Elevated whole blood chemiluminescence in patients with systemic sclerosis

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Abstract

Objective

Systemic sclerosis (SSc) is accompanied by oxidative stress that in turn may accelerate endothelium degeneration and thus disease progression. We tested whether phagocytes from SSc patients release more reactive oxygen species (ROS) and whether this release correlates with some clinical parameters.

Methods

ROS production by blood phagocytes was measured with the luminol enhanced whole blood chemiluminescence (CL). Resting and N-formyl-methionyl-leucyl-phenylalanine -induced CL (fMLP-induced CL) was measured in 30 patients with SSc and 30 healthy controls matched as to age, sex, and level of cigarette smoking.

Results

Resting CL and fMLP-induced CL calculated per 10^4 phagocytes present in the assayed blood sample were higher in patients with systemic sclerosis than in healthy controls (median; range, 0.88; 0.47-1.39 vs. 0.73; 0.13-1.07 aU/10^4p and 621; 293-3522 vs. 411; 289-810 aUxs/10^4p, $p<0.02$). Patients treated with cyclophosphamide and/or prednisone for 11; 3-168 months did not differ in respect to CL from those that never received the medications. Similarly, no significant differences were found between patients with limited and diffuse SSc. Resting CL correlated ($p<0.05$) with clinically manifested interstitial lung disease ($r=0.59$), single breath carbon monoxide diffusing capacity ($r= -0.56$) and serum autoantibodies titre ($r= 0.43$).

Conclusions

Blood phagocytes from patients with systemic sclerosis, especially from those with interstitial lung disease, generate elevated amounts of ROS as assessed with CL. This confirms the presence of systemic oxidative stress in SSc patients.

Key words

Systemic sclerosis, chemiluminescence, oxidative stress, interstitial lung disease.

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This study was supported by the institutional grant 503–104–4.

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Received on March 8, 2004; accepted in revised form on November 30, 2004.

Introduction

Patients with systemic sclerosis (SSc) are believed to be in a permanent systemic oxidative stress (1-4) that in turn may accelerate endothelial cell degeneration, putative SSc feature, and thus disease progression (4). This is a consequence of severe inflammatory response and may also result from episodes of ischemia/reperfusion related to Raynaud's phenomenon present in almost all affected subjects (1, 2, 5). Indeed, the highest levels of lipid peroxidation products, markers of oxidative stress, were observed in patients with interstitial lung disease (ILD) (3) and in patients with more frequent ischemia/reperfusion episodes (4). Potential source of increased release of reactive oxygen species with subsequent peroxidative damage to a variety of biomolecules (3, 4, 6, 7) in SSc patients are polymorphonuclear leukocytes (PMNs) (8), monocytes (9) and xanthine-xanthine oxidase system (4). Relative importance of these oxidative stress-generating systems in SSc was not examined so far. While, the xanthine-xanthine oxidase system stimulation should be taken into account as a main source of ROS in the Raynaud's phenomenon, like in other ischaemia/reperfusion syndromes (10), the phagocytes are under suspicion in inflammatory organ damage (e.g. ILD patients).

Monocytes and PMNs from SSc patients were reported to release more ROS than cells from healthy subjects upon stimulation (8, 9, 11, 12). However, there are conflicting results on resting and agonist induced ROS production. Moreover, almost all studies on ROS production by circulating phagocytes in SSc patients were performed with use of isolated cells (9, 11, 12). Because isolation procedures may enhance resting cell activity and prime phagocytes respiratory burst (13, 14), overestimation of phagocyte activity under these experimental conditions cannot be excluded. This necessitates evaluation of ROS production in SSc patients with more adequate method. Surprisingly, no associations between phagocyte function and disease activity and treatment have been addressed so far (8, 9, 11, 12, 15). Existence of such associations could further confirm pathogenic role of phagocyte-derived oxidative stress in scleroderma.

Luminol enhanced whole blood chemiluminescence assay is a rapid and reliable technique for measurement of ROS generation by circulating phagocytes. This method is as sensitive as isolated cells chemiluminescence and eliminates the risk of cell priming during the preparation procedure (16, 17). Moreover, CL allows monitoring of phagocyte ROS generation under conditions that more closely resemble the *in vivo* situation (16). Therefore, in this study we assessed the ability of peripheral blood phagocytes (PMNs and monocytes) to release ROS in SSc patients with CL.

The associations between CL and selected clinical and biochemical SSc features as well treatment with cyclophosphamide and/or prednisone were also examined.

Materials and methods

Study populations

The study was conducted at Department of Experimental and Clinical Physiology (whole blood chemiluminescence, lung function and blood cell count determination) and at Department of Dermatology, Medical University of Lodz (patient recruitment and clinical characteristics) from April 2002 to June 2003 and involved 30 patients with SSc (age: median 48, range 18-69 years, 25 women) that fulfilled the American College of Rheumatology criteria (18) and 30 healthy volunteers matched as to age, sex, and level of cigarette smoking (Table I and II).

SSc patients enrolled to the study had to fulfil the following inclusion criteria: (1) age ≥ 18 years; (2) negative pregnancy test (women of child-bearing potential); (3) disease duration of ≥ 6 months. The exclusion criteria were as follows: (1) pregnancy; (2) active alcohol or drug abuse; (3) presence of any coexistent chronic disease not related to SSc pathology; (4) liver function impairment (alanine-aminotransferase, aspartate-aminotransferase or alkaline phosphatase levels greater than 3 times the upper limit of normal); (5) history of any infectious disease

Table I. Characteristics of SSc patients and healthy controls.

Parameter/ Symptom	SSc group	DMD subgroup¶	NDMD subgroup ¶	Healthy controls
lSSc/dSSc	20/10	3/8	17/2	- - -
Age (yrs)	48; 18-69 (49±13)	45; 35-64 (48±9)	49; 18-69 (49±15)	49; 21-72 (48±14)
Sex F/M	25/5	7/4	18/1	24/6
Smokers/ nonsmokers	7/23	5/6	2/17	7/23
$DLCO_C$ %¶	68; 27-107** (69±27)	44; 36-105* (55±25)	90; 28-107*† (79±29)	102; 91-110 (104±11)
RV_{He}%	95; 50-160 (97±33)	80; 60-111* (83±18)	121; 50-160† (112±38)	104: 89-112 (107±11)
FVC %¶	81; 44-112** (82±21)	68; 53-99* (71±13)	97; 44-112*† (89±21)	100; 90-120 (103±14)
FEV_1%¶	66; 32-117** (69±24)	51; 32-103* (59±23)	70; 47-117*† (76±23)	99; 90-109 (99±11)
BMI¶	22; 18-33 (23±4)	21; 19-31 (24±5)	23; 18-33 (23±4)	25; 17-38 (26±5)
Disease duration (yrs.)	4; 0.5-23 (5±5)	3; 0.5-23 (5±6)	6; 2-20 (6±5)	- - -
Raynaud's duration (yrs.)	8; 1-46 (10±10)	4; 1-46 (9±13)	9; 2-30 (11±9)	- - -
No. of organs involved	1; 0-4	3; 1-4	1; 0-4	0
m. Rodnan skin score	17: 7-39 (19±6)	22; 13-39 (21±8)	14; 7-29 (16±5)	- - -

¶ DMD: patients currently treated with cyclophosphamide and/or prednisone; NDMD: patients never treated with disease modifying drugs, BMI: body mass index, FVC: forced vital capacity, FEV_1: forced expiratory volume in the first second, $DLCO_C$: single breath carbon monoxide diffusing capacity corrected for haemoglobin, RV_{He}: residual volume is expressed as a percentage of the predicted value. *p < 0.01; **p < 0.001 vs. healthy subjects, †p < 0.05 vs. DMD patients. Between parentheses mean ± SD.

Table II. Blood cell count, autoantibody titres and selected plasma chemistry parameters in SSc patients and healthy controls.

Parameter	SSc group	DMD subgroup	NDMD subgroup	Healthy controls
Haemoglobin g/dl	13.1; 9.0-16.3 (13.3±1.7)	13.2;12.7-16.3 (14.1±1.4)	13.0;9.0-15.8 (12.8±1.8)	13.3;11.3-16.5 (13.7±1.5)
RBC $x10^6$/μl	4.65;3.43-7.91 (4.63±0.85)	4.76;3.87-5.69 (4.69±0.68)	4.52;3.43-7.91 (4.60±0.95)	4.40;3.58-5.34 (4.46±0.48)
WBC $x10^3$/μl	6.38;4.16-13.36 (7.07±2.28)	6.05;4.16-13.36 (7.21±3.05)	6.40;4.62-10.39 (6.99±1.78)	5.95;3.90-11.10 (6.11±1.59)
PMNs $x10^3$/μl	4.25;2.19-10.12 (4.83±1.77)	4.20;3.10-10.12 (5.05±2.24)	4.30;2.19-7.48 (4.69±1.49)	3.65;2.70-8.15 (3.97±1.17)
Monocytes $x10^3$/μl	0.30; 0.10-1.10 (0.34±0.19)	0.32; 0.23-0.50 (0.34±0.09)	0.31; 0.10-1.10 (0.35±0.24)	0.40; 0.20-0.70 (0.38±0.14)
Auto-antibodies titre	1/2560; 1/320-1/5120	1/2560; 1/320-1/2560	1/2560; 1/640-1/5120	Not detectable
Albumin g/dl	4.2; 2.4-5.4 (4.2±0.7)	4.2; 3.5-5.4 (4.3±0.6	4.2; 2.4-5.4 (4.1±0.8)	4.4; 3.5-5.7 (4.3±0.4)
Bilirubin mg/dl	1.1; 0.6-2.2 (1.1±0.3)	1.1; 0.6-1.2 (1.1±0.2)	1.1; 0.6-2.2 (1.1±0.4)	1.0; 0.5-1.3 (0.9±0.3)
Uric acid mg/dl	3.6;3.0-8.0 (5.5±1.6)	4.6;3.2-7.7 (4.5±1.4)	3.6;3.0-8.0 (6.1±1.9)	3.9;3.1-5.9 (4.6±1.7)

Between parentheses mean ± SD. No significant differences were found between any of the groups.

within 3 months prior to the study; (6) intensive cigarette smoking (daily cigarette consumption of >20 cigarettes a day or cumulative cigarette consumption of >15 pack years); (7) any food intolerance; (8) vegetarianism and other elimination diets. Healthy control subjects were free of any medication and fulfilled all above mentioned inclusion and exclusion criteria except those related to SSc.

The disease classification as limited (lSSc) or diffuse (dSSc) was based on criteria of Le Roy *et al.* (19). Modified Rodnan skin score was calculated according to Clements *et al*. (20).

ILD was considered present if (a) bilateral reticulonodular shadowing involving predominantly the lower lobes was demonstrated in chest X-ray and/or high-resolution computed tomography (21); (b) haemoglobin-corrected lung diffusion capacity for carbon monoxide ($DLCO_c$) was <80% of predicted. In most patients these findings were accompanied by bilateral fine, mid-to-late inspiratory crackles on clinical chest examination.

Lung involvement (ILD), musculoskeletal dysfunction (muscle weakness, arthralgia and flexion contractures), esophageal dismotility (evaluated with esophageal manometry), cardiac disease (conduction disturbances and/or arrhythmias), and renal disease (elevated arterial blood pressure, elevated serum creatinine, abnormal urinalysis) were noted in 16, 17, 10, 7 and 1 patient, respectively. The number of involved organs in a patient ranged from 4 (n=3) to 0 (n=3). Disease duration was calculated from the onset of the first non Raynaud's symptom.

Patients with dSSc (n=10) differed from those with lSSc (n=20) in respect of the following parameters (median and range, $p<0.05$); skin score (23; 13-39 vs. 13; 7-28), forced vital capacity (FVC 71; 43-98% vs. 93; 50-112% predicted), forced expiratory volume in the first second (FEV_1 52; 31-87% vs. 70; 43-122% predicted), $DLCO_C$ (43; 36-105% vs. 86; 27-107% predicted), residual volume with helium dilution (RV_{He} 75; 49-110% vs. 118; 67-160% predicted), musculoskeletal dysfunction (9/10 vs. 8/20), and Raynaud's duration (4; 1-11 yrs vs. 9; 2-46 yrs).

Eleven of SSc subjects, labeled DMD (disease modifying drugs) subgroup, were on cyclophosphamide 50 mg daily or prednisone 15-20 mg daily or combination of both in the same doses

as in monotherapies (treatment duration, median 11 months; 3 to 168 months, mean 27 ± 48 months) due to interstitial lung disease (n=7) and musculoskeletal involvement (n=4).

Remaining 19 patients never received this medication (NDMD subgroup). Patients from both subgroups were treated with nifedipine 10 mg daily and vitamin E 400 mg daily for at least 5 months. Seven SSc patients and 7 healthy controls were current cigarette smokers and their mean daily (11 ± 4 vs. 13 ± 5 cigarettes a day) and cumulative (7.6 ± 2.1 vs. 8.4 ± 3.1 pack-years) cigarette consumption did not differ significantly.

Scl70 autoantibodies were detected in 19 SSc patients. ANA-, U3RNP-, RNP-autoantibodies were detected in 7, 3 and 1 patient with SSc, respectively. Two patients had both Scl70- and ACA autoantibodies.

All subjects gave informed consent on participation in the study. The study protocol was approved by Ethics Committee of Medical University of Lodz.

Lung tests

Patients attended the laboratory (8 a.m. – 10 a.m.) for lung function measurements (FVC, FEV_1, $DLCO_C$, RV_{He}) with Master-Laboratory Screen (Jaeger Toennies, Wuerzburg, Germany) (22, 23). All lung functions were expressed as a percentage of the predicted value (24, 25).

Luminol enhanced whole blood chemiluminescence

Resting and N-formyl-methionyl-leucyl-phenylalanine (fMLP)-induced CL was measured according to Kukovetz *et al*. (17) with some modifications (26). Addition of fMLP to specimen of diluted blood triggers respiratory burst of PMNs and monocytes with generation of ROS that oxidize luminol to aminophtalate producing intense light. The intensity of emitted light is considered to reflect intensity of ROS production by blood phagocytes (17, 27). Venous blood for chemiluminescence was drawn into 2 mL EDTA K3 Vacuette tubes (Greiner Labor Technik). Samples containing 3 μl of blood and 947 μl of mixture solution were placed in thermostatically controlled luminometer (Bio-Orbit® 1251, Turku, Finland) and incubated for 30 min at 37°C with continuous gentle rotation mixing. Afterwards, CL was recorded continuously for 1 min and then 50 μl fMLP solution was added to final concentration of $2x10^{-5}$M and measurement was continued for next 7 min. The following CL parameters were assessed: resting CL (rCL – mean CL during first 60 seconds of measurement before fMLP stimulation), peak chemiluminescence (pCL – maximal chemiluminescence signal reached after fMLP addition), increment in chemiluminescence intensity (ΔCL = pCL – rCL), total light emission (tCL - area under the chemiluminescence intensity curve after fMLP addition), and peaktime (time from fMLP addition to appearance of maximal chemiluminescence signal) (Fig. 1). Individual results are mean of triplicate experiments and rCL, pCL and ΔCL were expressed in arbitrary units (aU) per 10^4 phagocytes present in the assayed sample (aU/10^4 p). Peaktime and tCL were expressed in seconds (s) and aU x seconds per 10^4 phagocytes (aU x s/10^4 p), respectively. The mixture solution was prepared freshly before the assay from 1 ml Ringer solution without calcium, 5 ml 0.025% w/v luminol, 0.2 ml 5% w/v glucose solution, and 3.6 ml distilled water (17). fMLP addition, timing of entire procedure and calculations of chemiluminescence parameters were under control of PC software (Multi-Use v.2.01) and programmable dispenser controller (Bio-Orbit DC 1251-101) with precise dispenser (Bio-Orbit SVD 1251-100).

Other measurements

Blood cells count, including phagocytes (PMNs and monocytes) count, and haemoglobin concentration were performed with automatic analyser (ABX MICROS, AVL, Montpellier France) from the same blood sample as that used for CL measurements. For serum levels of bilirubin, uric acid, albumin, creatinine, activity of alanine-aminotransferase, asparate-aminotransferase, and alkaline phosphatase, and serum autoantibodies titres (antitopoisomerase I - Scl70, antifibrillarin - U3RNP, anticentromere - ACA, antinuclear - ANA, antiribonucleoprotein - RNP) blood was drawn into Z Serum Clot Activator Vacuette tubes (Greiner Labor Technik). All biochemical/serological measurements were performed, in both SSc patients and healthy subjects, with standard techniques in the Diagnostic Laboratory of Teaching Hospital of Medical University of Lodz.

Statistical analysis

All data are expressed as median and range as well as mean ± SD. The differences between groups were computed with Mann-Whitney U test. Correla-

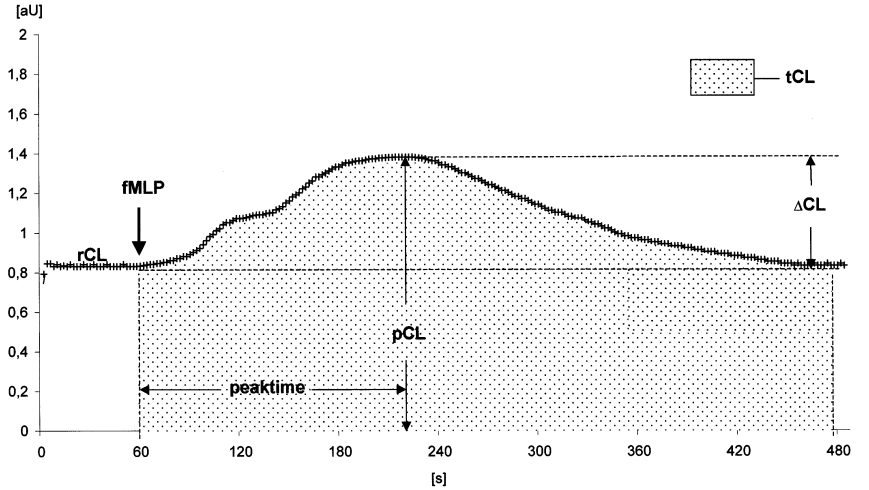

Fig. 1. Typical tracing of whole blood chemiluminescence of healthy subject. rCL: resting chemiluminescence; pCL: peak chemiluminescence; ΔCL: increment in CL intensity; tCL: total light emission; peaktime: time from fMLP addition to pCL appearance.

tions were calculated by the Spearman test. In all cases a p-value of < 0.05 was considered significant.

Results

Luminol enhanced whole blood chemiluminescence

The CL assay revealed higher ROS production by circulating phagocytes in SSc patients as compared to healthy controls. Resting CL in blood specimens was higher in SSc group than in controls ($p < 0.02$). Three (ΔCL, pCL and tCL) of four analysed parameters of fMLP-induced CL were also significantly elevated comparing to healthy subjects and the highest difference (2.7-fold, $p < 0.001$) was observed for ΔCL (Table III).

Current treatment with cyclophosphamide and/or prednisone did not influence resting and fMLP-provoked ROS production in the whole blood. There was very little non significant difference in pCL, ΔCL, and tCL between DMD and NDMD SSc patients subgroup (Table III). There were also no differences ($p > 0.05$) in any parameter of CL between dSSc and lSSc patients (e.g. rCL - 0.88; 0.47–1.39 aU/10^4p vs. 0.87; 0.51–1.34 aU/10^4p, pCl-1.90; 1.0–9.8 aU/10^4p vs. 2.1; 0.80–12.24 aU/10^4p and tCL – 577; 329–2735 aUxs/10^4p vs. 720; 293-3523 aUxs/10^4p).

No differences were found between CL parameters of SSc patients who were current cigarette smokers and those who had never smoked. Also in healthy controls cigarette smoking did not influence resting CL and after stimulation with fMLP (Table IV). However, both cigarette smokers with SSc and never smoked patients with SSc had higher CL than corresponding healthy subjects subgroups.

Correlations between chemiluminescence and clinical parameters

There was a significant positive correlation between rCL and serum autoantibodies titre ($r = 0.43$, $p = 0.02$). Moderate negative correlation between rCL and $DLCO_C$ ($r = -0.59$, $p = 0.02$) paralleled moderate positive correlation between this CL parameter and ILD occurrence ($r = 0.59$, $p = 0.02$). Clinical and demographic features listed in Tables I and II did not associate with rCL or any parameter of fMLP-induced CL (data not shown).

Table III. Comparison of fMLP-induced chemiluminescence parameters in healthy subjects and SSc patients.

Parameter	Healthy subjects	SSc-group	DMD subgroup	NDMD subgroup
rCL [aU/10^4p]	0.73;0.13-1.07 (0.69±0.22)	0.88;0.47-1.39† (0.88±0.27)	0.87;0.47-1.13* (0.87±0.28)	0.89;0.53-1.39* (0.89±0.28)
pCL [aU/10^4 p]	1.09;0.71-1.84 (1.18±0.31)	1.93;0.80-12.24** (2.91±2.68)	2.06;1.02-12.24† (2.83±3.18)	1.92;0.80-9.77† (2.95±2.44)
ΔCL [aU/10^4p]	0.40;0.13-1.06 (0.42±0.2)	1.07;0.15-11.28** (2.02±2.62)	1.19;0.19-11.28* (1.96±3.17)	1.04;0.15-8.38† (2.06±2.4)
tCL [aUxs/10^4p]	411;289-810 (437±120)	621;293-3522† (893±757)	643;329-3522† (865±897)	555;293-1861† (909±689)
peaktime [s]	230;166-272 (231±44)	237;91-299 (233±48)	226;171-299 (227±38)	248;91-295 (236±54)

rCL: resting chemiluminescence; pCL: peak chemiluminescence; ΔCL: increment in chemiluminescene intensity; tCL: total light emission; peaktime: time from fMLP addition to pCL appearance; aU: arbitrary units; aU x s /10^4 p: arbitrary units times seconds per 10^4 phagocytes. †$p < 0.02$; *$p < 0.05$; **$p < 0.001$ vs. healthy controls. Between parentheses mean ± SD.

Table IV. Effect of cigarette smoking on whole blood chemiluminescence in SSc patients and healthy controls.

Parameter	SSc patients		Healthy controls	
	Smokers	Non smokers	Smokers	Non smokers
rCL [aU/10^4p]	0.96; 0.49-1.39† (0.90±0.31)	0.87; 0.47-1.39* (0.88±0.27)	0.51; 0.49-0.78 (0.52±0.14)	0.76;0.13-1.07 (0.71±0.23)
pCL [aU/10^4 p]	1.87; 0.80-12.24† (3.27±4.05)	1.94; 0.98-9.77† (2.80±2.23)	0.99; 0.79-1.26 (0.89±0.18)	1.11; 0.71-1.84 (1.19±0.32)
ΔCL [aU/10^4p]	1.02; 0.15-11.28† (2.37±3.99)	1.09; 0.26-8.38† (1.92±2.16)	0.48; 0.29-0.66 (0.43±0.15)	0.39; 0.13-1.06 (0.39±0.23)
tCL [aUxs/10^4p]	599; 303-3522† (977±1142)	643; 293-2735* (867±630)	345; 289-443 (313±61)	438; 312-810 (458±132)
peaktime [s]	246; 203-299 (262±52)	233; 91-295 (223±45)	231; 166-254 (218±35)	229; 163-272 (234±46)

rCL: resting chemiluminescence; pCL: peak chemiluminescence; ΔCL: increment in chemiluminescene intensity; tCL: total light emission; peaktime: time from fMLP addition to pCL appearance; aU: arbitrary units; aU x s /10^4 p: arbitrary units times seconds per 10^4 phagocytes. †$p < 0.02$; *$p < 0.05$ vs. corresponding healthy controls subgroup. No significant differences were found between smokers and never smoked patients with SSc and also between never smoked healthy controls and asymptomatic cigarette smokers. Between parentheses mean ± SD.

Discussion

Patients with SSc revealed increased resting and fMLP-provoked CL in comparison with age- and sex-matched healthy subjects. This is compatible to previous reports on increased ROS production by isolated PMNs and monocytes from SSc patients (8, 9, 11, 12). We found for the first time the correlations between the increase in resting CL and selected clinical characteristics such as reduced $DLCO_C$, occurrence of ILD, and autoantibodies titre in SSc patients. These indicate that lung involvement in the course of SSc is accompanied by higher ROS production by circulating phagocytes which is in concordance with the occurrence of high plasma levels of lipid peroxidation markers in SSc patients with ILD (3).

CL examines production of superoxide

radical and other ROS including hypochloride and H_2O_2 (28). They are produced by circulating phagocytes as a consequence of NADPH oxidase activation. Mitochondria of leukocytes and plasma xanthine-xanthine oxidase system are also able to produce superoxide radical (28). The contribution of xanthine-xanthine oxidase system to CL seems to be negligible due to low plasma concentration of xanthine (29) and high blood specimen dilution used by us during measurements. Although luminol easily penetrates into leukocytes it hardly accumulates in mitochondria (28, 30). Therefore, CL reflects mainly ROS production by cellular NADPH oxidase system. Various plasma antioxidants may suppress CL. Serum levels of bilirubin, albumin, uric acid as well as the haemoglobin concentration and erythrocytes number that all can scavenge ROS (31, 32) did not differ between healthy controls and SSc patients. Thus, differences in the blood antioxidants seem not to be responsible for elevated CL in SSc patients.

Patients with SSc are reported to have increased plasma concentrations of pro-inflammatory cytokines (e.g TNF-alpha and IL-6) that positively correlate with the occurrence and severity of ILD and auto-antibodies titre (33, 34). It is possible that elevated CL in SSc patients could result from priming effect of these cytokines (35, 36). This may also explain association between resting CL and ILD and auto-antibodies titre.

In opposite to resting CL, fMLP-induced CL did not associate with any of analysed clinical parameter.

All patients were treated with vitamin E and nifedypine that both can inhibit ROS release from phagocytes (37, 38). Vitamin E can directly scavenge ROS generated by phagocytes or may suppress the respiratory burst of these cells by inhibition of NADPH-oxidase activation (37). Six-week administration of vitamin E (400 mg/day) was reported to result in the decrease in whole blood chemiluminescence in healthy subjects (39). Also combination of vitamins E and C (600 mg of each/day) is known to decrease ROS production by isolated PMNs in healthy subjects and patients with acute myocardial infarction (40, 41). Patients in our study received 400 mg/day of vitamin E for at least 5 months. Thus this treatment can suppress ROS production by phagocytes and could be responsible for lack of correlations between fMLP-induced CL and clinical parameters.

Prednisone and cyclophosphamide can inhibit phagocyte activity (42, 43). In previous study by others, 2 month administration of prednisone decreased ROS production by alveolar macrophages in children with ILD (44). Also 6 week treatment with cyclophosphamide decreased resting and fMLP-stimulated chemiluminescence of isolated granulocytes in patients with breast cancer (45). However, in present study, no significant differences in resting and agonist induced-CL were noted between DMD and NDMD patients. This is compatible to our previous study showing no effect of treatment with these drugs on elevated H_2O_2 exhalation by SSc patients (46). It cannot be excluded that; first – no effect of this treatment on CL could be a consequence of low efficacy of these drugs as disease modifying agents in SSc; second – the doses of prednisone and/or cyclophosphamide were too low to suppress phagocyte respiratory burst; and third - concomitant treatment with vitamin E and nifedypine by suppression of phagocyte respiratory burst could mask the effect of DMD drugs on resting and fMLP-induced CL. Additionally, there was a great inter-individual variability in the treatment duration (3 to 168 months) in present study and this can also be responsible for lack of differences between DMD and NDMD SSc patients.

On the other hand, our study protocol did not allow assessing fully the influence of treatment with DMD on CL because there was no comparison between CL before and after treatment institution.

In conclusion, we found that blood phagocytes from SSc patients generated elevated amounts of ROS under resting conditions and in response to fMLP. Resting ROS production correlated positively with ILD and serum autoantibodies titre. The former confirms the occurrence of systemic phagocyte-derived oxidative stress in patients with SSc. The latter suggests that increased resting phagocyte ROS production can be a marker of disease progression with ILD development. However, it requires further prospective studies.

Acknowledgements

The authors wish to thank Mrs Anna Włodarczyk for technical assistance during the experiments.

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