reproduce this finding [54]. While CCR2 is generally not expressed by fibroblasts, Carulli and colleagues found that CCR2 is expressed by a small population of fibroblasts derived from patients with early dcSSc [55]. CCL2's profibrotic effects may thus require interaction with other cell types that express CCR2, such as monocytes and T cells. In this regard, it has been shown in SSc patients that CCL2 induces infiltrating $CD4^+$ T cells to differentiate into T-helper 2 cells, which release higher amounts of IL-4 and stimulate fibroblasts to produce excess ECM [54]. In this scenario, versican functions as a local reservoir for CCL2 and, whether bound to or released from versican, CCL2 is capable of efficiently stimulating T cells. In addition to its role in this T-cell-mediated mechanism, CCL2 also contributes to fibrotic response by promoting the migration and accumulation of profibrotic monocytes at affected sites in SSc patients. All together, the upregulation of versican and CCL2 in circulating monocytes accelerates CCL2-mediated profibrotic responses in SSc patients.

What mechanisms assist in shaping circulating monocytes to the profibrotic phenotype seen in patients with SSc? One of histopathological hallmarks of SSc is the perivascular infiltration of monocytes early in the disease [4]. It is possible that intrinsically altered monocytes migrate into target organs and trigger a profibrotic response by stimulating resident fibroblasts. Alternatively, monocyte phenotypes may be altered in SSc patients by the strong profibrotic environment. Versican production is highly regulated by soluble factors and certain stimuli, and several studies have reported that profibrotic growth factors such as transforming growth factor beta, platelet-derived growth factor, and basic fibroblast growth factor upregulate versican synthesis [56-58]. These factors are also known to upregulate CCL2 expression [59,60]. In contrast, IFNγ and IL-1β reduce versican expression [61,62]. Hypoxia dramatically upregulates versican in macrophages via hypoxia-inducible factor signaling [38]. The profibrotic and hypoxic environment associated with SSc may modulate gene expression profiles of circulating monocytes.

Genes selected by initial screening via gene expression array but excluded by confirmatory analyses may be of some interest, because some of them have been reported as molecules associated with SSc pathogenesis. For example, circulating levels of soluble L-selectin and CXCL8 were increased in SSc patients versus healthy controls [63,64]. In addition, gene expression of CCR1 was shown to be upregulated in PBMCs derived from patients with lcSSc and pulmonary arterial hypertension [65].

There are several limitations to this study. First, we used total $CD14^+$ monocytes enriched form PBMCs, which contained $CD14^+$ fibrocyte precursors [9]. Since fibrocyte precursors are reported to be a rare cell population, comprising approximately 0.5% of circulating monocytes [66], contamination of them into $CD14^+$ cells should have minimal impact on gene and protein expression data. Second, prominent upregulation of versican and CCL2 in circulating monocytes was observed in a minority of SSc patients, raising a possibility that the monocytic versican-mediated pathogenic process is only one of the roles of circulating monocytes in the pathogenesis of SSc. Since all patients with an extremely high mRNA expression level of versican had dcSSc, this type of monocyte phenotypic change might be unique to patients with a prominent fibrotic phenotype. Finally, this study represented only an *in vitro* functional interaction between versican and CCL2, which may not reflect *in vivo* activity. In addition, overexpression of versican in circulating monocytes of SSc patients might be a bystander of other more important pathogenic process of SSc. Further investigations involving genetically manipulated animals- for example, mice lacking functional versican expression specifically in monocytes - is necessary to confirm a critical role of versican upregulated by circulating monocytes in SSc pathogenesis.

Conclusion

The cellular and molecular mechanisms underlying the SSc fibrotic process primarily involve the interaction of cells such as fibroblasts, endothelial cells, and circulating immune cells, orchestrated by profibrotic soluble mediators and ECM components. This concept is supported by our observation that circulating monocytes in SSc patients are phenotypically altered and amplify a positive feedback loop, mediated by versican and CCL2, between monocytes and fibroblasts. Further studies evaluating the roles of circulating monocytes in the pathogenic process of SSc should help to elucidate the complex pathophysiology of SSc and assist us to develop novel therapeutic strategies in this multisystem fibrotic disease.

Abbreviations

CS: chondroitin sulfate; dcSSC: diffuse cutaneous systemic sclerosis; ECM: extracellular matrix: ELISA: enzyme-linked immunosorbent assay; GAG: glycosaminoglycan; IFN: interferon; IL: interleukin; lcSSc: limited cutaneous systemic sclerosis; mAb: monoclonal antibody; PBMC: peripheral blood mononuclear cell; PCR: polymerase chain reaction; RT: reverse transcriptase; SSc: systemic sclerosis.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AM acquired: analyzed, and interpreted data, and wrote the manuscript. HY analyzed and interpreted data, and wrote the manuscript. TS, YO, and YY acquired data. MK designed the experiments, analyzed and interpreted data, and wrote the manuscript. All authors read and approved the final manuscript.

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ORIGINAL ARTICLE

Serum chemokine levels as prognostic markers in patients with early systemic sclerosis: a multicenter, prospective, observational study

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Abstract
Objective To assess the utility of serum chemokine levels as a prognostic indicator of disease progression in systemic sclerosis (SSc) patients with early onset disease.
Methods Seventy Japanese patients with early onset SSc presenting with diffuse skin sclerosis and/or interstitial lung disease were registered in a multicenter, observational study. Concentrations of CCL2, CCL5, CXCL8, CXCL9, and CXCL10 in serum samples from all patients were measured using cytometric beads array. In 33 patients, chemokine levels were measured each year for 4 years. The ability of baseline chemokine levels to predict changes in clinical features were evaluated statistically by multiple regression analysis.
Results At their first visit, serum levels of CCL2, CCL5, CXCL8, CXCL9, and CXCL10 were significantly elevated in patients with SSc compared with healthy controls. There were significant associations between CCL2 and CXCL8 levels and between CXCL9 and CXCL10 levels in patients. The initial serum CXCL8 levels were significantly associated with the HAQ-DI at the fourth year while the %VC of baseline tended to be negatively associated with HAQ-DI at the fourth year. Initial chemokine levels were not associated with other clinical features including skin thickness score and the respiratory function.
Conclusion Serum CXCL8 level may serve as a prognostic indicator of the physical dysfunction in SSc. Further

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longitudinal studies of larger populations are needed to confirm these findings.

Keywords Chemokine · CXCL8 · HAQ · Serum marker · Systemic sclerosis

Introduction

Systemic sclerosis (SSc) is a connective tissue disease characterized by tissue fibrosis in the skin and internal organs [1, 2]. Interstitial lung disease (ILD) develops in more than half of SSc patients and is one of the major SSc-related causes of death. Additionally, joint contracture due to extensive skin sclerosis and/or severe internal organ involvement results in impaired physical function.

SSc patients exhibit increased numbers and activation of monocytes/macrophages and T cells in the circulation and tissues [3, 4]. Infiltration of these cells into the skin or internal organs of SSc patients may promote endothelial damage and fibrosis, most likely through the production of soluble mediators including cytokines and chemokines. Several reports have shown chemokine abnormalities in SSc that might explain the altered accumulation of effector leukocyte subsets in affected tissues [5, 6].

In most patients, severe organ involvement occurs within the first 3 years of disease and skin sclerosis seldom progresses after 5 or 6 years [7, 8]. Thus, predicting disease progression is particularly important for SSc patients at their first visit; however, except for SSc-related autoantibodies [9], there are no definitive serum biomarkers available to estimate disease progression. We hypothesized that some chemokines may be related to underlying biologic process which is ongoing and which will change clinical features over time.

In the present study, we focused on five major chemokines (CCL2, CCL5, CXCL8, CXCL9, and CXCL10), since these chemokines have been reported to be elevated and are associated with disease activity in other connective tissue diseases such as systemic lupus erythematosus [10, 11]. We sought to determine if baseline serum chemokines levels could predict the progress of symptoms in early SSc patients diagnosed with disease no more than 3 years prior to the onset of the study. To this end, serum levels of five chemokines were evaluated in early SSc patients at baseline and every year over the course of 4 years.

Materials and methods

Patients

Patients were grouped according to the degree of skin involvement based upon the classification system proposed by LeRoy et al. [diffuse cutaneous SSc (dcSSc) versus limited cutaneous SSc (lcSSc), 12]. In this study, 70 Japanese patients with early SSc (disease duration defined by the period from the first symptom including Raynaud's phenomenon attributable to SSc to our first assessment $\leq$3 years) who had dcSSc and/or ILD were registered at nine major scleroderma centers in Japan (Gunma University Hospital, Kanazawa University Hospital, Keio University Hospital, Kitasato University Hospital, Kumamoto University Hospital, Nagasaki University Hospital, Tokyo University Hospital, Tokyo Women's Medical University Hospital, Tsukuba University Hospital). Patients with other inflammatory, infectious, or malignant diseases were not included in this study.

Among the patients, 39 patients had dcSSc with ILD, 23 patients had dcSSc without ILD, and 8 patients had lcSSc with ILD. Forty-seven patients were female and twenty-three patients were male. The median age was 53 years (range, 14–76 years). The median disease duration was 12 months (range, 1–36 months). All patients fulfilled the criteria for SSc proposed by the American College of Rheumatology [13]. With respect to the specificity of anti-nuclear antibodies (Abs) in the serum, 41 patients were positive for anti-topoisomerase I Ab and 8 patients were positive for anticentromere Ab. Fifteen age- and gender-matched healthy persons [11 females and 4 males, median age 49 years (range, 20-65 years)] were also included as normal controls in this study.

Among 70 patients, 33 patients could be followed every year for 4 years. Twenty-one patients had dcSSc with ILD, nine patients had dcSSc without ILD, and nine patients had lcSSc with ILD. Twenty-one patients were female and twelve patients were male. The median age was 55 years (range, 14–75 years). The median disease duration was 19 months (range, 1–36 months). With respect to the specificity of anti-nuclear Abs, 23 patients were positive for anti-topoisomerase I Ab and one patient was positive for anticentromere Ab. The ethical committee at each center approved all protocols and informed consent was obtained from all patients.

Clinical assessments

Patients had a physical examination and laboratory tests were performed at their first visit and at each subsequent year for 4 years. The degree of skin involvement was determined according to the modified Rodnan total skin thickness score (MRSS), as described elsewhere [14]. Organ system involvement was defined as described previously [15] with some modifications: ILD = bibasilar interstitial fibrosis or ground-glass shadow on computed tomogram (CT); pulmonary arterial hypertension (PAH) = clinical evidence of pulmonary hypertension and

elevated right ventricular systolic pressure (>45 mmHg) documented by echocardiography in the absence of severe pulmonary interstitial fibrosis; esophagus = apparent dysphagia, reflux symptoms, or hypomotility shown by barium radiography; heart = pericarditis, congestive heart failure, or arrhythmias requiring treatment; kidney = malignant hypertension and rapidly progressive renal failure unexplained by certain diseases other than SSc; joint = inflammatory polyarthralgias or arthritis; and muscle = proximal muscle weakness and elevated serum creatine kinase. A health assessment questionnaire-disability index (HAQ-DI) modified for Japanese patients [16] including digital ulcer, pitting scar, maximal oral aperture (the maximum vertical length of opened mouth), and skin pigmentation/depigmentation was also evaluated. Erythrocyte sedimentation rate (ESR) and pulmonary function, including vital capacity (VC) and diffusion capacity for carbon monoxide (DLco), were also tested.

Serum cytokine and chemokine assays

Fresh venous blood samples were taken from 70 patients and 15 healthy controls at their first visit (baseline). In 33 patients, blood samples were also taken at each subsequent year for 4 years. Samples were centrifuged shortly after clot formation. All serum samples were stored at −70 °C prior to use in assays. Serum levels of CCL2/monocyte chemoattractant protein-1 (MCP-1), CCL5/RANTES (regulated upon activation, normally expressed in T cells, and secreted), CXCL8/interleukin 8 (IL-8), CXCL9/monokine induced by interferon γ (MIG), and CXCL10/interferon γ-inducible protein-10 (IP-10) were measured by cytometric beads array (BD PharMingen, San Diego, CA) using a FACScan flow cytometer (BD PharMingen). Limit of detection was as follows; CCL2 2.7, CCL5 1.0, CXCL8 0.2, CXCL9 2.5, CXCL10 2.8 pg/ml.

Statistical analysis

JMP® Statistical Discovery Software (SAS Institute, Cary, NC) was used for analysis. Since the Shapiro–Wilk test did not indicate that serum chemokine concentration showed normal distribution, the data were converted to logarithm so that the data exhibited normal distribution. Then statistical analyses were performed using the Student's t test for the comparison of sample levels between two groups. The Pearson product-moment correlation coefficient was used to examine the relationship between two continuous variables. Potential prognostic factors for estimating subsequent MRSS, %VC, and HAQ-DI were statistically examined by multiple regression analysis. A P value <0.05 was considered statistically significant. All values are expressed as the median (range) otherwise indicated.

Results

Baseline serum chemokine levels were elevated in SSc patients

Serum samples taken from normal controls ($n = 15$) and all patients ($n = 70$) at their first visit were assayed and CCL2 levels were found to be significantly increased in SSc patients compared with healthy controls ($P < 0.0001$, Fig. 1). Similarly, the levels of serum CCL5, CXCL8, CXCL9, and CXCL10 were also significantly elevated in the SSc patients ($P < 0.0001$, respectively, Fig. 1). In this regard, serum levels of CCL2 were significantly associated with levels of CXCL8 in patients ($r = 0.76$, $P < 0.0001$). Similarly, serum CXCL9 levels were significantly associated with serum CXCL10 levels in patients ($r = 0.58$, $P < 0.0001$). Other combinations of chemokines were not significantly associated with each other.

At the initial visit, there was a significant association between serum CCL2 levels and HAQ-DI in patients with SSc ($r = 0.41$, $P = 0.010$). There was also a significant association between serum CXCL8 levels and HAQ-DI ($r = 0.40$, $P = 0.020$). However, there were no statistically significant differences in the chemokine levels between dcSSc and lcSSc patients or between patients with ILD and patients without ILD. Moreover, no significant correlations were found between the levels of any of the chemokines measured and any other clinical or laboratory findings. Steroid treatment did not significantly affect the levels of these chemokines [steroid (+) vs. steroid (−) (median (range) pg/ml); CCL2 131.0 (41.8–3806.6) vs. 167.7 (48.3–872.7); CCL5 7902.9 (144.7–27071.9) vs. 7276.5 (1482.2–25752.4); CXCL8 18.7 (<0.2–12166.9) vs. 14.0 (<0.2–6705.1); CXCL9 104.9 (4.2–1177.3) vs. 94.3 (7.5–813.4); CXCL10 156.6 (3.5–1554.5) vs. 201.3 (5.0–651.1)].

Longitudinal change of clinical features

To assess progression of SSc over time, thirty-three patients were followed-up every year for 4 years (Table 1). To assess the degree of skin involvement in patients, MRSS values were calculated, and %VC and %DLco were used to assess lung involvement. HAQ-DI was also obtained in order to evaluate the functional abilities of the patients. For the patient population as a whole, the median MRSS value decreased from 16 to 10 during the first year. The median MRSS was 7 at the end of year 2, 9 at the end year 3, and 8 at the end year 4. Median values for %VC did not significantly change during the 4-year evaluation period. In this regard, the %VC was 96 at first visit, 92 at the end of the first year, 96 at the end of the second year, 94 at the end of the third year, and 91 at the end of the fourth

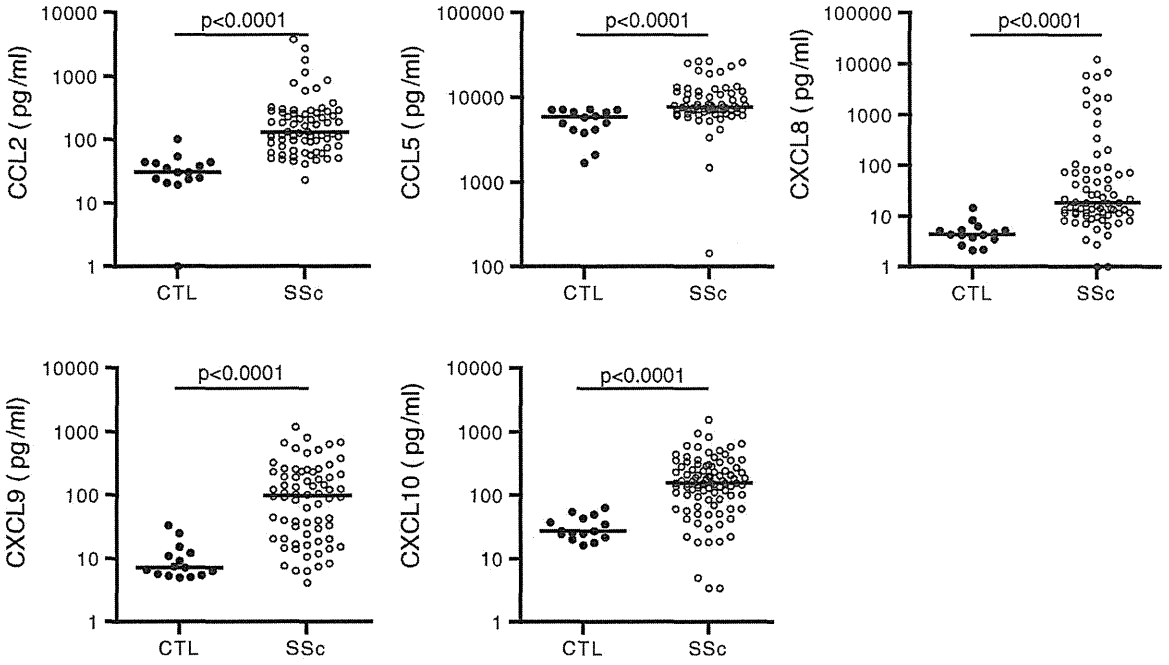

Fig. 1 Serum chemokine levels in early SSc patients (SSc) and healthy controls (CTL). The *horizontal bar* in each group indicates the median value

Table 1 The course of clinical and laboratory features in patients with SSc

	Baseline	1 year follow-up	2 year follow-up	3 year follow-up	4 year follow-up
MRSS	16 (2–39), $n = 33$	10 (0–38), $n = 33$	7 (1–25), $n = 33$	9 (1–25), $n = 33$	8 (0–29), $n = 33$
HAQ-DI	0.125 (0–1.5), $n = 3$	0.125 (0–1.75), $n = 33$	0.375 (0–2.5), $n = 33$	0.125 (0–1.875), $n = 33$	0.25 (0–1.75), $n = 33$
%VC	96 (53–144), $n = 27$	92 (62–120), $n = 20$	96 (61–144), $n = 20$	94 (56–137), $n = 22$	91 (58–136), $n = 24$
%DLco	70 (41–113), $n = 27$	68 (43–104), $n = 19$	70 (44–96), $n = 19$	69 (28–119), $n = 22$	63 (21–107), $n = 25$
ILD	24 (73), $n = 33$	24 (73), $n = 33$	25 (76), $n = 33$	28 (85), $n = 33$	28 (85), $n = 33$
PAH	0 (0), $n = 33$	1 (3.7), $n = 27$	1 (3.6), $n = 28$	0 (0), $n = 29$	0 (0), $n = 27$
Renal crisis	0 (0), $n = 33$	2 (6.1), $n = 33$	0 (0), $n = 33$	1 (3.0), $n = 33$	1 (3.0), $n = 33$
Corticosteroid therapy	23 (70), $n = 33$	28 (85), $n = 33$	31 (94), $n = 33$	31 (94), $n = 33$	28 (85), $n = 33$
Cyclophosphamide therapy	3 (9), $n = 33$	5 (15), $n = 33$	3 (9), $n = 33$	3 (9), $n = 33$	4 (12), $n = 33$
Other immunosuppressive agents therapy	1 (3), $n = 33$	1 (3), $n = 33$	4 (12), $n = 33$	4 (12), $n = 33$	5 (15), $n = 33$

Values are represented as median (range) or as number of positive cases with percentage within parentheses, in total patients in whom those data are available

year. Similarly, median values for %DLco did not significantly change during the 4-year evaluation period. Specifically, the %DLco was 70 at first visit, 68 at the end of the first year, 70 at the end of the second year, 69 at the end of the third year, and 63 at the end of the fourth year. The median HAQ-DI was 0.125 at the first visit, 0.125 at the end of year 1, 0.375 at the end of year 2, 0.125 at the end of year 3, and 0.25 at the end of year 4. The frequency of patients with ILD determined by CT was unchanged during the evaluation period. The development of PAH and renal crisis was rare during the course of the study. Orally administered prednisolone (~20 mg/day) use was common at baseline and subsequent years (70–94 %).

Longitudinal change of chemokine levels

The yearly changes in serum chemokine levels for each case are shown in Fig. 2. The dotted horizontal lines

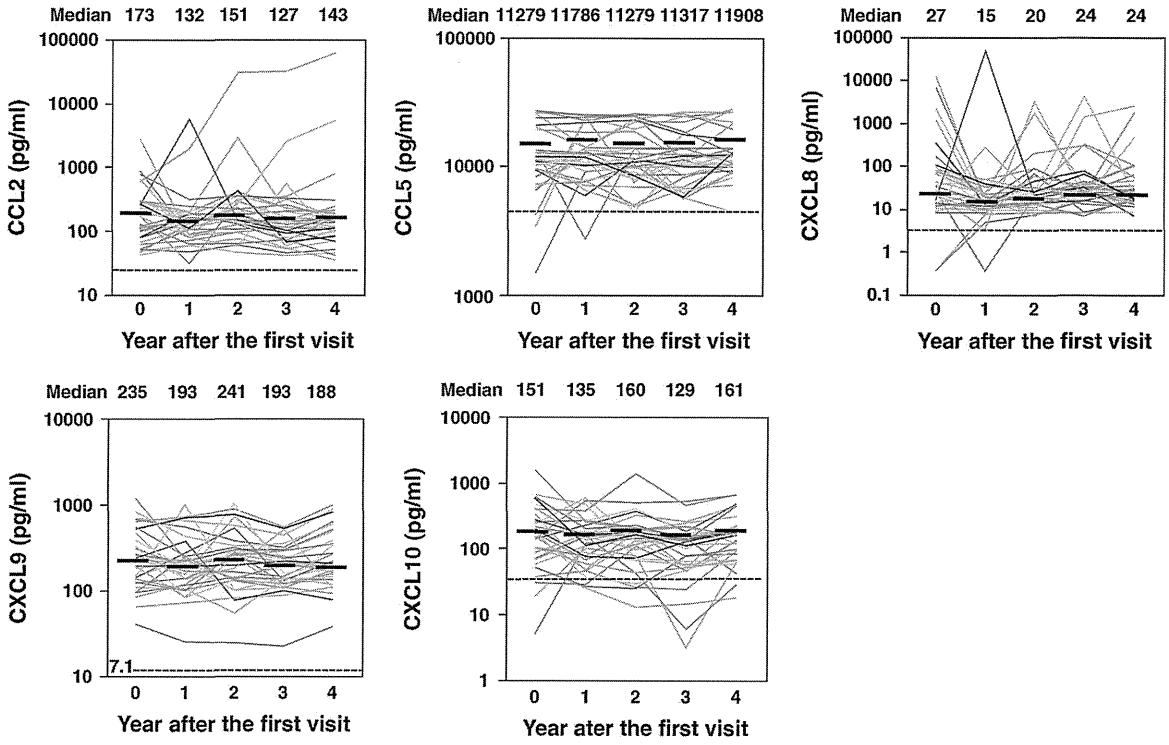

Fig. 2 Longitudinal change of serum chemokine levels in each patient during the 4 years of the study. The *horizontal dotted line* indicates the median value in healthy controls. The *horizontal bar* at each time point indicates the median value

indicate median values of healthy controls. Overall, the levels of each chemokine in the serum showed considerable variations in each patient; however, the median values of each of the five chemokines measured did not change significantly over time (Fig. 2). Furthermore, the variation in chemokine levels over time was not significantly associated with the variation of MRSS, %VC, %DLco, and HAQ-DI during the 4 years course of the study (data not shown).

Association between each chemokine level and clinical features of SSc

Next, we evaluated if baseline serum chemokine levels are associated with baseline and subsequent clinical features of SSc by univariate analysis. Baseline serum levels of CCL2 and CXCL8 were significantly associated with HAQ-DI values at baseline and subsequently every year for 4 years (Table 2). However, serum levels of CCL2 and CXCL8 did not significantly associated with other clinical features including skin thickness score and lung function. No significant associations between serum levels of CCL5, CXCL9, or CXCL10 and subsequent clinical features were found. These data indicate that serum levels of CCL2 and CXCL8 may be useful biomarkers for estimating the subsequent progression of physical disability.

Association between the level of each chemokine and clinical features

Finally, we utilized multiple regression analysis to evaluate the ability of serum chemokine levels to predict clinical or laboratory factors such as MRSS, %VC, and HAQ-DI of patients 4 years after the first visit. Selected variables were as follows: each chemokine level, anti-topoisomerase I Ab, anticentromere Ab, MRSS, %VC, %DLco, presence of ILD, HAQ-DI, ESR, corticosteroid treatment, and cyclophosphamide treatment at the first visit. We performed step-wise multiple regression analyses that specified the α level for either adding or removing a regression as 0.15. As a result, the multiple regression equation predicting the HAQ-DI of 4 year follow-up = $-0.24 + \log_{10}$ [serum CXCL8 levels (pg/ml)] of baseline + $-0.0061 \times$ %VC of baseline ($R^2 = 0.41$, root mean square error = 0.36, $P = 0.0016$, Table 3). Using our equation, we found that the HAQ-DI value at the fourth year was significantly associated with the CXCL8 level of baseline ($P = 0.0016$). Additionally, the HAQ-DI value of 4 year follow-up tended to be negatively associated with %VC of baseline ($P = 0.086$). ESR, MRSS, %VC, or %DLco at the fourth year was not significantly associated with any chemokine levels or clinical factors of baseline.

Table 2 The associations between baseline chemokine levels and subsequent clinical parameters in patients with SSc

	Baseline	1 year follow-up	2 year follow-up	3 year follow-up	4 year follow-up
Baseline CCL2 versus MRSS (baseline–4 years)	$r = 0.16$, $P = 0.36$	$r = 0.43$, $P = 0.013$	$r = 0.21$, $P = 0.25$	$r = 0.10$, $P = 0.56$	$r = 0.19$, $P = 0.29$
Baseline CCL2 versus %VC (baseline–4 years)	$r = -0.24$, $P = 0.23$	$r = -0.13$, $P = 0.67$	$r = -0.40$, $P = 0.079$	$r = -0.32$, $P = 0.15$	$r = -0.31$, $P = 0.14$
Baseline CCL2 versus HAQ-DI (baseline–4 years)	$r = 0.44$, $P = 0.011$	$r = 0.56$, $P = 0.0007$	$r = 0.52$, $P = 0.0019$	$r = 0.42$, $P = 0.014$	$r = 0.49$, $P = 0.0040$
Baseline CCL5 versus MRSS (baseline–4 years)	$r = 0.21$, $P = 0.25$	$r = 0.023$, $P = 0.90$	$r = 0.019$, $P = 0.92$	$r = 0.12$, $P = 0.51$	$r = 0.11$, $P = 0.54$
Baseline CCL5 versus %VC (baseline–4 years)	$r = -0.090$, $P = 0.66$	$r = -0.26$, $P = 0.39$	$r = -0.085$, $P = 0.72$	$r = -0.14$, $P = 0.54$	$r = -0.23$, $P = 0.27$
Baseline CCL5 versus HAQ-DI (baseline–4 years)	$r = 0.056$, $P = 0.76$	$r = -0.11$, $P = 0.56$	$r = -0.11$, $P = 0.95$	$r = 0.013$, $P = 0.94$	$r = -0.076$, $P = 0.68$
Baseline CXCL8 versus MRSS (baseline–4 years)	$r = -0.053$, $P = 0.77$	$r = 0.40$, $P = 0.021$	$r = 0.16$, $P = 0.39$	$r = 0.035$, $P = 0.85$	$r = 0.14$, $P = 0.44$
Baseline CXCL8 versus %VC (baseline–4 years)	$r = -0.081$, $P = 0.69$	$r = -0.21$, $P = 0.48$	$r = -0.14$, $P = 0.56$	$r = -0.010$, $P = 0.96$	$r = 0.0052$, $P = 0.98$
Baseline CXCL8 versus HAQ-DI (baseline–4 years)	$r = 0.47$, $P = 0.0009$	$r = 0.72$, $P < 0.0001$	$r = 0.74$, $P < 0.0001$	$r = 0.48$, $P = 0.0044$	$r = 0.45$, $P = 0.0081$
Baseline CXCL9 versus MRSS (baseline–4 years)	$r = -0.23$, $P = 0.24$	$r = -0.068$, $P = 0.71$	$r = -0.26$, $P = 0.14$	$r = -0.35$, $P = 0.042$	$r = -0.31$, $P = 0.082$
Baseline CXCL9 versus %VC (baseline–4 years)	$r = -0.13$, $P = 0.48$	$r = -0.004$, $P = 0.99$	$r = -0.25$, $P = 0.29$	$r = -0.37$, $P = 0.093$	$r = -0.29$, $P = 0.17$
Baseline CXCL9 versus HAQ-DI (baseline–4 years)	$r = 0.18$, $P = 0.31$	$r = 0.10$, $P = 0.58$	$r = 0.016$, $P = 0.93$	$r = 0.093$, $P = 0.61$	$r = 0.053$, $P = 0.77$
Baseline CXCL10 versus MRSS (baseline–4 years)	$r = -0.055$, $P = 0.76$	$r = -0.16$, $P = 0.38$	$r = -0.21$, $P = 0.25$	$r = -0.31$, $P = 0.082$	$r = -0.23$, $P = 0.21$
Baseline CXCL10 versus %VC (baseline–4 years)	$r = -0.045$, $P = 0.82$	$r = 0.16$, $P = 0.59$	$r = 0.17$, $P = 0.47$	$r = -0.28$, $P = 0.20$	$r = -0.23$, $P = 0.27$
Baseline CXCL10 versus HAQ-DI (baseline–4 years)	$r = 0.20$, $P = 0.27$	$r = 0.080$, $P = 0.66$	$r = -0.045$, $P = 0.80$	$r = -0.11$, $P = 0.53$	$r = -0.040$, $P = 0.82$

Table 3 Factors predicting HAQ-DI of 4 year follow-up determined by multiple regression analysis

	Estimate	Standard error	*P* value
Intercept	−0.24	0.49	0.63
Log_{10} (serum CXCL8 levels of baseline) pg/ml	0.81	0.23	0.0016
%VC of baseline	−0.0061	0.0033	0.086

The multiple regression equations predicting HAQ-DI of 4 year follow-up are as follows; HAQ-DI of 4 year follow-up = $-0.24 + 0.81 \times \log_{10}$ [serum CXCL8 levels (pg/ml) of baseline] + $-0.0061 \times$ %VC of baseline. R^2 (determination coefficient) = 0.41, root mean square error = 0.36, $P = 0.0016$

Discussion

In this study, we measured serum chemokine levels using a cytometric beads array, a method that has comparable analytical sensitivity, but a wider dynamic range than conventional ELISA [17]. Serum levels of CCL2, CCL5, CXCL8, CXCL9, and CXCL10 were significantly elevated in early SSc patients with diffuse skin sclerosis and/or ILD. In this multicenter, longitudinal, prospective study, a multiple regression equation was defined to predict symptoms 4 years after initial diagnosis using baseline serum levels of five chemokines and multiple clinical or laboratory factors presenting at the time of the first visit. In this regard, we found that the initial serum CXCL8 levels were significantly associated with the HAQ-DI at the fourth year while the %VC of baseline tended to be negatively associated with HAQ-DI at the fourth year.

CXCL8 is a member of the CXC chemokine family which has an ELR (glutamic acid–leucine–arginine) motif between the N-terminus and the first cysteine that attracts CXCL1- or CXCL2-expressing neutrophils [18]. Of note, CXCL8 gene polymorphisms are associated with an

increased risk of SSc [19] and elevated serum CXCL8 levels have been reported in SSc patients [20, 21]. A recent study demonstrated that plasma CXCL8 levels are specifically elevated in SSc patients with anti-topoisomerase I Ab [22]. Furthermore, expression levels of CXCL8 are increased in skin biopsy specimens from patients with SSc, especially patients with early SSc [23]. SSc dermal fibroblasts produce more CXCL8 than do fibroblasts from healthy controls in vitro [24]. CXCL8 is also increased in bronchoalveolar lavage (BAL) fluid from patients with SSc, suggesting a critical role for CXCL8 in ILD of SSc patients [25–27]. Taken together, elevated serum levels of CXCL8 measured in early SSc patients in our study are consistent with previous reports.

We found that baseline serum CXCL8 levels were significantly associated with severity of physical dysfunction at the baseline and subsequent years. Since serum CXCL8 levels were not significantly associated with specific organ involvement, CXCL8 may affect neutrophil infiltration into various organs, resulting in systemic inflammation and subsequent functional disability. In addition, endothelial cell activation has been considered a first step in the development of tissue fibrosis [2], and CXCL8 is synthesized by endothelial cells, which store this chemokine in storage vesicles known as Weibel–Palade bodies [28, 29]. Therefore, it is interesting that baseline serum CXCL8 levels were associated more closely with HAQ-DI of the first and second year compared with baseline HAQ-DI.

CCL2 is produced by monocytes, fibroblasts, endothelial cells and other cells, and is a predominant chemoattractant and activator of monocytes and T cells [18]. Increased CCL2 expression on SSc fibroblasts stimulates the chemotaxis of mononuclear cells [30]. In addition to its chemoattractant activities, CCL2 induces Th2 cell polarization and stimulates collagen production by fibroblasts via endogenous upregulation of TGF-β expression [31–33]. CCR2, the receptor for CCL2, is upregulated in the skin of patients with active SSc and CCR2 expression on SSc fibroblasts appears to positively regulate the expression of CCL2 and α-smooth muscle actin [34]. In the current study serum CCL2 levels were found to be increased, consistent with previous reports [35, 36], and the baseline CCL2 levels were significantly associated with HAQ-DI at baseline and subsequent every year until 4 years by univariate analysis. However, baseline CCL2 levels were not significantly associated HAQ-DI at the fourth year as determined by multiple regression analysis.

There are some discrepancies between the findings in this study and the results of our previous study [37]. In the previous study, we analyzed serum chemokine levels in Japanese early SSc patients of single center during 3 years. Although CXCL8 levels tended to be higher in patients than in controls at their first visit, the difference was not statistically significant. Levels of CCL2, CXCL9, and CXCL10 were significantly increased consistent with the current findings, although CCL5 were not measured in that study. Since the measuring procedures and measurers were the same in both studies, the difference is likely to due the difference of population. Previous study included only 31 patients of a single center. On the other hand, 70 non-overlapping patients of multicenter were examined in this study and this made it possible to detect a significant increase of CXCL8 in patients with SSc. Although the variations of CCL2 were significantly associated with the variations of skin thickness score and VC during 3 years in our previous study, we could not detect those findings in this study. Our current findings together with the previous results indicate that CXCL8 in addition to CCL2 are candidates of biomarkers in SSc.

In this study, serum CXCL8 levels strongly correlated with serum CCL2 levels at the patients' first visit. In general, CXCL8 and CCL2 are the most critical chemokines for the recruitment of neutrophils and monocytes, respectively. Further, the transition from neutrophil to monocyte accumulation during inflammation might be linked to a shift in chemokine synthesis from CXCL8 to CCL2, probably via IL-6 signaling [38]. Therefore, increased CXCL8 production may trigger initial inflammation while CCL2 may contribute to the subsequent chronic inflammation and fibrosis in SSc. Thus, CXCL8 levels, rather than CCL2 levels, can likely predict the progression of SSc, despite the finding that serum levels of CXCL8 and CCL2 were associated with each other.

CCL5 produced by T cells and other cell types is chemotactic for T cells and eosinophils [18]. Abundant expression of the mRNA and elevated protein levels of CCL5 have been detected in the skin of SSc patients, but not in the skin of healthy controls [39, 40]. Moreover, levels of CCL5 are increased in BAL fluids from SSc patients [26]. Although serum CCL5 levels in SSc patients have been reported to be comparable to those of healthy controls [41], the levels of CCL5 were found to be significantly elevated in early SSc patients in this study. Since our patients were selected as those with early SSc with diffuse skin sclerosis and/or ILD, it is likely that serum CCL5 is elevated in these active patients.

CXCL9 and CXCL10 belong to the family of CXC chemokines without an ELR motif and chemoattract CXCR3-expressed Th1 cells. These chemokines are produced by numerous cell types in response to IFN-γ, including monocytes and epithelial cells [18]. Serum levels of both CXCL9 and CXCL10 are reportedly increased in SSc patients compared to controls [36, 42, 43]. In one study, high values of CXCL10 were associated with a more severe clinical phenotype that was characterized by lung and kidney involvement [36]. Although our patients with

early SSc also showed significantly elevated CXCL9 and CXCL10 levels, the levels did not correlate with clinical features in our selected population.

Some limitations exist in this study including analysis of a relatively small population size as well as the fact that this is an observational study and, therefore, the treatment protocol is heterogeneous. Nonetheless, our data indicate that serum CXCL8 levels may be useful for predicting the decline in physical function in patients with progressive SSc. Further longitudinal studies in a larger population will clarify the utility of serum chemokine levels as prognostic indicators in SSc patients.

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ORIGINAL ARTICLE

Intravenous epoprostenol treatment of patients with connective tissue disease and pulmonary arterial hypertension at a single center

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Abstract
Objective To assess the efficacy of epoprostenol treatment in Japanese patients with pulmonary arterial hypertension (PAH) associated with connective tissue disease (CTD).
Methods Sixteen patients with PAH–CTD treated with continuous intravenous epoprostenol at a single center between 2000 and 2009 were enrolled. Baseline characteristics, short-term and long-term outcomes, predictors of mortality, and safety profiles were evaluated. For survival analysis, 16 controls were selected who matched the underlying CTD, World Health Organization functional class, and use of PAH drugs, except for epoprostenol.
Results Six patients had systemic lupus erythematosus, five had mixed CTD, four had systemic sclerosis, and one had primary Sjögren's syndrome. The mean pulmonary arterial pressure (mPAP), cardiac index (CI), pulmonary vascular resistance, and functional class were significantly improved during the first 6 months of epoprostenol treatment. Cumulative survival rates at 1, 2, and 3 years in epoprostenol-treated patients were 69, 69, and 55 %, respectively, and were significantly better than those of the controls. Functional class, CI at baseline, and reduction of mPAP at 6 months were identified as predictors of survival. Adverse events, including flushing and catheter-related infection, were frequent, but all patients tolerated the treatment.
Conclusion Based on the improvements in both short-term and long-term outcomes among our patient cohort, epoprostenol is an effective treatment for CTD patients with advanced PAH.

Keywords Epoprostenol · Functional class · Pulmonary arterial hypertension · Survival

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Introduction

Pulmonary arterial hypertension (PAH) is an intractable condition in patients with connective tissue disease (CTD) [1]. Over the past decade, PAH-specific vasodilative agents, including prostanoids, endothelin receptor antagonists (ERAs), and phosphodiesterase 5 inhibitors (PDE5Is), have become available for clinical use. A large prospective cohort study recently conducted in the USA demonstrated that modern treatments have substantially improved the short-term survival of patients with PAH associated with CTD (PAH–CTD) [2]. However, the long-term survival rates remain unsatisfactory. In recently reported studies conducted in Japan (by our group) [3], USA [4], France [5], UK [6], Sweden [7], and China [8], the 3-year survival rate was 76, 64 , 56, 47, 39, and 54 %, respectively.

Epoprostenol, a synthetic prostacyclin, is a potent vasodilator and inhibitor of platelet aggregation and smooth muscle cell growth. Due to its very short half-life, epoprostenol is administered by continuous intravenous infusion via a surgically implanted central venous catheter

using a portable pump. Because of this complex procedure, epoprostenol treatment is performed only at specialized pulmonary hypertension (PH) centers. Epoprostenol has been shown to improve the exercise capacity, hemodynamics, and short-term survival of patients with idiopathic PAH (IPAH) or PAH–CTD [9, 10].

In a randomized, open-label, controlled trial involving 111 patients with systemic sclerosis (SSc) and moderate-to-severe PAH, the 6-min walk distance, hemodynamic parameters, and World Health Organization (WHO) functional class were significantly better at 12 weeks in patients treated with epoprostenol than in those treated with conventional therapy alone [10]. Short-term efficacy for hemodynamic status and exercise capacity has also been reported in open-label studies of epoprostenol involving a small number of patients with non-SSc CTDs, such as systemic lupus erythematosus (SLE) [11, 12]. Based on its short-term efficacy together with the long-term survival benefits that have been demonstrated in large cohorts of patients with IPAH [13, 14], epoprostenol is strongly recommended as the first-line treatment regimen for PAH patients in WHO functional class III or IV, in the current evidence-based treatment algorithm [15]. In particular, intravenous epoprostenol is the only therapeutic option recommended as the first-line therapy for PAH patients in WHO functional class IV.

In contrast to the numerous findings showing the short-term and long-term efficacy of epoprostenol in patients with IPAH, only limited data are available on patients with PAH–CTD in the literature [16, 17]. In addition, to date, reports on evaluations of the efficacy and safety profiles of epoprostenol in Japanese patients with PAH–CTD, who have demographic and clinical characteristics different from patients in the USA and Europe [3], are lacking. In the study reported here, we evaluated the short-term and long-term treatment effects of epoprostenol, predictors of mortality, and safety profiles in patients with PAH–CTD who were treated with epoprostenol, using a cohort of one of the major tertiary PH referral centers in Japan.

Patients and methods

Study population

This retrospective study involved 16 patients with PAH–CTD who were treated with continuous intravenous epoprostenol. These patients were selected from the database of the specialized PH clinic of Keio University Hospital based on the following criteria: (1) initiation of treatment with continuous intravenous epoprostenol between 2000 and 2009 and (2) at least 6 months of follow-up unless the patient had died. Clinical diagnoses of SLE and SSc were made according to the American College of Rheumatology preliminary classification criteria [18, 19]. Mixed CTD (MCTD) was diagnosed according to the criteria proposed by Kasukawa and colleagues [20], and primary Sjögren's syndrome (SS) was diagnosed according to the revised criteria proposed by the American–European Consensus Group [21] without any apparent CTD. PAH was diagnosed as (1) a mean pulmonary arterial pressure (mPAP) of $\geq$25 mmHg at rest and a pulmonary capillary wedge pressure of $\leq$15 mmHg by right heart catheterization [22] and (2) PH was not attributable to left-sided heart disease, advanced interstitial lung disease (ILD) determined by a predicted forced vital capacity <70 %, or chronic thromboembolism. For survival analysis, 16 control PAH–CTD patients who matched the original patients in underlying CTD, WHO functional class, and use of PAH drugs after enrollment, but who had not received epoprostenol, were selected from our database. All clinical information and blood samples were obtained at first visit after the patients had given written informed consent; the study was approved by the Institutional Review Board.

Data collection

A complete medical history, physical examination, laboratory evaluations, and right heart catheterization were performed for each patient at the initiation of epoprostenol treatment. Hemodynamic parameters included mean pulmonary arterial pressure (mPAP), mean right atrial pressure (mRAP), cardiac output (CO), cardiac index (CI), pulmonary vascular resistance (PVR), and mixed venous oxygen saturation (SvO_2). The follow-up clinical and laboratory information was prospectively recorded in the database.

Epoprostenol was administered via a central venous catheter by an ambulatory infusion pump. Patients were started on a continuous infusion of epoprostenol at 0.5 or 1 ng/kg/min, and the dosage was gradually increased based on the patient's tolerance. Minimum target dosage at 6 months was 20 ng/kg/min. Adverse events and dosing information were recorded for all patients. Events most likely to be related to the underlying CTDs or the worsening of PAH were not considered to be drug-related. The short-term response was evaluated 6 months after the introduction of epoprostenol, based on hemodynamic parameters, WHO functional class status, and plasma brain natriuretic peptide (BNP) level. The serum antinuclear antibody (ANA) profile was obtained by an indirect immunofluorescence and immunoprecipitation assay [23]. All of the treatment regimens for PAH and the underlying CTD were recorded, and included PAH drugs (beraprost, epoprostenol, bosentan, and sildenafil) and immunosuppressive treatment, which consisted of corticosteroids

(>0.5 mg/kg prednisolone equivalent) with or without immunosuppressive agents, such as azathioprine and cyclophosphamide.

Statistical analysis

All continuous variables are shown as the mean ± standard deviation (SD). Unpaired and paired comparisons of continuous variables were made using the Mann–Whitney *U* test and Wilcoxon test, respectively. Categorical variables were compared by Fischer's exact test. Survival was analyzed using the Kaplan–Meier method, and the survival between two groups was compared using the log-rank test. The follow-up period for the analysis of survival data ended in December 2009. Univariate analysis with the Cox proportional hazards regression model was used to determine factors associated with an increased risk of mortality at 1, 3, and 5 years after the initiation of epoprostenol therapy. For this purpose, survival data in all patients were censored at year 1, 3, and 5, as described previously [3]. Twenty-two baseline characteristics were used as variables: presence or absence of Raynaud's phenomenon; ILD; underlying CTDs (SLE, MCTD, SSc, primary SS); ANA specificities (anti-U1RNP, anti-Sm, anti-SSA, anti-SSB, anticentromere, anti-topoisomerase I); WHO functional classes (II, III, and IV) as dichotomous variables; age, hemodynamic parameters (mPAP, PVR, CI, mRAP, and SvO_2), and BNP as continuous variables. Changes in hemodynamic parameters (mPAP, PVR, CI, mRAP, SvO_2) and BNP after 6 months of epoprostenol treatment were also used as continuous variables. Percent changes were calculated in comparison with the baseline values. With regard to factors selected to be associated with an increased risk of mortality by univariate analysis, cut-off values that best discriminated the favorable survival group from the poor survival group were further determined using receiver operating characteristic (ROC) curve analysis. Statistically significant results were presented as a hazard ratio (HR) with a 95 % confidence interval (95 % CI). Statistical analysis was performed using the SPSS ver. 19.0 statistical software (SPSS, Chicago, IL).

Table 1 Baseline demographic and clinical characteristics of PAH–CTD patients who were treated/not treated with epoprostenol

Demographic and clinical findings	Epoprostenol group ($n = 16$)	Control group ($n = 16$)	*P* value
Female	100 %	100 %	1.0
Age (years)	43 ± 14	36 ± 18	0.1
Raynaud's phenomenon	7 (44 %)	13 (81 %)	0.07
ILD	5 (31 %)	1 (6 %)	0.2
Underlying CTD			
SLE	6 (38 %)	6 (38 %)	1.0
MCTD	5 (31 %)	5 (31 %)	
SSc	4 (25 %)	4 (25 %)	
Primary SS	1 (6 %)	1 (6 %)	
Antinuclear antibody			
Anti-U1RNP	10 (62 %)	7 (44 %)	0.5
Anti-Sm	3 (19 %)	1 (6 %)	0.6
Anti-SSA	6 (38 %)	12 (75 %)	0.07
Anti-SSB	5 (31 %)	4 (25 %)	1.0
Anticentromere	3 (19 %)	4 (25 %)	1.0
Anti-topoisomerase I	1 (6 %)	0 (0 %)	1.0
WHO functional class (FC)			
II	1 (6 %)	1 (6 %)	1.0
III	8 (50 %)	8 (50 %)	
IV	7 (44 %)	7 (44 %)	
Hemodynamic parameters			
mPAP (mmHg)	56 ± 9	53 ± 12	0.6
mRAP (mmHg)	8.4 ± 5.0	6.3 ± 7.4	0.2
CO (L/min)	2.8 ± 1.4	3.5 ± 1.1	0.02
CI (L/min/m^2)	1.8 ± 0.7	2.4 ± 0.5	0.1
PVR (Wood units)	21 ± 9	14 ± 6	0.02
SvO_2 (%)	56 ± 12	59 ± 3	0.8
Plasma BNP (pg/ml)	1,033 ± 1,383	312 ± 286	0.2

PAH–CTD Pulmonary arterial hypertension associated with connective tissue disease, *ILD* interstitial lung disease, *SLE* systemic lupus erythematosus, *MCTD* mixed CTD, *SSc* systematic sclerosis, *SS* Sjögren's syndrome, *WHO* World Health Organization, *mPAP* mean pulmonary arterial pressure, *mRAP* mean right atrial pressure, *CO* cardiac output, *CI* cardiac index, *PVR* pulmonary vascular resistance, *SvO_2* mixed venous oxygen saturation, BNP, brain natriuretic peptide

Values are presented as the number with the percentage in parenthesis, or as the mean ± standard deviation (SD)

Results

Clinical characteristics at baseline

In our center, epoprostenol is indicated mainly for PAH patients with clinically apparent right heart failure, mPAP of ≥55 mmHg, and/or acute progression. Table 1 shows the demographic and clinical characteristics at baseline of 16 patients with PAH–CTD who were treated with epoprostenol. SLE, MCTD, and SSc were the major underlying CTDs, and this distribution was consistent with the entire cohort [3]. Anti-U1RNP antibody was the predominant ANA. All but one of the patients were classified as WHO functional class III or IV at the time of epoprostenol introduction. The hemodynamic parameters were severely impaired at epoprostenol introduction and included marked elevations of mPAP and mRAP, decreased CO and CI, a rise in PVR, and low SvO_2. The plasma BNP was also highly elevated. Prior treatment and outcomes in individual patients are listed in Table 2. Twelve patients

(75 %) had not been treated with any PAH drug or had been treated with beraprost alone. This is probably because beraprost and epoprostenol were the only drugs approved for PAH before 2005 in Japan. Epoprostenol therapy was initiated within 1 year after the diagnosis of PAH in the majority of the patients because there was no other treatment option at that time. Beraprost treatment was terminated upon the introduction of epoprostenol, but sildenafil and bosentan treatments were maintained. Thus, 12 patients received epoprostenol monotherapy, two received a combination therapy with sildenafil, and the remaining two patients received a combination therapy with sildenafil and bosentan. Seven patients had received immunosuppressive treatment in advance, but their PAH responded poorly or insufficiently to this treatment. The WHO functional class had been improved in one patient with MCTD (#15) from class III to II by treatment with high-dose prednisolone before the introduction of epoprostenol therapy. In none of the patients were the epoprostenol treatment and immunosuppressive treatment (either initiation or intensification) started simultaneously. Oxygen supplementation was started prior to the introduction of epoprostenol for all patients.

Short-term efficacy

We evaluated the short-term efficacy of epoprostenol after 6 months of treatment in only 12 patients because four patients had died during this period. Of the four patients who died, three patients had uncontrolled right heart failure and died within a few weeks after the initiation of epoprostenol therapy.

The dose escalation data for the first 6 months of epoprostenol treatment in the 12 patients are shown in Fig. 1. The dose was increased to 23 ± 4 (range 18–31) ng/kg/min by 6 months. The dose escalation pace was relatively slow due to some patients experiencing adverse effects, but nearly all patients were able to tolerate 20 ng/kg/min of epoprostenol, which was the minimum target dose. No PAH drug or immunosuppressive therapy was added to the therapeutic regimen during the first 6 months. The changes in hemodynamic parameters after 6 months of epoprostenol treatment are shown in Fig. 2. All the hemodynamic parameters and the BNP level showed overall trends toward improvement, but a few patients experienced worsening of these parameters. Significant improvement was observed in the mPAP (26 % reduction from baseline; $P = 0.006$), CI (36 % increase from baseline; $P = 0.02$), and PVR (41 % reduction from baseline; $P = 0.009$). In addition, the mRAP and BNP tended to decrease and SvO_2 tended to increase after 6 months of epoprostenol treatment. The distribution of WHO functional class at baseline and after 6 months of epoprostenol treatment is shown in Fig. 3. The WHO functional class improved or remained the same for all the patients. The change in functional class during the 6-month treatment period was statistically significant ($P = 0.02$).

We further evaluated potential associations between epoprostenol dosage at 6 months and changes in the hemodynamic parameters. When the 12 patients who were still alive after the first 6 months of epoprostenol were subgrouped into two groups based on epoprostenol dosage at 6 months that was higher or lower than the average dosage at this time (23 ng/kg/min), the reduction of mPAP from baseline was more prominent in patients who were able to increase the higher dosage, compared with those who were not (23 ± 6 vs. 8 ± 11 mmHg; $P = 0.02$).

Survival

All 16 patients enrolled in the study continued epoprostenol treatment during follow-up. When the four patients who died early in the course of epoprostenol treatment were excluded from the analysis (patients #1–4 in Table 2), the maximum dosage administered during follow-up was 31 ± 7 (range 22–43) ng/kg/min, and the duration of epoprostenol treatment was 52 ± 27 (range 9–90) months. Of the original 16 patients, 11 had died by 28 ± 27 (range 0.2–73) months following the initiation of epoprostenol treatment. All causes of death appeared to be related to PAH, including uncontrolled right heart failure and sudden death. During the disease course, sildenafil and bosentan were added to the epoprostenol therapeutic regimen of four patients and one patient, respectively (Table 2). The 1-, 2-, and 3-year survival rates in epoprostenol-treated patients were 69, 69, and 55 %, respectively (Fig. 4). To examine if epoprostenol treatment improved prognosis, the survival curve in epoprostenol-treated patients was compared with that of 16 control patients who matched the original patients in terms of underlying CTD, WHO functional class, and use of PAH drugs after enrollment, but who had not received epoprostenol. Comparison of the baseline characteristics of the epoprostenol versus control group revealed that the CO and PVR were worse in the epoprostenol group than in the control group ($P = 0.02$ for both comparisons), but other findings were comparable between these two groups (Table 1). In the control group, four patients and one patient were treated with sildenafil and bosentan, respectively, after enrollment. The survival rates in the control group were 56, 25, and 6 % at 1, 2, and 3 years, respectively, and the overall survival was significantly worse than that of the epoprostenol group ($P = 0.02$), even though hemodynamic parameters were worse in the control group.

Table 2 Prior treatment for PAH and outcomes in individual patients with PAH–CTD enrolled in this study

Case	Age at Epo introduction (years)	Underlying CTD	Prior treatment for PAH		WHO-FC at Epo introduction	Time between PAH diagnosis and Epo introduction (months)	Additional treatment during Epo treatment	Follow-up period (months)	Outcome
			PAH drugs	Immunosuppressive treatment					
#1	24	SLE	Ber	None	IV	12	None	1	Dead
#2	22	MCTD	Ber	PSL	IV	9	None	1	Dead
#3	68	SSc	Ber	None	IV	0	None	1	Dead
#4	27	SLE	Ber	PSL, IVCY	III	2	Sil	3	Dead
#5	47	SLE	Ber	None	III	0	Sil	9	Dead
#6	50	SLE	Ber, Sil, Bos	None	III	69	None	13	Alive
#7	48	SLE	Ber, Bos, Sil	PSL	IV	69	None	27	Dead
#8	57	MCTD	Ber	PSL	IV	9	None	32	Dead
#9	52	SSc	None	None	III	0	None	48	Dead
#10	40	SSc	Ber	None	III	0	Sil	55	Dead
#11	51	SSc	Ber, Sil	None	IV	2	None	58	Dead
#12	45	MCTD	Ber, Sil	None	III	14	None	71	Alive
#13	30	Primary SS	Ber	None	III	0	Bos	73	Alive
#14	62	MCTD	Ber	None	IV	0	Sil	73	Dead
#15	29	MCTD	Ber	PSL	II	0	None	77	Alive
#16	31	SLE	None	None	III	0	None	90	Alive

Epo epoprostenol, *Ber* beraprost, *Sil* sildenafil, *Bos* bosentan, *PSL* prednisolone, *IVCY* intravenous cyclophosphamide pulse therapy

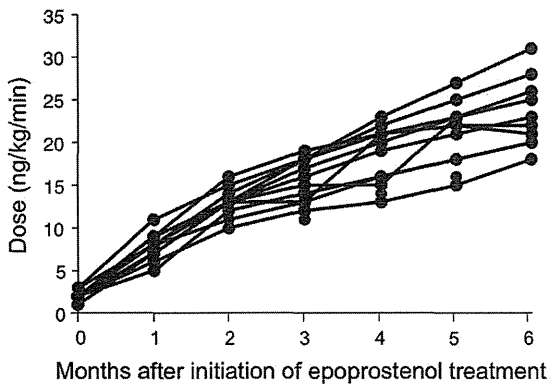

Fig. 1 Dose escalation during the first 6 months of epoprostenol treatment for the 12 patients who completed 6 months of continuous intravenous epoprostenol treatment. Epoprostenol was started at 0.5 or 1 ng/kg/min, and the dosage was increased gradually based on tolerability

Predictors of mortality

To identify variables that predict outcomes in patients with PAH–CTD who were treated with epoprostenol, we first analyzed 22 baseline parameters at the time of initiating epoprostenol therapy for all 16 patients. Since the majority of patients enrolled had low survival rates, outcomes were analyzed by setting the follow-up period at 1, 3, and 5 years, respectively, after the introduction of epoprostenol. This analysis enabled us to identify predictors of early and late mortality separately. The results of univariate analysis identified the WHO functional class and CI at baseline as sole predictors of mortality (Table 3). Patients in WHO functional class IV at baseline tended to have an increased risk for mortality throughout the disease course, but only the hazard ratio at 3 years reached statistical significance (HR 6.49; $P = 0.03$). In fact, patients in WHO functional class IV at baseline had a significantly worse survival rate than those in WHO functional classes II and III combined ($P = 0.02$) (Fig. 5a). In contrast, the baseline CI was associated with mortality 5 years after the initiation of epoprostenol treatment (HR 0.21; $P = 0.049$), but this variable showed less impact on shorter term survival, i.e., at 1 and 3 years. The cut-off value for baseline CI that best discriminated the favorable survival group from the poor survival one was determined to be 1.9 L/min/m^2 by ROC curve analysis. The cumulative survival rates were significantly different between groups stratified by a baseline CI higher and lower than 1.9 L/min/m^2 ($P = 0.01$) (Fig. 5b).

In the 12 surviving patients, we further investigated whether the short-term treatment response to epoprostenol

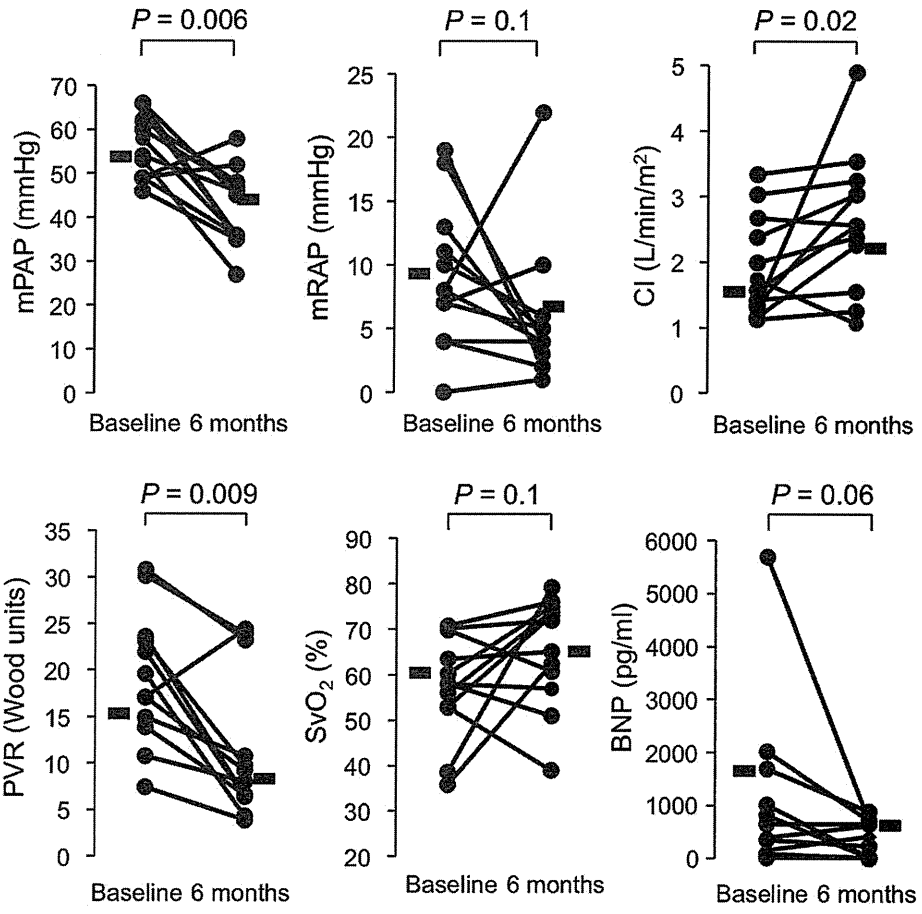

Fig. 2 Changes in hemodynamic parameters and the plasma brain natriuretic peptide (*BNP*) level after the first 6 months of epoprostenol treatment in the 12 patients who were still alive. *Bold lines* Mean of individual data. *mPAP* Mean pulmonary arterial pressure, *mRAP* mean right atrial pressure, *PVR* pulmonary vascular resistance, *CI* cardiac index, SvO_2 mixed venous oxygen saturation

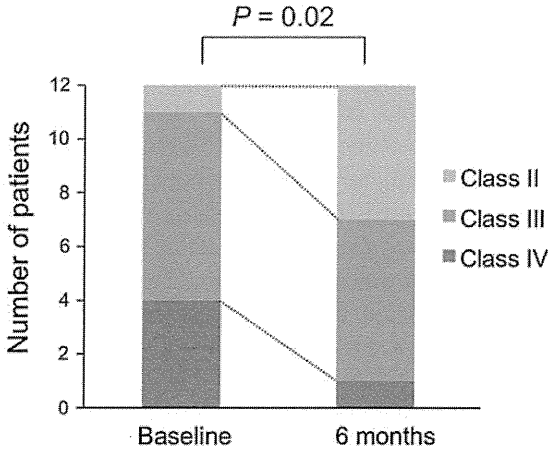

Fig. 3 Distribution of World Health Organization functional class at baseline and after 6 months of epoprostenol treatment in the 12 patients who were still alive after 6 months of treatment

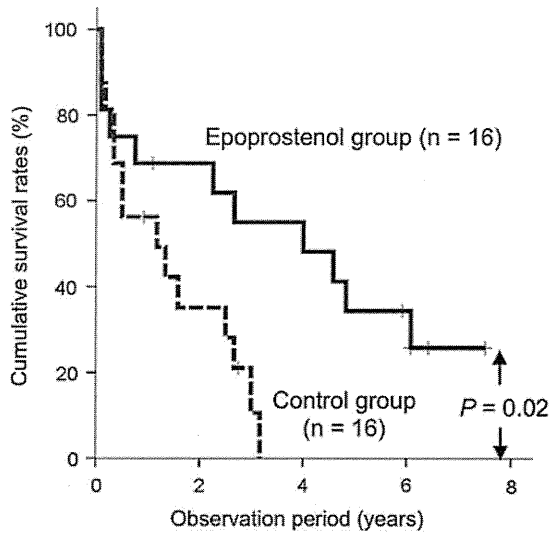

Fig. 4 Cumulative survival rates in 16 patients with PAH–CTD who were treated with epoprostenol (epoprostenol group) and 16 historical controls selected from our database that matched the patients in terms of underlying CTD, WHO functional class, and use of PAH drugs except for epoprostenol (control group). The groups were compared using the log-rank test

at 6 months was associated with subsequent outcomes. Using changes in hemodynamic parameters (mPAP, PVR, CI, mRAP, SvO_2) and BNP as variables, we identified a change in mPAP after 6 months of epoprostenol treatment as the sole predictor of mortality at 5 years (HR 0.95, 95 % CI 0.90–0.99, $P = 0.03$). The cut-off value for change in mPAP after 6 months that best discriminated the favorable survival group from the poor survival one was determined to be 25 % by the ROC curve analysis. Patients who had achieved a ≥25 % reduction of mPAP at 6 months had a

Table 3 Baseline predictors of mortality at 1, 3, and 5 years after initiation of epoprostenol treatment

Variable	Mortality					
	1 year		3 years		5 years	
	HR (95 % CI)	*P*	HR (95 % CI)	*P*	HR (95 % CI)	*P*
WHO functional class IV	8.88 (0.97–80.88)	0.053	6.49 (1.23–34.19)	0.03	3.27 (0.91–11.72)	0.07
CI (L/min/m^2)	0.40 (0.07–2.22)	0.3	0.23 (0.04–1.40)	0.1	0.21 (0.04–0.99)	0.049

HR hazard ratio, *95 % CI* 95 % confidence interval

better survival rate than those who did not ($P = 0.005$) (Fig. 6).

Safety profiles

All 16 patients reported at least one epoprostenol-related adverse event, and 46 events were recorded during 40 ± 32 months of follow-up (Table 4). The most common adverse event was flushing. Flushing, headache, and diarrhea occurred during the dose-escalating phase and were self-limiting, although these symptoms often led to a delay in the scheduled dose escalation. Seven patients (44 %) experienced a total of 25 episodes of catheter-related infection, which required hospitalization, catheter replacement, and treatment with intravenous antibiotics. Notably, the majority of patients with this event had repeated episodes, and three patients experienced more than four events. None of the patients died of this complication. Comparison of the characteristics between patients with and without catheter-related infection revealed no parameters associated with an increased risk. However, there was a trend toward an increased frequency of concomitant immunosuppressive treatment in patients with catheter-related infection than in those without (71 vs. 22 %; $P = 0.1$). Severe thrombocytopenia occurred in three patients with SLE. The platelet count in two patients partially recovered with an increased dosage of corticosteroids in combination with cyclosporin or intravenous immunoglobulin, but it did not reach the normal range while the patients were on epoprostenol treatment. The platelet count in the remaining patient was increased by reducing the epoprostenol dosage. None of the adverse events led to a discontinuation of epoprostenol.

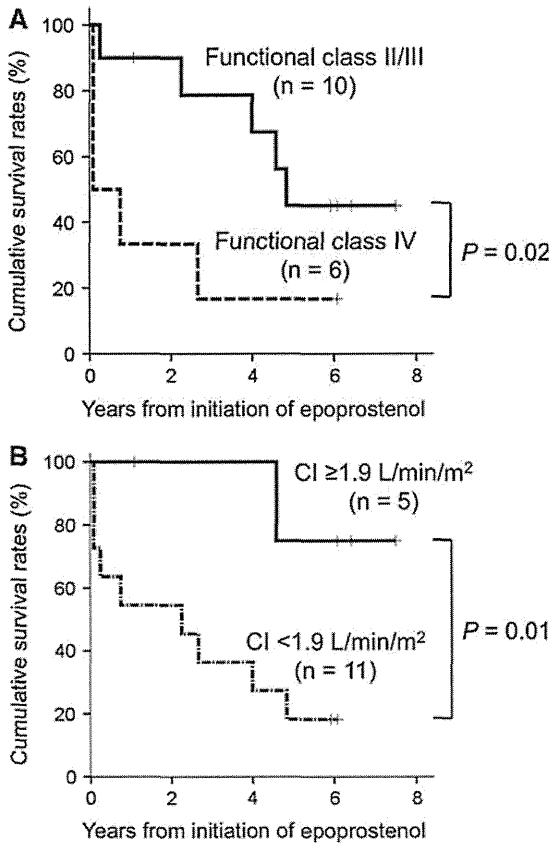

Fig. 5 Cumulative survival rates in 16 patients with PAH–CTD who were treated with epoprostenol, stratified by baseline WHO functional class (II/III vs. IV) (**a**) or baseline CI (≥1.9 vs. <1.9 L/min/m^2) (**b**). The two groups were compared using the log-rank test

Discussion

In this study, we observed a significant improvement in WHO functional class status and hemodynamic parameters, including the mPAP, CI, and PVR, after 6 months of epoprostenol treatment in CTD patients with advanced PAH who presented with severely impaired functional class and hemodynamics. Short-term hemodynamic improvement resulting from epoprostenol treatment in CTD patients with severe PAH was also reported in 111 patients with SSc at 12 weeks [10], 15 patients with various CTDs at 6 weeks [11], and four patients with SSc at 9–16 weeks [12]. In addition, we found that epoprostenol treatment prolonged the survival of patients with PAH–CTD compared with matched controls who had not received epoprostenol treatment. Adverse events related to vasodilation of the peripheral vasculature and catheter-related infection occurred frequently, but epoprostenol treatment was well tolerated in general. Our findings collectively confirm the efficacy of epoprostenol in Japanese patients with CTD and advanced PAH.

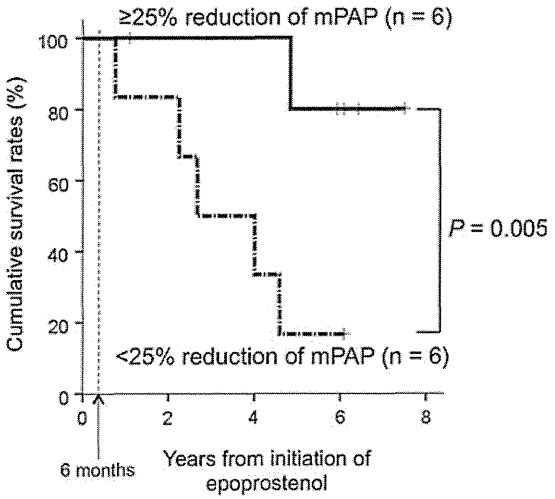

Fig. 6 Cumulative survival rates in 12 patients with PAH–CTD who had survived for 6 months after initiation of epoprostenol treatment, stratified by the change in mPAP after the first 6 months of epoprostenol treatment (≥25 % reduction vs. <25 % reduction). The two groups were compared using the log-rank test

Table 4 Adverse events related to epoprostenol treatment in 16 patients

Adverse event	Number of patients (%)	Total number of events
Flushing	13 (81)	13
Catheter-related infection	7 (44)	25
Severe thrombocytopenia (<30,000/μL)	3 (19)	3
Headache	3 (19)	3
Diarrhea	2 (13)	2

On the other hand, the long-term survival benefits in PAH–CTD patients treated with epoprostenol are less clear. In our study, the survival rates at 1, 2, and 3 years of epoprostenol treatment were 69, 69, and 55 %, respectively. These survival values are better than those of two earlier studies, which showed 1-, 2-, and 3-year survival rates of 58, 41, and 34 % [16] and 71, 52, and 48 %, respectively [17]; however, these previous results were derived solely from SSc patients, who generally have worse outcomes that patients with other forms of PAH [2, 6]. Moreover, it is notable that three of our patients died within a few weeks after the introduction of epoprostenol. Cases of early death were also reported in previous studies [10, 11, 24]. The majority of these patients died primarily of uncontrolled heart failure due to severely impaired hemodynamics, but the dilation of peripheral vasculature by epoprostenol may have contributed to the worsening circulatory insufficiency. Supportive therapies, such as catecholamine derivatives and/or percutaneous cardio-pulmonary support, should be included, especially for patients with very low CO and systemic blood pressure. It is important to recognize that the efficacy of epoprostenol is apparently limited in patients with end-stage PAH.

Our study also provided simple baseline predictors of long-term survival in epoprostenol-treated patients with PAH–CTD: WHO functional class II/III and a CI > 1.9 L/min/m^2 were associated with a favorable outcome. The WHO functional class, although it seems subjective, was a powerful predictor of survival, as also found by Kuhn and colleagues, whose study involved 91 patients with various forms of PAH, including 19 with SSc and five with SLE [16]. In addition, a ≥25 % reduction in the mPAP after the first 6 months of treatment with epoprostenol was a strong predictor of a good prognosis, suggesting that hemodynamic evaluation after 6 months of treatment provides a useful marker for stratifying the risk of death during long-term follow-up. In contrast, none of the baseline or follow-up hemodynamic parameters was selected as a predictor of future outcome in Kuhn's study [16], probably because these authors were analyzing patients with heterogeneous forms of PAH.

Our data can be readily translated into clinical practice. First, we demonstrated that in our patients with PAH–CTD long-term survival was favorable if epoprostenol was initiated when the functional status did not worsen beyond WHO functional class III and the CI was preserved. While the current treatment algorithm recommends epoprostenol, ERAs, and PDE5Is as the first-line treatment for patients in WHO functional class III [15], epoprostenol should be used in patients with severely impaired hemodynamics and/or acute progression. Second, we found that a prominent reduction in mPAP during the first 6 months of epoprostenol treatment was a predictor for favorable outcome. Thus, the dosage of epoprostenol should be escalated as sharply as possible during the first 6 months of treatment, unless the patient cannot tolerate this escalation. In this regard, epoprostenol's ability to reduce the mPAP in patients with IPAH has been shown to be dependent on its absolute dosage and dose-escalating pace [25]. Our study also showed an association between improvement of mPAP during the first 6 months of epoprostenol treatment and the dose-escalating pace of epoprostenol. Based on these findings, at our hospital we recently changed to a dose-escalating protocol for epoprostenol, i.e., dosage up to 40 ng/kg/min during the first 6 months of treatment. Finally, if the treatment response is insufficient at 6 months, even using an epoprostenol dosage that has been increased to the fullest extent possible or has not been increased due to intolerance, other PAH drugs should be readily added to the epoprostenol therapeutic regimen. A recent systematic review of combination therapy for IPAH and PAH–CTD showed that dual therapy improves multiple clinically relevant outcomes, particularly in patients