TITLE:

“DNASE 1 ACTIVITY IN SYSTEMIC LUPUS ERYTHEMATOSUS PATIENTS
WITH AND WITHOUT NEPHROPATHY”

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Running head: Dnase1 activity in SLE glomerulonephritis

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Objective: To determine whether the activity of DNase1 is associated to the presence of nephropathy in patients with SLE.

Methods: Forty-five patients affected with SLE and renal involvement were analyzed. The type of renal involvement was type III or IV glomerulonephritis. At least two serum samples were withdrawn from each patient, one obtained in a renal flare and the other obtained in a period of clinical stability. C3 and C4 complement levels and anti-DNA antibodies were determined. DNase1 activity was measured by using a radial enzyme diffusion method.

Results: When comparison of DNase1 activity was established between samples obtained during a phase of active renal involvement and those obtained in the clinically stable phase, we did not find statistically significant differences. When the comparison was performed with matched samples of the same patient, DNase1 activity was lower when patients had active renal involvement than when samples were taken in clinically stable phase ($21.21 \mu\text{g/ml} \pm 16.47$ vs. $25.62 \mu\text{g/ml} \pm 18.81$; $p < 0.05$). None difference in DNase1 activity was observed between samples positive or negative for anti-DNA antibodies. None difference in DNase1 activity was found in patients with normal or decreased levels of C3 ($25.09 \mu\text{g/ml} \pm 17.78$ versus $20.01 \mu\text{g/ml} \pm 16.15$ - $p = 0.073$ -) or C4 ($23.52 \mu\text{m/ml} \pm 16.60$ versus $19.62 \mu\text{g/ml} \pm 17.54$ - $p = 0.060$ -).

Conclusions: Low DNase1 activity is associated to the active phase of type III or IV nephropathy. Therefore, it is possible that this enzyme play an important role on the development of SLE nephropathy.

Abbreviations: systemic lupus erythematosus (SLE), double-stranded DNA (dsDNA), American College Rheumatology (ACR).

Keywords: DNase1 activity, Systemic Lupus Erythematosus, autoantibodies, nephropathy.

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease of unknown aetiology characterized by the production of a wide range of autoantibodies. More than 100 different autoantibodies have been identified in the sera of patients with SLE [1]. In particular, anti-DNA antibodies are a hallmark of SLE and may induce renal failure. It has been shown that anti-DNA antibodies and DNA-anti-DNA antibodies complexes are deposited on many organs, including the kidney, which leads to tissue inflammation [2, 3]. The incidence of clinical immune complex-mediated glomerulonephritis in SLE patients range between 30-50% [4-7]. Different studies suggest a strong correlation between increasing levels of anti-DNA antibodies and subsequent activation of the disease [8, 9]. An abrupt, marked increase of these auto-antibodies is usually followed by exacerbations of the disease. Exacerbations of glomerulonephritis, vasculitis, or both are the manifestations most likely to be heralded by rising titers of antibodies to ds-DNA.

Auto-antigens from apoptotic cells released during apoptosis are thought to drive the auto-immune response in human SLE [10-12]. During apoptosis, chromatin is cleaved by specific DNases and nucleosomes (the fundamental units of chromatin), are released [13]. Nucleosomes contain structures (DNA and histones) that are targeted in lupus and it provides a link between apoptosis and SLE.

DNase1, a $\text{Ca}^{2+}/\text{Mg}^{2+}$ - dependent enzyme, is the major nuclease found in body fluids such as serum and urine [14, 15]. DNase1 has also been regarded

as a candidate apoptotic endonuclease [16, 17]. We have previously measured DNase1 activity in different clinical and immunological settings in SLE patients [18]. In this work, we set out to test the hypothesis that DNase1 activity is related to the presence of lupus nephritis.

MATERIALS AND METHODS

The study was performed by the Department of Systemic Autoimmune Diseases at the Vall d'Hebron Hospital, a tertiary hospital in Barcelona, Spain, with a referral population of 400,000 people. Between September 2000 and October 2007 we collected 45 SLE patients with renal involvement (10 men - 22.2%- and 35 women -77.8%-). All the patients fulfilled at least four of the American College of Rheumatology (ACR) criteria. The type of renal involvement was type III or IV glomerulonephritis (according to the World Health Organization (WHO) classification), and it was diagnosed by altered urinoanalysis, proteinuria, decreased complement levels, and biopsy. Clinical stability was defined if SLEDAI was < 4 and no changes in BILAG score. Overall, we collected a total of 125 samples, 49 (39.2%) being obtained at renal flare and 76 (60.8%) being withdrawn during the stable phase. Serums were frozen and stored at -80°C. When more than one sample were obtained from a patient during a phase of stability or renal flare, we calculate a mean value. The study was approved by the ethical committee of our centre.

Anti-dsDNA antibodies levels were measured by an enzyme-linked immunosorbent assay (Quanta Lite TM dsDNA ELISA from Inova Diagnostics, Inc. San Diego, USA). Positivity was considered when achieving > 15 U/mL. Complement levels were measured by a nephelometric method (normal levels: 85.0 - 180.0 mg/dL for C3, and 10.0 - 40.0 mg/dL for C4).

DNase1 activity was measured by means of a radial enzyme-diffusion method described by Chitrabamrung *et al.* [19, 20], modified by Macanovic *et al.* [21] and adapted by us. The method is based on the hydrolysis of DNA in a DNA-agar plate. To perform the DNA-agar plate we used calf thymus DNA (Sigma, St Louis, USA) and low melting point agarose (GIBCO-BRL, United Kingdom). We also used recombinant DNase1 (Pulmozyme Dornasa Alfa, Roche Farma, Madrid, Spain) in order to create the standard curve. The area of hydrolysed DNA was identified after staining with Sibr Green I (Sigma, St Louis, USA). Digital images were acquired with a BioDoc-it™ UVP system.

Data were analyzed by the statistical program SPSS 12. Statistical analyses were performed by using the Kolmogorov-Smirnov test and the Mann-Whitney U test. To analyze matched samples we use the Wilcoxon test. We performed the Spearman test to determine the correlation between complement levels and DNase1 activity.

RESULTS

Twenty-six samples (21.66%) were negative for anti-DNA antibodies, whereas 94 samples (78.33%) were positive. Data from 5 samples were not available. The clinical stability phase was not always associated to an anti-DNA negative seroconversion.

With regard to the C3 complement factor we found that 72 samples had low levels (57.6%), whereas 53 (42.4%) samples had normal levels. Forty-five samples (36%) had low levels of C4 complement factor, whereas 80 (64%) samples had normal levels.

When comparison of DNase1 activity was established between the overall samples obtained during an active renal involvement and those obtained in the

clinically stable phase, we did not find statistically significant differences ($20.78 \pm 16.16 \mu\text{g/ml}$ vs. $22.17 \pm 17.20 \mu\text{g/ml}$) ($p=0.524$).

However, when the comparison was performed with matched samples of the same patient, we observed that DNase1 activity was lower when patients had active renal involvement than when they were clinically stable ($21.21 \pm 16.47 \mu\text{g/ml}$ vs. $25.62 \pm 18.81 \mu\text{g/ml}$; $p<0.05$).

None difference was observed in DNase1 activity of samples that were anti-DNA positive with respect to those which were negative ($20.85 \mu\text{g/ml} \pm 16.80$ vs. $26.77 \mu\text{g/ml} \pm 17.09$; $p=0.063$).

None difference in DNase1 activity was observed between patients with normal or decreased C3 levels ($25.09 \mu\text{g/ml} \pm 17.78$ versus $20.01 \mu\text{g/ml} \pm 16.15$ respectively; $p=0.073$). Similar results were obtained when considering C4 levels ($23.52 \mu\text{g/ml} \pm 16.60$ versus $19.62 \mu\text{g/ml} \pm 17.54$; $p=0.060$). No correlation could be established between C3 or C4 levels and DNase1 activity.

DISCUSSION

The exact aetiology of SLE is unknown but antibodies against nucleosomes and anti-DNA are a serological hallmark of this disease and have been clearly involved in the immune complex-mediated lesion in the kidneys [22]; they have also been widely used to evaluate the clinical activity in SLE [23, 24]. The formation of the SLE autoantibodies seems to be a T-cell dependent and an autoantigen-driven process [25].

Apoptotic cells are the unique source of nucleosomes, which are formed through cleavage of chromatin by nucleases [26-28]. At physiological condition, apoptotic cells, which maintain their membrane integrity for a certain time, have

to be cleared quickly and efficiently by phagocytes in order to prevent the release of tissue-damaging intracellular constituents [29].

Disturbances in either apoptosis or the phagocytosis of apoptotic cells have been proposed in the development of SLE [30-32].

DNase1, the major serum nuclease, is responsible for the degradation of the chromatin released by inappropriately cleared dead cells [16, 33]. Furthermore, DNase1 in cooperation with the plasminogen system guarantees a fast and an effective breakdown of chromatin during necrosis by the combined cleavage of DNA as well as DNA binding proteins [17]. DNase1 and C1q also cooperate in the degradation of chromatin from necrotic cells [34].

Observations *in vivo* and *in vitro* have pointed out that DNase1 and SLE have a close relationship. Chitrabamrung *et al* [20] described that DNase1 activity was low in the serum of patients with SLE. These authors also found that DNase1 activity was particularly low in patients with renal disease. Macanovic *et al* [21] observed that mean serum concentrations of DNase1 in the murine lupus model NZB/NZW were lower than in normal mice.

In agreement with the results obtained by Chitrabamrung *et al*. [20], we also found that the mean DNase1 activity of samples obtained through active renal disease was significantly lower than those obtained in the inactive renal disease period. Some authors have demonstrated that the low DNase1 activity observed in these samples is not due to the lost of DNase1 in the urine [20, 21]. None relationship between proteinuria and DNase1 activity has been detected by our group either [18].

Patients with type III and IV glomerulonephritis classically have an increase of anti-DNA antibodies levels, which play a major role in the development of

human lupus nephritis [35]. Decreased DNase1 activity is related to an inappropriately clearing of dead cells, which leads to an increase in the number of cells that undergo secondary necrosis, that furthermore will promote the maturation of dendritic cells to full antigen-presenting capacity [32, 36, 37]. The maturation of dendritic cells to full antigen-presenting cells and an increase of chromatin fragments that escape from non-ingested apoptotic cells (and that can be directly immunogenic [32]) are two mechanisms that can promote autoimmunity. Since nucleosomes, anti-nucleosomes antibodies, and anti-DNA antibodies are hallmarks of some types of glomerulonephritis in SLE patients, decreased Dnase1 activity can initiate the process that drive toward the apparition of nephropathy in SLE patients.

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