Concise Report

Disturbed angiogenesis in systemic sclerosis: high levels of soluble endoglin

J. Wipff1,2,*, J. Avouac1,2,*, D. Borderie3, D. Zerkak1, H. Lemarechal3, A. Kahan1, C. Boileau2,4 and Y. Allanore1,2

Objective. SSCs is a CTD characterized by early generalized microangiopathy with disturbed angiogenesis. Soluble endoglin (sENG), a serum anti-angiogenic protein, has recently been described as a major actor in pre-eclampsia, another severe vascular disease with abnormal angiogenesis. The aim of this study was to investigate, in a cross-sectional study, sENG levels together with other serum vascular markers.

Methods. Serum levels of sENG were assessed by ELISA in consecutive SSc patients and controls matched for age and sex. We also measured ELISA serum levels of VEGF and asymmetric dimethylarginine (ADMA), as respective markers of angiogenesis and endothelial dysfunction.

Results. We included 235 unrelated subjects: 187 SSc patients and 48 controls. Higher concentrations of sENG ($P=0.002$) and sVEGF ($P<0.0001$) were found in SSc patients compared with controls whereas there was no difference for ADMA. In multivariate analysis, sENG levels were significantly increased in SSc patients with cutaneous ulcerations ($P=0.0003$), positive for ACAs ($P=0.009$) and with abnormal diffusioning capacity for carbon monoxide divided by alveolar volume ($P=0.03$). Soluble ENG levels negatively correlated with ADMA, but no relationship was found between sENG and sVEGF.

Conclusion. This study shows increased values of sENG in a large SSc cohort and a relevant association with a vascular phenotype. The predictive value of the biomarker sENG and its potential role on cellular endothelial disturbances remain to be determined.

Key Words: Systemic sclerosis, Soluble endoglin, Asymmetric dimethylarginine, Soluble vascular endothelial growth factor.

Introduction

SSC is a CTD characterized by early generalized microangiopathy. Endothelium injury is considered as one of the earliest key steps throughout the disease course. In SSc, microvascular involvement results in a transient vasospastic phenomenon and culminates in myointimal proliferation causing microvascular obliterations. These abnormalities lead to chronic severe tissue hypoxia [1], which is a major stimulus for angiogenesis. However, adapted angiogenesis does not seem to occur in SSc patients [2] and defective vascularization contrasts with the overexpression of VEGF in serum [3] and in skin [1, 4] of SSc patients throughout various stages of the disease.

TGF is a multifunctional cytokine displaying a role in numerous physiological processes. The vascular effect of TGF-β in angiogenesis results in activation of endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) [5]. These effects are mainly mediated by endoglin (ENG, CD105), a coreceptor of TGF-β and vasodilation [6].

In pathology, ENG and sENG are involved in various vascular disorders. ENG gene is the second major gene of hereditary haemorrhagic telangiectasia (HHT), an autosomal dominant vascular disorder characterized by epistaxis, telangiectasia and vascular arteriovenous malformations. Several studies demonstrated the active contribution of sENG in the pathogenesis of pre-eclampsia, a severe vascular complication of pregnancy [6, 7]. In this latter disease, sENG interferes with the binding of TGF-β to its receptor and the downstream TGF-β signalling leading to negative effects on activation of endothelial nitric oxide synthase and vasodilation [6].

In SSc, scarce data are available but telangiectasia were found more frequently in SSc patients with elevated sENG [8] and we demonstrated an association between an ENG gene polymorphism and pulmonary arterial hypertension (PAH) [9].

Vasculopathy in SSc involves several factors including endothe- lin, cell adhesion molecules and nitric oxide (NO) [10]. NO is a potent vasodilator synthesized by NO synthase isoforms and negatively regulated by asymmetric dimethylarginine (ADMA). ADMA levels are considered as the reflection of the endothelial dysfunction observed in several cardiovascular disorders [11].

Our aim was to assess, in a cross-sectional study, the serum levels of sENG as a potential marker of SSc vasculopathy and its relationship with VEGF and ADMA concentrations in SSc patients compared with healthy controls.

Patients and methods

Patients

All subjects were recruited from Rheumatology A Department of Cochin Hospital. The local ethics committee approved the study and all the subjects gave written informed consent.

The following clinical data were collected: age, sex, disease duration (date of first non-Raynaud symptom), cutaneous SSc subtype according to the definition by LeRoy et al. [12], pulmonary involvement with pulmonary fibrosis on CT and restrictive syndrome defined by a forced vital capacity (FVC) <75%.
Vascular involvement was assessed by cutaneous ulcerations, PAH defined by right heart catheterism [13] and diffusing capacity for carbon monoxide divided by alveolar volume (DLCO/VA) abnormal in case of <75%. The following immunological tests were conducted: ACAs and anti-endothelial antibodies.

The control group consisted of 48 unrelated matched (for age and sex) subjects with French Caucasian origin who have no autoimmune diseases.

Methods

Serum of subjects were extracted from blood samples (10 ml) after centrifugation at 3000 g for 10 min and then were stored in aliquots at −80°C until use but the storage duration was <6 months. The investigators performing the assays (J.A. and J.W.) were blinded to individual status.

Quantitative sandwich ELISAs (R&D Systems, Minneapolis, MN, USA) was used to measure serum sENG (ng/ml) and sVEGF (pg/ml). Competitive ELISA (DLD Diagnostika GMBH, Hamburg, Germany) was used to measure ADMA (micromoles per litre) in all subjects. The detailed ELISA assay characteristics are provided in the supplementary data (supplementary data are available at Rheumatology Online).

In order to evaluate a potential link with the previously reported ENG variant [9], the insertion polymorphism (6bINS) of the ENG gene was investigated to assess its possible influence on sENG value. Ninety-four percent of the genotyped SSc patients belonging to our previous cohort were thus representative of the latter.

Comparisons of soluble protein levels between two groups were assessed using the non-parametric Mann–Whitney test. Correlations between soluble protein levels were performed with the Spearman’s rank correlation test. A probability value P < 0.05 was considered statistically significant. Hardy–Weinberg equilibrium was tested for the 6bINS ENG polymorphism.

Results

We included 235 consecutive unrelated subjects comprising 187 French SSc patients (157 were females and mean age was 55.9 ± 12.3 yrs) and 48 healthy matched controls (40 were females and mean age was 59.4 ± 11.6 yrs). The characteristics of the patient population were as follows: 57 SSc patients (29%) had history of digital ulcerations, 17 patients (9%) had PAH and 66 patients (35%) had decreased DLCO/VA <75%. Other characteristics are detailed in supplementary Table 1, available as supplementary data at Rheumatology Online. Among the 187 SSc patients, six had a ‘HHT-like’ vascular phenotype: four with ‘HHT-like’ vascular phenotype (watermelon stomach or telangiectasia) without detecting any difference as compared with other patients or controls.

We found higher concentration of soluble VEGF in SSc patients than in controls (445.5 ± 295.5 vs 261.2 ± 108.6 pg/ml, P < 0.0001). In SSc sub-groups, levels of sVEGF were found to be much higher in patients with PAH compared with those without (579.3 ± 270.0 vs 430.2 ± 295.3 ng/ml, P = 0.04), in SSc patients with pulmonary fibrosis than remainders (488.2 ± 322.4 vs 393.8 ± 268.2 ng/ml, P = 0.04) and in SSc patients with FVC <75% vs FVC >75% (529.8 ± 299.7 vs 423.8 ± 299.8 ng/ml, P = 0.02). These subgroups of SSc patients had also higher value of sVEGF than controls (Table 1). These associations were not confirmed by multivariate analysis.

ADMA levels in sera of SSc patients were found to be similar as in controls (0.86 ± 0.35 vs 0.89 ± 0.30 µmol/l, P = NS). There was no difference in SSc sub-group analyses for ADMA, and in particular no difference regarding disease duration.

Finally, correlation tests revealed a negative correlation between the sENG and ADMA in SSc patients (r = −0.25, P = 0.0009), but no correlation was found between sENG and VEGF.

From the initial 187 patients and 48 controls, respectively, 176 (14 SSc patients with PAH and 162 without) and 44 had interpretable data for the 6bINS polymorphism of the ENG gene. This polymorphism was in Hardy–Weinberg equilibrium for the control group. The frequency of genotypes carrying at least one 6bINS allele was 64/176 (36.4%) in SSc patients compared with 21/44 (47.7%) in controls (P = NS). There was also a significant lower frequency of these genotypes in SSc patients with PAH compared with controls (7.7% vs 47.7%, P = 0.009) and to the SSc patients without PAH (7.7% vs 38.9%, P = 0.04). There was no association between sENG serum levels and the different 6bINS genotype.

Discussion

Vasculature has a major effect in SSc prognosis, which depends mostly on the extent and severity of vascular lesions [14]. Thus, the understanding of the mechanisms of this vasculopathy and the roles of the different vascular factors in SSc is crucial. Recently, the pathogenic role of sENG, an anti-angiogenic protein, has largely been demonstrated in pre-eclampsia. In the latter, sENG is both a biomarker that heralds the onset of pre-eclampsia and an actor contributing to its pathogenesis [6]. Soluble ENG may impair binding of TGF-β to its receptors as well as the downstream TGF-β signalling resulting in vitro in inhibition of capillary formation on matrigel and in vivo in induction of pre-eclampsia in pregnant rats [6]. In our study, we detected significantly higher levels of sENG in sera of SSc patients than matched controls, highlighting a possible contribution of this anti-angiogenic protein in the SSc vascular disturbances. This hypothesis is supported by the results of multivariate analyses, which showed association between higher sENG levels and an SSc vascular phenotype that integrates the presence of digital ulcers and altered DLCO/VA. The association with the positivity of ACAs is in accordance with this hypothesis taking into account that vascular manifestations are considered to be prominent in the limited SSc cutaneous sub-type [15]. Our results strengthen the single previous report of sENG levels in SSc, which detected higher sENG levels in patients with the limited cutaneous sub-type [8]. In this latter report, telangiectasia seemed to be also more frequent in SSc patients exhibiting elevated sENG concentrations; however, it must be highlighted that measuring telangiectasia remains difficult. In our study, we tried to focus on patients with ‘HHT-like’ phenotypes but we could not identify significant sENG level differences.

Among the activator of sENG, the inflammatory cytokine TNF-α is known to induce sENG release [16]. We failed to detect any association between the acute-phase reactant, CRP, and sENG levels (data not shown). In SSc, oxidative stress is highly
suspected to play a key role. Recently, a close relationship between oxidative stress and sENG has been reported in implicating inducible enzyme haeme oxygenase-1 (HO-1) that acts as a negative regulator of sENG. Thus, HO-1 seems to be a good candidate for participating in the vascular abnormalities in SSc and sENG disturbances and this will need to be addressed in the future.

In accordance with previous reports, we found elevated sVEGF concentrations in SSc patients [4, 17]. VEGF is a strong proangiogenic factor with a crucial role in the disease [4]. Although we found no correlation between sVEGF and sENG, we may hypothesize that increased serum sENG levels is a response to the strong proangiogenic signal induced by VEGF.

There was no difference between SSc and controls for ADMA levels and no association with disease features. Our data may support either a decoupling between enhanced angiogenesis and endothelial dysfunction in SSc, or that endothelial dysfunction in SSc is not related to ADMA levels. There was a negative correlation between levels of sENG and ADMA in SSc patients; it may be hypothesized that prolonged endothelial lesions may decrease the capacity of endothelium to produce ADMA because of already established major damages. In order to test this hypothesis, we assessed statistical analysis taking into account the disease duration without detecting any association with this characteristic. Previously, Dooley et al. [18] described an association between the dSSc sub-type and ADMA levels. We could not confirm this association in our larger sample size cohort.

Finally, in a previous study, we showed an association between an insertion polymorphism (6bINS) of ENG gene and SSc-related PAH [9]. The genotyping results in the herein study is in accordance with our previous findings. We could not find any association between the 6bINS polymorphism and sENG levels. This does not support a functional role for the 6bINS polymorphism on sENG levels. However, this genetic variant may influence other biological regulations of ENG or it may be in linkage disequilibrium with another regional gene involved in this regulation.

### Conclusion

Soluble ENG appeared to be increased in SSc and particularly to be associated with vascular phenotype. This is particularly relevant regarding the role of sENG in other vascular diseases. This biomarker needs to be further studied as a predictive factor of vascular damages, and cellular investigations are required to further determine its role in endothelial disturbances.

### Acknowledgement

We acknowledge Mrs Carole Desbas for her expert secretarial assistance.

**Funding:** This work was funded by *Association des Sclérodérmines de France* and *Société Française de Rhumatologie* and was supported by *Groupe Français de la Recherche sur la Sclérodérmie* and *Agence Nationale pour la Recherche*, Grant number R07094K5.

**Disclosure statement:** The authors have declared no conflicts of interest.

### Supplementary Data

Supplementary data are available at *Rheumatology* Online.
References


