PATIENTS AND METHODS

Patients and controls. This study included 171 consecutive patients with SSc (15 men and 156 women) who visited an SSc clinic at Keio University Hospital between 2007 and 2013. All patients met the SSc classification criteria from the 2013 American College of Rheumatology (ACR)/European League against Rheumatism [10], and 137 patients (80%) also met the 1980 ACR preliminary classification criteria [11]. Peripheral blood samples were collected from all patients when they entered the study and from a subset of patients 2 years later. Peripheral blood samples were also collected from 19 healthy control subjects matched for sex and age (4 men and 15 women). The heparinized blood was immediately separated into platelet-poor plasma and mononuclear cells, which were used to count endothelial progenitor cells (EPCs). Serum and plasma samples were stored at -80°C until use. We obtained informed, written consent from all subjects prior to collecting samples, in accord with the tenets of the Declaration of Helsinki, and all study protocols were approved by the International Review Board of Keio University School of Medicine.

Clinical assessment. A complete medical history, physical examination, and laboratory workup was performed for each patient at the time of enrollment, and limited evaluations were conducted at each follow-up visit. Patients were carefully monitored for new-onset DU and PAH until December 2013. We collected the following data for each patient: age, sex, disease subset, disease duration from the first appearance of non-Raynaud's symptoms, disease duration from the first appearance of Raynaud's phenomenon, the modified Rodnan total skin thickness score (MRSS), and SSc-related organ involvement,

including digital ulcers (DU), interstitial lung disease (ILD), pulmonary arterial hypertension (PAH), and any heart, upper GI, lower GI, or renal involvement. We also recorded treatment profiles and any risk factors for atherosclerosis, including hypertension, dyslipidemia, diabetes mellitus, and whether the patient was currently a smoker. The SSc was classified as diffuse cutaneous SSc (dcSSc) or limited cutaneous SSc (lcSSc) according to Medsger [12]. SSc-related organ involvement was defined as described previously [13] with modifications: DU was defined as a loss of both epidermis and dermis in an area at least 2 mm in a diameter located at volar surface of the digit distal to the proximal interphalangeal digital crease [14]; ILD as bilateral reticulations, ground-glass opacity, and/or honeycombing on chest radiograph or high resolution computed tomographic scan of the lungs; PAH as a mean pulmonary arterial pressure ≥25 mmHg and pulmonary vascular resistance >3 Wood units, measured at rest using right heart catheterization, after excluding pulmonary hypertension due to left-sided heart disease (an end-expiratory pulmonary artery wedge pressure > 15 mmHg), advanced ILD (forced vital capacity predicted <70%), or chronic thromboembolism [15]; heart involvement as clinical evidence of symptomatic pericardial effusion, congestive heart failure, or arrhythmia considered to be due to SSc requiring treatment; upper GI involvement as distal esophageal hypomotility by cine esophagogram or manometry; lower GI involvement as radiologic evidence of wide-mouthed colonic sacculations or small intestinal dysmotility, malabsorption, or administration of antibiotics for small bowel bacterial overgrowth; and renal involvement as acute or subacute development of renal insufficiency often, but not always, associated with accelerated hypertension and/or microangiopathic hemolytic anemia. Extensive ILD was defined according to Goh et al [16].

SSc-related autoantibodies. The following antibodies were identified by indirect immunofluorescence using commercially prepared slides of monolayer HEp-2 cells (MBL, Nagano, Japan) and immunoprecipitation assays: anticentromere, anti-topoisomerase I, anti-RNA polymerase III, anti-U1RNP, anti-U3RNP, anti-U11/U12RNP, anti-Th/To, anti-PM-Scl, anti-Ku, and anti-RuvBL1/2 [17].

Measuring PTX3 and FGF2 in the circulation. Concentrations of PTX3 in plasma and FGF2 in serum were measured in duplicate using enzyme immunoassay kits according to the manufacturers' instructions (Perseus Proteomics, Tokyo, Japan, and R&D Systems, Minneapolis, MN, respectively). The lower limits of detection for PTX3 and FGF2 were 0.1 ng/mL and 3 pg/mL, respectively. Coefficient of variance of two values ranged from 0 to 0.25. In some analyses, the ratio of PTX3 (ng/mL) and FGF2 (ng/mL) was used as an indicator of the anti/pro-angiogenic activity of FGF2 signaling.

Quantifying EPCs. The absolute number of $CD34^{+}CD133^{+}CD309^{+}$ EPCs in peripheral blood was determined as described previously [18], and is shown as the number per 1 mL of peripheral blood, determined using FlowCount ™ fluorospheres (Beckman-Coulter, Fullerton, CA) as an internal calibrator.

EPC colony formation assays. The ability of mouse bone-marrow stem cells to differentiate into EPCs in the presence of a series of pro-angiogenic factors, including FGF2, was assessed by colony formation assays as described previously [19]. Briefly, bone-marrow mononuclear cells were isolated from the thigh bones of 10-12-week-old male C57BL/6J mice. The cells were suspended in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) and seeded on Pronectin

(Sigma-Aldrich)-coated 60 mm culture dishes. After 24 hours, non-adherent mononuclear cells were collected and cultured on Pronectin-coated *UpCell*® 6-well plates with EGM™-2 supplemented with SingleQuots™ (Lonza, Basel, Switzerland), which contains 2% fetal bovine serum and other components with no available information on individual concentrations (hydrocortisone, insulin-like growth factor-1 [IGF-1], epidermal growth factor [EGF], vascular endothelial growth factor [VEGF], FGF2, ascorbic acid, and heparin), either with or without recombinant mouse PTX3 (5 or 20 nM; R&D Systems). After 6 days, adherent cells were subjected in duplicate to a colony assay that is able to evaluate colony forming units-endothelial cells (CFU-EC) and colony forming units-granulocytes, erythrocytes, monocyte/macrophages, and megakaryocytes (CFU-GEMM) simultaneously. Culture medium used was MethoCult® (Stem Cell Technologies, Vancouver, Canada) with stem cell factor (100 ng/mL), IL-3 (20 ng/mL), IGF-1 (50 ng/mL), EGF (50 ng/mL), VEGF (50 ng/mL), FGF2 (50 ng/ml) (all from R&D Systems), insulin (10 µg/mL; PromoCell GmbH, Heidelberg, Germany), and transferrin (200 µg/mL; Sigma-Aldrich). After 14 days, CFU-EC and CFU-GEMM were individually counted with a microscope with a magnification of 1:20. Results are shown as the mean of the two measurements.

Statistical analysis. All continuous variables are shown as mean ± standard deviation (SD). Paired and unpaired comparisons of continuous variables were made using Wilcoxon tests or Mann-Whitney *U* tests, respectively. Categorical variables were compared with Fisher's exact test or a *chi*-square test when appropriate. The correlation coefficient (r) was calculated using Pearson's regression model. Variables that best explained the risk for DU and PAH were identified by multivariate logistic regression analysis combining clinical

parameters (sex, age at onset and at entry into the study, disease duration from the first non-Raynaud's symptoms and Raynaud's phenomenon, disease subset, Raynaud's phenomenon, individual organ involvement, and previous history of DU), autoantibodies, corticosteroid use, and circulating levels of PTX3 and FGF2. The results are presented as an odds ratio (OR) with a 95% confidence interval (CI). The Cox proportional hazards regression model was used to determine factors associated with an increased risk of the future development of DU. The results are presented as a hazard ratio (HR) with a 95% CI. The cut-off value that best discriminated two groups was determined by receiver operating characteristic (ROC) curve analysis. Survival analysis was performed using the Kaplan-Meier method, and survival was compared between two groups by log-rank tests. All statistical analyses were performed using SPSS 19.0 statistical software (SPSS, Chicago, IL).

RESULTS

Levels of circulating PTX3 and FGF2 in SSc patients. Demographic and clinical characteristics were recorded and blood samples obtained from each of the 171 SSc patients as they entered the study (Table 1). Our cohort consisted mainly of patients with lcSSc (74%). The mean disease duration from the first non-Raynaud's symptoms and Raynaud's phenomenon was 9.5 ± 7.2 and 14.0 ± 9.9 years, respectively. Figure 1 shows that, in comparison to healthy control subjects, SSc patients had significantly higher levels of circulating PTX3 (4.8 ± 3.3 versus 2.4 ± 1.7 ng/mL) and FGF2 (12.0 ± 9.4 versus 5.8 ± 5.0 pg/mL). There were no differences in PTX3 or FGF2 levels between SSc patients with dcSSc and those with lcSSc (Figure 1). The PTX3/FGF2 ratio, which we used as an indicator for the relative anti/pro-angiogenic activity of the FGF signaling, was no different between SSc patients and healthy controls. We did not find correlations between PTX3, FGF2, and CRP levels in patients with SSc ($r < 0.1$ for all comparisons).

Organ involvement association with circulating levels of PTX3 and FGF2. PTX3 and FGF2 levels were compared between SSc patients grouped by the presence or absence of specific organ involvement (see supplementary Table 1). Statistically significant differences were detected when patients were divided by the presence or absence of DU or PAH (Figure 2). PTX3 was significantly elevated in patients with DU compared to those without DU, while there was no difference in FGF2 concentrations. On the other hand, PAH was associated with increased PTX3 and decreased FGF2, compared with SSc patients without PAH, and FGF2 levels were comparable in healthy controls and SSc patients with PAH (5.8

± 5.0 versus 8.1 ±12.3 pg/mL, $P = 0.8$). The PTX3/FGF2 ratio was significantly higher in patients with DU or PAH than in those without, suggesting that FGF2 signaling was suppressed. When we assessed potential associations of PTX3 and FGF2 levels with risk factors for atherosclerosis and individual treatment drugs, PTX3 levels were significantly higher in SSc patients with corticosteroids than in those without ($P = 0.02$).

As the behavior of FGF2 appeared to change in association with DU or PAH, we divided SSc patients into 4 groups based on the presence or absence of DU and the presence or absence of PAH, and evaluated the distribution of PTX3 and FGF2 levels in each group (Figure 3). When PTX3 and FGF2 levels were divided as high or low relative to the mean (3.88 ng/mL for PTX3 and 9.82 pg/mL for FGF2), differences in patient distribution in the groups became apparent: all but one patient with DU was included in the high-PTX3 group, independent of the presence or absence of PAH, and this frequency was significantly greater than in patients without DU (94% versus 45%, $P < 0.001$). On the other hand, patients with PAH were more frequently included in the low-FGF2 group than those without PAH (86% versus 45%, $P < 0.001$). Thus, it is likely that different PTX3 and FGF2 profiles regulate the development of DU and PAH. However, the PTX3/FGF2 ratio was elevated in patients with both DU and PAH (Figure 2), indicating the presence of common anti-angiogenic properties.

PTX3 and FGF2 as independent parameters associated with DU or PAH. We further determined whether the circulating PTX3 and FGF2 levels were independently associated with the presence of DU or PAH using multivariate analysis (see supplementary Table 2). Univariate analyses identified disease duration from the first non-Raynaud's symptoms, dcSSc, a previous history of DU, upper GI involvement, and anti-topoisomerase I

antibody as parameters potentially associated with the presence of DU ($P < 0.1$). Multivariate logistic regression analysis of these parameters along with PTX3 and FGF2 levels identified the following as independent parameters positively associated with DU: PTX3 (OR = 1.50, 95% CI 1.22-1.85, $P < 0.001$), anti-topoisomerase I antibody (OR = 8.01, 95% CI 1.13-57.1, $P = 0.04$), and a previous history of DU (OR = 61.7, 95% CI = 7.09-537, $P < 0.001$). To identify independent parameters associated with PAH, we conducted multivariate analysis with 5 variables: the absence of anti-topoisomerase I antibody, which was selected by univariate analysis; disease duration from the first Raynaud's phenomenon and lcSSc, which were reported to be associated with PAH [20]; and PTX3 and FGF2 (see supplementary Table 3). PTX3 (OR = 1.23, 95% CI 1.08-1.40, $P = 0.002$) was again selected as the sole parameter positively associated with PAH, while FGF2 (OR = 0.92, 95% CI 0.85-0.99, $P = 0.02$) was identified as an independent parameter negatively associated with PAH. When these analyses were repeated using PTX3/FGF2 ratio instead of PTX3 and FGF2 levels, the PTX3/FGF2 ratio was selected as an independent parameter associated with DU and PAH (OR = 1.001, 95% CI 1.000-1.002, $P = 0.04$ and OR = 1.001, 95% CI 1.001-1.002, $P < 0.001$, respectively).

Predictors of a new occurrence of DU. Of 171 patients, 148 had never developed DU at the initial evaluation. These patients were prospectively followed for 3.4 ± 2.5 years, and 18 (12%) newly developed DU during the follow-up period. ROC analysis was used to determine whether circulating levels of PTX3 and FGF2, as well as the PTX3/FGF2 ratio, were predictive of new DU development (Figure 4A). PTX3 levels and the PTX3/FGF2 ratio were useful in predicting the future onset of DU, but FGF2 levels were not. The cut-off

values that best discriminated a risk for future DU development were 4.3 ng/mL for PTX3 and 344 for the PTX3/FGF2 ratio. When SSc patients were divided by high or low PTX3 levels relative to the cut-off values, the cumulative occurrence rate of new DU changed significantly (Figure 4B). We were unable to conduct a similar analysis of PAH predictors because only one patient developed PAH during the follow-up period.

We further assessed whether the circulating PTX3 level was an independent parameter predicting the future development of DU using the Cox proportional hazard regression model (see supplementary Table 4). In this analysis, the time origin was the time of enrollment. The variables included were dcSSc and anti-topoisomerase I antibody, which were selected by univariate analysis ($P < 0.1$), disease duration from the first non-Raynaud's symptoms, which was shown to be associated with DU [21, 22], and PTX3 and FGF2. This identified PTX3 (HR = 1.25, 95% CI 1.09-1.42, $P = 0.001$) as a sole independent predictors for the new development of DU. When the analysis was repeated using the PTX3/FGF2 ratio instead of PTX3 and FGF2, the PTX3/FGF2 ratio was selected as a sole predictor for the future development of DU (HR = 1.001, 95% CI 1.000-1.001, $P = 0.003$).

Changes of PTX3 and FGF2 levels after an interval of 2 years. Follow-up blood samples obtained 2 years after the initial evaluation were available for 37 SSc patients, in whom 9 developed DU during this period. The levels of circulating PTX3 and FGF2 were fairly stable during this period regardless of whether there was any new onset of DU (4.7 ± 3.3 versus 4.4 ± 3.2 ng/mL, and 10.7 ± 10.4 versus 10.9 ± 7.1 pg/mL, respectively). Together, these results suggest that elevated PTX3 levels and PTX3/FGF2 ratios are useful biomarkers to predict the future development of DU in SSc patients, and that high, persistent

concentrations of PTX3 in circulation may lead to the occurrence of DU.

Correlations between PTX3 and EPCs in circulation. It was recently reported that the number of circulating EPCs is inversely correlated with the presence of DU, and that a low EPC count in SSc patients is a risk factor for developing DU [23, 24]. Thus, we investigated the potential correlation between circulating PTX3 levels and EPC counts. Circulating EPC counts obtained at the same time as PTX3 and FGF2 levels were available for 70 patients. EPC counts were significantly lower in patients with DU than in those without DU, and in patients with PAH compared to those without PAH (Figure 5A). EPC counts were negatively correlated with circulating PTX3 levels and PTX3/FGF2 ratios, but there was no correlation with FGF2 levels (Figure 5B). These findings demonstrated an association between reduced EPC counts and high concentrations of PTX3 in circulation in the context of the occurrence of DU or PAH.

PTX3 inhibits EPC differentiation. To investigate the effect of PTX3 on EPC differentiation, we employed an *in vitro* mouse system in which non-adherent bone-marrow mononuclear cells were cultured in EPC differentiation conditions, which contained FGF2, in the presence or absence of PTX3, followed by colony-forming assays to count CFU-ECs and CFU-GEMMs (Figure 5C). In these cultures, PTX3 reduced the formation of CFU-ECs to approximately half, but had no effect on CFU-GEMM formation. Further increases in the concentration of PTX3 did not increase the inhibitory effect. This suggests that an FGF2-independent pathway is also involved in this *in vitro* EPC differentiation assay system, since PTX3 is known to directly inhibit binding of FGF2 to its receptor [5]. Thus, exposure to high concentrations of PTX3 may suppress EPC differentiation, leading to a negative

correlation between PTX3 levels and EPC counts.

DISCUSSION

In this study, we found that circulating PTX3 was elevated in SSc patients, irrespective of the disease subset. Interestingly, elevated PTX3 levels were associated with vascular manifestations such as DU and PAH, but not with the fibrotic aspects of the disease. This was confirmed by multivariate analysis, which selected PTX3 as an independent parameter for the presence of DU and PAH. Elevated PTX3 was also identified as a good predictor for the future development of DU. Together, these findings suggest that PTX3 primarily promotes SSc vasculopathy, which is consistent with previous studies [6, 25]. However, while Iwata and colleagues also found correlations between elevated PTX3 and various fibrotic aspects of SSc, including dcSSc, high MRSS, pulmonary fibrosis, and heart involvement, they did not find a correlation with PAH [6]. Although the exact reasons for this discrepancy are uncertain, it may be due to differences in the method of measuring PTX3 levels and the definitions for the involvement of various organs, as well as the lower proportion of patients with dcSSc in our study. Since circulating PTX3 levels stayed fairly stable over the course of this study, PTX3 should prove to be a useful biomarker for the future development of DU in SSc patients.

Since PTX3 exerts its anti-angiogenic effects primarily by competing with FGF2 for binding to its receptor, for which PTX3 has higher affinity [5], the balance between circulating PTX3 and FGF2 can reasonably be used to assess net anti/pro-angiogenic activity. Since FGF2 was also elevated in SSc patients compared to healthy subjects, as other studies have also reported [26, 27], the PTX3/FGF2 ratio remained comparable between SSc patients

and healthy controls. However, as with PTX3, our results clearly identified the PTX3/FGF2 ratio as an independent parameter for the presence of DU and PAH, as well as a predictor for the future development of DU.

FGF2's roles in neovascularization are mediated in part by the promotion of angiogenesis, a process in which endothelial cells proliferate and sprout from pre-existing vessels [28, 29]. It has been suggested that chronic tissue ischemia and a lack of compensatory angiogenesis leads to vascular manifestations in SSc patients [30]. In this regard, the angiogenic capacity of endothelial cells derived from SSc skin was reduced by FGF2 and VEGF *in vitro* [9]. On the other hand, we have also proposed that vasculogenesis, a vascularization process that involves the recruitment and *in situ* differentiation of bone marrow-derived EPCs, is also defective in SSc patients [31]; this is based on the reduced counts and impaired differentiation potential of EPCs in SSc patients [18, 32]. However, the underlying mechanisms of defective vasculogenesis in SSc patients are not well understood. In our present study, we showed that EPC counts were inversely correlated with the level of circulating PTX3 or the PTX3/FGF2 ratio, and that PTX3 could inhibit differentiation of stem cells into EPCs in *in vitro* cultures with FGF2. Therefore, it is likely that exposure to a high concentration of PTX3 suppresses the FGF2-mediated processes in both angiogenesis and vasculogenesis, increasing the risk of DU and PAH, although we did not show direct evidence of PTX3's inhibitory effect on FGF2 in our assay system.

While elevated PTX3 was associated with both DU and PAH, SSc patients with PAH uniquely exhibited FGF2 levels similar to those in healthy controls. This suggests that DU and PAH, in part, have distinct pathogenic processes in SSc patients. In this regard, French

and Canadian registries reported that the occurrence of DU did not necessarily correlate with that of PAH [21, 22], but the use of pulmonary vasodilators in patients with PAH may prevent the onset of DU. On the other hand, the relatively normal FGF2 levels seen in patients with SSc-PAH contrasts sharply with the elevated FGF2 seen in patients with idiopathic PAH (IPAH) [33]. In this regard, cultured pulmonary endothelial cells derived from patients with IPAH also overexpress FGF2 [34]. Furthermore, a series of studies using animal models for the medial hypertrophy of the pulmonary arterioles reported that FGF2 expression is enhanced in pulmonary arterioles, and that knocking down FGF2 or administering FGF receptor antagonists reverses pulmonary vascular remodeling [34-36].

Differences in FGF2 behavior in SSc-PAH or IPAH may be responsible for the distinct pulmonary vascular histologies seen in these two conditions. Specifically, SSc-PAH is characterized by intimal fibrosis of the pulmonary arterioles and venules (pulmonary veno-occlusive disease-like changes) and the absence of the plexogenic arteriopathy that is typical of IPAH [37]. Comprehensive gene expression profiling of lung samples showed that secreted protein acidic and rich in cysteine (SPARC) and thrombospondin 1 are upregulated in patients with SSc-PAH in comparison with those with IPAH [38]. Interestingly, these molecules are known to suppress autocrine and paracrine FGF2 production loops [39, 40]. Therefore, it is likely that levels of FGF2 signaling in the pathogenic process of PAH modify pulmonary vascular remodeling.

In summary, circulating PTX3 was elevated in SSc patients, and was a useful biomarker predicting the presence of DU and PAH as well as the future development of DU. In addition, PTX3 may contribute to SSc vasculopathy by inhibiting vasculogenesis-mediated

neovascularization through its suppressive effects on FGF2. Further studies are necessary to elucidate these roles and the complex interactions of anti/pro-angiogenic factors in the development of vascular manifestations of SSc.

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