Clinical Significance of Serum Anti-Annexin V Antibodies in Egyptian Patients with Scleroderma

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The pathogenesis of scleroderma encompasses vascular, immunological, and fibrotic processes, which contribute to clinical manifestations. We investigated the prevalence of anti-annexin V IgG and IgM antibodies in sera of scleroderma patients and their relation to the presence of other antibodies and development of disease morbidity. Sera of 40 scleroderma patients and 15 healthy controls were examined for IgG and IgM anti-annexin V antibodies by ELISA and anticentromere antibodies by indirect immunofluorescence. Serum level of anti-annexin V IgG antibodies in scleroderma patients was significantly higher than that of the control (*P*<0.001) and correlated significantly with the presence of digital ischemia (*P*=0.023) and pulmonary fibrosis (*P*=0.02). IgM isotype was comparable between patients and controls (*P*=0.317). Anticentromere antibodies are more prevalent in the limited cutaneous subtype (*P*=0.017). In conclusion, measurement of serum anti-annexin V IgG antibodies in scleroderma patients may be important for early diagnosis of vascular and pulmonary complications.

Scleroderma is a connective tissue disease characterized by microvascular damage and excessive fibrosis of the skin and various internal organs. There are variations of clinical expression in patients with scleroderma ranging from limited cutaneous subtype in which skin thickening is relatively restricted to the fingers and hands, and with less serious internal organ involvement, to diffuse cutaneous subtype in which skin lesions are extensive with earlier and more serious complications (Leroy *et al.,* 1988). The exact etiology of scleroderma is not known, however it is currently believed that it may represent an autoimmune response to an unknown antigen (Sapadin *et al.,* 2001). The pathogenesis of scleroderma related vascular manifestations is also not clearly understood. Vascular hyperreactivity is a hallmark of the disease, and smooth muscle and endothelial cell proliferation contribute to vascular occlusion, together with thrombotic events (Esposito *et al.,* 2005). Annexin V has an important role in the regulation of apoptosis and shows an *in vitro* anticoagulant effect (Andree *et al.,* 1992). Anti-annexin V antibodies have been observed to cause apoptosis of vascular endothelial cells with subsequent thrombosis (Nakamura *et al.,* 1998). We performed this study to determine the presence of anti-annexin V IgG and IgM antibodies in Egyptian patients with scleroderma and compared the level of such antibodies with the clinical manifestations of the disease.

Patients and Methods

Forty scleroderma patients were included in the study (36 females and 4 males). They were diagnosed at the Rheumatology and Rehabilitation department, Faculty of Medicine, Cairo University. The study was approved by Cairo University Ethical Committee and oral consent was taken from the participants. The diagnosis was established according to the criteria for scleroderma described by the Subcommittee for Scleroderma Criteria of the American College of Rheumatology (1980). Patients were classified into two groups limited cutaneous scleroderma and diffuse cutaneous scleroderma (Leroy *et al.,* 1988). Age matched fifteen healthy individuals (female: male=6: 1) were also included in the study as a control group.

All patients were subjected to complete history taking and full clinical examination adapted for scleroderma to provide details about the extent of the

disease, e.g. grading of the skin thickening was done according to the modified Rodnan's score (Clements *et al.,* 1993) and limbs were examined for the presence of digital ischemia and its complications including: pitting scars, ulcers and digital amputation. Pulmonary functions included forced vital capacity (FVC), forced expiratory volume in 1 second (FEV_1) and FEV_1/ FVC are measured using compact vitalograph spirometry (Vitalograph Ltd, Maids Moreton House, Buckingham, England). Results were interpreted as normal, restrictive or obstructive patterns (Owens *et al.,* 1983). Radiological investigations as high resolution computed tomography of the chest (HRCT) was done using a spiral CT scanner (LightSpeed 16; GE Healthcare, Paris, France) in high-resolution mode (Mayo *et al.,* 1987).

Anti-centromere antibodies were detected by indirect immunofluorescence using HEp-2 cells (Dick *et al.,* 1995). Briefly, fixed slide preparations of HEp-2 cells were incubated with patient serum. The slides were then washed, and fluorescein isothiocyanate-labeled secondary antibody directed against total human immunoglobulin was added. The slides were washed again, covered, and examined using a fluorescence microscope (Olympus Corp., Tokyo, Japan).

Anti Annexin V antibodies (Abs) were measured using a commercially available anti-annexin V IgG/IgM kit (ORGENTEC Diagnostika GmbH, Mainz, Germany) according to the manufacturer's instructions. The assay is an indirect solid phase enzyme immunometric assay (ELISA) designed for the quantitative measurement of IgG or IgM class autoantibodies directed against Annexin V. Briefly, Patients' sera were added to the microplate wells coated with a highly purified human recombinant annexin V. To detect the presence of specific Abs, if any, horseradish peroxidase labeled anti-human-IgG or IgM conjugate were added followed by a chromogenic substrate solution. Reactions' absorbance was measured photometrically at 450 nm. Concentration is determined by comparing the optical density to a standard curve prepared from 6 calibrators containing different concentrations of IgG and IgM anti-annexin V.

Interpretation of results: the following ranges have been established by the kits' manufacturer for both anti-annexin IgG and IgM: < 5 U/ml is considered negative, 5-8 U/ml border line and >8 U/ml positive.

Statistical Analysis

Data were computerized and analyzed by SPSS PC+, version 12 (SPSS corporation USA). Quantitative data were presented by means ± SD and compared using t-test. Pearson correlation was done for correlating quantitative variables. Qualitative data were presented in the form of number (frequency) and percent, compared by Chi square or Fischer's exact test when appropriate. A level of significance was set as $P \leq 0.05$ was considered significant, $P \leq 0.01$ was considered highly significant and $P > 0.05$ was considered not significant.

Results

This study included 40scleroderma patients (36 females and 4 males; mean age = 40.93 ± 13.18 years) and 15 healthy subjects (13 females and 2 males; mean age = 35.6 ± 10.46 years) serving as a control group. Scleroderma, like other autoimmune diseases, is seen more frequently in females (Mayes, 2003), we included the controls as to match gender of studied patients.

The mean level of serum IgG anti-annexin V antibodies in scleroderma patients (10.28±9.04 U/ml) was significantly higher (P<0.001) than the level observed in healthy controls (2.20±0.89U/ml) (Figure 1). On the other hand, no significant difference was noted in between the levels of anti-annexin V IgM antibodies in both groups (Figure 2).

Anticentromere antibodies were detected in 25% of the patients with scleroderma (n=10) and were not observed in sera obtained from the control subjects. The difference in positivity between the two groups was statistically significant (P=0.032).

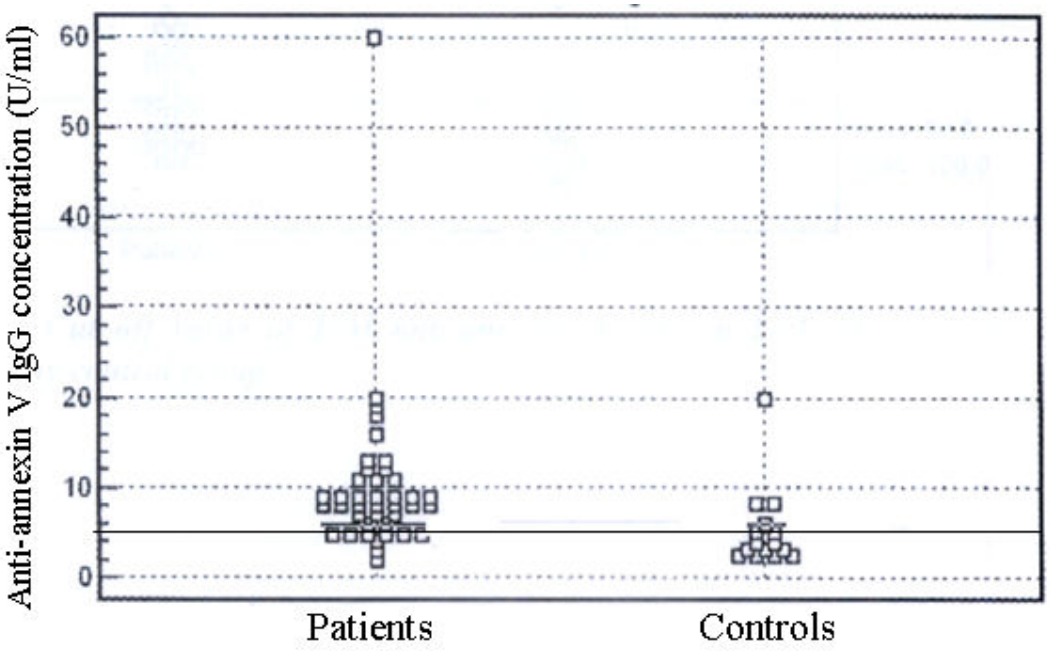

Figure 1. Anti-annexin V IgG concentration among patients and control groups, Values less than 5 U/ml are considered negative.

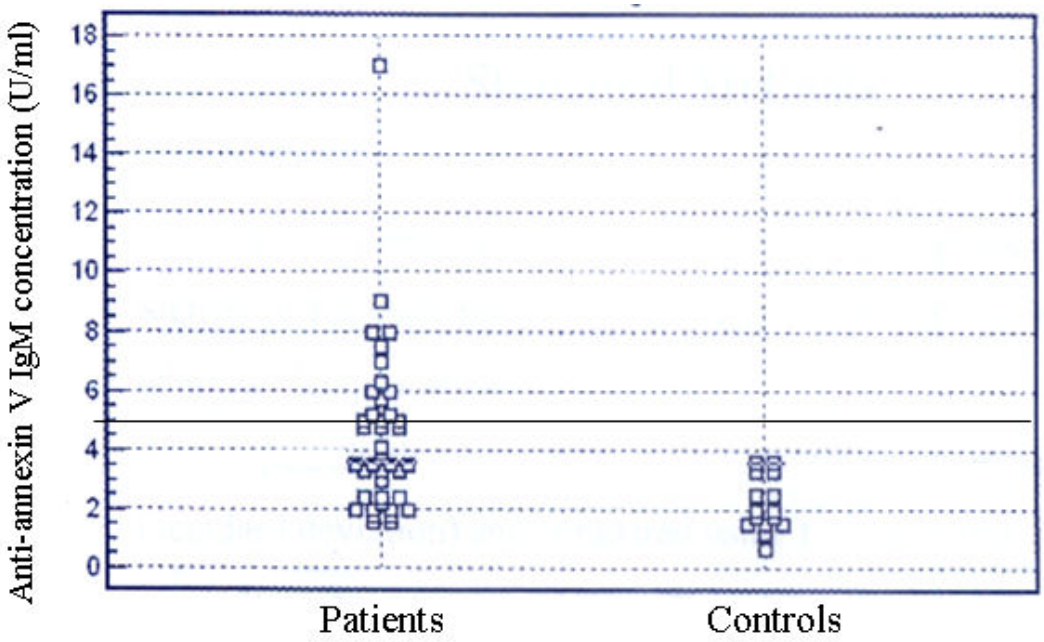

Figure 2. Anti-annexin V IgM concentration among patients and control groups, Values less than 5 U/ml are considered negative.

Correlation between mean serum level of anti-annexin V (IgG and IgM) antibodies and other variables was performed. Patients with digital ischemia (manifested as digital ulcers or autoamputation) had a significantly higher (*P*=0.023) serum level of anti-annexin V IgG antibodies (Table 1), although serum anti-annexin V IgM antibodies was higher in patients with digital ischemia than those without, however, the difference was not statistically significant (*P*=0.619). In addition, patients with pulmonary fibrosis according to the HRCT of the chest had significantly higher (*P*=0.02) level of serum anti-annexin V IgG antibodies than those without pulmonary fibrosis.

A highly significant correlation was found between anti-annexin V IgG antibodies and the presence of airflow limitation detected by FEV_1/ FVC ratio (*P*=0.008, r =-0.531).

No significant correlation was found between the mean serum level of anti-annexin V antibodies (IgG nor IgM) and the anticentromere antibodies within our group of scleroderma patients (*P*=0.876 and 0.249 respectively).

No significant correlation was observed between the level of anti-annexin V (IgG nor IgM) antibodies and patients' age, sex, disease duration and modified Rodnan's score.

We sub classified our patients into two subgroups; those with limited cutaneous scleroderma (n=19) and those with diffuse cutaneous scleroderma (n=21). The mean serum level of both IgG and IgM anti annexin V antibodies was higher in patients with the diffuse than in the limited cutaneous scleroderma, however the difference was not statistically significant (*P*= 0.157, 0.188 respectively), (Table 2). Eight out of the ten patients with positive anticentromere antibodies were of the limited scleroderma subtype, while the other two was of the diffuse scleroderma subtype and the difference between the two groups was significant statistically (*P*=0.017) (Table 2).

Table 1. Mean serum level of Anti-annexin V antibodies in scleroderma patients with and without digital ischemia

Anti-annexin V antibodies	Patients with digital ischemia (n=13)	Patients without digital ischemia (n=27)	*P*
IgG (Mean ± SD)	12.85±6.75	8.12±3.65	0.023
IgM (Mean ± SD)	4.66±3.24	4.18±1.73	NS

P<0.05 is significant. NS=not significant.

Table 2. Comparison between diffuse and limited scleroderma patients regarding serum level of Anti-annexin V antibodies and Anticentromere antibodies.

	Diffuse subtype (n=21)	Limited subtype (n=19)	*P*
Anti-annexin V IgG (Mean ± SD)	12.22±11.69	8.15±4.02	NS
Anti-annexin V IgM (Mean ± SD)	5.07±3.38	3.88±1.95	NS
Positive Anticentromere antibodies; n (%)	2 (9.5%)	8 (42%)	0.017

P<0.05 is significant. NS=not significant

Discussion

Annexin V is a glycoprotein that has an important role in the regulation of apoptosis (Gidon-Jeangirard *et al.,* 1999), it has a strong phospholipids binding activity and thus it shows an *in vitro* anticoagulant activity (van Heerde *et al.,* 1995). Anti-annexin V antibodies (especially the IgG isotype) have been detected in sera of patients with Systemic Lupus Erythematosus (Satoh *et al.,* 1999, Nojima *et al.,* 2001), rheumatoid arthritis (Dubois *et al.,* 1995, Rodriguez-Garcia *et al.,* 1996), autoimmune antiphospholipid syndrome (Ogawa *et al.,* 2000), recurrent fetal loss and preeclampsia (Matsuda *et al.,* 1994) and Takayasu's arteritis (Tripathy *et al.,* 2003). Unlike, the IgG isotype, only few studies evaluated the level of anti-annexin V IgM antibodies in sera of patients with autoimmune diseases. In addition, limited reports were available about the clinical significance of anti-annexin V in patients with scleroderma.

In this study we investigated serum level of anti-annexin V IgG and IgM Ab levels in patients with scleroderma and studied the possible association between the presence of such antibodies with various clinical manifestations.

In this study, we identified a significant increase in the level of serum anti-annexin V IgG antibodies in scleroderma patients than in the control group. This increase in anti-annexin V IgG was significantly associated with the presence of digital ischemia in patient with scleroderma. Our results are in agreement with the work of Sugiura & Muro (1999) who detected anti-annexinV IgG antibodies in 18.2% of patients with scleroderma but none in healthy controls; moreover, 75% of scleroderma patients with anti-annexin V IgG antibodies had digital ischemia. Such data suggest that anti-annexin V IgG antibodies may have a role in the pathogenesis of digital ischemia in scleroderma patients that can not be explained simply by thrombosis. It is possible that anti-annexin V antibodies interfere with annexin V functions and exert a detrimental role leading to thrombosis and apoptosis of vascular endothelial cells creating a procoagulant environment with more risk of thrombosis and vascular occlusion (Nakamura *et al.,* 1998).

Our study indicated a significant correlation between serum anti-annexin V IgG antibodies and the presence of airflow limitation detected by FEV_1/ FVC ratio, as well as with the presence of pulmonary fibrosis diagnosed by using HRCT of the chest. This may be explained by the fact that pulmonary fibrosis in scleroderma patients occurs secondary to the release of cytokines and growth factors in the lungs with subsequent activation of resident fibroblasts (Varga, 2008). Annexin was found to act as Fc receptor specific for IgG and thus in addition to binding their own specific autoantibodies at the antigen affinity sites, they may also clear body fluids from antibody complexes by binding their Fc region to the surface of macrophage and leucocytes. In effect, the elimination of circulating annexins by their own antibodies might be expected to aggravate the autoimmune condition by impeding the clearance of other autoantibody complexes (Rodriguez-Garcia *et al.,* 1996). These autoantibodes may play a role in pathogenesis by causing the release of cytokines that may contribute to vascular changes and pulmonary fibrosis (Sapadin *et al.,* 2001). In a recent study about the mechanism of scleroderma induced lung disease, du Bois (2007) concluded that the autoantibody profile carried by an individual patient with scleroderma has a significant role in determining the pattern of his lung involvement, however much remains to be learned about how does autoantibody

expression cause, trigger or mediate the scleroderma induced lung disease.

We found no significant correlation between serum anti-annexin V IgG and patients' age, sex or disease duration. Similarly, Dubois *et al.* (1995) found no correlation between anti-annexin V IgG antibodies and disease duration in patients with rheumatoid arthritis. We could not locate other studies that address the correlation between serum anti-annexin V and patients' age or sex.

We could not detect a significant difference in the level of anti-annexin V IgM antibodies in scleroderma patients and controls nor an association with various clinical manifestations. Similar results were obtained by Zammiti *et al*. (2006) who found only elevated levels of anti-annexin V IgG but not IgM among patients with recurrent spontaneous abortion, also Ogawa *et al.,* (2000) did not find a significant difference in positivity for anti-annexin V IgM isotypes in their group of patients with antiphospholipid syndrome relative to the healthy control group, while Rodriguez-Garcia *et al.,* (1996) found a normal range of anti-annexin V IgM antibodies in their group of rheumatoid arthritis patients in contrast to the IgG isotype. In these three studies and in concordance with our study, IgM isotype level was not correlated to clinical manifestations of the disease or to markers of disease activity.

We detected anticentromere antibody in 25% of scleroderma patients and none of the healthy controls and when we classify our patients into limited and diffuse subtypes, we found that 42% of the limited scleroderma patients had anticentromere antibody versus 9.5% of the diffuse subtype (P=0.017). These results were in concordance with other studies which reported a frequency of 20-30% for anticentromere antibody in scleroderma patients with a higher prevalence within the limited cutaneous subtype (Sato *et al.,* 1994; Furst, 2004; Steen, 2005). The presence of anticentromere antibodies have been strongly associated with the limited scleroderma subtype to the extent that when such antibodies are found in patients with Raynaud's phenomenon, it is predictive for the development of limited cutaneous scleroderma (Grassegger *et al.,* 2008). Anticentromere antibodies are rarely found in patients with other autoimmune diseases, family members of scleroderma patients, or healthy controls, and thus are very useful in the diagnosis of scleroderma (Reveille *et al.,* 2003).

We could not detect significant association between level of anti-annexin V antibodies (IgG nor IgM) and the anticentromere antibodies in our patients. These results are different from those obtained by Sugiura and Muro (1999) who found that most of scleroderma patients with anti-annexin V antibodies also had anticentromere antibodies. We can explain this finding because our patients have serum level of both IgG and IgM anti-annexin V antibodies higher in the diffuse than in the limited group-although not statistically significant- in contrast to the anticentromere antibodies that are significantly highly prevalent in the limited subgroup of patients.

In conclusion, although the exact mechanism underlying the generation of anti-annexin V in patients with scleroderma is not yet clear, data obtained in our study suggest that these antibodies-especially the IgG isotype- may play a role in the pathogenesis of the disease. The detection of systemic anti-annexin V IgG antibodies may be an early indicator for the diagnosis of vascular and pulmonary complications in scleroderma patients with subsequent avoidance of their debilitating symptoms; however this needs further confirmation in future studies including larger number of patients.

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