles the number of Chi3L1-expressing cells but does not affect myofibroblasts. (D) TGF-β depletes the expression of Chi3L1 but increases the abundance of myofibroblasts. Bars represent 100 μm.

population. Some myofibroblasts with characteristic actin stress fibers are evident in the cell preparations, but they do not co-localize with the Chi3L1 expressing cells (Fig. 5B). Upon OSM stimulation, the number of Chi3L1 expressing cells nearly doubled to 10.2–19.6% of the total cell population, with the cells still being unrelated to the myofibroblasts (Fig. 5C). In contrast, upon TGF-β stimulation, when many of the SSc skin cells are transformed to αSMA-expressing myofibroblasts, Chi3L1 expression is absent (Fig. 5D). This suggests that Chi3L1 expression in SSc dermal cells is up-regulated by pro-inflammatory stimulation but down-regulated by pro-fibrotic stimulation, and that cells other than typical fibroblasts and myofibroblasts are responsible for its production.

In recent years, it has been demonstrated that the dermis of SSc patients may contain a multiplicity of cell types other than fibroblasts and myofibroblasts. These cell types include bone marrow-derived mesenchymal stem cells [41], pericytes [42], endothelial progenitor cells [43], and circulating progenitor cells, such as fibrocytes and monocytes [41, 44, 45]. The SSc cell lines were therefore analyzed for the expression of the markers specific for these other cell types to determine whether they could be the source of Chi3L1 expression. Immunofluorescence dual localization revealed that the SSc dermal cells that endogenously expressed Chi3L1 also co-expressed the progenitor/stem cell marker, nestin (Fig. 6A). These cells also expressed STRO-1, CD73, TIE2, LSP-1 and NG2, but not CD34 and CD45 (results not shown). Cell counting showed that the nestin-positive cells constituted 4.9–9.9% of the total cell population. Dermal cells from healthy controls showed no expression of the aforementioned cell markers (results not shown). Thus endogenous Chi3L1 production in the SSc dermis appears to originate from cells with stem cell-like characteristics. It is, however, not clear if these cells represent a single cell type or are of multiple cell lineages.

While Chi3L1 expressing cells nearly doubled upon OSM stimulation, the number of cells expressing nestin (or STRO-1, CD73, TIE2, LSP-1 and NG2) remained relatively constant at 5.2–10.1% of the total cell population from patients with endogenous Chi3L1 expression. Interestingly, upon OSM stimulation, some of the cells that expressed Chi3L1 most exuberantly did not express the stem cell markers. However, these cells are observed to be closely associated with those that express both Chi3L1 and nestin (Fig. 6B).

In addition, five SSc dermal cell lines that did not endogenously express Chi3L1 but in which the protein was inducible by OSM were also studied. Immunofluorescence for Chi3L1 confirmed that there was no endogenous Chi3L1 expression, and indicated that upon OSM stimulation Chi3L1 expression was induced in approximately 4.1–7.9% of cells. Unlike cells that endogenously express Chi3L1, this group of cells were not stained for nestin (Fig. 6C) or any of the other progenitor/stem cell markers tested.

4. Discussion

In this study we investigated the capacity of skin cells from SSc patients and from healthy individuals to secrete Chi3L1 and we assessed the regulation of this process by growth factors and cytokines shown to be associated with the pathology of SSc. The results demonstrate that Chi3L1 is not secreted endogenously by normal skin cells nor is it

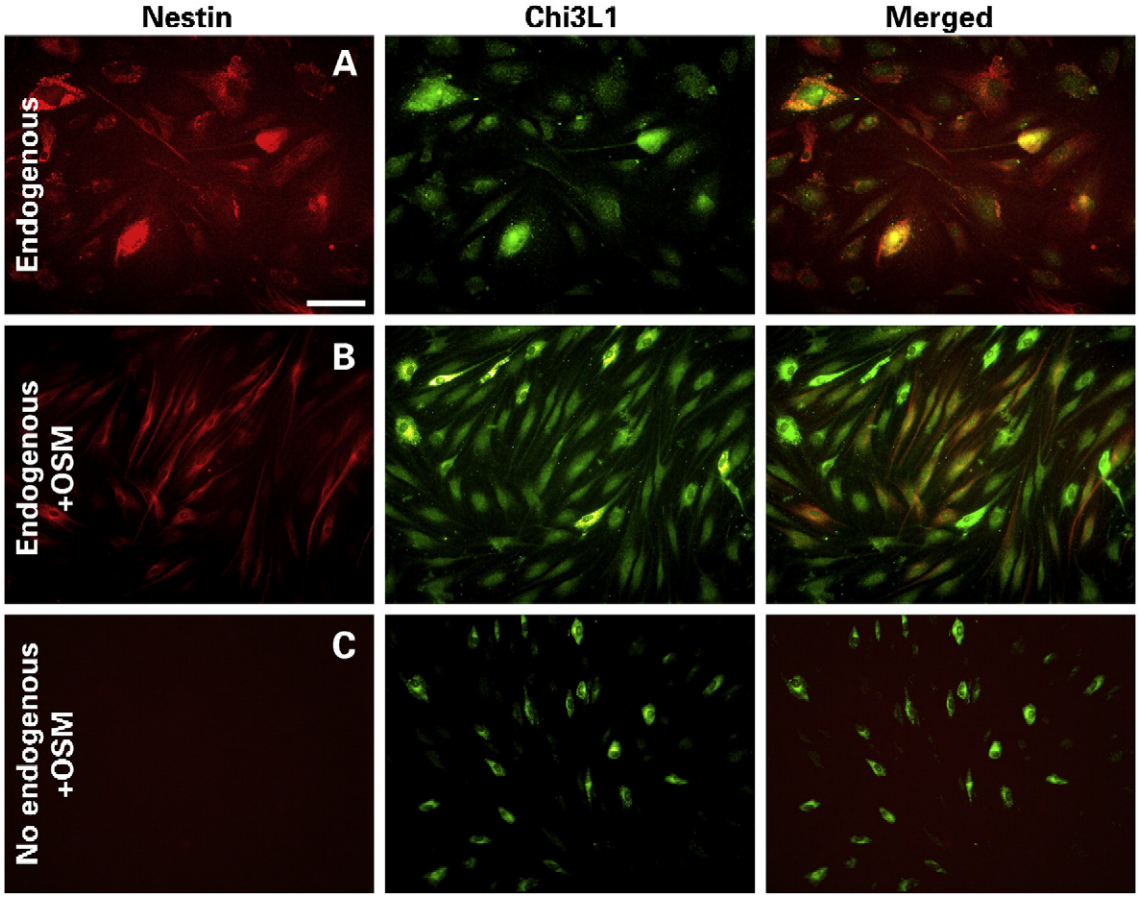

Fig. 6. Co-localization of Chi3L1-expressing cells and nestin by fluorescence microscopy. (A) SSc dermal cell lines that endogenously express Chi3L1 also express nestin. With both markers approximately 5–10% of the cells showed positive staining. One representative of 6 culture lines is shown for cells in passage 6. (B) Upon OSM stimulation of SSc dermal cell lines that endogenously expressed Chi3L1, the number of Chi3L1-expressing cells doubled while the number of cells expressing nestin remained unchanged. The Chi3L1 expressing cells that do not express nestin are in close proximity to those that express both nestin and Chi3L1. One representative of 6 culture lines is shown for cells in passage 6. (C) SSc dermal cell lines that do not endogenously express Chi3L1 also do not express nestin. Approximately 4–8% of these cells can be induced to express Chi3L1 upon stimulation of OSM. One representative of 3 culture lines is shown for cells in passage 6. Bar represents 100 μm.

induced by inflammatory cytokines such as OSM and IL-1. In contrast, all cell preparations obtained from peripheral skin biopsies of SSc patients responded to OSM with increased Chi3L1 secretion into the culture medium, and endogenous production was clearly detectable in a proportion of the cell preparations. This property was independent of clinical status, but cells from both arm and abdomen of subjects with early disease were more likely to demonstrate endogenous secretion of Chi3L1 than those with more advanced disease. The cells responsible for endogenous production of Chi3L1 did not appear to be typical fibroblasts or myofibroblasts, but consisted of about 5–10% of the SSc skin cell population that had stem cell-like features. The increased production of Chi3L1 in response to OSM, however, involved additional cells. Thus the cells involved in fibrosis and contracture appear to be distinct from those responsible for Chi3L1 production.

The lack of Chi3L1 production by normal skin fibroblasts in the absence or presence of growth factors or cytokines is consistent with literature reports [28, 39, 46, 47]. Thus the detection of Chi3L1 in culture media from SSc skin cell preparations was unexpected, indicating the presence of a cell population with an altered phenotype compared to healthy skin. Identification of Chi3L1 producing cells by immunofluorescence microscopy suggested that a relatively small proportion of the cells actually produced Chi3L1 even after exposure to OSM. The cells responsible for endogenous production of Chi3L1 expressed a variety of progenitor/stem cell markers, namely nestin, STRO-1, CD73, TIE2, LSP-1 and NG2. Nestin is a class VI intermediate filament protein reported to be expressed in neural tube-associated neural stem cells [48], but is also used as a general marker for progenitor cells, including skin-derived progenitor cells [49] and endothelial progenitor cells [50]. STRO-1 and CD73 are cell surface proteins commonly used as markers for bone marrow-derived mesenchymal stem cells [51]. TIE2 is an endothelial tyrosine kinase receptor that has been proposed to be the earliest mammalian endothelial cell lineage marker [52]. LSP-1 is a marker for fibrocytes — circulating bone marrow-derived mononuclear cells that have the capacity to deposit extracellular matrix in wound healing and numerous fibrotic disorders, including SSc [53]. NG2 is a cell surface proteoglycan widely expressed in both vasculogenic and angiogenic neovasculature, and is accepted as a marker for pericytes in microvessels [54]. The cells that express Chi3L1 did not stain for CD34 and CD45 (results not shown), suggesting that the cells do not have hematopoietic potential. At present, the origin of the cells co-expressing Chi3L1 and progenitor/stem cell markers is not known, but bone marrow appears to be the common source of such cells. It is also not clear if the ability of the progenitor/stem cells to react with a diversity of markers is a reflection of different transient stages within a single cell differentiation pathway or is an indication of different subpopulations. It is interesting to note that a recent study has shown that Chi3L1 expression is a property of both undifferentiated and differentiated mesenchymal stem cells [55].

The present study showed that OSM stimulation of SSc dermal cell cultures that endogenously express both Chi3L1 and progenitor/stem cell markers nearly doubled the number of Chi3L1 expressing cells but not cells that bear progenitor/stem cell markers. However, the induction of Chi3L1 upregulation is observed to occur in cells in very close proximity to the cells that co-express Chi3L1 and progenitor/stem cell markers. These OSM-responsive cells appear to be activated fibroblasts since they also co-express S100A4 (fibroblast-specific protein, FSP-1) (results not shown), and it is possible that they could represent the differentiated product of the progenitor/stem cells. OSM was also observed to induce expression of Chi3L1 in some SSc dermal cell cultures that did not show endogenous Chi3L1 expression and contained no cells with progenitor/stem cell markers. The cells that were induced by OSM to express Chi3L1 could also represent activated fibroblasts as they also co-express S100A4 (results not shown). As the patients without endogenous Chi3L1 expression tend to have a longer disease duration it is possible that all progenitor/stem cells that were present earlier have now differentiated. Unlike normal fibroblasts, these cells are primed to respond to inflammatory cytokines with increased Chi3L1 production.

While peripheral skin cells from SSc patients always produced Chi3L1 following OSM stimulation, abdominal cells behaved in a more sporadic fashion. This is not unexpected, as peripheral skin is more likely to be clinically involved in SSc than the abdomen. However, the present findings suggest that even when the skin on the forearm appears clinically normal, a pathophysiologic process characterized by the presence of cells capable of Chi3L1 secretion is ongoing. The finding that in matched samples endogenous Chi3L1 secretion tends to be more consistently upregulated in cells from both sites in SSc patients with a short disease duration, suggests that with time the widespread pathological process that is reflected by generalized endogenous Chi3L1 secretion diminishes and is replaced by more focal pathology. If Chi3L1 secretion does indeed reflect an active process, there is no a priori reason for a clear relationship between clinical skin thickening and Chi3L1 secretion. It is possible that Chi3L1 production is not reflected in clinically detectable skin thickening associated with fibrosis because it is either too early for this feature to present, or because turnover of ECM is initially able to keep pace with the increased ECM production associated with fibrosis. It is also unclear whether Chi3L1 has any direct effect on collagen production, though it may do so indirectly by stimulation of fibroblast proliferation [29].

The present study showed that endogenous secretion of Chi3L1 and/or inducibility by OSM did not distinguish patients with limited or diffuse SSc. Also, Chi3L1 secretion by SSc skin cells could not be related to clinical features, such as skin score, digital ulcers, interstitial lung disease, pulmonary hypertension and disease activity. However, other groups have reported that SSc patients with higher Chi3L1 serum levels exhibited an elevated incidence of pulmonary fibrosis, greater disease severity and shorter survival time [23]. An enhanced Chi3L1 serum level has also been reported to be associated with joint involvement in SSc patients [22, 24]. However, the cellular source of serum Chi3L1 is not known, but is likely to reflect variable levels of secretion from many sources. As such, it is not possible to relate the level of serum Chi3L1 to the level in any given tissue. Only direct analysis of specific tissues, as performed in this study, will permit this issue to be resolved.

While the biological functions of Chi3L1 have not been determined in SSc, the protein could be involved in modulating the inflammatory response. Previous work has shown that Chi3L1 can modulate the response of connective tissue cells to inflammatory cytokines by reducing the response of human chondrocytes to IL-1 or TNF-α [32]. This suggests that Chi3L1 is produced in response to pro-inflammatory stimulation, but once it is secreted, it can exert a profibrotic effect by reducing the turnover of extracellular matrix components. Thus, the induction and up-regulation of Chi3L1 by IL-1 and/or OSM in some SSc skin cells could occur during inflammation, where it may limit tissue damage.

Conflict of interest

Author declares that there are no conflict of interest.

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