

Brief Report

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Angiogenin expression in the sera and skin of patients with rheumatic diseases**Aki Kuwahara, Masatoshi Jinnin*, Takamitsu Makino, Ikko Kajihara, Katsunari Makino, Noritoshi Honda, Wakana Nakayama, Kuniko Inoue, Satoshi Fukushima, Hironobu Ihn***Department of Dermatology and Plastic Surgery, Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan.***Summary**

Vascular abnormalities are one of the common features in rheumatic diseases, but their pathogenesis is still not known. Angiogenin, a molecule implicated in the angiogenic process, may play some roles in such vascular changes. Serum angiogenin concentrations were measured in 21 scleroderma patients, 10 patients with systemic lupus erythematosus (SLE), 21 patients with dermatomyositis (DM), 5 patients with polymyositis (PM), 11 patients with clinically amyopathic DM (CADM) and 12 normal subjects, with specific enzyme-linked immunosorbent assays. Angiogenin mRNA *in vivo* was determined in skin tissues of 5 DM patients, 4 CADM patients, 5 SLE patients and 7 normal subjects using quantitative real-time polymerase chain reactions. We could not find any significant differences in the serum angiogenin levels among normal subjects and patients with rheumatic diseases. However, when we evaluated the correlation of serum angiogenin levels with clinical features of 32 DM/CADM patients, the patients with increased angiogenin levels had significantly higher aldolase levels than those with decreased levels. On the other hand, angiogenin mRNA is significantly up-regulated in the involved skin of DM and CADM, suggesting that angiogenin expression is up-regulated locally in the skin but not in sera of patients with DM and CADM. In conclusion, dysregulated angiogenin expression may contribute to the pathogenesis of myositis as well as skin involvement *via* the vascular change in DM/CADM. Further studies with an increased number of patients may help to clarify the relationship between angiogenin and vascular abnormalities in rheumatic diseases and to develop new therapeutic strategies.

Keywords: Collagen disease, vascular disease, angiogenesis

1. Introduction

Vascular abnormalities such as Raynaud's phenomenon, nailfold bleeding, skin ulcers, antiphospholipid antibody syndrome, and hyper γ -globulinemia are common features of rheumatic diseases. However, the cause of these changes is still unknown.

Angiogenin is a non-glycosylated polypeptide consisting of 123 amino acid residues in length and contains three intra-chain disulfide bonds.

This molecule is classified as a member of the RISBASE family of ribonucleases, which exhibit both ribonuclease activity and special biological actions (1-3). Various cell types including vascular endothelial cells, smooth muscle cells, fibroblasts, and lymphocytes are thought to express angiogenin (4,5). In addition, circulating angiogenin has been detected in normal serum (6,7).

Angiogenin is implicated in the angiogenic process. The molecule first binds to actin, followed by dissociation of the actin-angiogenin complex from the cell surface and subsequent activation of tissue plasminogen activator. This then generates plasmin, which is known to degrade matrix of basement membrane (8,9). Destruction of the existing basement membrane may be a prerequisite for endothelial cell migration during *de novo* vascularization (10,11).

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Angiogenin may play some role in the pathogenesis of vascular changes seen in rheumatic diseases. However, no link between angiogenin and rheumatic diseases has been established. Therefore, to prove our hypothesis, we examined angiogenin levels in the sera and skin of patients with various rheumatic diseases.

2. Materials and Methods

2.1. Clinical assessment and patient material

Patients with scleroderma (SSc) or systemic lupus erythematosus (SLE) fulfilled the criteria proposed by the American College of Rheumatology (ACR) (12-14). Polymyositis (PM) and dermatomyositis (DM) were diagnosed based on the criteria proposed by Bohan and Peter (15,16). Patients with clinically and histopathologically typical cutaneous lesions but without myositis were diagnosed as clinically amyopathic DM (CADM) according to the previous criteria (17). Clinical and laboratory data reported in this study were obtained at the time of sampling of tissue or serum.

Skin samples were obtained from 5 DM patients, 4 CADM patients and 5 SLE patients. Seven control skin samples were obtained from routinely discarded skin of healthy human subjects undergoing skin grafts. Immediately after removal, skin biopsy specimens were fixed with formalin and embedded in paraffin. Sera were obtained from 21 patients with SSc, 10 SLE patients, 21 DM patients, 5 PM patients, 11 CADM patients and 12 normal control subjects. All serum samples were stored at -80°C prior to use. Institutional review board approval and written informed consent were obtained before patients and normal subjects were entered into this study according to the Declaration of Helsinki.

2.2. Antinuclear antibodies

Antinuclear antibodies (ANA) were detected by indirect immunofluorescence using HEP-2 cells as the substrate and double immunodiffusion, as described previously (18).

2.3. Serum angiogenin levels

Levels of serum angiogenin were measured with a specific ELISA kit (R & D Systems) (19). Briefly, anti-angiogenin monoclonal antibodies were precoated onto microtiter wells. Aliquots of serum were added to each well, followed by peroxidase-conjugated antibodies to angiogenin. Color was developed with hydrogen peroxide and tetramethylbenzidine peroxidase, and the absorbance at 450 nm was measured. Wavelength correction was performed using absorbance at 540 nm. The concentration of angiogenin in each sample was

determined by interpolation from a standard curve.

2.4. RNA isolation and quantitative real-time polymerase chain reaction (PCR)

Total RNA isolation from paraffin-embedded sections was performed with an RNeasy FFPE kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. cDNA was synthesized from the total RNA with a PrimeScript RT reagent Kit (Takara Bio Inc, Shiga, Japan). Quantitative real-time PCR with Takara Thermal Cycler Dice (TP800)[®] used primers and templates mixed with the SYBR Premix Ex TaqII (Takara Bio Inc). Primer sets for angiogenin and 18S were purchased from Takara Bio Inc. DNA was amplified by denaturation for 5 sec at 95°C and annealing for 30 sec at 60°C . Data generated from each PCR reaction were analyzed using the Thermal Cycler Dice Real Time System ver2.10B (Takara Bio Inc.). The relative level of angiogenin was normalized to 18S levels in the same sample.

2.5. Statistical analysis

Statistical analysis was carried out with a Mann-Whitney's *U* test for the comparison of medians, and Fisher's exact probability test for the analysis of frequency. *p* values less than 0.05 were considered significant.

3. Results and Discussion

Serum samples were obtained from 32 DM patients including 11 CADM patients as well as 5 PM patients, 21 SSc patients, 10 SLE patients and 12 healthy normal subjects. The serum angiogenin levels in patients with these rheumatic diseases are shown in Figure 1. We could not find any significant difference in the angiogenin levels among normal subjects and patients with rheumatic diseases: the mean serum angiogenin in patients with DM (111.7 pg/mL) or CADM (107.7 pg/mL) was slightly lower than those in normal subjects (120.8 pg/mL), but not statistically significant.

Next, we evaluated the correlation of serum angiogenin levels with clinical features of 32 DM/CADM. The patients with increased angiogenin levels had significantly higher aldolase levels than those with decreased levels (47.2 vs. 7.1 U/I, $p < 0.05$, Table 1). Other myositis markers, CK and myoglobin, also tended to be higher in patients with increased angiogenin levels than those without, but were not statistically significant. There were no significant differences between the two patient groups in terms of other clinical/laboratory features including age at the time of serum sampling, duration of disease, the ratio of DM:CADM or IgG levels.

On the other hand, when skin samples were obtained

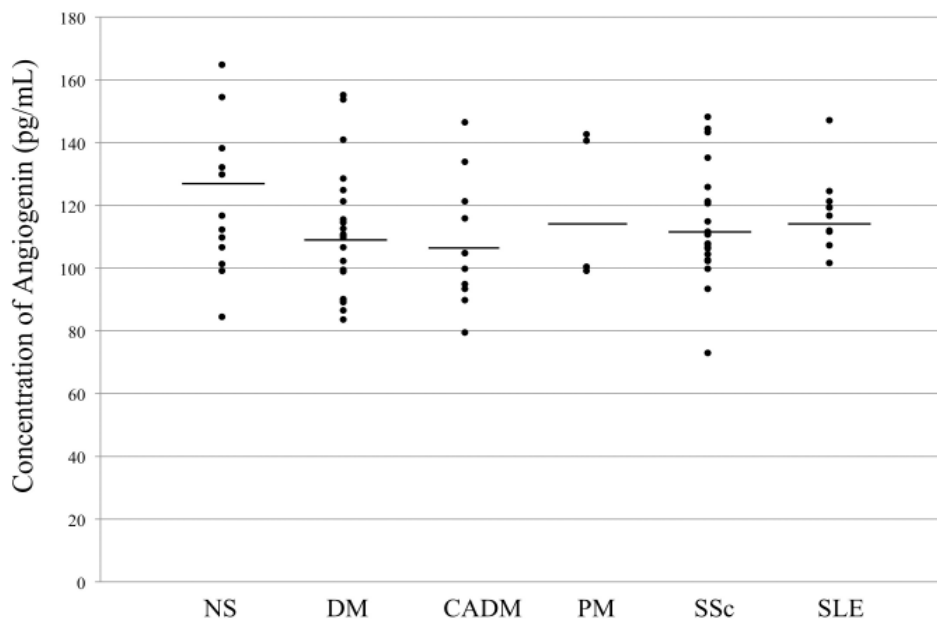


Figure 1. Serum concentrations of angiogenin in patients with rheumatic diseases. Serum concentrations of angiogenin determined by ELISA are shown on the ordinate; the horizontal bars show the mean value in each group. NS, normal subjects; DM, dermatomyositis; CADM, clinically amyopathic dermatomyositis; PM, polymyositis; SSc, systemic sclerosis; SLE, systemic lupus erythematosus.

Table 1. Correlation of serum angiogenin levels with clinical and serological features in patients with dermatomyositis (DM)

Items	Patients with decreased angiogenin levels (n = 16)	Patients with increased angiogenin levels (n = 16)
Age at the time of serum sampling (mean years)	58.3	54.6
Duration of disease (mean months)	6.1	5.2
Type (DM:CADM)	9:7	12:4
Clinical features		
Gottron's sign	62.5	62.5
Heliotrope	37.5	56.3
Laboratory features		
IgG (mg/dL)	1,453.6	1,523.9
CK (IU/L)	931.4	2,662.4
Myoglobin (ng/mL)	300.9	873.9
Aldolase (U/L)	7.1	47.2*
ANA	56.3	31.3
Organ involvement		
Muscle	50.0	68.8
Lung	43.8	25.0
Dysphagia	12.5	18.8
Joint	18.8	6.3

Unless indicated, values are percentages. DM, dermatomyositis; CADM, clinically amyopathic dermatomyositis; IgG, immunoglobulin; CK, creatine kinase; ANA, antinuclear antibodies. * $p < 0.05$ vs. patients with decreased angiogenin levels using Mann-Whitney *U* test.

from 5 DM patients, 4 CADM patients and 5 SLE patients, mean relative transcript levels of angiogenin in skin tissues from DM/CADM patients were significantly up-regulated compared with the values in normal skin ($p = 0.007$ and 0.014 , respectively) and SLE skin ($p = 0.009$ and 0.014 , respectively) (Figure 2). Our results suggest that angiogenin expression is up-regulated locally in the involved skin but not in sera of patients with DM and CADM.

In this study, we demonstrated three novel findings. First, there was no significant difference in the serum angiogenin levels among normal subjects and patients

with rheumatic diseases. Second, in DM/CADM patients, the levels of myopathic markers such as aldolase tended to be higher in patients with increased serum angiogenin levels than those without. Lastly, angiogenin mRNA is significantly up-regulated in the involved skin of DM and CADM patients *in vivo*.

It was reported that serum angiogenin levels increase in patients with cutaneous T cell lymphoma, and the molecule is thought to act as an inhibitor of polymorphonuclear leukocyte degranulation (20). Elevated levels of angiogenin have also been detected in the sera of patients with pancreatic cancer and

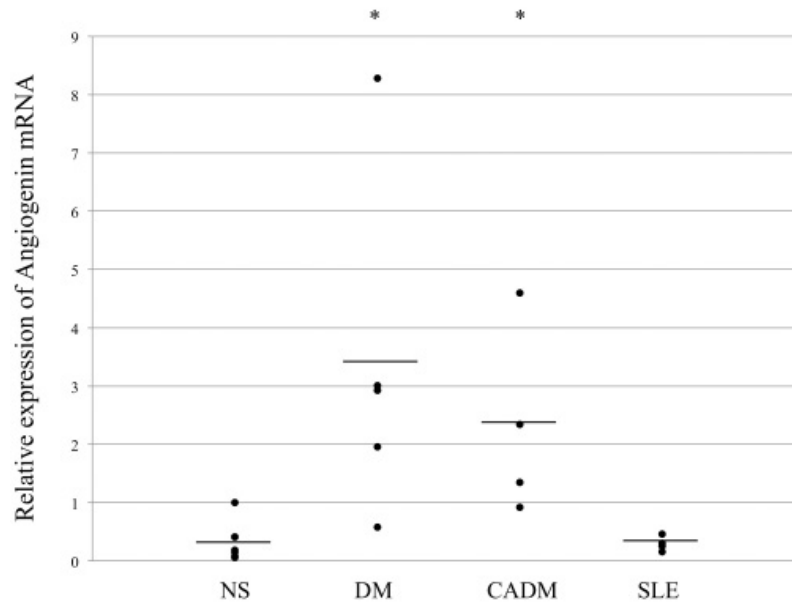


Figure 2. The mRNA expression of angiogenin in involved skin of rheumatic diseases. Mean relative transcript levels of angiogenin in skin tissues from 7 normal control subjects (NS), 5 dermatomyositis (DM) patients, 4 clinically amyopathic DM (CADM) patients, and 5 systemic lupus erythematosus (SLE) patients were determined by real-time quantitative PCR. The maximum value in samples from NS was set at 1.

arterial occlusive disease as well as inflammatory arthritis (21-24). Our research is the first to measure serum angiogenin levels in patients with PM/DM, SSc and SLE, but we did not find any significant difference between controls and these patients. This may be because of a small number of patients.

Vascular change is thought to be found frequently in the involved skin of DM/CADM; Crowson *et al.* described increased endothelial injury, increased vascular ectasia and reduced superficial vascular plexus density in skin lesions of DM/CADM compared with lupus erythematosus (25). Considering that uncontrolled activation of vascular endothelial growth factor (VEGF), one of the most potent angiogenic factors, rather than its inactivation is suggested to cause disturbed vessel morphology (26,27), and excessive expression of angiogenin in the skin of DM/CADM patients may also induce vascular change.

Similarly, histopathological findings of the muscles in DM are characterized by perivascular infiltration of CD4-positive T lymphocytes or macrophages (28,29). Furthermore, Pestronk *et al.* studied muscle specimens of DM and found reduction of vessel size and endothelial loss, as well as C5b9 complement deposition (30). Considering that patients with increased serum angiogenin had higher levels of myositis markers, the dysregulated angiogenin expression may contribute to the pathogenesis of muscle involvement *via* the vascular abnormality in DM. Further studies with an increased number of patients may help to clarify the relationship between angiogenin and vascular abnormalities in rheumatic diseases and to develop new therapeutic strategies.

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