Fluctuation of Anti-Endothelial Cell Antibody Titers in Mixed Connective Tissue Disease

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ABSTRACT: Background: Antibodies directed against endothelial cell surface antigens have been described in many disorders and have been associated with disease activity. Since the most prominent histopathologic feature in mixed connective tissue disease (MCTD) is the widespread and unique proliferative vascular lesion, our aim was to evaluate the frequency of anti-endothelial cell antibodies (AECA) in this condition. Objectives: To evaluate the frequency of AECA in this disease and assess its clinical and laboratory associations.

Methods: Seventy-three sera from 35 patients with MCTD (Kasukawa’s criteria), collected during a 7 year period, were tested for immunoglobulins G and M (IgG and IgM) AECA by cellular ELISA, using HUVEC (human umbilical vein endothelial cells). Sera from 37 patients with systemic lupus erythematosus (SLE), 22 with systemic sclerosis (SSc) and 36 sera from normal healthy individuals were used as controls. A cellular ELISA using HeLa cells was also performed as a laboratory control method.

Results: IgG-AECA was detected in 77% of MCTD patients, 54% of SLE patients, 36% of SSc patients and 6% of normal controls. In MCTD, IgG-AECA was associated with vasculitic manifestations, disease activity and lymphopenia, and was also a predictor of constant disease activity. Immunosuppressive drugs were shown to reduce IgG-AECA titers. Since antibodies directed to HeLa cell surface were negative, AECA was apparently unrelated to common epitopes present on epithelial cell lines.

Conclusions: AECA are present in a large proportion of patients with MCTD and these antibodies decrease after immunosuppressive treatment.

KEY WORDS: mixed connective tissue disease (MCTD), Sharp’s disease, anti-endothelial cell antibodies, endothelial cells, antibody fluctuation

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Mixed connective tissue disease, first described by Sharp et al. in 1972 [1], is an inflammatory disease clinically characterized by features of systemic lupus erythematosus, systemic sclerosis and polymyositis, and associated with high titers of circulating antinuclear antibody with specificity for nuclear ribonucleoprotein.

Anti-endothelial cell antibodies have been described in several connective tissue diseases such as SLE, SSc, rheumatoid arthritis, microscopic polyangiitis, Kawasaki disease, Wegener’s granulomatosis, and antiphospholipid syndrome [2,3]. Specifically in MCTD, some studies have shown that AECA might be present in the serum of patients with this disease [4-9]. In one study, high levels of AECA were associated with the presence of pulmonary fibrosis [4] and with disease activity [7]. However, little information is available regarding disease activity, and longitudinal evaluation of these antibodies has not yet been performed.

The objectives of the present study were to ascertain the frequency of AECA in patients with MCTD and to evaluate the association of these antibodies and their titers with clinical and laboratory manifestations of MCTD cross-sectionally and longitudinally.

PATIENTS AND METHODS

The study group comprised 33 consecutive patients with MCTD who were prospectively followed in our outpatient clinic at the Rheumatology Division of Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo. All patients fulfilled the classification criteria for MCTD according to Kasukawa [10]. Exclusion criteria included positivity for anti-Sm and anti-dsDNA antibodies (to exclude SLE). The control group comprised 37 patients with SLE, 22 with systemic sclerosis, and 36 healthy individuals. Clinical and laboratory data of the patients were collected through an extensive review of their medical charts and by clinical examination. All participants provided written informed consent and the study was approved by the local ethics committee. Blood samples were collected from all study participants.
DISEASE ACTIVITY
The disease was considered active if the patient presented two major manifestations (cutaneous vasculitis, increase of pulmonary fibrosis, central nervous system manifestations, severe myositis, or urinary casts/proteinuria), or one major plus two minor manifestations (arthritis, new rash, diarrhea, mild myositis, or weight loss), or three minor manifestations plus abnormal laboratory test (lymphopenia, thrombocytopenia, increased erythrocyte sedimentation rate or C-reactive protein, or increased gammaglobulin levels) [11].

For the data analysis, these patients were divided into two subgroups: group A – patients who had taken immunosuppressive drugs (chlorambucil 2–6 mg/day, intravenous cyclophosphamide or azathioprine 50–100 mg/day) associated or not with glucocorticoid (11/19); group B – patients who had received exclusively prednisone without other immunosuppressive drugs (8/19).

ANTI-ENDOTHELIAL CELL ANTIBODY ASSAY
ECV-304 endothelial cells were used to identify the anti-endothelial antibodies as previously described [12,13]. Briefly, these cells were expanded in plastic flasks appropriate for cell culture and seeded onto sterile polystyrene 96-well (6 mm in diameter) microplates at a concentration of 2 x 10⁴ cells/well in 100 μl medium 199 containing 10% fetal bovine serum. After diameter) microplates at a concentration of 2 x 10⁴ cells/well in 100 μl medium 199 containing 10% fetal bovine serum. After

STATISTICAL ANALYSIS
Results are presented as the mean and standard deviation for continuous variables and as the number (%) for categorical variables. Data were compared by t-tests or by the Mann-Whitney test for continuous variables to evaluate differences between groups. For categorical variables, differences were assessed by chi-square test or Fisher’s exact test. P values < 0.05 were considered significant.

RESULTS
CLINICAL MANIFESTATIONS
The mean age of the MCTD patients was 43 ± 11 years and they had a mean disease duration of 7.0 ± 4.0 years; 95% were female and 55% were Caucasian.

AECA FREQUENCY
IgG-AECA was detected in 77% of MCTD patients, 54% of SLE patients, 36% of SSc patients and 6% of healthy controls. Analysis of results showed that IgG-AECA was more frequently found in the studied diseases than in healthy controls (odds ratio = 10.53, P < 0.0001). Moreover, IgG-AECA was more common in MCTD than in SLE and SSc (P < 0.001) [Figure 1A]. There was no sig-

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Figure 1. Dot plots representing the ELISA binding. Titers were arbitrarily defined by the ratio between the sample optical density (OD) and the normal reference value (the OD obtained for 36 normal individuals plus three standard deviations) Values ≥ 1 were considered positive. [A and B] Binding of IgG and IgM class antibodies, respectively, to endothelial cells in sera from 35 patients with MCTD, 37 with SLE, 22 with SSc and 36 healthy controls. [Cand D] Binding of IgG and IgM class antibodies, respectively, to HeLa cells in sera from 35 patients with MCTD, 37 with SLE, 22 with SSc patients and 36 healthy controls.
significant difference between the presence of IgM-AECA [Figure 1B] and any of the studied groups (OR = 1.77, P > 0.05).

ANTI-HELA ANTIBODIES
IgG and IgM anti-HeLa cells using ELISA assay showed no statistical differences between the studied connective tissue diseases (MCTD, SLE and SSc) and healthy controls [Figures 1C and D].

AECA AND CLINICAL MANIFESTATIONS
A positive association of circulating IgG-AECA was found between IgG-AECA and fever, weakness, weight loss, cutaneous vasculitis, palmar erythema, current disease activity, persistent disease activity, lymphopenia and gamma-globulin (> 2 g/dl) (P < 0.001). Clinical manifestations of myositis, interstitial lung disease (vital capacity < 70%), arthritis and laboratory parameters such as elevated erythrocyte sedimentation rate, raised C-reactive protein levels, anemia and leukopenia were not associated with the presence of IgG-AECA.

AECA FLUCTUATION AND IMMUNOSUPPRESSIVE THERAPY
During a 5 year follow-up period, sera were collected from 19 of the 28 studied patients, allowing the evaluation of AECA oscillations and their relation with clinical and therapeutic manifestations. We observed that there was a tendency for IgG-AECA titers to decline in parallel to clinical activity reduction. In this context, patients from group A presented a significant decrease in IgG-AECA titers, when comparing levels at the start of the study with those measured during the follow-up period (P < 0.001) [Figure 2A]. On the other hand, in patients from group B, IgG-AECA titers also diminished during the study period, but this difference did not reach significance [Figure 2B]. As a whole, the interval between the collections of the two different samples of serum was 44 ± 17 months, which was not different between the two groups. It is noteworthy that the initial IgG-AECA levels were not different between groups A and B.

DISCUSSION
To the best of our knowledge, the present study demonstrates for the first time that IgG-AECA are more frequent in MCTD patients than in SLE or SSc patients, and these antibodies fluctuate following MCTD treatment. In addition, the presence of AECA is associated with a number of clinical and laboratory findings in MCTD.

The higher frequency of AECA in MCTD than in SLE or SSc suggests that the participation of the vascular system may play an important role in the striking features of this particular disease. The presence of AECA was less common in SSc than in MCTD. In SSc, denuded and deleted vessels are often found, a finding not described for MCTD [14]. Therefore, it is conceivable that the vascular changes encountered in SSc might result from circulating active molecules that lead to irreversible vascular damage [15], while in MCTD immunoglobulins with affinity for endothelial cells could somehow be involved in the vascular structural reorganization. Furthermore, our data showed that in MCTD the presence of IgG-AECA was associated with cutaneous vasculitis, active disease and persistent clinical activity, findings similar to those previously published by others and ourselves in SLE patients [16-18]. This point is particularly meaningful in MCTD, where few laboratory parameters are available to determine disease activity. Unlike SLE, complement levels are rarely altered in this disease. Anti-RNP titers, the serological marker of MCTD, do not oscillate much along time [1]. Perhaps, IgG-AECA may prove useful in this respect.
Data from the experiments with HeLa cells illustrate that the binding of immunoglobulins to endothelial cells was not related to epitopes commonly present on epithelial cell lines. This confirms specificity of the AECA assay in detecting altered circulating antibodies to vascular surface.

Previous studies showed that some sera with anti-endothelial cell activity may also recognize antigens present on fibroblasts, erythrocytes, monocytes, lymphocytes, platelets and subendothelial matrix [19]. We found an association between the presence of IgG-AECA and lymphopenia, but no association of IgG-AECA with anemia or thrombocytopenia. Therefore, it is possible that the former association simply reflects active disease and may not be directly implicated in the pathogenic process. Several articles that review AECA in various tissue connective diseases are available [20-25].

We found that significant decreases of IgG-AECA titers occurred with the use of immunosuppressive drugs. These observations, although preliminary, deserve further analysis in controlled trials.

In conclusion, this study showed that IgG-AECA were more frequent in MCTD than in other connective diseases, and these antibodies were associated with various clinical and laboratory findings in MCTD; titers of AECA can decrease with use of immunosuppressive drugs.

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References

“No great discovery was ever made without a bold guess”
Isaac Newton (1642-1727), English physicist, mathematician, astronomer, natural philosopher, alchemist, and theologian, considered by many to be the greatest and most influential scientist who ever lived. In his monograph Philosophiae Naturalis Principia Mathematica, Newton described universal gravitation and the three laws of motion, which dominated the scientific view of the physical universe for the next three centuries.