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Research Paper

A Comparative Study Showing Systemic Lupus Erthematosus (SLE) Autoantibody Binding to Native Calf Thymus DNA, Native Chromatin and Nitric Oxide Modified Chromatin

Medical Science

KEYWORDS : Autoantibodies, Chromatin, NO-chromatin, Nitric oxide, SLE.

Dr.Sarah Afaq

Assistant Professor, Department of Clinical Biochemistry, College of Medicine, King Khalid University, Abha, Kingdom of Saudi Arabia

ABSTRACT

Objective: Antibodies to double stranded DNA (dsDNA) is a classic antibody that characterizes SLE. However; some SLE patients lack serum anti-dsDNA reactivity throughout the course of their disease. The dominant presence of antibodies against the exposed conformational epitopes on chromatin strongly suggests that the pathogenic immune response in lupus is driven by chromatin. Also, retrospective studies have suggested a role of NO in the pathogenesis of SLE by demonstrating elevated levels of NO in SLE patients.

Therefore, in the present study, the binding of SLE autoantibodies to native and NO modified chromatin has been investigated.

Method: In the present study, the binding of SLE autoantibodies with native calf thymus DNA, native chromatin and NO modified chromatin (NO-chromatin) were analyzed. As assessed by direct binding and inhibition ELISA, circulating SLE autoantibodies exhibited a high degree of specificity towards NO-chromatin in comparison to native chromatin and native calf thymus DNA ($p<0.001$) . Gel retardation assay further substantiated the enhanced recognition of NO-chromatin by SLE autoantibodies as compared to native chromatin.

Result: The data suggests that the NO radical modification of chromatin causes perturbations, resulting in the generation of neo-epitopes, and making it a potential immunogen.

Conclusion: The NO-modified chromatin or nucleosomes might be the antigen for the production of circulating autoantibodies in SLE.

Introduction

Lupus research has dramatically increased in recent years but the exact cause of the disease is unknown and there is still no consensus on whether it is a single condition or a group of related diseases. SLE is a chronic inflammatory disease believed to be a type III hypersensitivity response with potential type II involvement, characterized by the body's production of antibodies against the nuclear components of its own cells. In the United States alone, it is estimated that between 270,000 and 1.5 million people have lupus, making it more common than cystic fibrosis or cerebral palsy. The disease affects both females and males, though young women are diagnosed nine times more often than men. Characteristically, inflammatory skin lesions and multiple organ damage occur, thus, SLE has become a prototype of systemic autoimmune disease . Antibodies to double stranded DNA (dsDNA) are disease-specific and closely associated with renal involvement and disease flares[1] .However; some SLE patients lack serum anti-dsDNA reactivity throughout the course of their disease. Nevertheless, they are not free from renal disorders and disease flares [2].It is well known that DNA is not present in its naked form in the circulation of SLE patients, but is instead complexed with histones in the form of oligonucleosomes [3].There are relevant studies that confirm the pathogenic immune response in lupus is driven by chromatin. It has been proposed that a defect in the catabolism of chromatin may render it immunogenic. The change of chromatin's antigenicity by environmental factors and genetic background may be the common pathway to SLE pathogenesis [4].

Nitric oxide (NO) is a well known environmental pollutant and a multifaceted bioregulatory agent exhibiting an astonishing range of physiological and pathological functions ranging from suppression of pathogens to being a cytotoxic agent leading to DNA damage and cell death via apoptosis in both NO-generating and neighboring cells . Studies have shown that NO deaminates deoxynucleosides, deoxynucleotides and intact DNA at physiological pH and cause DNA strand breaks [5]. Previous studies have suggested a role of NO in the pathogenesis of SLE by demonstrating elevated levels of NO in these patients [6]. Murine models of SLE demonstrate abnormally high levels of NO compared with normal mice, while systemic blockade of NO production reduces disease severity .Futher ,in SLE patients increased apoptosis of circulating lymphocytes have been reported , which could lead to an overflow of the apoptotic cell material including nuclear components both normal and modified e.g. dsDNA, nucleosomes or histones in the serum of these patients. In addition, dysregulated apoptosis and/or phagocytosis may trigger and provide survival signals for autoreactive B cells [7].

It has been reported from our laboratory that damage to DNA by NO results in an increased binding of SLE autoantibodies [8, 9].Therefore, the binding of SLE autoantibodies to native and NO modified chromatin has been investigated.

Materials and Methods:

Materials

Sodium nitrite and Sodium dithionite were purchased from Bio-Rad laboratories (California, USA). Calf thymus DNA, anti-human IgG-alkaline phosphatase conjugate, p-nitrophenyl phosphate, Tween-20, Triton X-100 were procured from Sigma-Aldrich (St.Louis, MO, USA). Protein A-sepharose CL-4b was purchased from Genei (India). Polystyrene microtitre flat bottom ELISA plates were procured from NUNC (Denmark).

Methods:

Serum samples

Sera from SLE patients, who met at least four of the 1997 American College of Rheumatology (ACR) criteria for lupus, were chosen for the study. No subject had an active infection, known malignancy, tuberculosis, pregnancy or cirrhosis. Controls were age- and sex-matched to lupus patients and met the same exclusion criteria. Serum samples were heated at 56°C for 30 min before use of inactive complements.

Isolation of chromatin

Chromatin was isolated from fresh goat liver by the method of Bonner et al (10) with slight modifications. Briefly, 10 g of goat liver was homogenized with 200ml of saline-EDTA (0.075M NaCl, and 0.024M EDTA, pH 8.0). The homogenate was strained through 6-8 layer of cheese cloth. The filtrate was centrifuged at 1500g for 15 min. the pellet was homogenized in 40 ml of Tris buffer (0.05M, pH8.0) and sedimented at 10,000g for 15 min. This step was repeated once. The final pellet was suspended in 30 ml of Tris buffer (0.05 M, pH 8.0). Five milliliter aliquots of the above suspension were layered on 25 ml portions of 1.7 M sucrose (in 0.01 M Tris buffer, pH 8.0) contained in centrifuge tubes. The upper two thirds of each tube were gently mixed and the tubes were then centrifuged at 21,000 rpm for 3h in F0650 rotor (Beckman) at 4C. The pellets were suspended in 0.01 M Tris buffer, pH8.0, and dialyzed overnight against the same buffer. The dialyzed suspension was sheared in Virits homogenizer for 90 s, stirred for 30 min and then centrifuged at 10,000g for 30 min. The supernatant of the above is referred to as sheared liver chromatin.

Modification of chromatin by nitric oxide

Isolated chromatin was modified with nitric oxide generated by the reduction of sodium nitrite with sodium dithionite . Aqueous solution of chromatin in PBS (A260=2), 20 MM Tris-HCl buffer, pH 7.5, sodium nitrite (100 mM) and sodium dithionite (10 mM) was kept at 37C for 3 h. Extensive dialysis of the sample was done immediately at the end of incubation time to remove the salts. Native chromatin incubated with salts alone served as control.

Circular dichroic(CD) spectra of native chromatin and NO-chromatin

CD measurements of native and NO-chromatin in 0.01 MTris,pH8 were performed in 10 mm path length cells at a constant temperature of 4C.The wavelength chosen(a)200-250 nm(b)250-300nm.Concentration of chromatin was kept A260=1,O.D 1.7.Buffer was used to set baseline. Molar ellipticity $[\Theta]=(M/10cl)\Theta$ .Where, M is average molecular weight of nucleiotide or amino acid residue,c,concentration of DNA or protein in g/ml, l is path length of the cell(cm),Θ is measured ellipticity of the sample. M is 309 for DNA and 115 for protein. The unit of $[\Theta]$ is $[(\mathrm{deg\ cm}^2)/\mathrm{dmol}]$.

ELISA

An enzyme linked immunosorbent assay (ELISA) was performed on flat bottom 96 well polystyrene immunoplates . Briefly, the plates were coated with 100 ul of respective antigen (2.5 ug/ml) for 2h at room temperature and over night at 4C. After washing three times with TBS-T (20mM Tris, 2.68 mM KCl, 150 mM NaCl, pH 7.4 containing 0.05 % Tween 20), unoccupied sites were blocked with 1.5% bovine serum albumin in TBS (10 mM Tris, 150 mM NaCl, pH 7.4) for 4-6 h at room temperature. The test serum serially diluted in TBS-T (100ul/well) was adsorbed for 2 h at room temperature and overnight at 4C. Bound antibodies were assayed with anti-human IgG alkaline phosphatase conjugate using p-nitrophenyl phosphate as substrate. The absorbance of each well was monitored at 410 nm on an automatic microplate reader. Equal number of wells devoid of antigen coating served as controls and the plate was thereafter processed similarly.

Competition ELISA

Antibody specificity was ascertained by competition binding assay.Varying concentrations of inhibitors (0-20 ug/ml) were allowed to interact with a constant amount of antibody (1:100 diluted serum) for 2 h at room temperature and overnight at 4C. The mixture was added to antigen coated plates and the bound antibody was detected as in the direct binding ELISA.

Band shift assay

Band shift assay was performed for the visual detection of antigen antibody binding and immune complex formation . Electrophoresis was performed on 1 % Agarose gel in 40 mM Tris-acetate buffer (TAE), pH 8.0. A constant amount of antigen (2.0 ug) was incubated with increasing concentration (0-80 ug) of affinity purified IgG for 2h at room temperature and overnight at 4C before loading onto the gel. The gel was run at 30 mA for 2h, stained with ethidium bromide and visualized under UV light.

Purification of immunoglobulin G

Immunoglobulin G was isolated from SLE sera on a Protein A-Sepharose 4B affinity column. The homogeneity of isolated IgG was checked by polyacrylamide gel electrophoresis.

Results

Chromatin on modification with nitric oxide showed various structural changes as compared with its native form:

Chromatin when exposed to nitric oxide showed loss of peak sharpness and hyperchromicity (51%) at 260 nm as compared to native chromatin indicating structural modification, generation of DNA strand breaks which was further confirmed by nuclease S1 digestion, fluorescence and thermal denaturation studies of native and NO-chromatin (data not shown).

The study was done at (a)200-250nm ,the CD of chromatin in this region due to peptide/protein chromophores (b)250-300nm,the CD in this region due to DNA(bound DNA).There was change in ellipticity and peak shifts which indicated chromatin damage caused by nitric oxide. This was accompanied with changes in protein/peptide and/or protein-DNA organization and base modification along with formation of DNA strand breaks (Figure 1).

Recognition of NO-Chromatin by SLE autoantibodies

To probe the possible role of the NO radical in the pathogenesis of SLE, 10 sera showing high titer of anti-DNA autoantibodies were selected for binding to native and NO-chromatin.

Direct binding ELISA showed higher binding of SLE autoantibodies with NO- chromatin as compared with native chromatin:

Direct binding ELISA results exhibited substantially higher binding of these SLE autoantibodies with NO-chromatin relative to native chromatin (Figure 2). With normal human sera no appreciable binding was observed.

SLE IgG was purified by affinity chromatography on Protein A-Sepharose CL-4B column. The purified IgG was found to elute in a single symmetrical peak. SDS-PAGE of purified IgG under non–reducing conditions showed a single homogenous band (data not shown).

Competitive binding ELISA showed higher inhibition of SLE antibodies with NO-chromatin as compared with native chromatin or native calf thymus DNA :

The binding specificity of IgGs was investigated by inhibition ELISA using native chromatin, NO-chromatin and native calf thymus DNA as inhibitors. The microtitre plates were coated with calf thymus DNA. The average percent inhibition (+SD) of 10 purified SLE IgG binding to native calf thymus DNA, native chromatin and NO-chromatin was 49.5+7.8, 51.9+6.1 and 62.8+5.3 respectively (Table 1). On comparing the inhibition values of native chromatin, NO-chromatin, a p-value of <0.001 indicates significant difference in the binding of these two antigens. No significant change in the recognition of native DNA and native chromatin by SLE autoantibodies was observed.

The inhibition ELISA data indicate that NO-chromatin is a better inhibitor of naturally occurring autoantibodies in the majority of SLE IgG in comparison to native chromatin and native calf thymus DNA.

Visual detection of immune complexes between SLE IgG and NO-chromatin showed decrease antigen mobility and formation of high molecular weight immune complexes as compared with native chromatin:

The formation of immune complexes between native and NO-chromatin samples with SLE IgG was ascertained visually by band shift assay. A constant amount of antigen was incubated with varying amount of purified SLE IgG (0-80 ug) (Figure 3). In case of NO-chromatin, a decrease in antigen mobility and formation of high molecular weight immune complexes was observed (Figure 3.2). Simultaneously, a substantial decrease in the fluorescence of residual chromatin samples was observed. However, for native chromatin only retarded mobility was observed and no immune complex formation could be visualized (Figure 3 .1). Normal human IgG incubated with native and NO-chromatin samples did not show any immune complex formation (data not shown).

Discussion

SLE is a systemic autoimmune disorder characterized by a marked diversity of organ involvement and fluctuations in disease activity, although the pathogenesis process of SLE has not yet been clearly established . Serum antibodies reacting with chromatin or its predominant components, histones and DNA have been found in SLE, drug-induced lupus, several other disease states and in murine models of SLE (11). The nucleosome (or chromatin) is emerging as the most reactive substrate among nuclear antigens, 70-80% of SLE patients being positive (12,13). Anti-nucleosome or anti-chromatin antibodies might be a good marker of anti-DNA antibodies in negative SLE cases (14).

Nitric oxide (NO) is involved in the pathogenesis of infectious diseases, tumors, autoimmune processes and chronic degenerative diseases . Plasma nitrite concentrations were also found to be significantly higher in SLE patients compared with control subjects (15). In the presence of inflammatory stimuli, NO production is significantly increased through the amplified transcription of the NOS system (iNOS2). At high concentration, NO eliminates intracellular pathogens and kills tumor cells. But at the concentration at which NO is effective there it also damages normal cellular macromolecules for example by deamination of DNA, inhibition of DNA synthesis and ATP production . Apoptosis is an important mechanism of cell destruction by nitric oxide . Also, nucleosomes have been shown to be exposed at the cell surface in apoptosis leading to autoantibodies generation (16).

In view of the above findings the binding of SLE autoantibodies with native chromatin and NO-chromatin was examined. Isolated chromatin was modified by nitric oxide exposure. The CD spectra were taken at two different wavelengths, one for protein part of chromatin and second for DNA part in chromatin. The study indicated the loss of protein and DNA damage caused by nitric oxide along with changes in protein/peptide and/or protein-DNA organization and base modification/formation of strand breaks in DNA. Nitric oxide is known cause such damage. (5,17).

It was thought desirable to investigate the binding characteristics of naturally occurring SLE autoantibodies to native calf thymus DNA, native chromatin and NO-chromatin so that the possible involvement of NO-chromatin in SLE could be ascertained. Sera of SLE patients having high titer anti-DNA antibodies were collected. Of these, ten SLE IgG were found to show inhibition ranging from 35% to 60% with native calf thymus DNA. The preferential recognition of NO-chromatin (56%-73% inhibition) over native chromatin (45%-60% inhibition) was observed. Band shift assay further substantiated the preferential recognition of NO-chromatin over native chromatin with affinity purified SLE IgG.

It has been reported previously that DNA and polynucleotide after exposure to nitric oxide present a more discriminating antigen for the binding of SLE autoantibodies. Attempts to immunize mice with nucleosomes prepared in vitro have failed giving support to the notion that qualitative modifications of nucleosomes are necessary for autoantibody induction (18,19).

Therefore nitric oxide which known to cause cell death via apoptosis and serum nitrite levels are also reported to be elevated in SLE patients with increased apoptosis of circulating lymphocytes have been reported previously . This increased rate of apoptosis could lead to an overflow of the apoptotic cell material including both normal and modified nuclear components example dsDNA, nucleosomes or histones in sera of SLE patients. In addition, dysregualated apoptosis and /or phagocytosis may trigger and provide survival signals for auto reactive B-cells. Thus we conclude that chromatin after modification with nitric oxide presents unique antigenic determinants for the production of SLE autoantibodies.

Figure 1. Circular dichroic spectra of native(—) and NO-chromatin(---)(a)wavelength range for protein residues<250 nm,(b) wavelength range chosen for DNA bases>250nm.

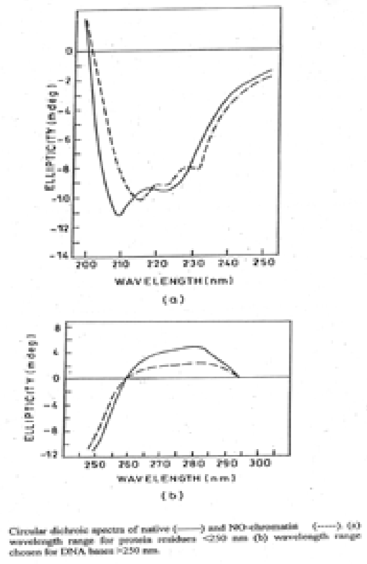


Figure 2. Direct binding ELISA of human SLE antibodies to native chromatin (□) and NO-chromatin (■).) Normal human sera (NHS) showed lower binding. Microtitre plates were coated with 2.5 ug/ml of respective antigen.

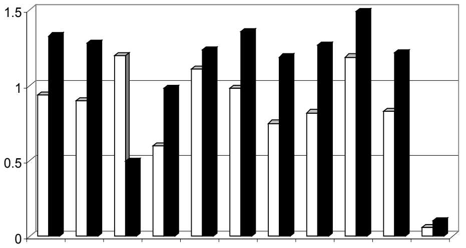


Figure 3. Band shift assay of SLE IgG binding to (a) native chromatin and (b) NO-chromatin. Antigen (2 ug) was incubated with buffer (lane 10 and 20, 40, 60 and 80 ug of IgG) through lane numbered 2-5 respectively in (a) .While 20, 60 and 80 ug of SLE IgG through lane numbered 2-4 in (b). The immune complex was incubated for 2 hr at 37° C and over night at 4°C. Electrophoresis was performed on 1% Agarose gel for two hours at 30 mA.

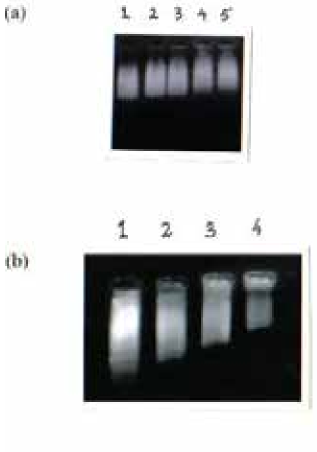
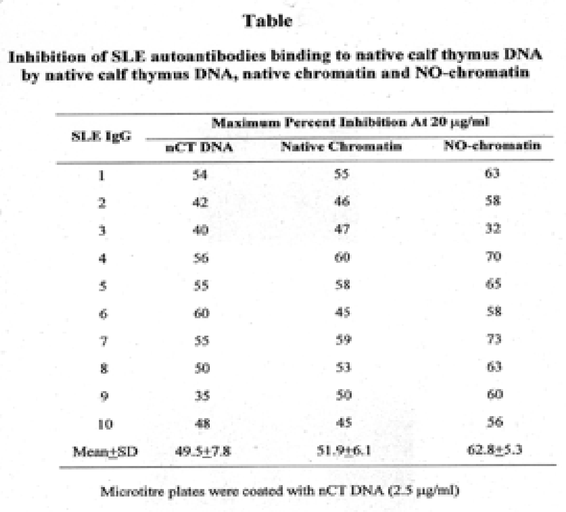


Figure 3

Volume : 2 | Issue : 5 | May 2013 • ISSN No 2277 - 8179



Table

Inhibition of SLE autoantibodies binding to native calf thymus DNA by native calf thymus DNA, native chromatin and NO-chromatin

SLE IgG	Maximum Percent Inhibition At 20 μg/ml		
	nCT DNA	Native Chromatin	NO-chromatin
1	54	55	63
2	42	46	58
3	40	47	32
4	56	60	70
5	55	58	65
6	60	45	58
7	55	59	73
8	50	53	63
9	35	50	60
10	48	45	56
Mean±SD	49.5±7.8	51.9±6.1	62.8±5.3

Microtitre plates were coated with nCT DNA (2.5 μg/ml)

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