Monocytic Angiotensin and Endothelin Receptor Imbalance Modulate Secretion of the Profibrotic Chemokine Ligand 18

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ABSTRACT. ***Objective.*** To assess monocytic expression and ratio of angiotensin and endothelin receptors in systemic sclerosis (SSc) and their functional relevance.

Methods. Receptor expression was measured by flow cytometry. Chemokine ligand 18 (CCL18) concentration in supernatants of peripheral blood mononuclear cells stimulated with immunoglobulin G was measured by ELISA.

Results. Monocytes of patients with SSc presented an increased angiotensin II Type 1 receptor (AT1R)/AT2R ratio compared with those of healthy donors. Patients with lung fibrosis and patients with high modified Rodnan skin score showed a reduced endothelin 1 Type A receptor (ETAR)/ETBR ratio. High AT1R/AT2R, but low ETAR/ETBR ratios corresponded to higher CCL18 secretion.

Conclusion. Altered angiotensin and endothelin receptor ratios observed in SSc influence autoantibody-mediated effects such as secretion of profibrotic CCL18. (J Rheumatol First Release January 15 2016; doi:10.3899/jrheum.150474)

Key Indexing Terms:
SYSTEMIC SCLERODERMA AUTOANTIBODIES HUMAN CCL18 PROTEIN
ANGIOTENSIN RECEPTORS ENDOTHELIN RECEPTORS MONOCYTES

Circulating monocytes may differentiate into macrophages, collagen-producing fibrocytes, and myofibroblasts contributing to the cellular infiltration in affected systemic sclerosis (SSc) tissue and fibrosis[1]. Markers for alternative activation of monocytes and macrophages such as chemokine ligand 18 (CCL18) are elevated in SSc blood and bronchoalveolar lavage fluid[2]. CCL18 is mainly produced by alveolar macrophages, but also by monocytes[3]. It stimulates collagen production, and increased CCL18 serum levels are associated with a higher incidence of SSc-related interstitial lung disease and predict mortality[4].

As shown in our previous studies, monocytes show highest expression of angiotensin II (ATII) and endothelin receptors (ETR) compared with other peripheral blood mononuclear cells (PBMC)[5]. The natural ligands ATII and endothelin 1 (ET-1), as well as stimulating autoantibodies against angiotensin II Type 1 receptor (AT1R) and endothelin 1 Type A receptor (ETAR), are elevated in SSc[6,7,8]. The latter are associated with mortality and SSc complications such as lung fibrosis[8]. SSc-immunoglobulin G (IgG) positive for anti-AT1R and anti-ETAR autoantibodies induces CCL18 secretion in PBMC through the respective receptors. This effect is significantly reduced by AT1R and ETAR blockers[5]. Altered ratios of ATR and ETR subtypes result in modified signal transduction[9,10]. Therefore, besides autoantibody levels, receptor expression might determine autoantibody-mediated effects[11].

The objective of our study was to identify whether monocytic expression levels of AT1R and ETAR and their functional counterparts AT2R and ETBR are altered in SSc, and whether they are associated with distinct clinical manifestations. Moreover, functional relevance of differences in receptor expression profiles was analyzed by measuring autoantibody-mediated CCL18 secretion.

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Supported by the intramural grant of the University Hospital Charité, by the German Systemic Sclerosis Network, the Scleroderma Foundation, the Mirjam Lichy Foundation, and the German Research Foundation grant RI 1056-11.

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Accepted for publication November 2, 2015.

MATERIALS AND METHODS

Patients with SSc and healthy donors (HD). To compare receptor expression profiles, 18 patients with SSc and 18 age- and sex-matched HD were included. For correlation analysis of receptor expression with clinical variables, 39 patients with SSc fulfilling the 2013 American College of Rheumatology and European League Against Rheumatism (EULAR) criteria[12] were chosen and clinically assessed in accordance with the EULAR Scleroderma Trials and Research (EUSTAR) standard protocols[13]. Detailed patient and HD characteristics are summarized in Table 1. Lung fibrosis was diagnosed by high-resolution computed tomography. Our study was conducted in compliance with the Helsinki Declaration and local ethics approval (EA1/042/09, EA1/013/05, EA1/160/10). Patients gave informed written consent.

Flow cytometry and PBMC stimulation. Surface CD14 as well as ATR and ETR expression on isolated PBMC were measured by flow cytometry. PBMC of 10 patients with SSc and 11 HD were stimulated with pooled IgG of either patients with SSc or HD. After IgG stimulation, CCL18 concentration in the supernatants was measured by ELISA (detailed descriptions in Supplementary Data, available from the authors on request).

Statistical analysis. Statistics were performed using Prism5 software (Graphpad Inc.). Because of the lack of Gaussian distribution, statistics were assessed with Spearman rank correlation, Mann-Whitney U test, or Wilcoxon signed-rank test. A value of $p < 0.05$ was interpreted as statistically significant.

RESULTS

AT1R/AT2R ratio is increased in SSc, and PBMC stimulation with IgG of SSc results in higher CCL18 concentrations. Ratios of the frequencies of AT1R/AT2R-positive cells were increased in SSc compared with HD (Figure 1A), whereas ETAR/ETBR ratios were not different. Patients with SSc presented higher density of AT1R, AT2R, ETAR, and ETBR compared with HD and increased frequencies of receptor-positive monocytes (Supplementary Figure 2, available from the authors on request). Neither endothelin receptor expression nor ETAR/ETBR ratios were different in patients with SSc who had endothelin-receptor antagonistic treatment compared with those without (data not shown).

To assess the functional relevance of different monocytic ATR and ETR expression and their respective ratios, their association with autoantibody-mediated CCL18 induction was examined. PBMC of SSc and HD stimulated with SSc-IgG showed a higher concentration of CCL18 in supernatants (1673 pg/ml ± 180 pg/ml) than those stimulated with HD-IgG (900 pg/ml ± 79 pg/ml; Figure 1B). CCL18 in supernatants of unstimulated PBMC was below detection limit. Stimulation of PBMC of HD with SSc-IgG resulted in higher CCL18 concentration than stimulation of PBMC of SSc (Figure 1B), whereas there was no difference regarding the stimulation with HD-IgG.

ETR imbalance is associated with fibrosis. Twenty-three out of 39 patients in the study had lung fibrosis (59.0%, female/male 15/8). These patients had a significantly higher ETBR density on monocytes and hence a reduced ETAR/ETBR ratio when compared with patients without lung fibrosis (Figure 1C). Four patients without lung fibrosis presented high ETBR density, but their lung function variables were not impaired. Time since lung fibrosis onset correlated positively with monocytic ETBR density (Spearman $r = 0.52$, $p = 0.01$) and negatively with the ratio of ETAR and ETBR density ($r = –0.39$, $p = 0.08$; data not shown). No correlation was observed between lung fibrosis severity, measured by lung function variables such as DLCO and forced vital capacity, and ETR expression. Moreover, ETAR/ETBR ratio of densities correlated negatively with the modified Rodnan skin score (mRSS; data for 37 patients; Figure 1D).

Table 1. Patients with SSc and HD characteristics. Values are n (%) unless otherwise specified.

Characteristics	Receptor Expression Profiles		Clinical Data	Stimulation Experiments	
	Patients with SSc, n = 18	HD, n = 18	Patients with SSc, n = 39	Patients with SSc, n = 10	HD, n = 11
Variables					
Age, yrs, mean (SD)	46.7 (15.5)	43.6 (11.4)	51.8 (14.6)	57.2 (14.9)	26.2 (3.3)
Females/males, n	10/8 (56/44)	10/8 (56/44)	25/14 (64/36)	8/2 (80/20)	5/6 (45/55)
Diffuse cutaneous form/limited cutaneous form/other, n	5/13/0		22/14/3	5/4/1	
mRSS, mean (SD)	9.9 (5.1)		8.0 (6.9)	13.7 (9.4)	
Pulmonary arterial hypertension	1 (6)		3 (8)	1 (10)	
Lung fibrosis	11 (61)		23 (59)	8 (80)	
Immunosuppressive treatment	16 out of 17 (94)		33 out of 37 (89)	8 (80)	
Bosentan, ambrisentan	7 (41)		9 (24), 1 (3)	1 (10)	
Prednisolone	9 (53)		20 (54)	3 (30)	
MTX	5 (29)		9 (24)	3 (30)	
Hydroxychloroquine	0		4 (11)	1 (10)	
Azathioprine	2 (12)		9 (24)	2 (20)	
Cyclosporine	4 (24)		1 (3)	3 (30)	
Leflunomide	0		1 (3)	0	
Tocilizumab	1 (6)		1 (3)	0	
Cyclophosphamide	3 (18)		4 (11)	0	

SSc: systemic sclerosis; HD: healthy donors; mRSS: modified Rodnan skin score; MTX: methotrexate.

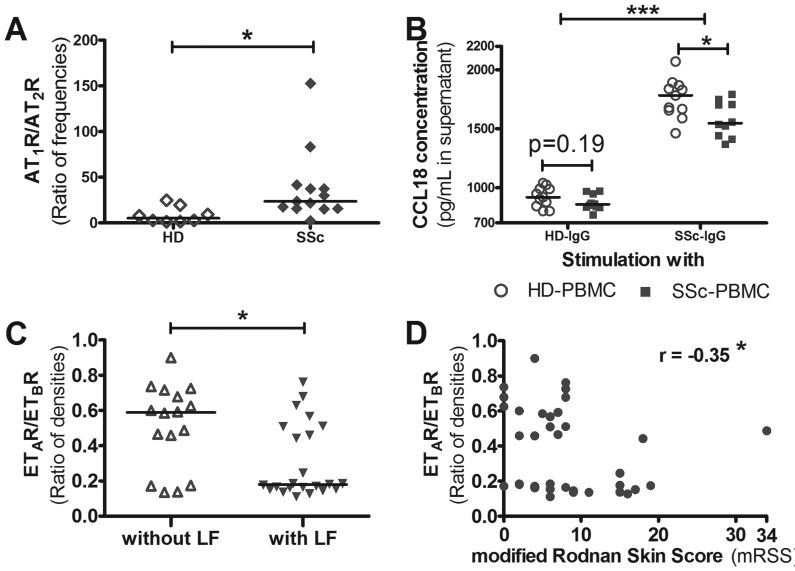

Figure 1. AT1R/AT2R ratio is higher in patients with SSc, and stimulation of PBMC with IgG of patients with SSc results in higher CCL18 concentrations than IgG of HD. ETAR/ETBR ratio is associated with clinical findings: receptor protein expression on monocytes analyzed by flow cytometry. A. Ratio of AT1R/AT2R positive monocytes compared to isotype control (18 patients with SSc and 18 age- and sex-matched HD. AT2R frequency > 0 available for 8 HD and 13 SSc). B. CCL18 concentration in supernatants of PBMC from SSc (filled rectangles) and HD (open circles) stimulated with IgG of patients with SSc and HD. C. ETAR/ETBR ratio of densities is lower in patients with SSc with LF. Results derived from the Mann-Whitney U test. Medians are shown. D. Ratio of ETAR/ETBR densities correlates negatively with mRSS, Spearman correlation coefficient r indicated. * P < 0.05. *** P < 0.001. AT1R: angiotensin II Type 1 receptor; AT2R: angiotensin II Type 2 receptor; SSc: systemic sclerosis; PBMC: peripheral blood mononuclear cells; IgG: immunoglobulin G; CCL18: chemokine ligand 18; HD: healthy donors; ETAR: endothelin 1 Type A receptor; ETBR: endothelin 1 Type B receptor; LF: lung fibrosis; mRSS: modified Rodnan skin score.

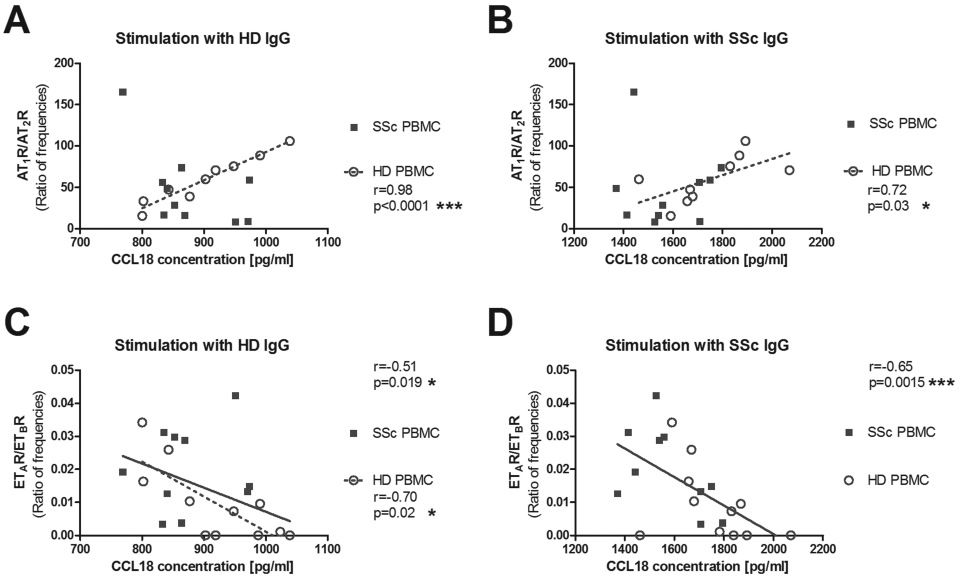

Figure 2. CCL18 induction correlates with AT1R/AT2R and inversely with ETAR/ETBR ratio. Ratio of (A) AT1R/AT2R frequency of receptor-positive monocytes stimulated with IgG of HD (HD-IgG) and (B) stimulated with IgG of patients with SSc (SSc-IgG), as well as (C) ratio of ETAR/ETBR frequency of receptor-positive monocytes stimulated with HD-IgG and (D) with SSc-IgG were correlated to CCL18 concentration in supernatants of PBMC from patients with SSc (filled rectangles) and HD (open circles). Spearman correlation coefficient r and tendency lines are shown for statistically significant correlations of HD-PBMC (dotted line) and PBMC from HD and SSc together (continuous line). * P < 0.05. *** P < 0.001. CCL18: chemokine ligand 18; AT1R: angiotensin II Type 1 receptor; AT2R: angiotensin II Type 2 receptor; ETAR: endothelin 1 Type A receptor; ETBR: endothelin 1 Type B receptor; IgG: immunoglobulin G; HD: healthy donors; SSc: systemic sclerosis; PBMC: peripheral blood mononuclear cells.

CCL18 induction of PBMC correlates with ratios of ATR and ETR expression. CCL18 concentrations induced by HD-IgG and SSc-IgG correlated with AT1R/AT2R ratio of frequency of receptor-positive monocytes. This correlation was statistically significant for PBMC of HD (Figures 2A and 2B). Contrarily, the ratio of ETAR-positive and ETBR-positive monocytes of the 11 HD and 10 patients with SSc showed a negative correlation with CCL18 levels (Figures 2C and 2D).

DISCUSSION

In our study, patients with SSc presented with an imbalance of monocytic expression of ATR subtypes when compared with HD. Although this imbalance was not observed for ETR altogether, low ETAR/ETBR ratios were associated with the presence of lung fibrosis and correlated with a higher mRSS. Different receptor profiles seem to modulate monocyte function toward an activated phenotype as shown by the secretion of the profibrotic cytokine CCL18 upon stimulation with autoantibodies against AT1R and ETAR.

Increased ATR and ETR expression together with modified subtype relation in SSc is in line with findings from other groups. Recently, higher percentages of septal and vessel cells positive for AT1R and AT2R in fibrotic lungs of patients with SSc with active lung fibrosis have been described[14]. Moreover, skin and lung tissue from patients with SSc are known to show higher ET-1 binding sites[7,15]. Increased total ETR mRNA expression along with reduced ETAR and raised ETBR mRNA levels were found in SSc-associated fibrotic lung tissue, indicating the relevance of ETR subtype ratios[7,15].

Changes in ETAR/ETBR ratios could influence the functional response to ET-1[10,16]. As shown here, they also modulate the functional response to stimulating receptor autoantibodies, such as induction of the profibrotic CCL18. Confirming previous studies, SSc-IgG induced higher CCL18 secretion compared with HD-IgG (Figure 1B and Figure 2). CCL18 concentration was higher in the supernatant of stimulated PBMC of HD. A possible explanation for this finding is that SSc PBMC, contrarily to those of HD, are already *in vivo* stimulated by IgG of the respective patient with SSc, which might result in higher CCL18 serum concentrations and/or receptor desensitization[5,9,10]. Thus, HD monocytes might be able to release higher amounts of formerly stored CCL18, or they might be more vulnerable to novel autoantibody stimulation. This could explain why CCL18 induction showed a stronger correlation with ATR and ETR subtypes ratios of HD monocytes.

High AT1R/AT2R but low ETAR/ETBR ratios corresponded to the highest CCL18 concentration, suggesting a link between ATR and ETR imbalance and monocytic function (Figure 2). These data imply a crosstalk between AT1R and AT2R and between ETAR and ETBR, respectively, as other groups have suggested before[6,17].

Increasing ETBR density during the course of lung fibrosis together with a decreasing ETAR/ETBR ratio (Figure 2C) might reflect a more powerful autoantibody stimulation at onset of this manifestation.

In our present manuscript, patients with SSc showed higher AT1R, AT2R, ETAR, and ETBR expression on monocytes (Supplementary Figure 2, available from the authors on request). Interestingly, in our former study, patients with SSc had reduced AT1R expression. This difference might be because the current cohort included more patients with lung fibrosis (59% of patients vs 39% in former cohort) and active SSc (mean EUSTAR activity score 1.3 vs 0.2). Receptor upregulation might be a feature of active and severe disease, but the small number of patients with SSc who were analyzed limits the conclusions that can be drawn from our study.

Interactions between the angiotensin and the endothelin system and their receptors are known[18]. Further, receptor colocalization and AT1R/ETBR heterodimers have been shown in a hypertensive rat model[19]. Whether monocytic receptor upregulation in SSc might be because of the formation of heterodimers and/or reciprocal stimulation remains highly speculative. Nevertheless, it seems important to evaluate further therapeutic concepts targeting both ATR and ETR as well as the pathogenic autoantibodies.

Monocytic receptor expression and imbalance of respective receptor subtypes might reflect systemic activation of the angiotensin and the endothelin systems in SSc and might serve as a marker for fibrosis. The association of receptor imbalance with autoantibody-induced CCL18 secretion and clinical phenotype of monocyte donors could be the result of an autoantibody-induced activation of monocytes mediated through ATR and ETR, a situation that might contribute to skin and lung fibrosis in SSc.

ACKNOWLEDGMENT

We thank Anika Klaus, Tuula Geske, Heidi Hecker-Kia, Anette Peddinghaus, and Heidi Schliemann for their excellent technical assistance. This work is part of the doctoral thesis of JR.

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