Increased serum fractalkine in systemic sclerosis. Down-regulation by prostaglandin E1

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Abstract

Objectives

To evaluate serum levels of fractalkine (FKN), a mediator of leukocyte transmigration, C-reactive protein (CRP) and expression of integrins CD11a and CD49d on peripheral blood lymphocytes in systemic sclerosis (SSc) and to investigate whether they are modulated by intravenous prostaglandin E1 (PGE1).

Methods

Serum levels of fractalkine and C-reactive protein and expression of CD11a and CD49d on peripheral blood lymphocytes were assessed in 50 SSc patients and in 18 healthy controls. In 25 SSc patients studied parameters were evaluated also after 3 consecutive daily PGE1 infusions (20 μg-40 μg-60 μg) and after 4 weeks.

Results

In SSc fractalkine basal level was significantly higher than in controls (9.04±1.79 ng/ml vs. 1.17±0.1 ng/ml; p<0.0001) and decreased significantly after PGE1 (5.16±1.27 ng/ml, p<0.05). After four weeks fractalkine level was still significantly lower than baseline 7.70±2.19 ng/ml (p<0.05). Basal percentage of CD11a (+) nor CD49d (+) lymphocytes in SSc (82.38±1.60%, 70.74±1.68%, respectively) did not differ from controls (85.73±2.04%, 75.62±2.48%; respectively, p>0.05). PGE1 treatment resulted in decrease of both CD11a (+) (67.72±3.34%, p<0.0001) and CD49d (+) lymphocytes (65.32±1.62%, p<0.0001). After 4 weeks the percentage of CD11a (+) and CD49d (+) lymphocytes remained significantly lower than at baseline (77.80±2.47% and 65.32±1.62%, respectively, both p<0.001). In SSc CRP basal level was significantly higher than in controls (4.70±2.01 mg/dl vs. 1.40±1.79 mg/dl, p<0.005) and reduced significantly after PGE1 (3.39±2.06 mg/dl, p<0.05). After 4 weeks, CRP level (4.38±2.19 ng/ml) was significantly lower than baseline (p<0.05).

Conclusion

Fractalkine may play an important role in the pathogenesis of vascular dysfunction in systemic sclerosis. Prostaglandin E1 down-regulates serum fractalkine level, as well as CD11a and CD49d expression on peripheral blood lymphocytes, which suggests additional mechanisms in which this vasodilatatory agent exerts its efficacy in systemic sclerosis.

Key words

CD11a antigen, CD49d antigen, endothelial cells, fractalkine, prostaglandins, scleroderma.

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Introduction

Systemic sclerosis (SSc) is a chronic connective tissue disease of unknown etiology, characterized by micro- and macrovascular damage, immunologic disturbances and fibrosis of the skin and internal organs (1). Endothelial injury, perivascular infiltration of inflammatory cells as well as collagen overproduction are the morphologic hallmarks of SSc. Structural changes are accompanied by Raynaud's phenomenon which is the most common clinical manifestation of SSc vascular dysfunction (2). Regulatory mechanisms of transendothelial migration of inflammatory cells, mainly activated lymphocytes, have been an issue of interest for many years (3, 4).

Fractalkine, a member of CX3C chemokine family was first described by Bazan *et al*. (5). The expression of FKN was found on tumor necrosis factor α (TNF-α) and IL-1 stimulated endothelial cells (6, 7). The extracellular domain of fractalkine is released as a result of enzymatic cleavage of the mucin stalk by the TNF-α converting enzyme (TACE, ADAM-17) (8, 9). Unlike other chemokines, membrane-bound FKN can mediate firm adhesion and initiate leukocyte capture (10, 11) while soluble FKN exhibits chemotactic activity for T cells, monocytes/macrophages and natural killer cells (12).

FKN expression on endothelial cells not only stimulates lymphocyte activation process, but also may mediate all stages of lymphocyte migration trough vessel walls: tethering, rolling and firm adhesion (13, 14). Fractalkine is detectable in many vasculopathies including atherosclerosis, diabetes mellitus and transplant vascular disease, while it was not expressed in normal vessels (15). FKN is also found in primary biliary cirrhosis, where it probably is responsible for the formation of lymphocyte infiltrates and may stimulate interstitial lesions in human crescentic glomerulonephritis (16, 17). There are few reports on the role of fractalkine in the pathogenesis of connective tissue diseases. In systemic lupus erythematosus, significant correlations between serum sFKN levels and disease activity and neuropsychiatric involvement were reported (18). Upregulation of metalloproteinase-2 production in synovial fibroblasts upon fractalkine stimulation *in vitro* supports the hypothesis of a proinflammatory role of this chemokine in rheumatoid arthritis (19). It has also been shown that prematurely aged $CD4^+$ T cells that accumulate in rheumatoid arthritis aberrantly express CX(3)CR1 (20). Membrane-anchored FKN was proved to enhance $CD4^+$ T cell adhesion to synoviocytes: it provided survival signals and co-stimulated the production of proinflammatory cytokines as well as granule release.

The role of fractalkine in systemic sclerosis has not been fully studied. Hasegawa *et al*. reported increased numbers of $CX(3)CR1^+$ cells in the lesional skin and lung tissue in diffuse cutaneous SSc (21). The authors also report pronounced FKN expression on endothelial cells in the affected skin and lung tissue as well as raised serum levels of fractalkine associated with raised erythrocyte sedimentation rates, digital ischemia, and severity of pulmonary fibrosis.

Adhesion molecules play an important role in leukocyte migration through vessel wall. Lymphocyte-function-associated antigen-1 (LFA-1, CD11a) and very late antigen-4 (VLA-4, CD49d) present on leucocytes bind to intercellular adhesion molecule-1 (ICAM-1, CD54) and vascular cell adhesion-1 (VCAM-1, CD106), respectively. CD11a and CD49d are predominant integrins for strong adhesion of leukocyte required for vessel wall infiltration. It has been reported that SSc lymphocytes display increased expression of LFA-1 and VLA-4, which additionally was shown to correlate with pulmonary involvement (22-24).

Therefore, the aim of the study was to evaluate serum fractalkine level and leukocyte expression of CD11a and CD49d in patients with systemic sclerosis before and after intravenous prostaglandin E1 treatment for digital ischemia. C-reactive protein levels were also assessed.

Patients and methods

Patients

Fifty consecutive Caucasian SSc patients with moderate/severe Raynaud's phenomenon (minimum 3 episodes a

day), especially with accompanying ischemic ulcers on distal parts of extremities (46 females, 4 males, mean age 49.5±12.9 years) were included in the study.

The diagnosis of limited SSc (18 patients) and diffuse SSc (32 patients) was based on the criteria proposed by LeRoy (25). Additionally, patients were classified according to Rand Maricq and Valter (26). Eighteen healthy subjects matched for age and sex served as control group (16 females, 2 males, mean age 47.2±13.6 years).

The study design was approved by the CSK MSWiA Hospital Ethical Committee. Patients and controls were required to provide written informed consent.

Exclusion criteria were: age less than 18 years, pregnancy, breastfeeding, myocardial ischemia, myocardial infarction in the preceding 6 months, advanced cardiopulmonary disease, active renal crisis, malignancy and no treatment with parenteral prostanoids within 6 months preceding the study. Treatment for systemic sclerosis *(e.g.,* methotrexate, corticosteroids, cyclophosphamide) could be continued, but new treatment was not introduced during any phase of the study. Clinical features of SSc patients are shown in Table I.

Three of the 25 patients (all with dSSc) were classified as early stage of SSc. The diagnosis of early systemic sclerosis was made according to Medsger's proposal for (dSSc <3 years, lSSc <5 years, both measured from the time of the first symptom attributable to SSc) (27). At the entry, all patients were assessed as clinically stable, not in the improving phase of the disease.

The patients included in the study had been evaluated for organ involvement within 1 month before the beginning of the study. The organ involvement was assessed as follows: lung: fibrosis on chest radiography and high resolution tomography; heart: arrhythmia/conduction defect requiring pharmacological medication, congestive heart failure, pericarditis or pericardiac effusion in two-dimensional ultrasound evaluation; esophagus: distal esophageal hypomotility/amotility on barium radiograph; kidney: abnormal creatinine clearance, isolated proteinuria >1g/d or presence of malignant hypertension. Arthralgia/myalgia were evaluated based on anamnesis. Skin involvement was evaluated according to Rodnan's modified skin score (28).

Table I. Clinical features of SSc patients.

	dSSc (n=32)	lSSc (n=18)
Age (years)	49.5 ± 12.0	49.5 ± 14.9
Sex (females/males)	28/4	18/0
Disease duration (years)	10.9 ± 7.3	12.0 ± 8.8
Duration of RP (years)	17 ± 10.0	19.9 ± 11.5
Onset of skin indurations (years)	11.7 ± 7.1	15.1 ± 9.5
Skin indurations	100%	88%
Modified Rodnan skin score	13.1 ± 7.5	7.8 ± 6.5
Fingertip ulcers	30%	16%
ANA	30/32 (94%)	17/18 (94%)
ACA	4/42 (13%)	6/18 (33%)
TOPO 1	22/32 (69%)	10/18 (56%)
Other ANA (Ro, La, RNP, Pol RNA I, II, III)	4/32 (13%)	1/18 (5%)
Esophagus involvement	59%	50%
Lung fibrosis	38%	21%
Heart involvement	38%	28%
Kidney involvement	15%	6%
Arthralgia/myalgia	78%	55%

In one female dSSc patient ACA and TOPO 1 coexisted.
RP: Raynaud phenomenon; ANA: antinuclear antibodies; ACA: anti-centromere antibodies: TOPO 1: anti-topoisomerase 1 antibodies.

Treatment regimen

Following the baseline evaluation, 25 patients were randomly assigned to receive prostaglandin E1 intravenously on 3 consecutive days (20 μg-40 μg-60 μg PGE1 diluted in 50 ml of 0.9% saline was administered in a 5-hour infusion).

Laboratory analysis

In all patients and in healthy controls, freshly drawn venous blood samples were maintained cooled in 4ºC for 30-60 minutes, centrifuged (5000g, 15 minutes) and then conserved in -80ºC until enzyme-linked immunosorbent assay (ELISA) was performed. In SSc patients treated with PGE1, blood was drawn before infusions (day 0), after third infusion (day 3) and after 4 weeks (day 28).

Serum fractalkine levels were assessed by ELISA. Reagents were obtained from R&D Systems (Minneapolis, MN). Soluble FKN (sFKN) levels were measured in serum samples, by using 96 well polystyrene plates coated overnight at 25ºC with 2 mg/ml of purified goat antihuman FKN IgG. After being washed, plates were blocked for 60 minutes at 20ºC with phosphate buffered saline containing 1% bovine serum albumin and 5% sucrose. Recombinant human FKN and serum samples were added in duplicate, then the plates were incubated for 2 hours at 20ºC. After further incubation with streptavidin-peroxidase for 1 hour at 20ºC, samples were developed by adding 0.1 ml/well of tetramethylbenzidine substrate diluted in citrate-phosphate buffer. Reactions were stopped by adding 1 M H2SO4 and absorbance was read at 450 nm.

Lymphocyte CD11a and CD49d expression was assessed by flow cytometry. Heparinised blood (5ml blood with 10,000 international units of heparin) samples were incubated with anti-CD49d/FITC and anti-CD11a/PE (Beckton&Dickinson). Additional staining with anti-CD3 and anti-CD4 (Beckton & Dickinson) for determination of cell population was performed. Blood samples were stained for 30 minutes at room temperature in a dark room. Blood erythrocytes were then lysed with Uti-Lyse for Flow (Dako) according to manufacturer's instructions. Samples were analyzed with Coulter Epics XL flow cytometer C-reactive protein level was analyzed by standard laboratory procedure with Coulter LH 750.

Statistical analysis

The results were expressed as the mean ± SE (standard error). The Shapiro-Wilk Normality Test was used to determine whether the variables followed a normal distribution. For comparison of non-parametric values Wilcoxon's rank test was used. In addition, correlations between disease parameters were assessed by Spearman's rank correlation test. *P*-values less than 0.05 were considered significant. Mean values were compared using Student's test.

Results

Effect of prostaglandin E1 on serum fractalkine

In patients with SSc the mean sFKN level (9.04±1.79 ng/ml.) was higher than in controls (1.17±0.1 ng/ml; $p<0.0001$). sFKN level was significantly higher in patients with dSSc (10.79 ± 2.46) than in lSSc (5.94±1.28; $p<0.05$).

In patients treated with prostaglandin E1, sFKN level significantly reduced after 3 day PGE1 treatment (5.16±1.27 ng/ml *vs*. baseline 10.74±2.76 ng/ml, $p<0.05$). After 4 weeks, sFKN level (7.70±2.19 ng/ml) was still significantly lower than baseline ($p<0.05$).

Effect of prostaglandin E1 on CD11a and CD49d lymphocyte expression

In patients with SSc CD11a expression on peripheral blood lymphocytes was 82.38±1.60%; dSSc 82.24±2.06%, lSSc 82.59±2.62% ($p>0.05$). In controls CD11a expression was 85.73±2.04% ($p>0.05$).

Significant reduction of CD11a lymphocyte expression after 3 day PGE1 treatment was detected (81.28±1.86% *vs*. 67.72±3.34%; $p<0.0001$). After 4 weeks, CD11a lymphocyte expression (77.80±2.47%) was still significantly lower than baseline ($p<0.001$).

Effect of prostaglandin E1 on CD49d lymphocyte expression

In patients with SSc CD49d expression on isolated peripheral blood lymphocytes was 70.74±1.68%; dSSc 71.14±2.27%, lSSc 70.02±2.39%. In controls CD49d expression was 75.62 ±2.48%. Differences between groups were insignificant ($p>0.05$)

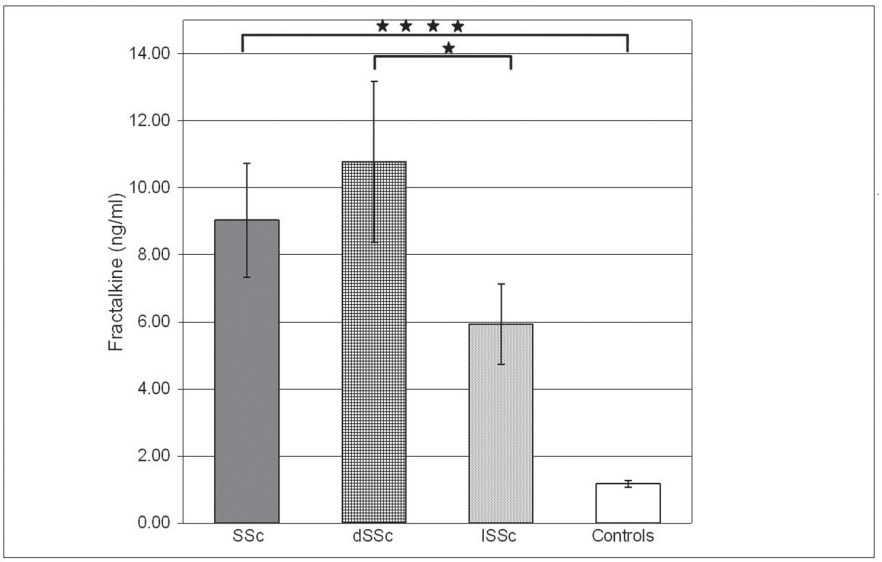

Fig. 1. Baseline serum fractalkine level (ng/ml) and lymphocyte expression of CD11a and CD49d (%). Mean values and standard error (SE) shown. $^{****}p<0.0001$, $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$.

Significant reduction of CD49d lymphocyte expression after 3 day PGE1 treatment was detected (71.46±1.46% *vs*. 57.33±2.81%; $p<0.0001$). After 4 weeks, CD49d lymphocyte expression (65.32±1.62%) was still significantly lower than baseline ($p<0.001$).

Effect of prostaglandin E1 on C-reactive protein *(CRP) level*

In SSc, CRP basal level was significantly higher than in controls (4.70±2.01 mg/dl *vs*. 1.40±1.79 mg/dl, $p<0.005$). CRP level was significantly higher in patients with dSSc (5.44±2.37 mg/dl) than in lSSc (3.40±2.89 mg/dl, $p<0.05$).

In patients treated with prostaglandin E1, CRP level reduced significantly after 3 day PGE1 treatment (3.39±2.06 mg/dl *vs*. 4.82±2.39 mg/dl at baseline, $p<0.05$). After 4 weeks, CRP level (4.68±2.19 ng/ml) was significantly lower than baseline ($p<0.05$).

Correlations of sFKN and CRP levels, CD11a and CD49d lymphocyte expression with clinical features

Serum FKN level inversely correlated with patients' age ($r=-0.366$, $p<0.05$) and the duration of Raynaud phenomenon ($r=-0.330$, $p<0.05$).

No correlation was found between CRP level and clinical features, but greater decreases correlated with shorter disease duration ($r=0.327$, $p<0.05$).

CD11a lymphocyte expression correlated with CD49d lymphocyte expression (r=0.814, p<0.0001) and skin score ($r=0.316$, $p<0.05$). Additionally, CD49d lymphocyte expression correlated inversely with age ($r=-0.325$, $p<0.05$).

Discussion

Pronounced vascular disease clinically evident as Raynaud's phenomenon and often accompanied by digital ulcers provides the need for intensive vasoactive treatment in systemic sclerosis. Besides endothelin receptor antagonists and phosphodiesterase-5 inhibitors, prostanoids remain an important therapeutical option in patients with severe Raynaud's phenomenon.

Clinical features of SSc patients were analyzed. The incidence of TOPO I was comparable to those previously observed by other authors who studied Polish SSc population. In one of the largest studies of Polish SSc patients (n=478) Topo I was found in 62.8% dSSc patients and 54.7% lSSc patients (29). In other studies for dSSc incidence was 72% and 85% and for lSSc -46% in both studies (30, 31).

We observed clinical improvement (RP reduction) in patients treated with prostaglandin E1. There are several mechanisms by which prostanoids exert their clinical efficacy. The vasodilatatory effect is of great therapeutic value, even

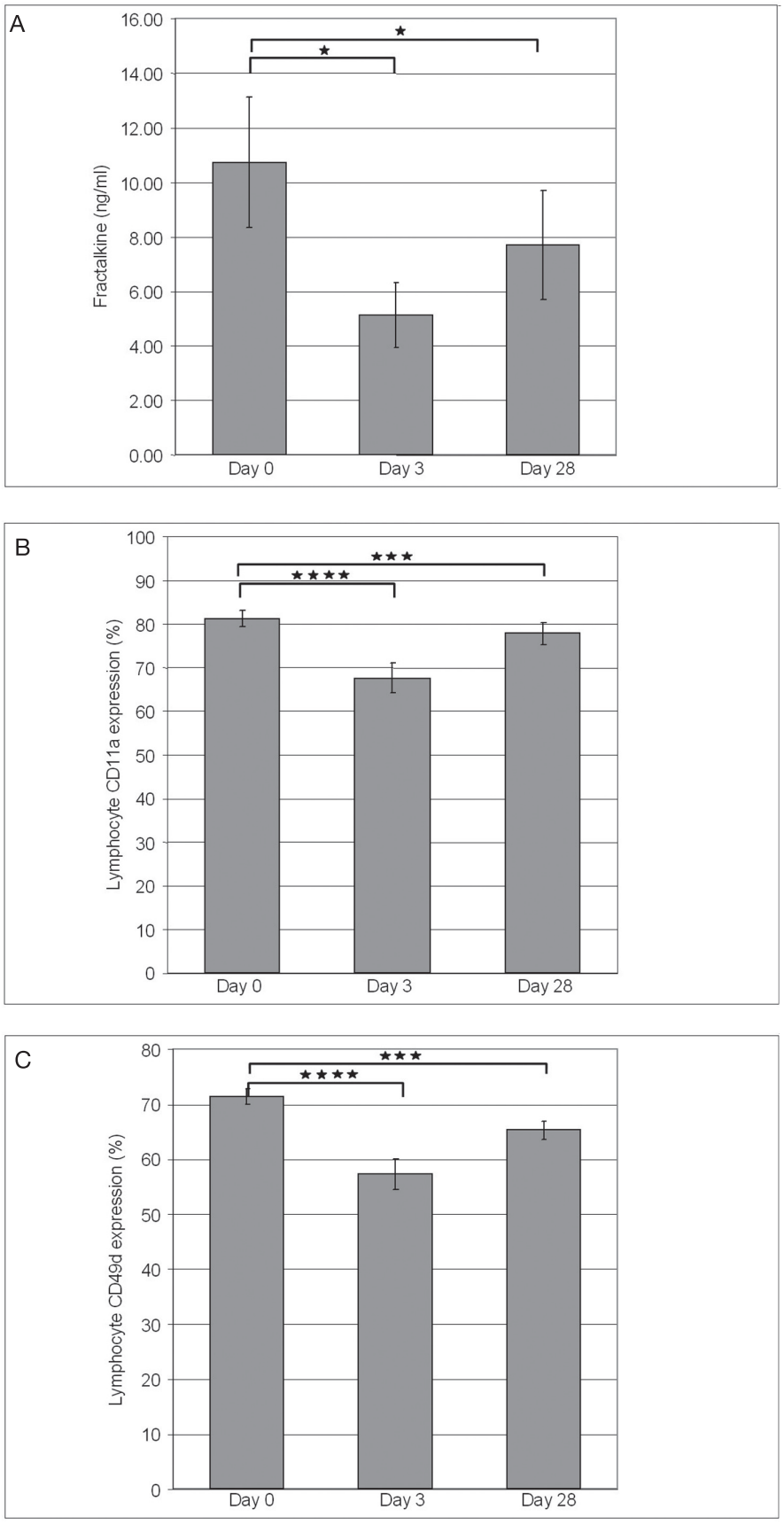

Fig. 2. Effect of PGE1 on lymphocyte expression of CD11a (%) and CD49d (%) and serum fractalkine level (ng/ml). Mean values and standard error (SE) shown.
$^{****}p<0.0001$, $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$.

though it is transient (32). Inhibition of platelet adhesion and activation, modulation of neutrophile activation as well as modulation of fibrinolytic activity are also involved (33, 34). Gardinali *et al*. suggested that the long-lasting therapeutic effect of prostaglandin E1 was related to functional improvement by presenting decreased plasma levels of markers of endothelial damage such as circulating intercellular adhesion molecule-1 (cICAM-1) and tissue-type plasminogen activator (t-PA) after PGE1 treatment (35). Treatment with prostaglandin E1 effects both endothelium and lymphocytes. PGE1 seems to have an antinflammatory effect related to the inhibition of leukotriene B4 release and superoxide anion generation from human polymorphonuclear leukocytes (PMN) (36, 37).

We presented reduction of lymphocyte integrin expression after PGE1 treatment. These effects add up to known vasodilatatory and antiplatelet effect of PGE1 (38).

We observed raised FKN levels in SSc patients in comparison to healthy controls. Significant decrease of soluble fractalkine level observed in patients treated with PGE1, which is probably associated with reduction of endothelium-bound FKN, reduces chemoattraction of CX3CR1+ lymphocytes in FKN-dependent mechanism and transmigration which stimulates immunologically induced fibrosis.

The possible effect of FKN level reduction might be caused by the fact, that CX3R1 expression is characteristic for NK cells possessing high levels of intracellular granzyme B and perforin (39, 40). As these two substances are known to be responsible for endothelial injury in cell-mediated cytotoxicity, we suggest that FKN level reduction might effect not only the adhesion and transmigration of the cells, but also cytotoxic, NK cell-mediated endothelium injury. It has also been shown that FKN-mediated monocyte/macrophage infiltration of the vessel wall plays a role in the pathogenesis of atherosclerosis (41). This suggests that blocking FKN-CX3CR1 pathway may also protect endothelium against atherosclerotic lesions.

Fractalkine was shown to play a regulatory role in apoptosis. Boehme *et al*. showed fractalkine modulates Fas-mediated apoptosis of microglia cells in central nervous system (42). Morita's studies revealed the fractalkine-CX3CR1 system was responsible for apoptosis of bone marrow progenitor cells in myelodysplastic syndrome (43). In pulmonary arterial hypertension, up-regulated expression of CX_3CR1 on T cells, increased plasma fractalkine concentrations as well as elevated fractalkine mRNA and protein product in pulmonary artery endothelial cells were found (44). In patients with atopic dermatitis, FKN was strongly expressed on endothelial cells in skin lesions, serum sFKN levels and the number of CX_3CR1+ cells were increased and correlated with the disease severity and decreased with the improvement of skin lesions (45). This suggests FKN system plays an important role in the trafficking of CX_3CR1+ cells during the inflammatory process. The evidence for fractalkine role in vascular injury is supported by FKN-deficient mice model: after transient focal cerebral ischemia, FKN-deficient mice developed less pronounced postischemic brain injury in comparison to wild-type controls (46).

Prostaglandin E1 may decrease FKN-mediated apoptosis, however this concept should be confirmed by further studies. The precise role of fractalkine in the whole network of cytokines implied in the pathogenesis of systemic sclerosis is to be determined. The work of Hasegawa *et al*. revealed raised levels of fractalkine receptor CX_3CR1 on peripheral monocytes/macrophages and T cells in patients with dc SSc. The numbers of CX_3CR1 expressing cells were increased in the lesional skin and lung tissues from patients with dcSSc. Additionally, strong fractalkine expression was found on endothelial cells in the affected skin and lung tissue. Hasegawa reported association between increased soluble fractalkine levels and raised erythrocyte sedimentation rate, presence of digital ischemia, and severity of pulmonary fibrosis. Functional studies detected a chemotactic activity of soluble fractalkine for T lymphocytes, monocytes and IL-2 activated NK cells (47-49). Decrease of serum fractalkine level after PGE1 treatment might result from the inhibition of fractalkine expression in human endothelial cells as shown for 15-deoxy-D12, 14-prostaglandin J2 (50).

Raised serum fractalkine levels were observed in other connective tissue diseases.

Serum sFKN levels were found to be significantly increased in patients with rheumatoid arthritis and rheumatoid vasculitis as compared to healthy controls (51). Yaima *et al*. reported serum FKN levels were significantly higher in patients with systemic lupus erythematosus (SLE) than in patients with rheumatoid arthritis or healthy controls (52).

Yaima *et al*. have shown sFKN levels correlate with the SLEDAI (Systemic Lupus Erythematosus Disease Activity Index), the SLICC/ACR (Systemic Lupus International Collaborative Clinics/ American College of Rheumatology) Damage Index, anti-double-stranded DNA and anti-Sm antibody titers, immune complex levels and serum complement levels. Treatment reduced serum FKN level in patients with SLE.

Our study showed that PGE1 infusions reduced expression of CD11a (LFA-1) and CD49d (VLA-4) on lymphocytes in patients with systemic sclerosis. This might also augment the ultimate effect of decreased lymphocyte transmigration. Other authors report PGE1 infusions decreased plasma cICAM-1 concentrations *in vivo* (35). We have observed that the reduction of levels of serum FKN and CD11a and CD49d persisted to day 28 which suggests possibility of obtaining further improvement during repeated therapy with PGE1. Even though there is no direct link between CD11 and CD49d and serum (or membrane-bound) fractalkine, all the aforementioned molecules play a role in the process of transendothelial migration of lymphocytes. This process depends on expression of adhesion molecules on endothelial cells, as well as on migrating cells. Integrins, including LFA-1 (CD11a) and VLA-4 (CD49d) are needed for firm adhesion in the transmigration process, while fractalkine mediates both leukocyte migration and adhesion (10). The resulting infiltration of T cells and monocytes/macrophages may promote tissue fibrosis. We showed reduction of CD11a and CD49d expression after PGE1 treatment to present the synergistic effect of the studied drug.

In conclusion, we have demonstrated that SSc patients exhibit increased serum levels of fractalkine which probably plays a significant role in endothelium injury in these patients. We also showed that intravenous prostaglandin E1 down-regulates fractalkine toward normal values, and thus may contribute to reduction of vascular dysfunction in SSc.

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