Up regulated expression of fractalkine/CX₃CL1 and CX₃CR1 in patients with systemic sclerosis

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Background: Fractalkine expressed on endothelial cells mediates activation and adhesion of leucocytes expressing its receptor, CX₃CR1. Soluble fractalkine exhibits chemotactic activity for leucocytes expressing CX₃CR1.

Objective: To determine the role of fractalkine and its receptor in systemic sclerosis (SSc) by assessing their expression levels in patients with this disease.

Methods: The expression of fractalkine and CX₃CR1 in the skin and lung tissues was immunohistochemically examined. Circulating soluble fractalkine levels were examined by enzyme linked immunosorbent assay (ELISA). Blood samples from patients with SSc were stained for CX₃CR1 with flow cytometric analysis.

Results: CX₃CR1 levels on peripheral monocytes/macrophages and T cells were found to be raised in patients with diffuse cutaneous SSc. The numbers of cells expressing CX₃CR1, including monocytes/macrophages, were significantly increased in the lesional skin and lung tissues from patients with diffuse cutaneous SSc. Fractalkine was strongly expressed on endothelial cells in the affected skin and lung tissues. Soluble fractalkine levels were significantly higher in sera and were associated with raised erythrocyte sedimentation rates, digital ischaemia, and severity of pulmonary fibrosis.

Conclusions: Up regulated expression of fractalkine and CX₃CR1 cooperatively augments the recruitment of mononuclear cells expressing CX₃CR1 into the affected tissue of SSc, leading to inflammation and vascular injury.

Systeimic sclerosis (SSc) is a multsystem disorder of connective tissue characterised by excessive fibrosis and vascular changes in the skin and various internal organs, with an autoimmune background. Endothelial injury associated with perivascular cellular infiltration is seen in SSc even before the development of tissue fibrosis. Although the pathogenesis of SSc remains unknown, it has been suggested that immunological abnormalities have an important role. Recent investigations have identified many potential molecules, including chemokines, that regulate the migration and recruitment of specific leucocytes to the inflammatory regions. Fractalkine (FKN)/CX₃CL1 is expressed on endothelial cells stimulated with proinflammatory cytokines such as interleukin 1, tumour necrosis factor α (TNFα), and interferon γ. FKN localised on the endothelial cells not only promotes leucocyte activation but, unlike other chemokines, can also mediate each individual step of the leucocyte adhesion cascade, including capture, rolling, and firm adhesion. Specifically, FKN interacts with its unique receptor, CX₃CR1, to effect firm adhesion of monocytes/macrophages, natural killer (NK) cells, and a subpopulation of T cells (CD8⁺ T cells >CD4⁺ T cells). TNFα converting enzyme, a disintegrin and metalloproteinase (ADAM) 17, can cleave the mucin stalk of FKN and release soluble FKN (sFKN). Soluble FKN exhibits efficient chemotactic activity for monocytes/macrophages, NK cells, and T cells expressing CX₃CR1. Accumulating evidence suggests that FKN-CX₃CR1 interaction might contribute to the development of vascular injury and inflammatory diseases, by recruiting activated leucocytes. Therefore, we suggested that FKN-CX₃CR1 interaction may have some role in the inflammatory processes and vascular injuries of SSc. We evaluated the expression of FKN and CX₃CR1 in patients with SSc to clarify the role of FKN in the inflammation of SSc.

MATERIALS AND METHODS

Serum samples

Serum samples were obtained from 67 Japanese patients (58 women, 9 men). All patients fulfilled the criteria proposed by the American College of Rheumatology. These patients were grouped according to the classification of LeRoy et al: 36 patients (32 women, 4 men) had limited cutaneous SSc (ISSLc) and 31 (26 women, 5 men) had diffuse cutaneous SSc (dSSc). The mean (SD) age of the patients was 49 (15) years; patients with ISSLc were aged 53 (12) years and patients with dSSc 46 (18) years. Antitopoisomerase 1 antibody (Ab) was positive in 24 (5 ISSLc, 19 dSSc), anticentromere Ab in 26 (24 ISSLc, 2 dSSc), and anti-U1RNP Ab in 5 (2 ISSLc, 3 dSSc) cases. Other Abs detected included anti-RNA polymerase Ab (3 dSSc), anti-U3RNP Ab (2 dSSc), and anti-7-2RNA Ab (2 ISSLc). Four patients (1 ISSLc, 3 dSSc) were found to have antinuclear Abs by indirect immunofluorescence using Hep-2 cells as substrate, but their specificities were not identified by autoantibody-specific enzyme linked immunosorbent assay (ELISA) and immunoprecipitation. The mean (SD) disease duration of the patients with ISSLc and dSSc was 8.2 (10.8) and 46.7 (2) years, respectively. None of the patients with

Abbreviations: Ab, antibody; dSSc, diffuse cutaneous SSc; ELISA, enzyme linked immunosorbent assay; FKN, fractalkine; ISSLc, limited cutaneous SSc; mTSS, modified Rodnan total skin thickness score; NK, natural killer; PBMC, peripheral blood mononuclear cells; SSc, systemic sclerosis; TLC, carbon monoxide transfer factor; TNF, tumour necrosis factor; VC, vital capacity

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SSc were treated with steroid, D-penicillamine, or immunosuppressive drugs at their first examination. None had a recent history of infection or other inflammatory diseases. Twenty healthy subjects matched for age and sex served as normal controls (18 women, 2 men; age 48 (15) years). Fresh venous blood samples were centrifuged shortly after clot formation. All samples were stored at −70°C before use.

**Clinical assessment**

At their first visit, all patients had a physical examination, underwent laboratory tests, and provided a complete medical history. A skin score—the modified Rodnan total skin thickness score (mTSS)24—was measured. Organ system involvement was defined as described previously25-26: lung, bibasilar fibrosis on chest radiography and high resolution computed tomography; isolated pulmonary hypertension, clinical evidence of pulmonary hypertension and increased mean pulmonary arterial pressure (>35 mm Hg) documented by echocardiography, in the absence of severe pulmonary interstitial fibrosis; oesophagus, hypomotility shown by barium radiography; joint, inflammatory polyarthralgias or arthritis; heart, pericarditis, congestive heart failure, or arrhythmias requiring treatment; kidney, renal crisis defined as malignant hypertension and rapidly progressive renal failure without any other explanation; and muscle, proximal muscle weakness and raised serum creatine kinase. A pulmonary function test, including vital capacity (VC) and carbon monoxide transfer factor (TLCO), was also carried out. When the TLCO and VC were <75% and <80%, respectively, of the predicted normal values, they were considered to be abnormal. The protocol was approved by the Kanazawa University Graduate School of Medical Science, and informed consent was obtained from all patients.

**Immunohistochemical staining**

Skin biopsy samples were taken from the dorsal aspect of the mid-forearm of five female patients with dSSc with rapidly progressing skin thickening (age 43 (15) years) and four healthy female volunteers (age 46 (19) years). Open lung biopsy specimens were obtained for clinical staging from six female patients with dSSc with active pulmonary fibrosis (age 46 (14) years) and were compared with control open lung biopsy specimens taken from the unaffected regions from five female subjects undergoing thoracotomy for tumour resection (age 49 (18) years). The control skin and lung tissue were normal upon histological examination under light microscopy after routine histological staining.

FKN and CX3CR1 levels in the skin and lung tissues were determined by immunohistological staining, as previously described.27 Briefly, formalin fixed, paraffin embedded dermal and lung tissues were fixed in acetone and then incubated with 10% normal rat serum in phosphate buffered...
saline (10 minutes, 37 °C) to block non-specific staining. Sections were stained with goat polyclonal IgG Ab specific for human FKN (Santa Cruz Biotechnology Inc, Santa Cruz, CA) or rabbit polyclonal IgG specific for human CX3CR1 (Chemicon International, Temecula, CA). Goat IgG (Santa Cruz Biotechnology Inc) and rabbit IgG (Santa Cruz Biotechnology Inc) were used as controls for non-specific staining of FKN and CX3CR1, respectively. Sections were incubated sequentially (20 minutes, 37 °C) with biotinylated rabbit antigoat IgG secondary Abs for FKN staining or with biotinylated goat antirabbit IgG secondary Abs for CX3CR1 (Vectastain avidin-biotin complex methods; Vector Laboratories, Burlingame, CA), then with horseradish peroxidase conjugated avidin-biotin complexes (Vectastain ABC methods; Vector Laboratories). Sections were finally developed with 3, 3′-diaminobenzidine tetrahydrochloride and hydrogen peroxide and counterstained with methyl green. Similarly, the serial tissues of skin and lung were stained with anti-CD3, anti-CD14, anti-CD16, anti-CD20, and anti-CD68 Abs (Dako Cytomation Co Ltd. Glostrup, Denmark) to identify the leucocyte subset of cells expressing CX3CR1.

Flow cytometric analysis
CX3CR1 expression levels by peripheral blood mononuclear cells (PBMC) were examined in 20 patients with SSc (10 lSSc, 10 dSSc; 17 women, 3 men; age 42 (20) years) and 12 normal controls (10 women, 2 men; age 40 (16) years). Heparinised blood samples were collected and placed on ice. Two colour analysis was performed with a combination of FITC conjugated anti-CX3CR1 (Medical and Biological Laboratories Corp, Nagoya, Japan) and phycoerythrin conjugated anti-CD4 (Coulter Corp, Miami, FL), anti-CD8 (Coulter Corp), anti-CD14 (Coulter Corp), or anti-CD16 monoclonal Abs (Coulter Corp). The blood samples were stained at 4 °C with a predetermined optimal concentration of the test monoclonal Ab for 20 minutes, as previously described.28 Blood erythrocytes were lysed after staining with the Coulter whole blood immunolyse kit as detailed by the manufacturer (Coulter Corp). Cells were washed and analysed with a FACSscan flow cytometer (BD PharMingen, San Diego, CA). Positive and negative populations of cells were determined with unreactive isotype matched monoclonal Abs (Coulter Corp) as controls for background staining.

ELISA for sFKN
Unless indicated otherwise, reagents were obtained from R&D Systems (Minneapolis, MN). Human sFKN levels were measured in serum samples by ELISA, using 96 well polystyrene plates coated overnight at 25 °C with 2 mg/ml of purified goat antihuman FKN IgG. After being washed, plates were blocked for 1 hour at 20 °C with phosphate buffered saline containing 1% bovine serum albumin and 5% sucrose. Recombinant human FKN and serum samples were added in triplicate, and the plates were incubated for 2 hours at 20 °C. After further washing, plates were incubated with biotinylated goat antihuman FKN Ab (250 ng/ml) for 2 hours at 20 °C and then with streptavidin-peroxidase for 1 hour at 20 °C. Samples were developed with 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.

Statistical analysis
Statistical analysis was performed using the Mann-Whitney U test for the comparison of concentration, and Fisher’s exact probability test for the comparison of frequencies. Spearman’s rank correlation coefficient was used to examine the relationship between two continuous variables. A p value <0.01 without adjustment for multiple comparisons was considered significant. All data are shown as mean (SD) unless otherwise indicated.

RESULTS
Immunohistochemical examination of CX3CR1+ cells
Firstly, we assessed the CX3CR1 expression on infiltrating inflammatory cells in the lesional skin and lung tissue of patients with dSSc (fig 1). CX3CR1+ cells were rarely detected by immunohistochemical staining in normal control skin.
tissues (3 (4)% of infiltrating mononuclear cells; fig 1A). In contrast, a considerable proportion of infiltrating cells were immunoreactive for CX3CR1 in the affected skin tissues from patients with dSSc with progressing skin thickening (33 (15)%; p<0.01; fig 1A). CX3CR1+ cells were less commonly found in affected skin tissues from patients with lSSc (15 (12)%). In patients with dSSc with active pulmonary fibrosis, large numbers of CX3CR1+ cells were detected in inflammatory cells of affected lung tissues (42 (11)%), but few such cells were detected in normal control lung tissues (8 (5)%; p<0.01; fig 1B). Most of the CX3CR1+ cells were identified as monocytes/macrophages morphologically with anti-CD14 Ab and anti-CD68 Ab in the skin and lung tissues from patients (data not shown). Staining with control polyclonal rabbit IgG showed the absence of non-specific staining in the affected skin and lung tissues of SSc. Thus, infiltration of CX3CR1+ cells was increased in the affected skin and lung tissues from patients with SSc.

**CX3CR1 expression on PBMC in patients with SSc**

Then, CX3CR1 expression on PBMC from patients with SSc was assessed by immunofluorescence staining with flow cytometry (figs 2 and 3). The frequency of CD4+ T cells expressing CX3CR1 in PBMC was significantly increased in patients with dSSc (3.7 (1.1)%; p<0.01) but not in patients with lSSc (2.9 (1.2)%; p<0.01) compared with normal controls (2.3 (0.9)%). CD4+ T cells from patients with dSSc had 1.3-fold higher mean CX3CR1 expression levels than those from normal controls (p<0.01). Similarly, the frequency of CD8+ T cells expressing CX3CR1 was significantly increased in patients with dSSc (5.5 (2.3)%; p<0.01) but not in patients with lSSc (3.4 (2.0)%; p<0.01) compared with normal controls (2.5 (1.7)%). Mean CX3CR1 expression on CD8+ T cells was raised by 1.4-fold in patients with dSSc compared with controls (p<0.01). Furthermore, the percentage of CD14+ monocytes expressing CX3CR1 was also significantly increased in patients with dSSc (6.9 (3.0)%; p<0.01) but not in patients with lSSc (5.4 (3.1)%; p<0.01) compared with normal controls (3.5 (1.9)%). CX3CR1 expression levels on CD14+ monocytes were significantly 1.5-fold higher in patients with dSSc than in normal controls (p<0.01). However, the frequency of CD16+ NK cells expressing CX3CR1 was similar in patients with dSSc (13.5 (7.2)%); patients with lSSc (12.9 (5.1)%), and normal controls (12.3 (6)%; p<0.01). CX3CR1 expression levels on CD16+ NK cells were not significantly different among patients with dSSc, patients with lSSc, and normal controls. Thus, the frequency of cells expressing CX3CR1 and CX3CR1 expression levels were increased in circulating CD4+ T cells, CD8+ T cells, and CD14+ monocytes/macrophages from patients with dSSc, although the increased frequency was probably due to the raised CX3CR1 expression in each subset.

**Immunohistochemical expression of FKN**

Next, we determined the FKN expression in the skin and lung tissues used for immunostaining of CX3CR1 (fig 4). In the skin from normal subjects, FKN expression was faintly detected in vascular endothelial cells. However, in the lesional skin of patients with dSSc with rapidly progressing skin thickening, endothelial cells strongly expressed FKN. FKN expression was moderately enhanced in the affected skin tissues from lSSc compared with that of normal controls (data not shown). Similarly, FKN was detected more intensely at endothelial cells in the lesional lung tissues from patients with dSSc with active pulmonary fibrosis than in normal control lung tissues. Staining with control polyclonal goat IgG showed the absence of non-specific staining in the affected skin and lung tissues of SSc. Thus, augmented FKN expression was found in the lesional skin and lungs from patients with SSc.
Soluble FKN levels were assessed in serum samples from patients with SSc (fig 5). Serum sFKN levels were four times higher in all patients with SSc and in the lSSc and dSSc subsets than in normal controls ($p<0.01$). Patients with dSSc had serum sFKN levels that were 2.4 times higher than those in patients with lSSc, although the difference was not significant. Patients with SSc with pulmonary fibrosis had sFKN levels about four times higher than those without pulmonary fibrosis ($p<0.01$).

Values higher than the mean $+3SD$ (that is, 216.6 pg/ml) of the control serum samples were considered to be raised in this study. Raised serum sFKN levels were seen in 25/67 (37%) patients with SSc (11/36 (31%) patients with lSSc and 14/31 (45%) patients with dSSc). Patients with SSc with raised sFKN levels more frequently had pitting scars or skin ulcers ($p<0.01$), and a raised erythrocyte sedimentation rate ($p<0.01$), than those patients with normal sFKN levels (table 1). Patients with raised serum sFKN levels had pulmonary fibrosis more frequently than those with normal sFKN levels ($p<0.01$). Consistently, patients with raised serum sFKN levels showed decreased %VC and %T LCO more often than those with normal sFKN levels ($p<0.01$). Serum sFKN levels correlated inversely with %VC ($r = -0.50$, $p<0.0001$) or %T LCO ($r = -0.40$, $p<0.0001$; fig 6).

However, serum sFKN levels did not correlate significantly with the mTSS, a semiquantitative measure of skin sclerosis.

To determine the treatment effect on sFKN levels, nine patients with dSSc were examined for serum sFKN levels before and after treatment. All patients were treated with systemic steroid ($-20$ mg/day of prednisolone) during the follow up (37 (15) months). Furthermore, four patients with active pulmonary fibrosis received six courses of intravenous cyclophosphamide pulse therapy ($500$–$1000$ mg/day). The skin sclerosis improved (mTSS changed from 23 (12) to 13 (8), $p=0.05$) and progression of pulmonary fibrosis was suppressed by the treatment. None of nine patients developed new organ involvement. Raised serum sFKN levels in these patients with SSc were significantly decreased during the follow up (from 1140 (1045) to 88 (53) pg/ml, $p<0.01$). Thus, serum sFKN levels correlated with systemic inflammation, the presence of vascular injury, and severity of lung fibrosis in patients with SSc. Furthermore, raised sFKN levels were considerably decreased during the follow up, probably owing to the immunosuppressive treatment.

**DISCUSSION**

In this study FKN expression was increased in the vascular endothelial cells of the lesional skin and lung tissues from patients with SSc (fig 4). CX3CR1 expression was enhanced...
on peripheral CD4+ T cells, CD8+ T cells, and CD14+ monocytes/macrophages in patients with dSSc (figs 2 and 3). Accordingly, the number of mononuclear cells expressing CX3CR1 was increased in the affected skin and lung tissues (fig 1). Reflecting the overexpression of FKN in the skin and lung, sFKN levels were increased in serum from patients with SSc (fig 5). These findings suggest that enhanced FKN-CX3CR1 interaction contributes to the disease process in SSc.

In SSc, the number of infiltrating cells expressing CX3CR1 was significantly increased in the affected skin (fig 1A). Increased FKN expression in the lesional tissue has been shown in some inflammatory or vascular diseases, including glomerulonephritis.91 12 9 However, as far as we know, up regulated CX3CR1 expression in peripheral blood mononuclear cells has not been reported in other diseases. Although the significance of overexpressed CX3CR1 is unknown, our data suggest that up regulated expression of FKN in the vascular endothelial cells and overexpression of CX3CR1 on peripheral mononuclear cells cooperatively promote migration of CX3CR1+ cells to the skin of patients with SSc (figs 2–4). Previous reports demonstrated that the number of monocytes/macrophages was increased in the skin of patients with SSc, especially in severely affected skin or early in the disease.30 31 Therefore, it is likely that up regulated FKN expression in endothelial cells promotes the infiltration of CX3CR1+ cells, including monocytes/macrophages, leading to initiation of inflammation.

Soluble FKN levels were raised in serum samples from patients with SSc (fig 5). This may be just reflecting the enhanced endothelial FKN expression in SSc. However, possibly, raised sFKN promotes CX3CR1+ cell infiltration into the affected tissue leading to tissue inflammation, because sFKN enhances the chemotactic activity of cells expressing CX3CR1.17 34 Serum sFKN levels were significantly associated with the inflammatory marker, erythrocyte sedimentation rate (table 1), and were significantly decreased by immunosuppressive treatment. Although the biological significance of sFKN shedding from the cell surface and circulating sFKN remains unclear, serum sFKN levels may reflect the inflammatory response through FKN-CX3CR1 interaction in SSc.

FKN expression was also augmented on the endothelial cells of the lung in patients with SSc and active pulmonary
fibrosis (fig 4B). In these patients, the number of infiltrating cells expressing CX3CR1 was significantly increased in the lung tissues (fig 1B). Serum sFKN levels were significantly associated with the involvement and severity of pulmonary fibrosis in SSc (figs 5B and 6, table 1). Although the pathogenesis of pulmonary fibrosis in SSc is poorly understood, monocytes/macrophages are present in abundance and are functionally activated within the lungs of patients with SSc.42 43 Our results indicate that at least a part of the infiltration of the lung by monocytes/macrophages may result from augmented local expression of FKN in the affected lungs, because most monocytes/macrophages express CX3CR1 in SSc. Monocytes/macrophages secrete interleukin 6, TNFα, and transforming growth factor β, and these cytokines are expressed in pulmonary fibrosis in SSc. Therefore, FKN may have an important role in the induction and/or development of pulmonary fibrosis in SSc by recruiting CX3CR1+ cells to the affected lungs.

In the current study, raised sFKN levels were associated with peripheral ischaemia, such as digital ischaemia and skin ulcer (table 1). Although the origin and mechanisms of endothelial cell injury in SSc remain obscure, previous findings indicated that circulating soluble proteases such as granzyme may be linked to the pathogenesis of endothelial injury in SSc.44 45 Recently, it has been demonstrated that CX3CR1 expression defines mononuclear cells possessing high levels of intracellular perforin and granzyme B.46 FKN dependent adhesion of NK cells to endothelial cells has been shown to promote endothelial cell damage.47 These previous findings suggest that FKN regulates recruitment of cytotoxic cells through inflamed endothelium. Therefore, FKN may have a critical role in cytotoxic, cell mediated endothelium damage, which may result in vascular injury. Indeed, it has been demonstrated that a polymorphism in CX3CR1 decreases the incidence of coronary artery disease as a consequence of lower susceptibility to vascular inflammation.48 49 Furthermore, monocyte/macrophage infiltration mediated by FKN plays a critical part in atherosclerosis, suggesting that amplified monocyte recruitment through the FKN-CX3CR1 pathway can have deleterious consequences in vivo.50 51 Thus, enhanced FKN-CX3CR1 interaction may be an important biological factor in promoting endothelial injury in SSc.

In conclusion, we have demonstrated the enhanced expression of FKN and CX3CR1 in patients with SSc. Our results suggest that FKN plays a part in the disease processes in SSc, including inflammation and vascular injury. However, further studies will be needed to determine the relative importance of FKN compared with other chemokines in SSc, and to clarify the specific role of FKN in SSc which is distinct from its role in other inflammatory diseases.

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