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IMPACT OF AGE AND AUTOANTIBODY STATUS ON THE GENE EXPRESSION OF SCLERODERMA FIBROBLASTS IN RESPONSE TO SILICA STIMULATION

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Abstract

Environmental factors are believed to play an important role in the pathogenesis of systemic sclerosis (SSc). Silica exposure has been implicated as potentially hazardous in epidemiological studies of SSc. It can activate fibroblasts to express profibrotic genes at certain conditions. The aim of this study is to examine whether the fibroblasts of SSc patients respond to silica particles with specific gene expressions differentially from normal control fibroblasts. The fibroblasts obtained from skin biopsies of 96 SSc patients and 104 controls were examined. Silica particles were used to perturb the cultures of the fibroblasts in time-course and dose-response assays. The transcript levels of COL1A2, COL3A1, MIVIP1, MMP3, TIMP3 and CTGF genes of the fibroblasts were measured with quantitative RT-PCR. The results showed that the expressions of all six genes in SSc fibroblasts under silica perturbation appeared significantly different from normal control fibroblasts. In age stratified analysis, compared to control fibroblasts, SSc fibroblasts from patients at age 30–40 years and 50–60 years displayed significantly decreased expressions of MMP1 gene in all dosage assays and increased expression of COL3A1 genes started at low dosages perturbation of silica particles, respectively. In autoantibody stratified analysis, specific gene expression patterns were significantly associated with autoantibody-subgroups of fibroblasts. A common feature of SSc fibroblasts was unstable and a wide range of gene expression changes in response to silica perturbation. Our studies may suggest an altered intrinsic dynamic control in SSc fibroblasts. In addition, sensitivity and specificity of SSc

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fibroblasts to potentially hazardous environmental trigger is age and autoantibody-subgroup-dependent. The fibroblasts of SSc patients at age 30–60 years may be more sensitive to silica perturbation toward a profibrotic gene expression.

Keywords

scleroderma; fibroblasts; silica; gene expression; autoantibody

Systemic Scleroderma or scleroderma (SSc) is a multi-system disorder of connective tissue characterized by extensive cutaneous and visceral fibrosis. It can be clinically classified into limited cutaneous (lcSSc) and diffuse cutaneous (dcSSc) SSc. The latter is characterized by more rapid progression of skin and visceral involvement, as well as poorer prognosis (1). SSc also is an autoimmune disease with different autoantibody subsets. The most common of these autoantibodies are directed against DNA topoisomerase I (ATA), centromeric proteins (ACA), RNA polymerases III (anti-RNAP3) and ribonucleoprotein (anti-RNP). The autoantibodies are clinically relevant. For instance, the presence of ATA is strongly associated with diffuse skin involvement and pulmonary fibrosis (2, 3). In contrast, the ACA is associated with limited SSc and less serious organ involvement, except for late development of pulmonary hypertension (PAH) or primary biliary cirrhosis (PBC) in a minority (4, 5). Anti-RNAP3 occurs in various combinations, but more frequently in diffuse skin disease, and is also associated with pulmonary fibrosis and renal crisis in SSc (6–8).

Although, etiopathogenesis of SSc is still unclear, silica particles appear to be an important SSc trigger, which has been persistently suggested by epidemiological studies. In particular, stonemasons and gold miners who were often exposed to silica particles were more likely to develop SSc (9, 10). The relative risk for developing SSc was reported 25–39-fold higher in patients with radiologically documented silicosis (11). Circulating ATA were found in 9 of the 14 patients with silica-exposed SSc (12). Moreover, subcutaneous injections of silica were reported to induce sclerodermatous skin changes and activation of skin fibroblasts (13). Skin fibroblasts can be activated by silica to produce excessive amounts of the extracellular matrix (ECM) (14, 15). Fibroblasts synthesize, secrete, and maintain all major ECM components, the expressions of which are disturbed in fibrotic tissues of SSc patients. In particular, COL1A2 and COL3A1 are two major genes encoding structure proteins collagen type I and III, respectively, that are frequently over-expressed in the fibroblasts, and are accumulated in fibrotic tissue of SSc patients. Matrix metalloproteinases (MMP) and tissue inhibitor of metalloproteinases (TIMP) are counterpart in controlling the degradation of the collagens and other ECM components, the unbalanced expressions of which are involved in the fibrotic process in SSc. CTGF is a matricellular protein that is also over-expressed in SSc patients, and it induces other ECM production (16).

Considering the heterogeneous feature of SSc, we proposed that fibroblasts from different SSc patients may be distinct from each other in responding to silica stimulation. Perturbed expression of some major ECM genes have been implicated in fibroblasts of SSc patients. In this study, we examined the major ECM expressions of primary cultured fibroblasts of different SSc patients and controls under stimulations of silica particles.

MATERIALS AND METHODS

Fibroblasts

We enrolled 200 subjects in this study, of whom 104 were controls and 96 were SSc patients. Of these, 138 were females, 62 were males; 39 were Blacks, 127 were Caucasians, 29 were Hispanics, 2 were Asians, 3 were Choctaws. The average age was 46.32 (21–85 years). All patients fulfilled the American College of Rheumatology criteria for SSc (17). All normal controls were individuals with no history of autoimmune diseases. The study was approved by the Committee for the Protection of Human Subjects at the University of Texas Health Science Center at Houston. Informed consent was obtained from all patients and controls involved in the study.

Each fibroblast strain was obtained from skin biopsy of each study subject. All biopsies were obtained from the upper arm of non-lesional patients and controls. Briefly, cultured fibroblast strains were established by mincing tissues and placing them into 60 mm culture dishes secured by glass cover-slips. The primary cultures were maintained in Dulbecco's Modified Essential Media (DMEM) with 10% FBS and supplemented with antibiotic and antimycotic. The 5th passage of fibroblast cell strains were plated at a density of 2.5×10^5 cells in a 35 mm dish and grown until confluency. The culture medium was replaced with DMEM without FBS before stimulation assays.

Measurement of autoantibodies

Patient's sera were tested for antinuclear antibodies (ANA) by indirect immunofluorescence using HEp-2 cells as antigen substrate (Antibodies, Davis, CA). ATA and anti-RNP were detected by passive immunodiffusion against calf thymus extracts (INOVA, Diagnostics). ACA was determined by indirect immunofluorescence using HEp-2 cells. Anti-RNAP3 was detected utilizing commercially available kits (NBL, Nagoya, Japan).

Silica stimulation on fibroblasts

The 5th passage of fibroblast strains were plated at a density of 2.5×10^5 cells in a 35 mm dish and grown until 80% confluence. Culture media then were replaced with FCS-free DMEM containing different doses (1, 5, 10, 25 and 50 $\mu\text{g/ml}$) of silica particles obtained from Sigma-Aldrich, St Louis, MO, USA. The cultures were also grouped into different time-courses including 0-, 24-, 48-, 72-, 96-, 120-h stimulation with 10 $\mu\text{g/ml}$ silica. After stimulation, the fibroblasts were harvested for extraction of RNA. The RNAs were examined with RT-PCR for gene expression of COL1A2, COL3A1, CTGF, MMP1, MMP3 and TIMP3.

Measurement of AQchange

We used "AQchange" as the phenotype variable in association analysis, defined as

$$\log_2 \left(\frac{\text{gene expression after stimulation at time } t}{\text{gene expression without stimulation at time } t} \right)$$

For better understanding, AQChange = 0 means gene expression remained the same after stimulation, while AQChange = 1 means gene expression was doubled after stimulation and AQChange = -1 means gene expression was reduced by half after stimulation. AQChange = 0.5 means that after stimulation gene expression was 1.414 times higher than before. AQchange can be any continuous value.

Measurement of the ECM gene expression

The transcripts of six ECM genes (CTGF, MMP1, MMP3, COL1A2, COL3A1 and TIMP3) were examined in the studies. Quantitative real time RT-PCR was performed using an ABI 7900 sequence detector (Applied Biosystems, Foster City, CA, USA). The specific primers and probes for each gene were purchased through Assays-on-Demand from Applied Biosystems. As described previously (15), total RNA from each sample was extracted from cultured fibroblasts described above using TRIzol reagent (Invitrogen Life Technology) and treated with Dnase I (Ambion, Austin, TX). cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen Life Technology). Synthesized cDNAs were mixed with primers/probes in the 2x Taqman universal PCR buffer, and then assayed on an ABI 7900. The data obtained from assays were analyzed with SDS 2.1 software (Applied Biosystems).

Linear Mixed Model (LMM) to examine differences between specific age-groups of SSc and control fibroblasts in gene expression responding to time-course and dosage changes of silica stimulation

The linear mixed model (LMM) was used to perform age-stratified analysis and test interactions between age and time/dose for each of the six ECM genes. The LMM takes into account the correlations between repeated measurements within the same subject by introducing population-level fixed effects and subject-specific random effects (random intercept and slope), and enjoys the parsimonious modeling of correlated outcomes and the ease of accommodating missing values (18, 19). We specified the LMM for time-course gene expression as follows: $y_{ij} = \beta_0 + X_i\beta + b_{0i} + b_{1i} t_{ij} + \varepsilon_{ij}$, where y_{ij} is a given gene's expression level in subject i at the j th measurement ($i = 1, \dots, n$ and $j = 1, \dots, J$), X_i is the covariate vector for the fixed effects β including continuous time t_{ij} , continuous t_{ij}^2 if tested necessary, disease status (binary). b_{0i} and b_{1i} are the random intercept and slope of time effect for subject i , jointly following a bivariate normal distribution, while ε_{ij} is a random error following a normal distribution. We tested the difference between disease and normal adjusting for their ages. We used likelihood Ratio test to test age $\times$ time of silica exposure or dosage change of silica interaction in the model and did age stratified analysis as well to confirm what was most risky age group for each of the six ECM genes. The sample size after stratifying subjects into five age groups: 20–30 years, 30–40 years, 40–50 years, 50–60 years and 60–85 years were 35, 40, 51, 41 and 33, respectively (8, 15, 29, 21, 23 in cases and 27, 25, 22, 20, 10 in controls, respectively).

LMM to examine differences between specific autoantibody subgroup and control fibroblasts in gene expression changes responding to time-course and dosage changes of silica stimulation

Similarly, the LMM was also used to examine the difference between a particular autoantibody subgroup and control fibroblasts. We used likelihood Ratio test to test the significance of a particular interaction term given the original main effect model; after two tests between interaction model and main effect model, we determine the best model to use. Depending on which model selected, we reported the significance of either the main effect of autoantibody-subgroup difference in ECM gene expression changes, or interaction effect in ECM gene expression changes of the age $\times$ autoantibody subgroup/time of exposure to silica $\times$ autoantibody subgroup, or age $\times$ autoantibody subgroup/dosage change of silica $\times$ autoantibody subgroup. Due to limited sample size of autoantibody subgroups (ATA:17, ACA:9, anti-RNAP3:13, anti-RNP:5), testing two types of interaction of one model simultaneously was not carried out (under power).

RESULTS

Specific autoantibody testing showed that there were seventeen patients with ATA, nine with ACA, thirteen with anti-RNAP3 and five with anti-RNP.

Overall comparisons of time-course and dose responses showed that expression changes of all 6 tested genes, except TIMP3 in time course and COL1A2/COL3A1 in dose response, were significantly different between SSc and control fibroblasts from the main effect model output (without justification for age and subgroup of SSc) (Table I).

In age stratified analysis of time-course studies, marginally significant differences of gene-expression changes between SSc and control fibroblasts were observed in the 40–50-year age group for MMP1 and TIMP3 ($p=0.082$ and 0.091 , respectively), 50–60-year age group for CTGF and TIMP3 ($p=0.069$ and 0.071 , respectively) and 60–85-year age group for MMP1 and CTGF ($p=0.092$ and 0.096 , respectively). Others did not show significant changes.

In age stratified analysis of dose-response assays, significant difference of gene expressions were observed in specific age groups between SSc and control fibroblasts. In particular, in the 30–40-year age group, expression changes of MMP1 gene in SSc fibroblasts showed weak responses to silica stimulations ($p = 0.0018$); in the 50–60-year age group, the COL3A1 expression was significantly increased at lower dosage of silica stimulation, but dropped back to original level at dosage of 25 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$ of silica ($p = 0.0499$); in the 60–85-year age group, the CTGF expression was significantly low in SSc fibroblasts in all dosages of silica stimulation ($p = 0.0442$) (Table II, Fig. 1).

Analysis of subsets of SSc also showed some significant differences of gene expression changes of the fibroblasts in response to silica stimulations. In general, expression changes of some ECM genes in SSc fibroblasts of specific subsets showed zigzag lines in time-course and/or dose responses to silica stimulation, which is in contrast to smooth lines in control fibroblasts (Fig. 2). Some of these changes were statistically significant. In

particular, ATA-positive patients showed the changes of COL3A1 expression were significantly different from the controls (Table III) in time-course responses to silica, ACA-positive patients showed CTGF and COL3A1 expression in both time-course and dose response to silica were significantly altered; anti-RNAP3 patients with CTGF in dose response, and anti-RNP with TIMP3 in time-course and COL1A2 in dose response to silica also were significantly altered (Table III and Fig. 2).

DISCUSSION

Like many other human complex diseases, SSc is believed to arise from dysregulation of biological systems perturbed by environmental triggers. Multiple reports have indicated potentially hazardous impact of silica particles in development of SSc (9–15). However, sensitivity and specificity of cellular responses to silica-exposure may be distinct in different individuals. Indeed, the present study demonstrated that age and subtypes of SSc patients would affect specific gene expression of fibroblasts in response to silica stimulation. The strongest association observed in the studies was the fibroblasts of SSc patients at age 30–40 years and disturbed expression of MMP1 gene. Compared to control fibroblasts, SSc fibroblasts from patients at age 30–40 years displayed a decreased expression of MMP1 gene in response to all dosages of silica stimulation. Since MMP1 controls the degradation of the collagens and other ECM structure proteins, insufficient expression of MMP1 may contribute to profibrotic process. In contrast, a significantly increased expression COL3A1 gene of the fibroblasts was observed in SSc patients at age 50–60 years in response to low doses of silica, which seemed highly sensitive. The COL3A1 encodes type III collagen that is a fibrillar-forming collagen, a major structure protein of the ECM, and accumulation of which leads to tissue fibrosis. It is worth noting that peak occurrence of SSc is at age 35–60 years (20). The above-mentioned observations suggested that this age group of SSc individuals is more sensitive to silica perturbation through disturbed expressions of MMP1 or COL3A1 genes of the fibroblasts, although this notion warrants further confirmation and functional investigations.

Another age-related expression change of the fibroblasts is a weak response of CTGF gene in patients at age 60–85 years to all dosages of silica particles. CTGF is a profibrotic cytokine that is widely believed to play important roles in activating and maintaining tissue fibrosis in SSc (16, 21, 22). However, functional roles of CTGF in SSc patients at later stage of disease have not been investigated. This is the first report showing a discrepancy between profibrotic nature and lower expression level of CTGF gene in the fibroblasts of later SSc patients under silica perturbation.

In addition to age-related differences of SSc fibroblasts, patients with specific autoantibodies also displayed distinct patterns of gene expression in response to silica. Eight groups of such correlation appeared to be significant (Fig. 2). Specific gene expression patterns of autoantibody-related SSc fibroblasts include ATA-positive patients with a relatively lower COL3A1 expression in the dose-response assays, ACA-positive patients with higher expressions of COL3A1 gene in response to higher dosages of silica stimulations, but with decreased levels of COL3A1 and CTGF transcripts in the time-course assays, anti-RNAP3-positive patients with a reduced transcript level of CTGF in the time-

course assay, anti-RNP-positive patients with increased expressions of COL1A2 and TIMP3 genes in higher dosages and later responses to silica, respectively (Fig. 2). A common feature of gene expressions of SSc fibroblasts in all correlation groups appeared zigzag lines in response to either dosage or time increase of silica stimulation.

Although, a clear explanation for the observed differences between SSc and control fibroblasts in response to silica perturbation is impossible based on only current studies, altered intrinsic properties of specific SSc fibroblasts may be operative. Using state-space equations to model biological responses of TGF- β network of human fibroblasts to silica, and using expectation-maximization (EM) algorithms and Kalman filter to estimate the parameters in the models, our previous study demonstrated that intrinsic stability and controllability of SSc fibroblasts were significantly reduced by comparing normal fibroblasts (23). Intrinsic stability and controllability are two dynamic properties of human cells to maintain their functions and performance under a broad range of random internal and external perturbations. The observed features of specific SSc fibroblasts, especially a wide range of gene expression changes represented by zigzag lines in response to silica perturbation seemed supportive to this notion. On the other hand, this is a first attempt to examine age- and autoantibody-related features of SSc fibroblasts under perturbation of silica particles. Although the results are appealing and the statistical p-values of which greatly exceed random expectation, further confirmation studies and more theoretical analysis for biological mechanisms are urgently needed. This is particularly important for the associations reported in autoantibody subsets of SSc in which study subjects were limited in number.

In conclusion, the expression of the ECM genes in SSc fibroblasts under environmental perturbation appeared significantly different from normal control fibroblasts. SSc fibroblasts from patients of different age and autoantibodies displayed distinct gene expression patterns in time and/or dose changes of silica stimulation. These observations may suggest that sensitivity and specificity of SSc fibroblasts to potentially hazardous environmental triggering is age and autoantibody-subgroup dependent.

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Figure 1. Changes of specific gene expression of SSc and control fibroblasts of specific age groups in response to silica stimulation. Dashed line: control fibroblasts; solid line: SSc fibroblasts

Figure 2.

Changes of specific gene expression of SSc with autoantibody subgroup and control fibroblasts in response to silica stimulation. Dashed line: control fibroblasts; solid line: SSc fibroblasts

Table I

Comparison between SSc patients and controls; fibroblasts showed different expression-level changes of all 6 tested genes in response to silica stimulations.

Gene	<u>Time-course</u>	<u>Dose response</u>
	p-value	p-value
CTGF	0.0028	4.39×10^{-5}
MMP1	0.001	7.32×10^{-5}
MMP3	2.28×10^{-7}	3.17×10^{-5}
COL1A2	0.037	NS
COL3A1	0.045	NS
TIMP3	NS	0.025

Data were examined with the main effect model output adjusted for age. NS: not significant

Table II

Significant difference between SSc and control fibroblasts in specific gene expressions responding to silica at different age groups.

SSc versus control	Dose-response	
Age group (years)	Gene	p-value
age 30–40	MMP1	0.0018
age 50–60	COL3A1	0.04995
age 60–85	CTGF	0.0442

Table III

Significant difference between autoantibody subgroup of SSc and control fibroblasts in specific gene expressions in response to silica.

Subgroup of SSc /controls	Time-course		Dose-response	
	Gene	<i>p</i> -value	Gene	<i>p</i> -value
ATA/control	COL3A1	0.0006		
ACA/control	CTGF	0.0385*	CTGF	0.0159
	COL3A1	0.0004*	COL3A1	0.0157
Anti-RNAP3/control			CTGF	0.0202
Anti-RNP/control	TIMP3	0.0394*	COL1A2	0.0241**

p-value: disease main effect; p-value*: time $\times$ SSc subgroup interaction; p-value**: age $\times$ SSc subgroup interaction.