Abnormalities in the Regulators of Angiogenesis in Patients with Scleroderma

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ABSTRACT. ***Objective.*** To determine plasma levels of regulators of angiogenesis in patients with scleroderma and to correlate those levels with manifestations of scleroderma-related vascular disease.

Methods. Plasma levels of vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor-2 (FGF-2), matrix metalloproteinase-9 (MMP-9), endostatin, pro-MMP-1, hepatocyte growth factor (HGF), placental growth factor (PlGF), and FGF-4 were examined by ELISA in a cross-sectional study of 113 patients with scleroderma and 27 healthy controls. Simple and multivariate regression models were used to look for associations between factor levels and clinical disease characteristics.

Results. There were marked differences in the levels of pro-angiogenic growth factors between patients with scleroderma and controls, with significant elevations of VEGF, PDGF, FGF-2, and PlGF among patients with scleroderma ($p < 0.0001$). Levels of MMP were also higher in scleroderma patients compared to controls (MMP-9 and pro-MMP-1) ($p < 0.0001$). Levels of the pro-angiogenic and anti-fibrotic factor, HGF, were noted to be lower in patients with scleroderma, but had a positive correlation with right ventricular systolic pressure (RVSP) as measured by echocardiogram ($p < 0.0001$) and the Raynaud Severity Score ($p = 0.05$). Endostatin (an anti-angiogenic factor) was notably higher in patients with scleroderma ($p < 0.0001$) and also correlated positively with RVSP ($p = 0.023$).

Conclusion. These results demonstrate striking abnormalities in the circulating regulators of angiogenesis in patients with scleroderma. The levels of some factors correlate with measures of vascular disease among patients with scleroderma. Dysregulated angiogenesis may play a role in the development of scleroderma vascular disease. (First Release Feb 15 2009; J Rheumatol 2009;36:576–82; doi:10.3899/ jrheum.080516)

Key Indexing Terms:
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Clinical and pathological evidence supports the concept that systemic sclerosis (scleroderma) is primarily a vascular disease that is mediated by autoimmunity and results in tissue fibrosis. Raynaud's phenomenon (RP) secondary to digital artery disease and microcirculatory abnormalities in skin is almost always the earliest clinical manifestation of scleroderma. Vascular insufficiency from a progressive obliteration of both small arteries and microvessels is associated with a state of chronic tissue hypoxia[1,2]. Recurrent episodes of ischemia-reperfusion injury to skin and other organ systems may contribute to tissue fibrosis, organ dysfunction, and significant morbidity and mortality[3,4]. A number of patients with scleroderma exhibit severe consequences of this vascular disease, with catastrophic events such as digital ischemia with ulceration or amputation, scleroderma renal crisis, or pulmonary arterial hypertension. Currently there are no biological measurements (biomarkers) to assess the subclinical vascular activity in patients with scleroderma, and therefore the clinician often does not detect disease until a late and irreversible stage.

On histological examination, microvascular changes can be seen in virtually all organ systems and include perturbation of endothelial cells, neointimal formation, increased numbers of myofibroblasts, pericyte activation, and perivascular lymphocyte infiltration[5-8]. Small arteries and arterioles (50–500 μm in diameter) develop a fibrous, concentric intimal lesion that can obliterate the vessel lumen. Endothelial cell injury seems to occur at an early stage, with evidence of endothelial apoptosis, chronic platelet activation, and subsequent vascular thrombosis[9,10]. A characteristic clinical find-

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ing is capillary dilation and capillary atrophy noted in the skin by nailfold capillary microscopy. Similar attenuation of the microvessels is seen in the involved organs. A question in scleroderma is why the obliterated arteries and capillaries are not replaced by new vessels via angiogenesis or vasculogenesis. In fact, the pathology suggests significant loss of the peripheral vascular network, with a defect in both the vascular repair and the expected increase in new vessel growth (angiogenesis, arteriogenesis); the net result is tissue ischemia, fibrosis, and organ failure[11,12].

The initiating factors or cause of the vascular insult in scleroderma remain unknown. However, once vascular perturbation has occurred there is evidence that ischemia-reperfusion injury is an ongoing pathologic process[13,14]. The local tissue response to vascular injury involves activation of matrix metalloproteinases (MMP) leading to the extracellular matrix breakdown; release of angiogenic growth factors such as fibroblast growth factors (FGF) and vascular endothelial growth factors (VEGF); and the increase in adhesion molecules that attract circulating mononuclear cells. Systemic factors influencing local tissue angiogenesis include circulating growth factors, inhibitors of angiogenesis such as endostatin and angiostatin, and, potentially, the level of circulating angiogenesis accessory cells[15]. In addition, tissue fibrosis can be the consequence of abnormal angiogenesis[16].

We and others have suspected that the regulators responding to vascular injury and repair are abnormal in scleroderma. We hypothesized that an imbalance of regulators of angiogenesis may contribute to the progressive vascular disease and small-vessel loss present in scleroderma. To test this, we screened 113 patients with scleroderma for the presence of a variety of known measurable circulating pro- and anti-angiogenic factors, and compared these levels to healthy controls. We also speculated that the group of patients with more severe vascular manifestations (pulmonary hypertension, severe RP) would have a more pronounced imbalance of these regulatory factors.

MATERIALS AND METHODS

Plasma samples were drawn from unselected consecutive, consenting scleroderma subjects during routine appointments at the Johns Hopkins Scleroderma Center from April to July 2003. All subjects either met the American College of Rheumatology (ACR) criteria for the diagnosis of scleroderma[17] or had at least 3 of the 5 features of CREST (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, telangiectasias) syndrome. Patients were classified as described by LeRoy and Medsger[18]. Patients with limited scleroderma have skin changes distal to the elbows and knees and do not have involvement of the trunk. Patients are classified as diffuse if they have skin thickening on the chest, abdomen, or extremities proximal to the knees and elbows.

All clinical data were obtained from the comprehensive database at the Johns Hopkins Scleroderma Center, which contains information collected longitudinally on disease manifestation, disease severity, demographic variables, and data from pulmonary function testing (PFT) and 2D-echocardiography (ECHO). Data on estimated right ventricular systolic pressure (eRVSP) as measured by ECHO were used as a marker of pulmonary vascular disease. Any data from right-heart catheterization were also collected as confirmation of a diagnosis of pulmonary arterial hypertension. The ECHO were performed for clinical reasons at various sites including Johns Hopkins Medical Institutions. We examined the closest ECHO in time to the sample collection. Forced vital capacity (FVC) was used as a surrogate for severity of interstitial lung disease and the PFT closest to the time of sample collection was used in the analysis. Severity of other organ involvement was assessed by the Medsger Severity Score[19]. For this study, we particularly focused on the severity of RP. Raynaud Severity Score equals 0 if the patient has no history of RP, 1 if the patient has RP without digital pitting or ulcerations, 2 if the patient has digital pitting scars, 3 if the patient has digital tip ulceration, and 4 if the patient has digital gangrene. The score closest to the time of sample collection was used in the analyses. Disease duration was defined by both the period of time from the time of physician diagnosis to the time of sample draw and the date of first non-Raynaud's symptom to time of sample draw.

Plasma samples were stored at –80°F from the time they were collected until the time they were analyzed. Plasma samples of subjects and healthy controls were assayed by ELISA for angiogenic and angiostatic factors using commercial ELISA kits. Quantikine basic FGF (bFGF, FGF-2), FGF-4, hepatocyte growth factor (HGF), pro-MMP-1, MMP-9, platelet-derived growth factor (PDGF-BB), placental growth factor (PlGF), and VEGF kits were from R&D Systems, Inc. (Minneapolis, MN, USA). Accucyte Endostatin kits were from CytImmune Sciences, Inc. (College Park, MD, USA).

Data are presented as means ± standard deviation (SD) and the nonparametric Mann-Whitney U-test was used to compare control and scleroderma groups. These values were corrected for multiple comparisons using the method of Bonferroni. Among the scleroderma subjects, Spearman's rank correlation, simple and multivariate analysis (linear regression and ordered logistic regression) was used to analyze correlations between angiogenic factors and clinical manifestations. All data analysis was performed using Stata, Version 9 (Stata Corp., College Station, TX, USA).

Our study was approved by the Institutional Review Board at Johns Hopkins University.

RESULTS

Characteristics of the patient population. Data were collected from 113 patients with scleroderma and 27 healthy controls. The controls had a mean age of 57.5 ± 2.8 years and were 63% women. The clinical characteristics of the subjects with scleroderma are summarized in Table 1; their mean age at diagnosis was 45.3 ± 11.9 years, and the mean disease duration (from time of first non-Raynaud symptom) at study time was 9.6 ± 7.1 years. The population was 8:1 women to men. The majority of patients had a limited disease subtype (72% limited, 28% diffuse), 91% met American College of Rheumatology criteria for the diagnosis of scleroderma, and the average skin score was 8.5 ± 8.4. One hundred ten subjects had ECHO data available. The mean eRVSP was 31.7 ± 12.8 mm Hg. Eleven percent of the group were current smokers at the time of study.

Angiogenesis factors. Scleroderma compared to controls. Scleroderma subjects, compared to controls, demonstrated a significant increase in the level of angiogenic growth factors including VEGF, FGF-2, PDGF-BB, and PlGF. There were also significant elevations in plasma levels of matrix degradation factors (MMP-9, pro-MMP-1; Table 2). At the same time, scleroderma patients also displayed significantly higher circulating levels of an inhibitor of angiogenesis, endo-

Table 1. Clinical characteristics of 113 patients with scleroderma.

Characteristics	Scleroderma Patients, n = 113
Age at time of diagnosis, yrs ± SD	45.3 ± 11.9
Age at time of study, yrs ± SD	53.0 ± 12.2
Duration from physician diagnosis, yrs ± SD	7.7 ± 6.0
Duration from first non-RP symptom, yrs ± SD	9.6 ± 7.1
Meet ACR criteria, n (%)	103 (91.2)
Female: male	8:1
Race, n (%)	
Caucasian	92 (81.4)
African American	18 (15.9)
Other	3 (2.7)
Limited:Diffuse disease	2.5:1
Skin score (mRSS), mean ± SD	8.5 ± 8.4
Mean RVSP (n = 110), mm Hg	31.7 ± 12.8
Mean FVC, % predicted	87.8 ± 12.1
Raynaud Severity Score, n (%)	
0	3 (2.7)
1	71 (62.8)
2	29 (25.7)
3	9 (8.0)
4	0 (0)
Current smokers at time of study, n (%)	12 (10.6)
Centromere-positive (n = 85), n (%)	22 (25.9)
Topoisomerase-positive (n = 77), n (%)	14 (18.2)

RP: Raynaud's phenomenon; ACR: American College of Rheumatology; RVSP: right ventricular systolic pressure; FVC: forced vital capacity; MRSS: modified Rodnan skin score.

statin, compared to controls (21.3 ± 7.8 vs 11.7 ± 3.7 ng/ml; p < 0.0001). All of these differences remained highly statistically significant when corrected for multiple comparisons. HGF, a potent pro-angiogenic and anti-fibrotic factor, is noted to be lower in subjects with scleroderma but the difference did not reach statistical significance (868 vs 2041 pg/ml; p = 0.267; Table 2).

Correlation of factors with clinical characteristics in scleroderma subjects. Clinical characteristics of the scleroderma patients were examined to see if any specific disease manifestations, particularly vascular disease, correlated with any of the measured factors. We found a positive association between eRVSP and several of the measured factors [endostatin (p = 0.023), HGF (p < 0.0001), and PlGF (p = 0.037)] using a simple linear regression (Figure 1) and by nonparametric Spearman's correlation for endostatin and HGF (R = 0.26 and R = 0.33, respectively). In a multivariate model that included sex, race, disease duration, disease subset (limited or diffuse), and antibody status, HGF and endostatin remained significantly associated with eRVSP (p = 0.006 and p = 0.002, respectively). There were no other significant associations noted in this model. When eRVSP was dichotomized into those patients with eRVSP < 40 mm Hg and those with eRVSP ≥ 40 mm Hg, there was a significant difference in HGF and endostatin between groups (Mann-Whitney U-test, p < 0.0001 and p = 0.015, respectively; Figure 2). Using followup data from the time of sample collection until the end of 2007, 14/25 patients with eRVSP > 40 mm Hg developed clinical and right-heart catheterization-diagnosed pulmonary hypertension. In addition, 8/83 patients with eRVSP < 40 mm Hg at the time of study also developed pulmonary hypertension. When this group (14 plus 8) was analyzed, there was still a marked difference in levels of HGF and endostatin between groups (p = 0.001 and p = 0.045; Figure 3).

Using ordered logistic regression using the angiogenic factors as single independent variables and Raynaud severity as the dependent variable, a positive association was also seen between Raynaud Severity Score and HGF (p = 0.05). This association persisted when included in a multivariate model including sex, race, disease subtype, and disease duration (p = 0.006). In this model there was also a positive association between Raynaud severity and disease duration (p = 0.013) and an inverse relationship with age at sample collection (p = 0.003), suggesting that those with more severe RP are younger and had longer disease duration.

HGF also increases with age (p < 0.001), male sex (p = 0.005), in current smokers (p = 0.017), and with skin score (p = 0.035), but not with disease duration, race, disease sub-

Table 2. Comparison of angiogenesis factors in scleroderma compared to normal controls.

Angiogenesis Factor mean ± SD	Scleroderma, n = 113	Controls, n = 27	p
VEGF, pg/ml	163.5 ± 176.4	26.1 ± 22.4	< 0.0001*
PDGF-BB, pg/ml	659.7 ± 716.8	80.6 ± 66.6	< 0.0001*
FGF-2, pg/ml	6.6 ± 9.1	0.46 ± 1.69	< 0.0001*
MMP-9, ng/ml	4.4 ± 6.4	0.95 ± 0.72	< 0.0001*
Endostatin, ng/ml	21.3 ± 7.8	11.7 ± 3.7	< 0.0001*
Pro-MMP-1, ng/ml	1.9 ± 1.6	0.49 ± 0.39	< 0.0001*
HGF, pg/ml	868.0 ± 328.2	2040.9 ± 3365.9	0.267
PlGF, pg/ml	17.6 ± 6.3	12.4 ± 3.5	< 0.0001*
FGF-4, pg/ml	0.31 ± 3.3	0 ± 0	0.632

* p < 0.009 when corrected for multiple comparisons. VEGF: vascular endothelial growth factor; PDGF: platelet-derived growth factor; FGF: fibroblast growth factor; MMP: matrix metalloproteinase; HGF: hepatocyte growth factor; PlGF: placental growth factor.

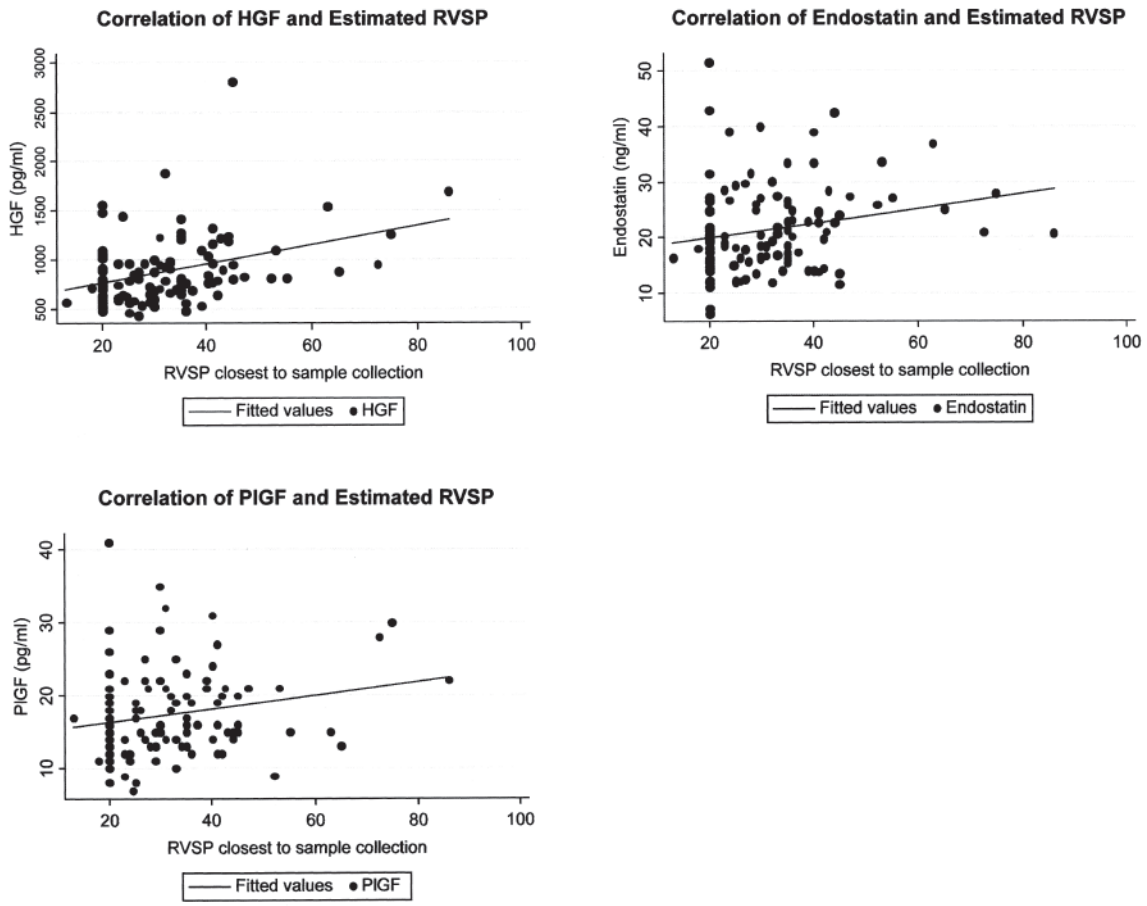

Figure 1. Associations between right ventricular systolic pressure (RVSP) and levels of endostatin (ng/ml), hepatocyte growth factor (HGF; pg/ml), and placental growth factor (PlGF; pg/ml). Correlation coefficients are R = 0.26, R = 0.33, and R = 0.23 for endostatin, HGF, and PlGF, respectively. Significant difference between groups in HGF levels persists when the one outlier value (HGF = 2815) is removed from the analysis.

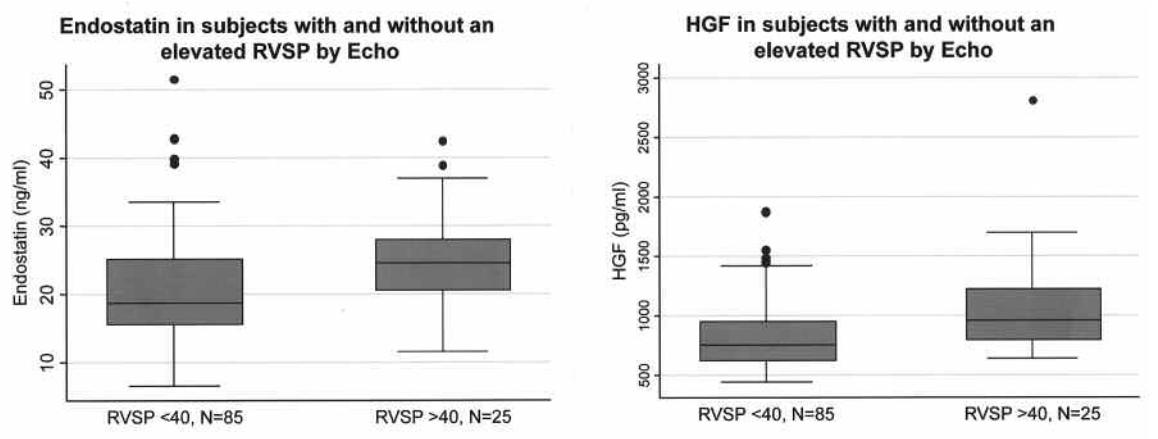

Figure 2. Endostatin and HGF levels in patients dichotomized for the presence of elevated estimated right ventricular systolic pressure (eRVSP), where pressure ≥ 40 mm Hg was considered elevated. The significant difference between groups in HGF levels persists when the one outlier value (HGF = 2815) is removed from the analysis.

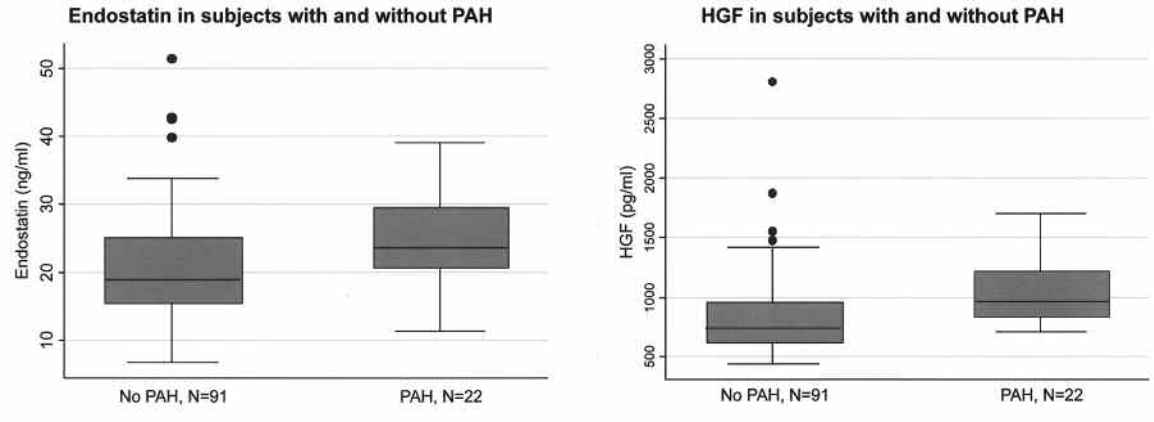

Figure 3. Endostatin and HGF levels in patients who developed pulmonary arterial hypertension diagnosed by right-heart catheterization.

type, FVC, or autoantibody status. When HGF was used as the dependent variable and subject characteristics as independent variables in a multivariate analysis, age, sex, Raynaud severity, and eRVSP continued to show an association.

In evaluating for associations with other measured factors and clinical characteristics, we found an inverse relationship between endostatin level and FVC ($p = 0.028$) and a higher level of endostatin in African American versus Caucasian subjects ($p = 0.019$). We also noted that PlGF was higher in male subjects ($p = 0.009$) and lower in those with topoisomerase antibodies ($p = 0.037$), and pro-MMP-1 increased with age ($p = 0.017$). No other associations were seen with any other demographic or clinical characteristic and measured factors.

DISCUSSION

The major finding of our investigation was a clear demonstration of abnormal plasma levels of several pro- and anti-angiogenic factors among patients with scleroderma compared with healthy controls. There was a higher level of the angiogenic growth factors VEGF, FGF2, PDGF-BB, and PlGF measured in patients with scleroderma. The notable exception was HGF, which was lower in the scleroderma group compared to controls, although the difference was not statistically significant. There was, however, a significant correlation of HGF levels with the presence of vascular disease among patients with scleroderma.

An increase in the levels of pro-angiogenic growth factors may represent an ongoing attempt at angiogenic tissue repair or may reflect growth factor release from damaged tissues. Elevations in circulating VEGF have been noted by other investigators in patients with scleroderma[20-22] and have the potential to be modified by therapy[20]. Unlike previous investigations, we did not find a correlation between VEGF and disease subtype (limited vs diffuse) or disease duration[21], nor could we find an association with significant vascular disease (pulmonary hypertension and severe RP). Previous investigations have shown controversial results with regard to FGF-2. Distler, *et al*[21] found no difference between patients with scleroderma and controls, while Kadono, *et al*[23] demonstrated elevations among 31 of 74 patients with scleroderma. We found the levels of FGF-2 were significantly higher in our patients than in controls, but could not correlate elevations with any of our clinical measures, including vascular disease.

The decreased level of circulating HGF in our patient population was of particular interest because of this molecule's potent pro-angiogenic factor and anti-fibrotic effects[24]. A deficiency or reduction in HGF may prevent vascular repair and increase tissue fibrosis, pathological features that are typical of scleroderma[24,25]. HGF production by scleroderma fibroblasts is noted to be increased compared to control fibroblasts and studies demonstrate that c-met (receptor for HGF) is spontaneously expressed in scleroderma fibroblasts and not in control fibroblasts[26]. These findings suggest that HGF is produced in an attempt at an anti-fibrotic response. Indeed, in this *in vitro* system, the addition of HGF diminished collagen production[26]. Also, in the bleomycin mouse model, transfection of human HGF c-DNA into mouse skeletal muscle seemed to both prevent and treat bleomycin-induced fibrosis[25]. In our study we did not find a correlation of levels of HGF with measures of fibrotic disease (skin score and FVC), although this association has been noted by others[27]. Therapeutically, HGF is being investigated for its ability to induce angiogenesis in atherosclerotic diseases[28]. In our study we found the levels of HGF were decreased in patients with scleroderma on the whole, but levels consistently appeared to increase, relative to the degree of scleroderma vascular disease severity, implying a failing attempt at vessel repair.

We noted that blood levels of endostatin, a potent inhibitor of angiogenesis, were increased in the scleroderma group compared to control patients. Our findings are consistent with several previous reports[29,30], although Distler, *et al* did not note differences in scleroderma versus controls[21].

Two prior studies also demonstrated correlation of endostatin levels and clinical disease characteristics. Hebbar, *et al* noted increased levels in those with diffuse skin disease, evidence of lung fibrosis, and more severe peripheral vascular disease[29]. Others have also noted an increase in endostatin among scleroderma patients with cardiovascular disease[30]. This finding of elevated endostatin level is also consistent with our observation of elevated levels of another circulating angiogenesis inhibitor, angiostatin, in a different scleroderma patient cohort[31]. Our finding of high levels of endostatin coupled with low levels of HGF suggests that an imbalance of the regulators of angiogenesis is present in scleroderma that could lead to disordered vascular repair mechanisms and prevent the normal responses of ischemia-reperfusion injury[32].

We also identified subgroups of patients with more severe vascular disease: those with pulmonary hypertension and those with severe digital ischemia. We hypothesize that these patients have ongoing vascular damage and that circulating angiogenic factors would be measures of this vascular "activity." Ideally, a biomarker of vascular disease would be present in the preclinical state, involved directly in the pathogenic process, and correlate well with future vascular outcomes defined by gold-standard testing. We have demonstrated correlation of multiple assayed factors and these more severe vascular phenotypes in this cross-section of patients with scleroderma, and found some robust associations. This suggests that these circulating factors could be viable biomarkers. To further validate this concept, however, we would need a prospective cohort of patients followed longitudinally.

There are several shortcomings of our investigations. First, this was a cross-sectional study of a relatively limited number of patients; however, these patients were representative of typical patients with scleroderma followed at academic centers. A cross-sectional survey does not provide temporal data to define if the findings are the cause or consequence of disease; it is possible that these measurable blood factors either reflect ongoing disease activity or are a measure of vascular damage with aberrant repair mechanism(s). In addition, we did not necessarily have a clinical assessment of the vascular disease exactly at the time of sample collection, which may impair our ability to distinguish these factors as markers of disease activity. Currently, little is known about how to assess ongoing vascular disease activity, apart from the clinical outcomes caused by cumulative vascular damage. We used clinical data to identify a subset of patients with more severe vascular disease (those with a history of an elevated eRVSP by ECHO and those with a history of severe peripheral vascular events). This group, however, may represent late-stage vascular disease and may not be the group with "active" ongoing vascular insult. The factors we measured could be solely indicators of vascular damage rather than progressive vascular disease activity. Longitudinal studies would be needed to answer this question.

We found significant abnormalities in the circulating levels of the regulators of angiogenesis in patients with scleroderma compared to healthy controls. Low levels of HGF and high levels of endostatin correlated with clinical measurements of more severe scleroderma vascular disease. These data are consistent with our hypothesis that biomarkers of vascular disease are circulating, and can be measured in patients with scleroderma, and may provide measures of disease activity. In addition, our data provide insight into the pathogenesis of scleroderma, with evidence of defective angiogenesis and imbalance in factors regulating vascular repair.

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