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Abstract

Molecular factors modulating angiogenic responses are dysregulated in patients with MCTD and SSc with increases of VEGF in MCTD and SSc and selective upregulation of endostatin in MCTD. Furthermore, high serum levels of VEGF might characterise patients with MCTD with a more severe course of the disease with increased prevalence of PAH and myositis.
Dysbalance of angiogenic and angiostatic mediators in patients with mixed connective tissue disease

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Abstract

Objective: Vascular disease is common in mixed connective tissue disease (MCTD). The aim of the present study was to investigate, whether dysbalance of angiogenic and angiostatic factors occurs in MCTD.

Methods: Thirty-eight patients with MCTD and 40 patients with systemic sclerosis (SSc) for comparison were included. Four centers contributed to this cross-sectional analysis. Sixty-six healthy volunteers were used as controls. The serum levels of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and endostatin were determined by ELISA. For comparisons between controls and MCTD patients and detection of associations of serum levels with dichotomous clinical parameters in MCTD patients the Mann–Whitney test was used.

Results: Serum levels of the angiogenic factor VEGF were significantly elevated in patients with MCTD and SSc. We also detected significantly increased levels of the angiostatic factor endostatin in MCTD, but not in SSc. No differences were observed for bFGF. Levels of VEGF were higher in MCTD patients with pulmonary arterial hypertension (PAH), acrosclerosis and myositis. In multivariate linear regression analysis, an additive model of PAH, myositis and lymphadenopathy accounted for 79 % of the variability of the VEGF levels (r = 0.889).

Conclusion: Molecular factors modulating angiogenic responses are dysregulated in patients with MCTD and SSc with increases of VEGF in MCTD and SSc and selective upregulation of endostatin in MCTD. Furthermore, high serum levels of VEGF might characterize MCTD patients with a more severe course of the disease with increased prevalence of PAH and myositis.
Introduction

Mixed connective tissue disease (MCTD) was first described in 1972 by Sharp and colleagues as a connective tissue disease with features of systemic lupus erythematosus (SLE), systemic sclerosis (SSc) and polymyositis characterized by the presence of high titres of anti-U1-RNP autoantibodies [1]. A high prevalence of arthritis resembling rheumatoid arthritis (RA) was later observed in patients with MCTD. Common manifestations are myositis, arthritis, puffy hands, oesophageal dysfunction and reduced DLCO on lung function testing [2]. However, the most common and most severe clinical manifestations do not result from inflammation or fibrosis, but from vascular alterations [3]. The pattern of vascular involvement resembles that of SSc with potential involvement of pulmonary arteries and frequent microvascular changes. PAH is common in MCTD. While right heart catheterization data are missing, the prevalence of PAH based on data from echocardiography has been suggested to be as high as 23 % [2], and PAH is a major cause of death in patients with MCTD. Raynaud’s phenomenon occurs in 75 – 96 % of patients and is observed early in the course of the disease [2]. Although the vascular changes in MCTD are well documented, the underlying pathophysiology remains obscure.

The formation of new vessels in the adult can occur by angiogenesis or vasculogenesis [4], both of which are perturbed in rheumatic diseases such as SSc, SLE and RA [5, 6]. Angiogenesis and vasculogenesis are highly complex, multi-step processes. To avoid an uncontrolled formation of new vessels, all steps are controlled by an array of angiogenic and angiostatic factors [7]. However, dysregulation of one of these factors might have profound impact and result in severe defects of angiogenesis and vasculogenesis [6]. Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) have been characterized as key molecules for the formation of new vessels. VEGF regulates several steps of angiogenesis and vasculogenesis including proliferation, survival and migration of endothelial cells and precursor cells [7]. The biological effects of VEGF are dose- and time
dependent. As indicated by transgenic mice overexpressing VEGF, the uncontrolled overexpression of VEGF might have deleterious rather than beneficial effects as it may result in the formation of irregular vessels with impaired blood flow [8]. Similar to VEGF, bFGF stimulates proliferation, migration and differentiation of endothelial cells in vitro and in vivo [7, 9]. Endostatin is a very potent inhibitor of angiogenesis that is generated upon cleavage of collagen type XVIII. Endostatin inhibits angiogenesis by reducing endothelial cell proliferation and migration, potentially by inhibiting membrane type 1 matrix metalloproteinase and matrix metalloproteinase-2 [7, 10].

The microvascular alterations in MCTD might be caused by a dysbalance between angiogenic and angiostatic factors or by an uncontrolled overexpression of angiogenic factors. The aim of the present study was to analyze, whether a decrease of the angiogenic factors VEGF and bFGF and an increase of the angiostatic factor endostatin contribute to the vascular disease in MCTD, and whether the levels of VEGF, bFGF or endostatin correlate with the disease phenotype of MCTD.
Material and methods

MCTD Patients

Thirty-eight consecutive, unselected patients with MCTD were recruited at the Department of Internal Medicine 3 and Institute for Clinical Immunology of the University of Erlangen-Nuremberg, at the Department of Rheumatology of the University Hospital Zurich, at the Division of Rheumatology of the Medical University of Vienna and at the Department of Internal Medicine and Rheumatology of the Justus-Liebig-University of Giessen. The MCTD cohort comprised 34 women and 4 men. The median age was 47 years (range 18-78 years). All patients fulfilled the criteria of Alarcon-Segovia for MCTD [11]. The criteria of Alarcon-Segovia (Table 1) were chosen, because of their very high specificity for MCTD [12, 13]. Patients fulfilling classification criteria for other connective tissue diseases were excluded from the study as were patients fulfilling classification criteria for two connective tissue diseases at the same time (“overlap syndromes”). The control group consisted of 44 women and 22 men with a median age of 39 years (range 19 – 74 years). All patients and controls were of Caucasian origin and signed a consent form approved by the local ethical committees. Since MCTD and control group significantly differed in their median age by 8 years, and age could not be precluded to influence the levels of growth factors, a selection of control and of MCTD group matched by age categories in 5-years steps was drawn for additional analyses. These age-matched samples consisted of n=30 patients with a common median age of 46 years (range MCTD 18-71 years, range controls 19-74 years).

SSc patients

For comparison, twenty patients with limited cutaneous SSc (ISSc) and 20 patients with diffuse cutaneous SSc (dSSc) were recruited at the Department of Internal Medicine 3 and Institute for Clinical Immunology of the University of Erlangen-Nuremberg. Both groups were matched for age and sex. The SSc cohort comprised 34 women and 4 men. The median
age was 46 years (range 19-77 years). All patients fulfilled the criteria of LeRoy [14]. Patients fulfilling classification criteria for other connective tissue diseases were excluded.

Clinical assessment

An extensive clinical profile was established for each MCTD patient. The characteristics of the patients are summarized in Table 2. Muscular involvement was analyzed by clinical assessment of muscle strength, determination of serum creatine kinase (CK), electromyography and muscle biopsy. In accordance with the criteria of Alarcon-Segovia, myositis was diagnosed, when at least twofold elevated serum CK levels and characteristic electromyographic changes were detected or when myositis was proven histologically. In this cohort, myositis was confirmed by muscle biopsy in 14 of 21 MCTD patients with myositis. The presence of joint involvement with arthritis, arthralgias, morning stiffness and rheumatoid nodules were evaluated by trained rheumatologists. Patients were also evaluated by clinical examination for the presence of lymphadenopathy, malar rash, alopecia, Raynaud’s phenomenon, puffy hands and acrosclerosis. Interstitial pulmonary involvement was examined in all patients by the carbon monoxide diffusion capacity using the single-breath method standardized for hemoglobin, by determination of the forced vital capacity (FVC) and by x-ray. Suspected interstitial lung disease was further evaluated by high resolution computed tomography (HRCT). Echocardiography was performed to screen for PAH, and sPAP was calculated. As patients with elevated sPAP cannot be automatically considered as P(A)H patients, PAH was diagnosed in patients with a mean pulmonary arterial pressure of > 25 mmHg at rest with a wedge pressure of ≤ 15 mmHg on right heart catheterization. Upon clinical suspicion, the presence or absence of pleuritis and pericarditis was analyzed by ultrasound, x-ray or HRCT. In patients with clinical symptoms suspicious of oesophageal involvement, the incidence of oesophageal dysmotility was determined by barium swallow or manometry. Trigeminal neuralgia was diagnosed according to the International Headache
Society criteria. Antinuclear antibodies were determined by immunofluorescence according to local practice and U1-RNP-antibodies antibodies were determined by ELISA (Erlangen/Zurich: Anti-RNP 70 Kit 632, Orgentec, Mainz, Germany; Giessen: Anti-nRNP/Sm-ELISA, Euroimmun, Lübeck, Germany; Vienna: ELiATM U1RNP Kit, Phadia, Freiburg, Germany).

**Enzyme linked Immunosorbent Assay (ELISA)**

After obtaining informed consent, blood samples were drawn from patients as well as from healthy controls from the antecubital vein. Samples were centrifuged, and the obtained sera were stored in aliquots at -80°C until analysis. Serum levels of VEGF, bFGF and endostatin were measured using commercially available colorimetric sandwich ELISAs (R&D Systems, Minneapolis, USA) as described [15]. Concentrations were calculated using a standard curve generated with specific standards provided by the manufacturer. The ELISA for VEGF recognizes human VEGF$_{165}$ with cross-reactivity to VEGF$_{121}$, but not to factors related to VEGF such as human placental growth factor and platelet-derived growth factor. Inter-assay and intra-assay variances were less than 10%. The assay for bFGF showed minimal cross-reactivity to FGF-4 (0.02 %), but not to other factors related to bFGF such as acidic fibroblast growth factor. The assay for endostatin did not show any cross-reactivity with World Health Organization standards for any human cytokine.

**Statistical analysis**

Statistical analysis was performed by the biostatistician of the group (DH). Data are expressed as median and interquartile range (IQR). Differences in serum levels of growth factors were tested between groups composed by categorical parameters to assess association of disease parameters with serum levels of VEGF, bFGF and endostatin. The Mann Whitney U-test was used to compare two groups. To assess correlation of continuous variables with the
levels of the analyzed growth factors, Pearson’s and Spearman’s rank correlation coefficients were calculated. P-values < 0.05 were considered statistically significant. Since we tested many variables in a small data set, multivariate linear regression models were applied in a stepwise forward and stepwise backward manner to determine a robust combination of variables predicting the levels of VEGF in MCTD patients. ROC analysis was used to identify a cut off for VEGF levels to predict PAH in MCTD patients.
**Results**

**Increased levels of circulating VEGF and endostatin in patients with MCTD**

Levels of the potent angiogenic growth factor VEGF were significantly increased in the blood of patients with MCTD (p=0.017). Interestingly, we also observed elevated levels of the anti-angiogenic mediator endostatin (p<0.001). Since we assumed a possible confounding by the differing age between groups, comparisons were repeated in the age-matched samples. But confirmative, VEGF levels were significantly increased in MCTD patients (median 218 pg/ml; IQR 141 – 310 pg/ml) compared to healthy volunteers (median 120 pg/ml; IQR 67 – 269 pg/ml) (p = 0.045) (Figure 1a). The levels of circulating endostatin were almost twice as high in MCTD patients (median 30600 pg/ml; IQR 25290 – 48040 pg/ml) as in controls (median 18840 pg/ml; IQR 6770 – 25490 pg/ml) of similar age (p < 0.001) (Figure 1b). No differences were detected for bFGF between patients with MCTD and controls (MCTD: median 64 pg/ml; IQR 14 – 180 pg/ml / controls: median 55 pg/ml; IQR 14 – 90 pg/ml) (p = 0.80).

For further comparison to other connective tissue diseases, we analyzed the levels of circulating VEGF and endostatin in an additional cohort of age- and sex matched SSc patients, which consisted of 20 patients with limited cutaneous SSc and 20 patients with diffuse cutaneous SSc. The mean levels of VEGF were higher in both SSc groups (dcSSc: median 351 pg/ml; IQR 96 – 802 pg/ml; lcSSc: median 310 pg/ml; IQR 75 – 624 pg/ml) than in patients with MCTD. However, this difference did not reach statistical significance (p = 0.34). Similarly, the differences between diffuse and limited SSc were not significant (p = 0.66). In contrast, the levels of endostatin were only elevated in patients with MCTD, but not in patients with lcSSc or dcSSc (dcSSc: median 15450 pg/ml; IQR 5960 – 27890 pg/ml; lcSSc: median 17230 pg/ml; IQR 8540 – 32410 pg/ml; healthy: median 18840 pg/ml; IQR 6770 – 25490 pg/ml; p = 0.54 and p = 0.87, respectively).
Higher levels of VEGF in MCTD patients with acrosclerosis

Increased concentrations of VEGF have been reported by several independent investigators in patients with SSc [7, 16]. A subset of patients with MCTD also shows SSc-like manifestations with skin fibrosis at the fingers and acrosclerosis. Consistent with the results in SSc patients, we measured elevated blood levels of VEGF in MCTD patients with acrosclerosis (median 282 pg/ml; IQR 220 – 492 pg/ml) compared to other MCTD patients (median 171 pg/ml; IQR 113 – 253 pg/ml) (p = 0.015) (Figure 2). In contrast, no association between the levels of bFGF or endostatin and acrosclerosis were observed. As all SSc patients had acrosclerosis, subanalysis was not possible in this cohort. There was no significant correlation with the modified Rodnan skin score in patients with SSc.

Increased levels of VEGF in MCTD patients with myositis

High levels of VEGF correlated with the presence of myositis. Significantly increased levels of VEGF were measured in MCTD patients with myositis (median 261 pg/ml; IQR 195 – 492 pg/ml) compared to MCTD patients without myositis (median 144 pg/ml; IQR 105 – 277 pg/ml) (p = 0.009) (Figure 3a). Consistent with the diagnosis of myositis, elevated levels of VEGF were also detected in patients with a history of at least twofold increased serum levels of CK (elevated CK: median 352 pg/ml; IQR 236 – 646 pg/ml / normal CK: median 143 pg/ml; IQR 105 – 264 pg/ml) (p < 0.001) (Figure 3b). Interestingly, the increased levels of VEGF persisted even when the myositis was well controlled and CK levels had returned to normal, suggesting that increased levels of VEGF might be a risk marker rather than an activity marker. No associations between the presence of myositis or a history of elevated CK and the levels of bFGF or endostatin were observed.
**Association between the levels of circulating VEGF and PAH in MCTD patients**

VEGF has recently been implicated in the pathogenesis of PAH [17]. We found significantly increased levels of VEGF in patients with MCTD, in whom PAH had been diagnosed by RHC (median 371 pg/ml; IQR 277 – 718 pg/ml) compared to MCTD patients without PAH (median 159 pg/ml; IQR 120 – 254 pg/ml) (p = 0.005) (Figure 4a). In contrast to VEGF, no associations were found between bFGF or endostatin and PAH (p = 0.32 and p = 0.16, respectively).

**Multivariate analysis of clinical parameters and levels of VEGF in MCTD**

Next, we calculated a multivariate linear regression model with these 5 associated clinical parameters (acrosclerosis, presence of myositis as defined in methods, increased CK levels, PAH by RHC and increased PASP on echocardiography) to predict VEGF levels. In addition, lymphadenopathy and rheumatoid factor levels were considered for modeling, since they had shown a trend to be associated with VEGF levels in univariate analysis. Also sex and age were considered as possible confounders in the regression analysis. The best forward selection method led to a model (constant = 71.918) consisting of PAH by RHC (b = 332.275), increased CK levels (b = 185.485) and lymphadenopathy (b = 325.770). The backward selection method led to the same model, which therefore proved to be robust in this data set. The correlation coefficient of this model (R = 0.889) results in a coefficient of determination (R²) of 0.791. Thus, these results demonstrate that in patients with MCTD as much as 79 % of the variability of the levels of VEGF can be explained by a combination of PAH, myositis and lymphadenopathy.

We also examined cut-off values for the diagnosis of PAH in our patient cohort. VEGF levels greater than 270 pg/ml had a sensitivity of 86 % and a specificity of 88 % for PAH in our patient collective. Moreover, 94 % of MCTD patients without PAH had VEGF levels below 366 pg/ml.
Discussion

In the present study, we report for the first time a dysbalance of angiogenic and angiostatic mediators in patients with MCTD. VEGF, a potent angiogenic factor that stimulates migration, proliferation and survival of endothelial cells and endothelial precursor cells was significantly increased in the blood of patients with MCTD. Although the upregulation of VEGF appears to be in contrast with the loss of capillaries and the insufficient formation in MCTD, it is known that an uncontrolled overexpression of VEGF causes deleterious rather than beneficial effects on angiogenesis. Even modestly increased levels of VEGF induced by transgenic overexpression of VEGF in mice resulted in a microangiopathy that resembled closely the findings in human MCTD and SSc [8]. Thus, the increased levels of VEGF might directly contribute to the vascular disease in MCTD. In contrast to SSc, the levels of endostatin were also significantly increased in patients with MCTD. A previous study also did not find differences in the levels of endostatin in patients with SSc, suggesting that the upregulation of endostatin might be specific for MCTD [7]. However, the results for endostatin in SSc are inconsistent [18, 19]. Endostatin exerts potent anti-angiogenic effects and inhibits endothelial cell migration and proliferation. The upregulation of endostatin might thus further perturb the balance between angiogenic and angiostatic growth factors and exacerbate the microangiopathy in MCTD. Thus, the combined increase of VEGF and endostatin might play a crucial role in the vascular pathogenesis of MCTD. However, a limitation of our study is the lack of direct correlations between findings on nailfold capillaroscopy and the levels of VEGF and endostatin, which could provide additional support of the concept of perturbed angiogenesis in MCTD and needs to be analyzed in further studies.

MCTD is characterized by a spectrum of different clinical manifestations and disease severity. Markers predicting the highly variable clinical course and identifying patients at high risk for severe disease are currently lacking. Our study provides first evidence that patients
with high levels of VEGF might have an increased risk of developing severe disease. High levels of VEGF were associated with PAH, the most life-threatening complication of MCTD that might occur in a significant percentage of MCTD patients. Consistent with the association between VEGF and PAH, increased levels of VEGF were also observed in patients with increased PASP on echocardiography. We found that VEGF levels of higher than 270 pg/ml had a sensitivity of 86 % and a specificity of 88 % for PAH in our patients. This level of accuracy is better than those obtained for echocardiography in patients with SSc [20]. Significantly higher levels of VEGF were also observed in MCTD patients with myositis. The validity of this association was supported by increased levels of VEGF in patients with a history of increased serum levels of CK. The levels of VEGF remained elevated in MCTD patients with myositis, even when the myositis was in remission, suggesting that increased VEGF may characterize patients with previous myositis rather than serve as a marker for myositis activity. These associations between the levels of VEGF and PAH and myositis obtained from the univariate analysis were confirmed by the multivariate analysis, in which PAH and myositis, together with lymphadenopathy, accounted for as much as 79 % of the variability of VEGF levels. Furthermore, the presence of acrosclerosis was also associated with increased levels of VEGF, indicating high levels of VEGF might also be a feature of the subgroup of MCTD patients with SSc-like manifestations. Although confirmation of our results in larger patient cohorts is needed, our study provides the first evidence for elevated blood levels of VEGF as a novel risk marker for a more severe, potentially life-threatening course of MCTD. These findings may have direct clinical implications as those patients likely require close follow-up and monitoring for PAH and myositis.

In summary, we show an uncontrolled overexpression of angiogenic and angiostatic factors such as VEGF and endostatin. Furthermore, we demonstrate that high serum levels of VEGF are associated with PAH and myositis in MCTD suggesting that increased levels of
VEGF might be a potential marker for patients with increased risk for severe, life-threatening disease. However, prospective studies with increased numbers of patients are needed for confirmation.
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COMPETING INTERESTS

The authors declare no conflicts of interest.

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References

### Clinical criteria

1. Puffy hands
2. Acrosclerosis with or without proximal SSc
3. Raynaud’s phenomenon
4. Myositis: biologically or histologically proven
5. Synovitis

### Serologic criterion

- Anti-RNP-antibodies with a titre of > 1:1600 at the hemagglutinin assay

Diagnosis of MCTD, if the serologic criterion is present and ≥ 3 clinical criteria (if 1, 2 and 3 are present, 4 and 5 are also required to distinguish MCTD from SSc)

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<td>Raynaud’s phenomenon</td>
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<td>Synovitis</td>
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<tr>
<td>Oesophageal hypomobility</td>
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Table 1: Alarcon-Segovia’s criteria for MCTD
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Table 2: Clinical characteristics of the patients included into the study; n = 38.
Figure legends

**Figure 1**: Increased levels of VEGF and endostatin in the blood of MCTD patients. The serum levels of the angiogenic cytokine VEGF (**Figure 1a**) as well as the levels of the angiostatic factor endostatin (**Figure 1b**) were significantly increased in patients with MCTD compared to age- and sex-matched healthy volunteers. In contrast to MCTD, only the levels of VEGF, but not the levels of endostatin, were elevated in patients with lSSc and dSSc (**Figures 1a and 1b**). The levels of VEGF and endostatin are shown as box plots. Each box represents the 25th to 75th percentiles. Lines outside the boxes represent the 10th and the 90th percentiles. Lines inside the boxes represent the median, circles the outliers, and star the extreme values.

**Figure 2**: Elevated levels of VEGF in MCTD patients with acrosclerosis. Consistent with previous reports on increased levels of VEGF in SSc patients, VEGF levels were higher in the subset of MCTD patients with acrosclerosis. The levels of VEGF are shown as box plots. Each box represents the 25th to 75th percentiles. Lines outside the boxes represent the 10th and the 90th percentiles. Lines inside the boxes represent the median, circles the outliers, and star the extreme values.

**Figure 3**: Higher levels of VEGF in patients with myositis. MCTD patients with a history of myositis (**Figure 3a**) or significantly elevated CK (**Figure 3b**) presented with increased serum levels of VEGF compared to MCTD patients without myositis. The levels of VEGF are shown as box plots. Each box represents the 25th to 75th percentiles. Lines outside the boxes represent the 10th and the 90th percentiles. Lines inside the boxes represent the median, circles the outliers, and star the extreme values.
Figure 4: Increased levels of circulating VEGF in MCTD patients with PAH. Levels of VEGF were significantly higher in MCTD patients diagnosed with PAH by right heart catheterization than in those without PAH. The levels of VEGF are shown as box plots. Each box represents the 25th to 75th percentiles. Lines outside the boxes represent the 10th and the 90th percentiles. Lines inside the boxes represent the median, circles the outliers, and star the extreme values.