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Transcellular distribution heterogeneity of Annexin A5 represents a protective response to lupus-related thrombophilia: A pilot Proteomics-based study

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ABSTRACT

Lupus-related vascular events are becoming a formidable obstacle to the improvement of long-term prognosis of systemic lupus erythematosus (SLE) and the existent findings lack for systematization. Proteomics is a strategic approach but its applications in this regard are rare and primarily involve proteome acquisition or biomarker screening, rather than functional identification. To provide further insight, we investigated the proteomic diversity of peripheral blood mononuclear cells (PBMCs) in SLE and the possible role of the identified Annexin A5 (AnxA5) in pathogenesis. The study involved 214 SLE and 183 healthy women. The two-dimensional electrophoresis gel images showed 649 ± 25 and 676 ± 19 protein spots from the PBMCs of the patients and controls, respectively. From these protein spots, 30 differentially expressed proteins were chosen, and 16 of these proteins were identified by mass spectrometer. Western blotting confirmed the over-expressed candidate, AnxA5, from the PBMCs of the patients (SLE:control = 1.607:1, $P = 0.0004$), but ELISAs indicated decreased levels of sera AnxA5 in the patients compared to healthy donors (SLE vs. control = 26.8 ± 3.0 vs. 49.0 ± 3.3 ng/mL, $P < 0.0001$). A positive correlation was demonstrated between the manifestation of thrombosis and AnxA5 (Mann–Whitney $Z = -2.084$, $P = 0.037$), not anti-AnxA5, while searching for correlations between clinical parameters and the two molecular levels of patient sera. The coagulation assays using plasma from SLE patients revealed that elevated AnxA5 could shorten prothrombin time, activated partial thromboplastin time and prolonged thrombin time ($P < 0.001$). Our data demonstrated the proteomic differences in the PBMCs between SLE patients and healthy persons. Moreover, the heterogeneous transcellular distribution, increased intracellular concentrations and decreased serum levels of AnxA5 represent a protective response to lupus-related thrombophilia; AnxA5 mostly participate in the common coagulation pathway in the thrombogenesis of SLE.

1. Introduction

Systemic lupus erythematosus (SLE) is an important model of autoimmunity characterized by multiple organ involvement and capricious clinical manifestation. Before the 1960s, SLE was a catastrophic disease worldwide, with an approximately 50% five-year survival rate [1]. In subsequent decades, the pathogenesis studies and the use of corticosteroids, cytotoxic drugs and antibiotics led to a dramatic reduction in its severity and mortality, especially nephritis and infection [2,3]. Unfortunately, although these regimens prolonged the patient's life spans, some unpredictable complications also followed. Lupus nephritis and infection are no longer the most frequent primary causes of death [4]. Instead, patients now suffer from increasing thrombosis-related symptoms, which can be very serious, sometimes even lethal [5].

To clarify the etiopathogenesis, which remains unclear, current studies focused on how the autoimmunity begins, with respect to genetic susceptibility, invasive autoantibody alteration, obstacles to the elimination of apoptosis, cytokine and immune regulation pathway dysfunction [6–8]. Vascular events have hindered the improvement of long-term prognosis and there are meaningful detections on potential biomarkers such as antiphospholipid antibodies, Annexin A5 (AnxA5) and β2 glycoprotein I [9,10]. However, these findings are not abundant enough to verify the cascade of lupus-related vascular events.

Abbreviations: AnxA5, annexin A5; APS, antiphospholipid syndrome; aPTT, activated partial thromboplastin time; CTX, cyclophosphamide; ELISA, enzyme-linked immunosorbent assay; Fib, fibrinogen; IEF, isoelectric focusing; Ig, immunoglobulin; INR, international normalized ratio; IPG, immobilized pH gradient; mAb, monoclonal antibody; MS, mass spectrometer; MW, molecular weight; PBMCs, peripheral blood mononuclear cells; pH, power of hydrogen; pI, pH of isoelectric point; PPP, platelet-poor plasma; PSTs, peptide sequence tags; PT, prothrombin time; SDS–PAGE, sodium dodecyl sulphate polyacrylamide gel; SLE, systemic lupus erythematosus; TT, thrombin time; 2-DE, two-dimensional electrophoresis.

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Among the extant approaches, Proteomics has the capacity for high-throughput screening and possesses unique advantages. The combination of proteome isolation, identification and functional analyses has enabled the study of protein function in the post-genomic era. To our knowledge, the applications of Proteomics in this field are rare, and the few studies undertaken were suspended mostly at the stage of proteome acquisition or biomarker screening, rather than function probing [11].

In the present study, we used Proteomics to identify differentially regulated proteins between SLE patients and healthy controls. Then, using clinical parameters and bioinformatics analyses, we hypothesized that AnxA5, a candidate of intracellular overexpression in SLE, might possess the capacity for protection; this protection may avoid SLE patients plagued by antiphospholipid syndrome (APS), a typical thrombotic disease. We performed pilot studies to confirm the heterogeneity in the transcellular distribution of AnxA5. This heterogeneity may be also correlated with thrombus-related manifestations.

2. Materials and methods

2.1. Reagents and antibodies

Most of the reagents and equipments were purchased from Pharmacia (GE Healthcare, UK) and Bio-Rad (USA) unless otherwise stated. Several consumables were from Roche (Switzerland), Millipore, Sigma–Aldrich and Thermo Scientific (USA). The mouse anti-human AnxA5 mAb was from Abnova, Taiwan. Antibodies against AnxA5 were obtained from R&D Systems (USA) and precoated by Biovalue as a quantitative enzyme-linked immunosorbent assay (ELISA) system. Human anti-AnxA5 Platinum ELISA kits were from Bender MedSystems (eBioscience, Austria). Mouse anti-βactin IgG and peroxidase-conjugated goat anti-mouse IgG were home products of Zhongshan Biology. Basic inorganic chemicals were native made from Kelong and Boyi Chemistry.

2.2. Study subjects and sample applications

The study participants consisted of 214 Mongoloid female SLE patients from the Department of Dermatology and 183 age-gender-matched volunteers from the Health Examination Center and Blood Transfusion Center, Southwest Hospital, Chongqing, China. The participants were recruited from July 2007 to November 2011. Some participants with proven infections and other systemic or autoimmune diseases were excluded during screening. Each patient fulfilled at least 4 of the 11 diagnostic criteria defined by the American College of Rheumatology [12], and the severity of the SLE was evaluated using the SLE Disease Activity Index 2000 score [13]. The clinical characteristics are summarized in Table 1. Peripheral blood mononuclear cells (PBMCs) from 14 patients and 9 controls were isolated from approximately 20 mL heparinized venous blood by Ficoll-Hypaque centrifugation. These samples were used for isoelectric focusing (IEF) and two-dimensional electrophoresis (2-DE). The PBMCs of other 47 patients and 31 volunteers were separately isolated from approximately 5 mL blood for use in Western blotting. The sera of 123 patients and 113 volunteers were collected in succession before use. So did the platelet-poor plasma (PPP) from 30 patients and 30 volunteers. This study was approved by the ethics committee of the hospital and a written formal consent of the participant was signed before every acquisition.

Table 1
Characteristics of the SLE patients involved in the study.

Parameter	No.
Sum	214
Gender	Female
Age: Mean (SD) [range] years	34.0 (11.0) [11–60]
Duration: Mean (SD) [range] months	45.4 (50.7) [1–348]
Rash	119 (55.6%)
Photosensitivity	50 (23.4%)
Raynaud phenomenon	30 (14.0%)
Mucosal ulcers	8 (3.7%)
Arthritis	57 (26.6%)
Serositis	5 (2.3%)
Hematuria	35 (16.4%)
Proteinuria (positive or >150 mg/d)	58 (27.1%)
Pyuria	43 (20.1%)
Neurologic disorder	3 (1.4%)
Vasculitis	8 (3.7%)
Thrombosis	9 (4.2%)
Fever (>38 °C)	10 (4.7%)
Anti-nuclear antibodies	208 (97.2%)
Thrombocytopenia ($<100 \times 10^9$/L)	105/18 (17.1%)[a]
Leukopenia ($<4 \times 10^9$/L)	106/33 (31.1%)[a]
Anti-dsDNA antibodies	212/50 (23.6%)[a]
Anti-Sm antibodies	212/88 (41.5%)[a]
Anti-Cardiolipin IgG	138/52 (37.7%)[a]
Low complement 3 (<0.6 g/L)	102/41 (40.2%)[a]
Prednisone: Mean (SD) [range] mg	14.0 (17.6) [0–60]
Immunosuppressant	82 (38.3%)

[a] Because some participants from outpatient had not received these laboratory examinations, the three values sequentially represent the patients who received these examinations, the positive patients and the percentage.

2.3. IEF and 2-DE

Following the protocols used previously with few modifications [14,15], the soluble proteins of the PBMCs were extracted by detergent lysis. Once quantitated by the 2-D Quant Kit, the individual lysate from every participant was rehydrated with 18 cm IPG non-linear strips overnight. IEF was performed according to the following parameters: 3 h at 30 V, 1 h at 200 V, 1 h at 500 V, 1 h at 1000 V, upgrading to 8000 V within 30 min and held constant until the total voltage × time reached approximately 60 kV h. After equilibrated twice, the strips were loaded on a 12.5% sodium dodecyl sulfate polyacrylamide gel (SDS–PAGE) and electrophoresed at 40 mA per gel. All electrophoresed gels were stained using the Silver Stain method improved by Gharahdaghi et al. [16] and scanned with a GS-800 Calibrated Densitometer.

2.4. Image analysis and MS identification

The scanned images were analyzed using PDQuest software version 7.1. The image pairs of higher quality were chosen as primary match gels, which were used to check the match rates of the spots. Based on the analytical match rates of the software, the highest quality pair among the primary gels was chosen as the actual match gels. Spots with a gradation ratio greater than 2 or less than 0.5 were marked on the match gels. The spots from the other images in both groups were compared to the marked spots on the match gels. After statistically confirming the reproducibility of the gradation diversity, spot excision and tryptic digestion were appropriately executed [15]. The supernatant of the prepared digested peptides was loaded on an ion-trap high-performance liquid chromatography chip tandem mass spectrometer (MS), according to manufacturer's manual (Agilent Technologies, USA). Peptide sequence tags (PSTs) from each spot were obtained. Acquired PSTs were searched in the UniProtKB/Swiss-Prot database. After excluding duplicate and unreasonable results based on the molecular weight (MW) gradient, pH of isoelectric point (pI) level and distribution *in vivo*, the proteins with the greatest number of matched PSTs were considered of actually differential expression.