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BAL FLUID DERIVED FIBROBLASTS DIFFER FROM BIOPSY DERIVED FIBROBLASTS IN SYSTEMIC SCLEROSIS

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ABSTRACT

Growth of fibroblasts from bronchoalveolar lavage fluid (BALF) in patients with systemic sclerosis (SSc) has previously been described. The purpose of this study was to characterize fibroblasts from BALF and bronchial biopsies from SSc patients with alveolitis and from controls, to analyse fibroblast proliferation, migration, stress fibres and proteoglycan production.

BALF and bronchial biopsies were collected from 10 patients with SSc and alveolitis and from 15 controls.

Outgrowth of fibroblasts was observed from the BALF of 4 patients, particularly in patients with a markedly increased percentage of eosinophils in BALF, but in no member of the control group. Increased levels of granulocyte macrophage-colony stimulating factor (GM-CSF), correlating with the percentage of eosinophils in BALF, were found in the patients when compared to the controls. Fibroblasts from BALF showed an elongated, mobile phenotype and increased proteoglycan production compared to the corresponding biopsy fibroblasts.

In conclusion, we report outgrowth of fibroblasts with an altered phenotype from BALF in SSc patients with alveolitis and an increased percentage of eosinophils in the BALF. These findings indicate a possible role for eosinophil-fibroblast interaction in pulmonary fibrosis in SSc.

INTRODUCTION

Systemic sclerosis (SSc, scleroderma) is an autoimmune systemic disease, characterized by microangiopathy and fibrosis in skin and internal organs. Pulmonary fibrosis, with or without pulmonary hypertension, is the major cause of mortality (1) and serious morbidity. Interstitial lung disease, fibrosis and/or alveolitis**,** may be found during post mortem examinations or on high resolution computed tomography (HRCT) in about 90 % of patients (2). Several studies suggest that alveolitis precedes pulmonary fibrosis in SSc patients and may be diagnosed by HRCT or by bronchoalveolar lavage (BAL) in which increased amounts of inflammatory cells in BALF indicate alveolitis (2). Increased percentages of neutrophils and/or eosinophils have been found to be associated with decreased lung diffusing capacity for carbon monoxide (DLCO) (2). Recently, Bouros and co-workers reported that higher levels of eosinophils in BALF were associated with increased mortality in SSc patients (3). High numbers of eosinophils are also found to be associated with poor prognosis in idiopathic pulmonary fibrosis (4).

In our previous study of SSc patients with short disease duration, the production of versican, biglycan and decorin from skin fibroblasts correlated to changes in skin thickness and echogenicity measured by ultrasound (5). In asthma, a disease characterized by peribronchial fibrosis, increased amounts of several proteoglycans are reported in culture medium from bronchial fibroblasts, where also some of these proteoglycans could be related to hyperreactivity (6). Recently we furthermore reported that fibroblasts of a thin, elongated phenotype could be cultured from the BALF of asthmatic patients with increased amounts of eosinophils (7). These fibroblasts were activated α-smooth muscle actin (α-SMA) expressing myofibroblasts and showed increased migration and proteoglycan production when compared with corresponding biopsy fibroblasts. Growth of fibroblasts has been noted from the BALF

of SSc patients with active lung disease (8). These fibroblasts were reported to have a higher expression of collagen and fibronectin compared to normal, adult lung fibroblasts obtained from autopsy.

Cytokines and growth factors, such as endothelin-1 and transforming growth factor–β (TGF-β), released from T-cells and endothelial cells, may have the capacity to activate the fibroblasts to increased proliferation and matrix production (9). Granulocyte-macrophage colony stimulating factor (GM-CSF) is a growth factor, synthesized and released especially by eosinophils, endothelial cells and fibroblasts. GM-CSF has capacity to influence proliferation, differentiation and recruitment of eosinophils and is also shown to affect fibroblasts and endothelial cells, resulting potentially in tissue remodelling and angiogenesis 10). Increased levels of GM-CSF in BALF were found in patients with interstitial lung disease with eosinophilia (11).

The purpose of this study was to characterize fibroblasts from the BALF and from the bronchial biopsies obtained from SSc patients with alveolitis and from controls and to analyse cell content and concentrations of cytokines in the BALF, fibroblast proliferation, migration, stress fibres and proteoglycan production.

MATERIAL

Ten patients, 4 men and 6 women aged between 29 and 69, with a median of 58 years, and suffering from SSc and alveolitis diagnosed by HRCT, were included in the study (Table 1). All patients fulfilled the American College of Rheumatology criteria for systemic sclerosis; 4 patients had diffuse cutaneous systemic sclerosis (dcSSc) (12), and 6 had limited cutaneous

systemic sclerosis (lcSSc). In the majority of patients the disease duration was short, with a median of 1,5 years and a range of 0,5 to 32 years. Nine patients of the 10 were non-smokers. No patient was treated with any putative disease modifying drug. Fifteen healthy non-smoking volunteers, aged between 24 and 58 years, were selected as the control group. The controls were screened with lung function tests and a metacholine inhalation test PC_{20} in order to exclude persons with a pulmonary disease. Pulmonary function in the patients was assessed by vital capacity (VC), as measured by a dry spirometer, and by DLCO, as measured by the single breath method. Both were expressed as a percentage of predicted value (p %). Skin involvement was assessed by a modified Rodnan skin score.

METHODS

After informed consent, a BAL was performed with 100 -150 ml (20 ml x 5-7) of 0.9% NaCl solution. The volume of the recovered lavage fluid varied between 26,5 – 70 ml. After the lavage, bronchial biopsies were taken from the central bronchial part of the lung of 9 patients and of all controls. To grade the alveolitis, differential cell counts of BALF were performed on cytospin preparations stained by the May-Grünewald-Giemsa method. In total, 400 cells were counted by the same person (MW). The HRCT scans were scored by a "blinded" observer (SE) who evaluated the percentage involvement of whole lung of both ground glass opacity (GGO) and reticulation and regions of both GGO and reticulation. In addition for each of these 3 patterns the extent of disease was scored in terms of the number of regions involved (min = 0, max = 6). The scans were split into 6 regions, 3 for each lung, below pulmonary venous confluence, between pulmonary venous confluence and carina, and above carina (Table 2).

For the establishment of fibroblast cultures, BALF and biopsies were incubated in 25 cm^2 tissue culture flasks in Dulbeco modified Eagles's medium, DMEM (Sigma Chemicals, St Louis/MO, USA) supplemented with 10 % fetal bovine serum (Hyclone, Logan/Utah, USA), 2 mM L- glutamin (Sigma), 5 µg/ml Amphotericin B and 50µg/ml Gentamicin. The flasks were stored at 37˚ C in a humidified cell incubator with 5 % CO_2 and 95% atmosphere. When confluent, the cells were loosened from the bottom of the flask by treatment with 0,05% trypsin for 70 seconds and divided into two new bottles.

Concentrations in the BALF of two cytokines were analysed with ELISA; granulocyte-macrophage colony-stimulating factor (GM-CSF), and endothelin-1 (ET-1). The ELISA methods used were Quantikine (R&D system, Abingdon, UK). The detection limits of the tests were 0,26 pg/ml for GM-CSF and 1.0 pg/ml for ET-1.

Proliferation, migration and morphological characterisation

The growth capacity of fibroblasts was evaluated after 72 hours using crystal violet as earlier described (7). For migration assay, 30,000 fibroblasts were seeded and allowed to adhere for 6 hours in a cylinder, usually used for cloning, which was placed into a chamber. The cylinder was removed and the fibroblasts were allowed to migrate for 48 hours. The cells were fixed for 30 minutes in 1 % glutaraldehyde, stained for 2 hours in 0,5 % crystal violet and washed. The distance for 200 cells was counted by measuring the distance from the border of the removed cylinder. Morphological characterisation was performed with crystal violet (13). Cell shape was recorded as the ratio of the length against the width of 200 cells.

Stress fibres

For stress fibre analysis, cells were fixed in 4% paraformaldehyde in PBS. After permeabilisation in 0,5% Triton X-100 and blocking in 1 % BSA in PBS, the cells were

incubated with Alexa Fluor^{TM}488 phalloidin probe (Molecular Probes, Netherlands) diluted in blocking buffer. A fluorescence microscope was used to examine the cells (13).

Immunostaining of α-SMA

Monoclonal mouse antibody against human α- smooth muscle actin (α-SMA) (M0851, Dako, Dakopatts AB, Älvsjö, Sweden) was used followed by Alexa Fluor^{R} 584 goat anti-mouse IgG (Molecular Probes, Netherlands)(13).

Western Blot

The cell lysate was separated by 4-12 % Bis-Tris Gel (Invitrogen, Uppsala, Sweden) in MOPS running-buffer. The separated proteins were transfered to PVDF-membranes and incubated with primary antibodies against α-SMA (DAKO, Glostrup, Denmark). A secondary HRP-Labelled rabbit-antimouse (DAKO, Glostrup, Denmark) antibody was used and the intensity of the bands were analysed using the Gel-Pro^{TM} Analyser software (Media Cybernetics, Silver Spring, MD) (14).

Proteoglycan production

Confluent cultures in passages three to six were labelled 100 μCi/ml medium of (^{35}S)-sulphate for 24 hours. The proteoglycans were isolated using ion-exchange gel (6) and further separated into decorin, biglycan, HSPG and versican on SDS-PAGE (6) and the amounts were calculated per μg protein using a BSA method.

ETHICAL CONSIDERATIONS

The study was reviewed and approved by the local ethical committee (Nr LU 193-01) for patients and controls.

STATISTICS

Levels of significant differences between the two groups were calculated using the Mann Whitney U test for unpaired observations. The relations between variables were calculated with Spearman´s rho.

RESULTS

Cell profile of BALF

Outgrowth of fibroblasts was in SSc patients found in 50 % from biopsies and in 45 % from BALF. In contrast, outgrowth of fibroblasts from biopsies from controls was found in 82 % but no growth was obtained from BALF from any of the controls (Table 2). The total number of cells in BALF in all SSc patients was 0,5-12 x 10^6 (median 2,5, Table 2) compared to 0,8-2,8 x 10^6 (median 1,05) in the controls (p=0,14). Differential counts of BALF in SSc patients divided according to growth of fibroblasts in BALF and in controls are shown in Figure 1**.** SSc patients had increased percentages of eosinophils (median 2,0 %, p<0,01) and neutrophils (median 9,0 %, p<0,05) compared to the controls (median 0 % and 1% respectively), whereas no significant difference was found in the percentage of lymphocytes. High percentages of eosinophils and neutrophils were seen especially in patients with fibroblast growth in BALF (Table 2). There was no correlation between the percentages of neutrophils and eosinophils and VC or DLCO. The two patients in whom an increased BALF percentage of eosinophils were found but no fibroblast growth, were the two most severely affected patients with markedly decreased pulmonary function despite short disease duration. After assessment the patients were treated for one year, 4 with cyclophosphamide, 5 with azathioprine and one,

who was included in a study, with Bosentan. Lung function after one year of therapy is shown in Table 1. VC and DLCO were median 73 and 65 % p before and 85 and 73 % p after therapy in the 7 patients evaluable after one year of therapy.

Levels of GM-CSF and endothelin-1 in BALF.

Concentrations of GM-CSF in BALF were higher in the patients than in the controls ($p<0.02$ A correlation was found between percentage of eosinophils and GM-CSF (Fig 2, $p<0,01$, rho=0,81). The percentage of neutrophils was not related to GM-CSF. When GM-CSF was corrected for % of eosinophils, patients with growth of fibroblasts (N=4) had higher values than patients with no growth (N=3, $p<0,05$). Two patients and 9 controls had no eosinophils and GM-CSF/eos could thus not be calculated. Concentration of total protein was increased in patients compared to controls as expected ($p<0,01$), but was not related to eosinophils. No significant difference was found in BALF concentrations of endothelin-1 (ET-1), median 2.25 (range 0-7,9) pg/ml in patients and 3.0 (range 1,15-9,5) pg/ml in controls (data not shown).

Characterization of BALF fibroblasts

SSc fibroblasts cultured from BALF tended to migrate a longer distance (median 477 μm, n=3) than the biopsy fibroblasts (median 386 μm, Figure 3**).** The degree of elongation of fibroblasts, as determined by the ratio of cell length to cell width, was calculated. The BALF fibroblasts displayed a thin elongated phenotype (ratio median 6,93, n=3), when compared to the fibroblasts cultured from the biopsies (ratio median 3,1, n=3). The fibroblasts were stained to make visible the stress fibres. In BALF fibroblasts, long filaments were seen that were verified to contain long thick F-actin bundles (Figure 4a) which were not seen in corresponding biopsy fibroblasts (Figure 4b). The presence of the myofibroblast marker α-SMA in intact cell cultures is shown in Fig 4 c and d. α-SMA showed no significant

difference between BALF and biopsy derived fibroblasts when measured by Western Blot (median 55 and 57 intensity / ug protein respectively). A higher 72 hour proliferation of BALF fibroblasts (median 2,05, n=3) was found than of biopsy fibroblasts (1,44, n=3). The production of biglycan, decorin, versican and perlecan in BALF fibroblasts and corresponding biopsy fibroblasts in an SSc patient is shown in Figure 5**.** For all four proteoglycans, the production was higher in the BALF fibroblasts than in biopsy fibroblasts
Notably, the fibroblasts from the bronchial biopsies of the SSc patients showed no difference from those of the controls with respect to proliferation, migration, and cell phenotype or proteoglycan production.

DISCUSSION

Our study aimed to characterize BALF derived fibroblasts in SSc patients with alveolitis. We show a) that these fibroblasts differ from fibroblasts obtained by bronchial biopsies in morphology, in capacity to migrate and to produce extra cellular matrix proteoglycans; b) that fibroblast growth from BALF is associated with an increased amount of eosinophils in BALF and c) that the amount of eosinophils in BALF correlates positively with levels of GM-CSF in BALF.

To obtain fibroblasts from lung tissue we performed both BAL and bronchial biopsies. Open lung biopsy would have been preferable but is associated with significant morbidity also when performed thorascopically. Since also healthy controls were included in the study this was not an option. Biopsy sampling is a complicated procedure influenced by several factors such as localisation of the biopsy which may not be representative for the lung. This problem constitutes one of the greatest draw backs with biopsies. Outgrowth of fibroblasts from biopsies was found in 50 % of SSc patients compared to fibroblast outgrowth in 82 % of

controls. The reason for this discrepancy is not clear but similar results were found in patients with asthma with outgrowth of fibroblasts in 55% of biopsies (unpublished observations). We speculate that biopsy fibroblasts may represent resident lung fibroblasts and that either the amount of residential fibroblasts or the local milieu in the tissue may have favoured the increased outgrowth of these fibroblasts in healthy controls during cell culture. In similarity with normal lung fibroblasts (15), biopsy fibroblasts in SSc stained positively for α-SMA and had similar features as biopsy fibroblasts from the healthy controls.

In the present study, outgrowth of α-SMA positive fibroblasts from BALF was observed in patients with SSc and alveolitis, but not in the controls. These observations confirm previous reports of growth of fibroblasts with human smooth muscle cell differentiation features from BALF in 9 of 19 SSc patients, but not in members of the control group (8). BALF fibroblasts were characterized by an elongated, mobile phenotype, with long extended actin bundles, corresponding to the findings in the asthma patients (7). In support of a mobile phenotype of BALF fibroblasts compared to biopsy fibroblasts is the recent finding of an increased expression of the small GTPases RhoA and Rac 1, known to induce stress fibres and focal adhesions, both in patients with SSc and asthma (14). In addition, an increased production of extra cellular matrix proteoglycans in BALF fibroblasts compared to biopsy fibroblasts suggests that BALF fibroblasts may represent one possible source for the increased extra cellular matrix accumulation and fibrosis not only in SSc but also in asthma (7). However, BALF fibroblasts from SSc patients differ from those obtained from asthmatic patients in protein expression pattern of cytoskeletal and scavenger proteins suggesting additional differences and mechanism in the pathogenesis of fibrosis in SSc (14).

The cellular origin of fibroblasts in BALF is not clear. They may represent differentiated resident fibroblasts from the mesenchyme or result from transdifferentiation of other cell types (16). Finally they may have developed from haematopoietic stem cells, such as the previously described fibrocytes recruited by different chemotactic substances (17). Fibrocytes are circulating cells which express both haematopoietic markers such as the surface markers CD34, CD45 and CXCR4 as well as mesenchymal markers such as collagen I and α-SMA. Several factors IL-1, TGF-β, chemokines and serum amyloid P (SAP) modulate the appearance and function of fibrocytes (18). In presence of TGF- β, fibrocytes are differentiated to assume a myofibroblast phenotype expressing α-SMA (19). In a mouse model of allergic asthma, circulating fibrocytes were shown to be recruited into bronchial tissue at allergen exposure and to differentiate to myofibroblasts (20).

In the present study, we noted that fibroblasts could be cultured from BALF in patients with increased levels of eosinophils, similarly to previous observations in asthmatic patients (7). Although he link between high eosinophil count in BALF and outgrowth of fibroblasts is not clear, interaction between eosinophils and fibroblasts has been studied in vitro suggesting a potential profibrotic liaison. Firstly, in co-culture models eosinophils stimulate fibroblast proliferation, collagen production and lattice contraction mediated at least in part by TGF-β (21). Secondly, eosinophils store and release TGF-β, which has been shown to induce myofibroblast differentiation in both fibroblasts and epithelial cells (10,16). Interestingly, GM-CSF derived from human lung fibroblast enhances survival of eosinophils in vitro (22), and GM-CSF added to fibroblast eosinophil co-cultures regulates eosinophil density and function (23). Eosinophils are believed to be important for tissue remodelling in asthma and may also play a role in interstitial pulmonary disease (10). Regarding SSc, increased serum levels of eosinophil cationic protein indicating eosinophil activation has been associated with

decreased lung function (24). However, the role of eosinophils in the pathogenesis of fibrosis in SSc remains to be elucidated. Thus, it is not known whether eosinophils might favour recruitment and differentiation of fibroblasts and fibrocytes into myofibroblasts or on the contrary whether activated fibroblasts release chemokines and cytokines such as eotaxins and IL-15 which favour recruitment and/or survival of eosinophils.

We observed outgrowth of BALF fibroblasts with an altered fibroblast phenotype in patients with both increased eosinophil counts and increased levels of GM-CSF in BALF. Similarly, increased GM-CSF levels were observed in BALF of patients with idiopathic pulmonary fibrosis that was associated with increased eosinophilia (11). These findings may be explained by effects of GM-CSF on eosinophil survival or eosinophil specific recruitment (25), which is reflected by the relationship that was observed between GM-CSF and eosinophils, but not neutrophils. Of interest for the pathogenesis of fibrosis in SSc is the notion that BALF neutrophils correlate to the extent of fibrosis on HRCT, while the percentage of eosinophils correlates to the extent of ground glass opacity, representing alveolitis that is assumed to precede fibrosis development in SSc (26). The majority of patients in the present study had short disease duration and relatively well preserved VC (median 70%), indicating that fibrosis development restricting the pulmonary capacity had not taken place yet.

GM-CSF has been studied in context of fibrosis development especially in asthma and IPF, suggesting a profibrotic effect of GM-CSF (27), e.g. adenoviral vector mediated gene transfer of GM-CSF to rat lung was reported to induce eosinophili and fibrosis and markedly increased TGF-β levels in BALF (28). Until recently GM-CSF has not been studied in the context of SSc. Expression of the GM-CSF receptor-α was confirmed in SSc skin fibroblast in-vitro cultures and GM-CSF had an inhibitory effect on Collagen I protein and mRNA

production suggesting rather an anti-fibrotic effect (29). In contrast, the above data showing increased levels of GM-CSF in BALF of patients with systemic sclerosis associated with increased eosinophilia and an increased outgrowth of proteoglycan producing fibroblasts with a mobile phenotype suggest a more profibrotic function of GM-CSF. GM-CSF could therefore be involved in the recruitment of inflammatory cells eosinophils and /or fibrocytes to the pulmonary interstitium. However, further studies are required to address the exact mechanism of GM-CSF on eosinophil and fibroblast interaction and to clarify the role of GM-CSF in the pathogenesis of SSc.

Our study suggests the coexistence of two different types of fibroblasts in the bronchial tree of patients with SSc. Firstly, “normal” resident lung fibroblasts as obtained by bronchial biopsies and secondly mobile, proteoglycan producing fibroblasts that were associated with high eosinophil counts and increased levels of GM-CSF in BALF . Speculatively, these BALF fibroblasts could be derived from circulating fibrocytes. Surprisingly, no growth was found in the two most severely affected patients with high eosinophil counts, one of whom also had a chronic obstructive pulmonary disease which may have influenced the evaluation of the HRCT scan. Therefore other factors than GM-CSF may be important for promotion of fibroblasts in BALF in SSc, e.g. SAP that has been reported to be the factor in serum that inhibits fibrocyte differentiation (30). SSc patients had lower serum levels of SAP suggesting that low levels of SAP may augment pathological processes leading to fibrosis. The variation within the SSc group was great possibly reflecting the wide spectrum of disease manifestations in SSc .

Systemic sclerosis is rare disease which is difficult to diagnose early and has a wide disease spectrum, which hamper studies of the early disease process. Although the material in the

present study is small and interpretation of the results is difficult, the results might indicate that eosinophil-fibroblast interaction could have an important role in the tissue remodelling in pulmonary fibrosis in SSc. The increased mortality reported by Bouros and co-workers in patients with SSc and high BAL eosinophils (5) and the above findings call for further studies to clarify the role of eosinophils and eosinophil-fibroblast interplay in the pathogenesis of scleroderma pulmonary fibrosis.

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Table 1. Clinical characteristics of the 10 included SSc patients

Form	F/M	Dis.dur (years)	Age (years)	Autoantibodies ANA	ACA	ENA	Skin score	Lung function Baseline			Therapy	Lung function After 1 year therapy	
								VC(%p)	DL_{CO}(%p)	FEV(%)		VC (%p)	DL_{co}(%p)
lcSSc	F	1	29	+	+	-	2	66	61	96	Aza	89	73
lcSSc	F	8	55	+	+	-	4	78	65	76	Aza	88	76
lcSSc	M	32	60	+	-	SSA,SSB,RNP	4	101	80	75	Aza	111	70
dcSSc	M	1	41	-	-	-	9	73	32	82	Aza	80	42
dcSSc	M	0,5	56	-	-	anti-Scl70	19	56	32	90	Cyc	†	
lcSSc	F	7	68	-	-	anti-Scl70	8	73	57	86	Aza	*	
lcSSc	F	4	68	+	-	anti-Scl70	8	69	49	81	Cyc	**	
lcSSc	F	1	69	+	-	anti-Jo	3	106	85	79	Cyc	120	94
dcSSc	M	1	59	+	-	-	34	62	28	83	Bos	71	39
dcSSc	F	2	57	+	-	-	20	70	81	83	Cyc	81	88

lcSSc = limited form, dcSSc = diffuse form, VC = Vital capacity DL_{CO} = Carbon monoxide diffusing capacity %p = % of predicted value
† died within the first year. * CNS lymphoma ** Progressed on HRCT, Apergillus infection.
Aza=Azathioprine, Cyc=Cyclophosphamide, Bos = Bosentan

Table 2. Growth of fibroblasts, recovery , differential counts and HRCT scores in 10 SSc patients with alveolitis.

Dis.form	Growth of fibroblasts	Recovery		Differential counts			HRCT GGO	
	BAL/biopsy	ml (%)	Cells 10^6	Lymph (%)	Neutr (%)	Eos (%)	% of lungs	Nr of regions
lcSSc	-/ nd	70 (50)	0,5	0	0	0	3	4
lcSSc	-/-	50 (36)	2,0	9	0	0	20	4
lcSSc	+/+	38 (25)	12	8	42	2	5	2
dcSSc	+/+	27 (18)	0,8	13	10	4,5	60	6
dcSSc	-/+	48 (48)	6,4	9	4	5	nd	nd
lcSSc	-/-	70 (64)	2,9	8	3,5	0,5	5	2
lcSSc	*	57 (71)	5,7	12	19,5	3,5	70	6
lcSSc	+/+	41 (34)	3,6	25,5	23	4	25	5
dcSSc	-/-	50 (50)	1,5	11	9	5	30	6
dcSSc	+/-	40 (50)	1,2	8,5	28	2	10	2

* Cultures discarded because of contamination nd = not determined

GGO = ground glass opacity

Legends

Fig 1. Differential counts of bronchial lavage fluid (BALF) (mean and SEM) in 9 SSc patients with alveolitis and in 13 controls. The patients are divided according to growth of fibroblasts (n=4) or no growth (n=5) from BALF. The fluid from one patient was discarded because of contamination.

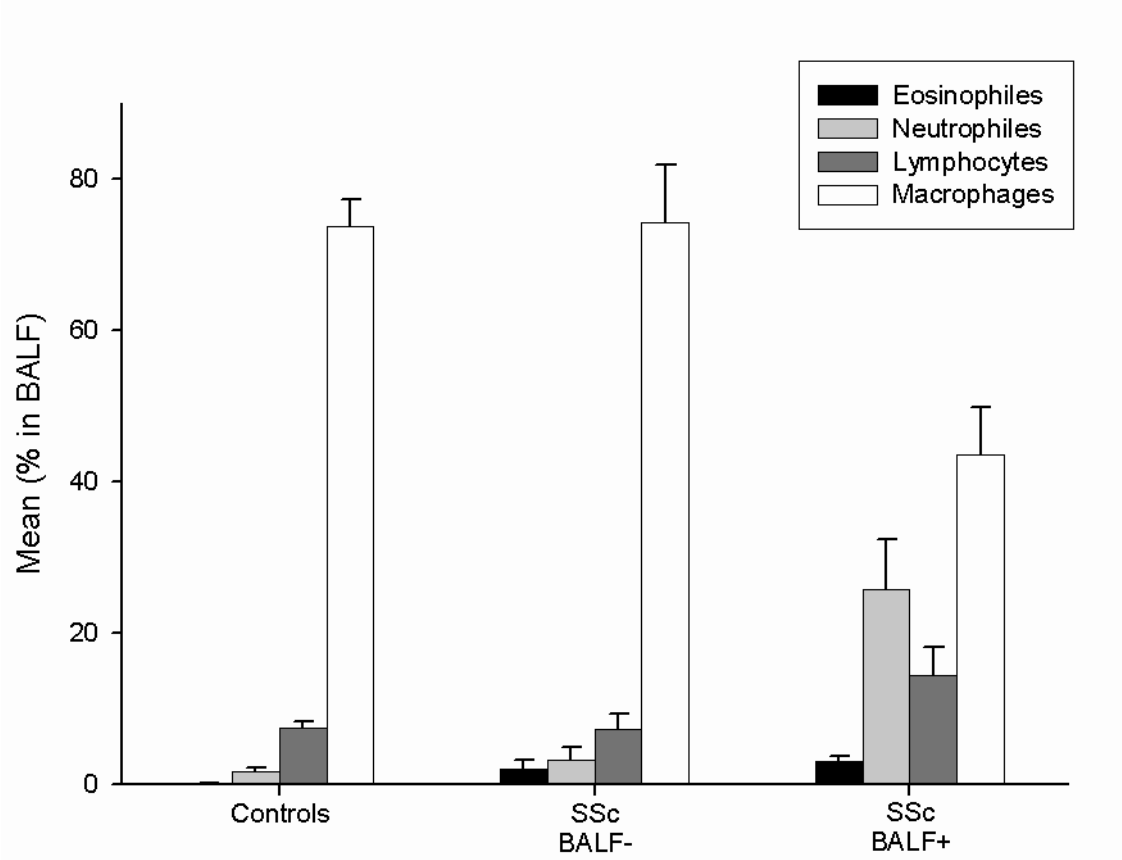


Fig 2. Correlation between concentration of GM-CSF (pg/ml) and percentage of eosinophils in BALF in 9 patients with SSc and alveolitis. The patients with growth of fibroblasts from BALF are marked with open symbols in the figure.

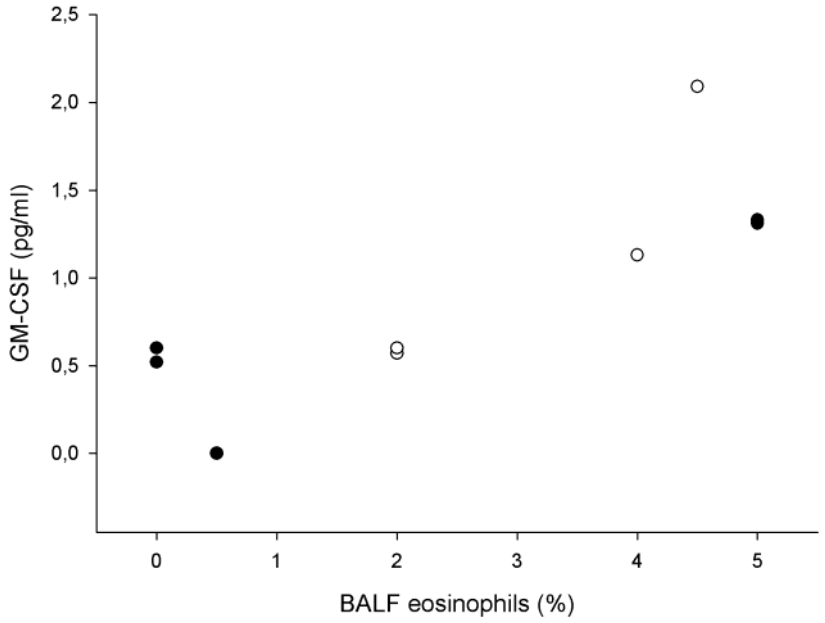

2,5
2,0
1,5
1,0
0,5
0,0
GM-CSF (pg/ml)
0
1
2
3
4
5
BALF eosinophils (%)

Fig 3. Migration (μm) of fibroblasts cultured from BALF and from bronchial biopsy in SSc patients with alveolitis. Medians are marked in the figure.

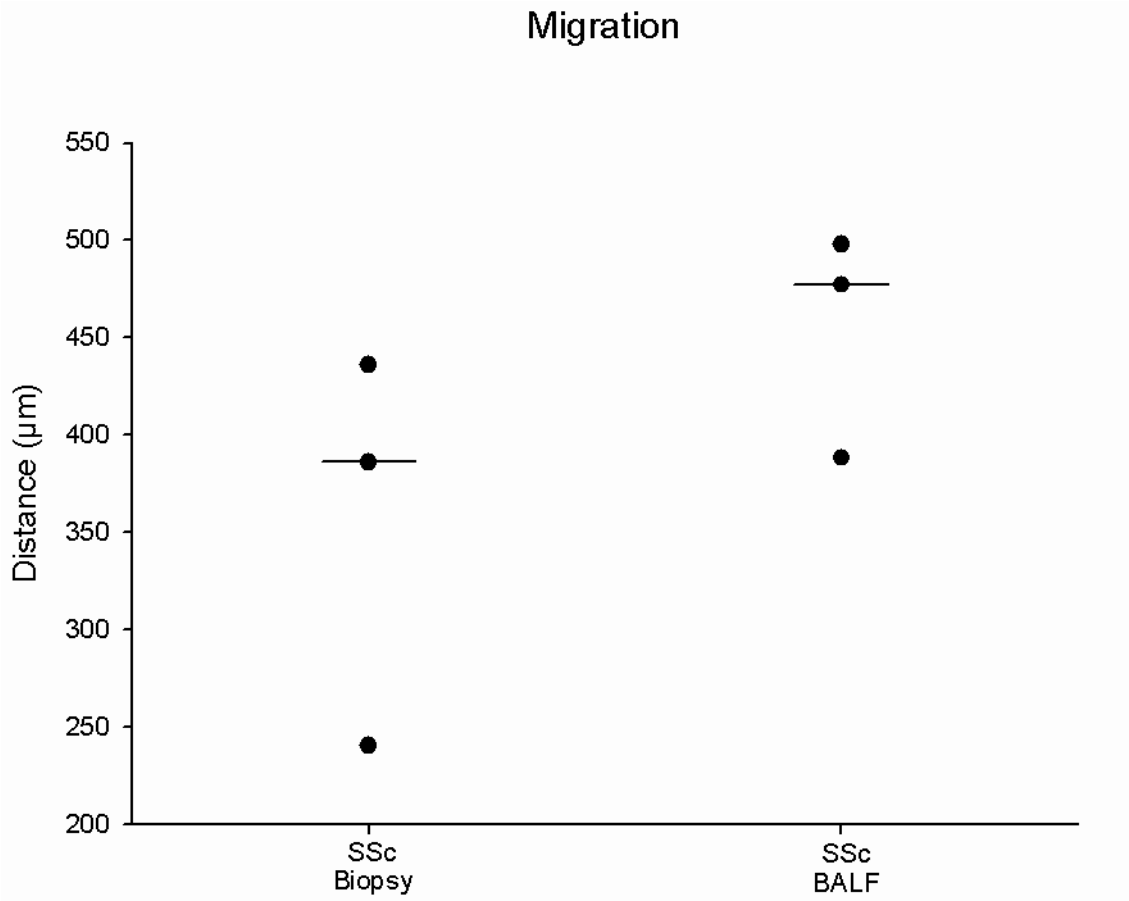
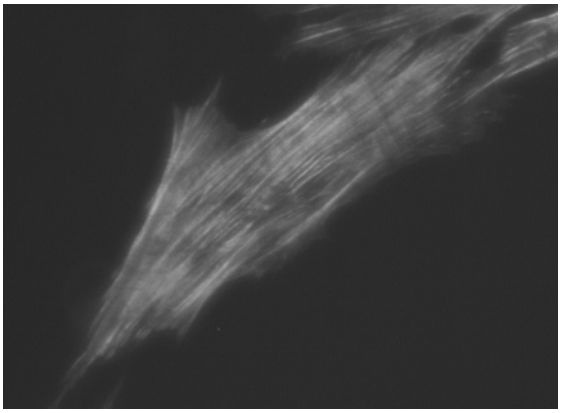
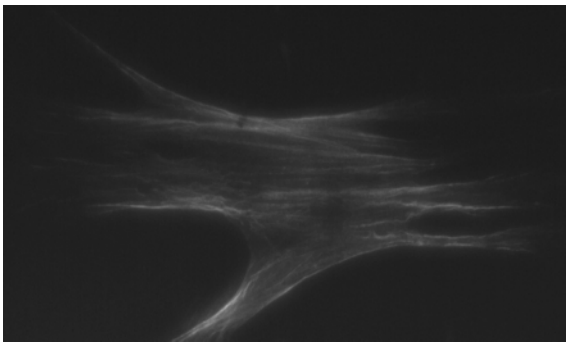
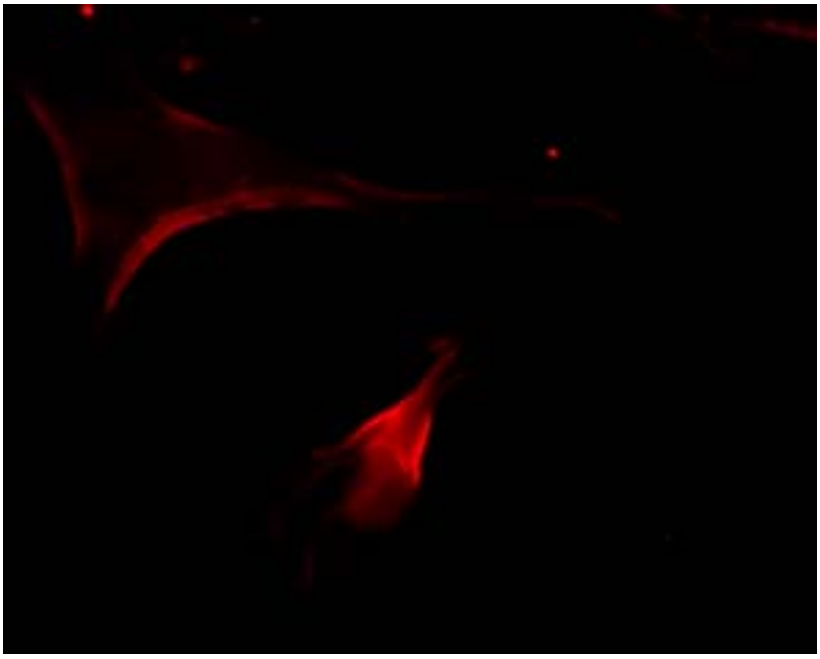


Fig 4. Stress fibres (bundles of F-actin) in fibroblasts cultured from BALF (**Fig 4a**) and bronchial biopsy (**Fig 4b**) in a SSc patient. stained with AlexaFluor™ 488 phallioidin. A-SMA in fibroblasts from BALF (**Fig 4c**) and bronchial biopsy (**Fig 4d**) incubated with monoclonal mouse antibody against α-SMA followed by AlexaFluor® 584 goat antimouse IgG







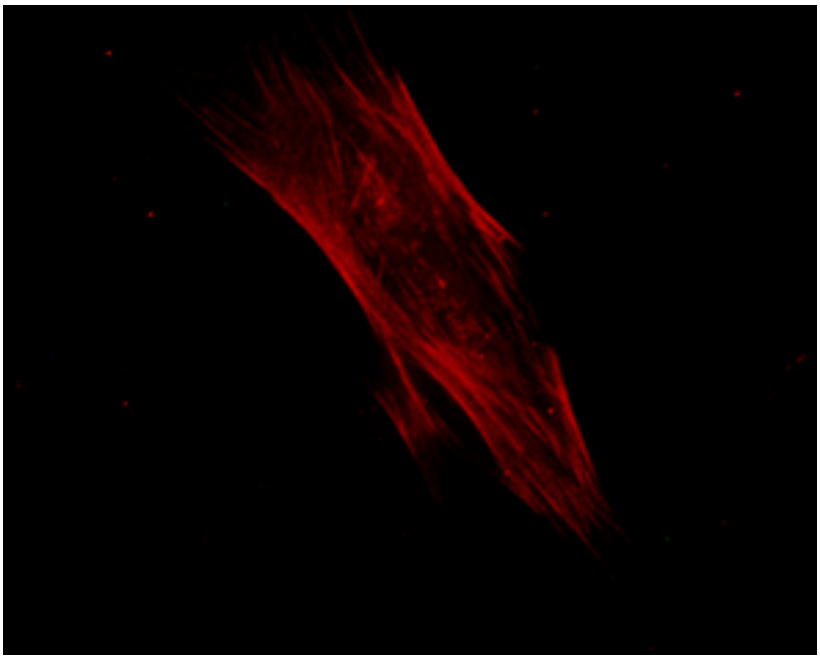
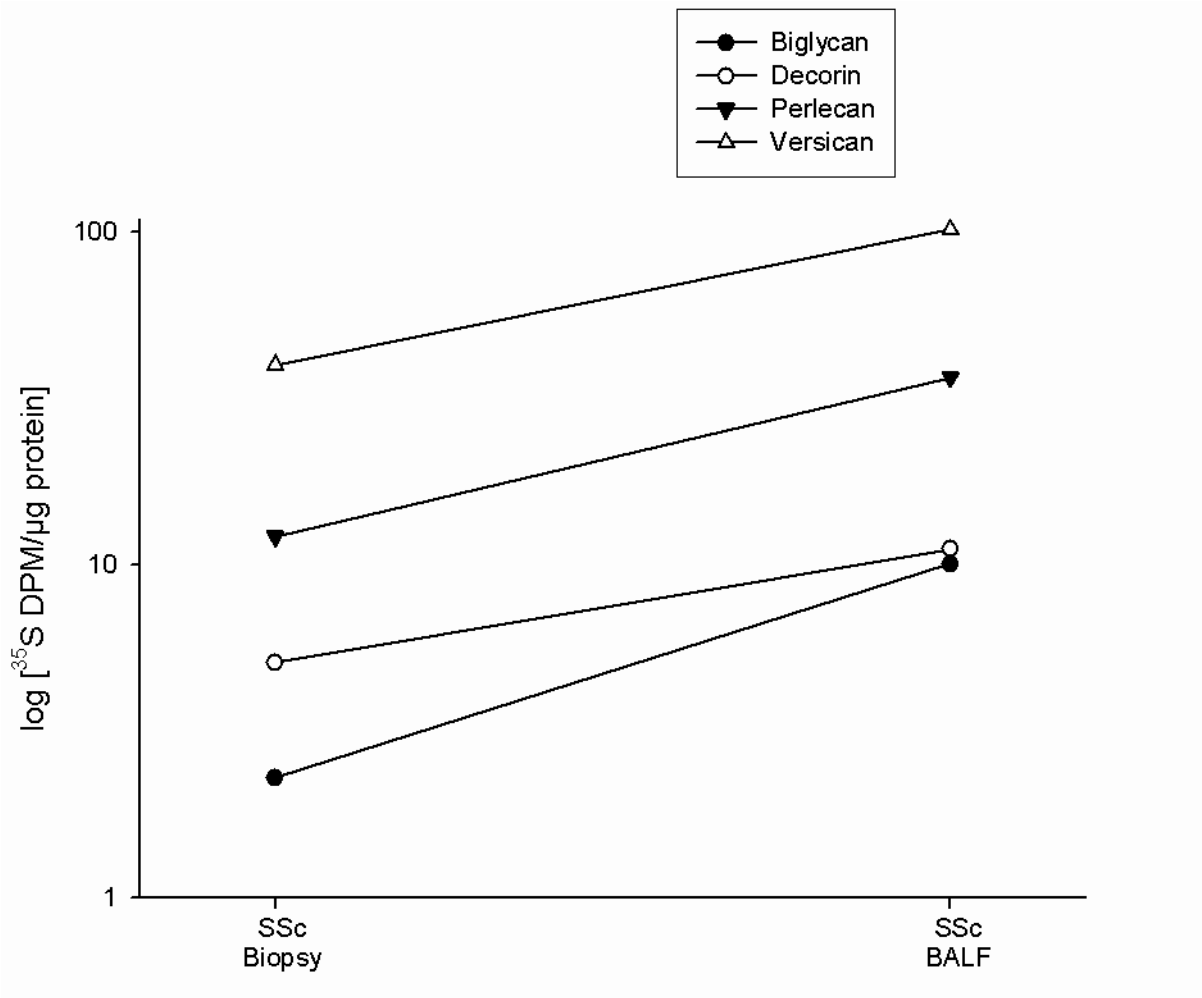


Fig 5. Production of proteoglycans; biglycan, decorin, versican and perlecan ($log^{35}S$ DPM/ μg protein), in fibroblasts cultured from BALF and from a biopsy from a SSc patient**.**


Biglycan
Decorin
Perlecan
Versican
100
10
1
log [35S DPM/µg protein]
SSc
Biopsy
SSc
BALF