PLASMA OSTEOPONTIN CONCENTRATION IN LUPUS NEPHRITIS

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KEY WORDS: PLASMA OSTEOPONTIN, LUPUS NEPHRITIS.

ABSTRACT

Aim: To measure plasma concentration of osteopontin and to correlate these levels with clinical, laboratory, disease activity and histopathological parameters in SLE patients.

Methodology: This study was performed on 20 SLE patients and 10 normal control subjects. Plasma osteopontin concentrations were measured by ELISA technique for both patients and control groups. All patients underwent renal biopsies within 3 months of onset of proteinuria or hematuria.

Results: There was a highly significant difference between patients and control groups as regards plasma osteopontin concentration (p<0.001). There was a significant positive correlation between osteopontin and SLEDAI (p<0.001) and activity index of renal biopsies (p<0.05). Also there was a highly significant differences as regard osteopontin plasma concentration between patients with and without renal affection (p<0.001).

Conclusion: Osteopontin has been shown at least partly to account for SLE nephritis probably through predominance of Th1-type response in both peripheral and renal tissue.

Recommendations: Further investigation of this mechanism in lupus nephritis may allow the design of new therapeutic strategies of lupus nephritis such as manipulation of Th1/Th2 and down-regulation of Th1-response.
INTRODUCTION

Systemic lupus erythematosus (SLE) is a prototypic systemic autoimmune disease characterized by various immunological abnormalities, including dysregulated activation of both T and B lymphocytes with the production of a large quantity of autoreactive antibodies (Kotzin, 1996).

It is widely recognized that Th cells when activated differentiate into at least 2 subjects, Th1 and Th2, characterized by distinct profiles of cytokine production. The balance between Th1 and Th2 cells seems to be important in much animal and human autoimmune disease. For instance Th2 type response is prevalent in SLE (Prete, 1998).

Renal involvement in SLE is one of the most serious complications and is of highly variable nature. Diffuse proliferative lupus nephritis (DPLN) is the most common, severe and important form of lupus nephritis. High percentage of patients with DPLN eventually progress to renal failure despite aggressive treatment (Kashgarian, 1997).

Akahoshi et al. (1999) identified a shift in cytokine expression in DPLN with predominance of IFN-γ- producing T cells. These data suggest an important role for cell-mediated immunity in pathogenesis of DPLN. Masutani et al. (2001) suggested that Th1 immune response is quite important in the development and progression of DPLN. Thus highlighting the importance of cellular immune mechanism in lupus nephritis.

Osteopontin (OPN) is an extracellular matrix cell adhesion phosphoprotein. It is also called T-lymphocyte activation protein (Eta-1) because of its early production upon cell activation and has been shown to enhance Th1 but inhibit Th2 response (Ashkar et al., 2000). It is expressed in mineralized tissue (bone and teeth) and damaged renal tissue (Chabas et al., 2001).

Renal biopsy provides the most accurate window into the kidney, because it provides information about class, severity, activity and chronicity of renal disease that cannot be accurately predicted on the basis of clinical manifestations (Edmund et al., 2001).

The aim of this work is to measure the plasma concentration of osteopontin and to correlate these levels with clinical, laboratory, disease activity and histopathological parameters in SLE patients.

PATIENTS AND METHODS

Twenty SLE patients diagnosed according to 1982 revised criteria of American College of Rheumatology (ACR) for diagnosis of SLE (Tan et al.,
were included in this study. They used to attend the outpatient clinic of Rheumatology and Rehabilitation Department as well as the Internal Medicine Department. Also, ten normal healthy subjects were included as a control group. They were matched for age and sex with SLE patients.

All subjects were subjected to the following:

- Full history taking.
- Thorough clinical examination.
- Laboratory determination include complete blood picture using Coulter Counter T660, ESR by Westergren method, serum creatinine with Synchroon CX5 autoanalyzer, complete urine analysis, quantitative 24-hours urinary protein excretion. ANA and anti-dsDNA with indirect immunofluorescence technique.
- Disease activity was evaluated with SLEDAI (Bombardier et al., 1992).
- Estimation of plasma osteopontin with ELISA:

The osteopontin levels in the plasma were measured with the osteopontin ELISA kit (Quantikine, R&D system, MN, USA). This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for OPN has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any OPN present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for OPN is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of OPN bound in the initial step. The color development is stopped and the intensity of the color is measured. Normal value (49-175 ng/ml).

- Renal biopsy was done for all patients within 3 months of the onset of proteinuria or hematuria. Informed consent for the procedure was obtained from each patient before renal biopsy.

The histopathology of lupus nephritis is extremely pleomorphic, the diversity is evident when comparing adjacent glomeruli in a single biopsy, and also histopathological features have the capacity to transform from one pattern to another either spontaneously or as a result of therapy (Brain et al., 2001). Histopathological features of lupus nephritis are classified by means
Activity and chronicity index were interpreted and scored by a pathologist (Austin et al., 1984). A semiquantitative histologic scoring system for lupus nephritis has been developed in an effort to predict renal outcome. The score of the activity index was the sum of: The scores (on a scale of 1-3) for endocapillary proliferation, karyorrhexis and fibrinoid necrosis (with the score for fibrinoid necrosis multiplied by 2), cellular crescents (with the score multiplied by 2), hyaline deposits, leukocyte exudation, and interstitial inflammation. The score on the chronicity index was the sum of the scores (on a scale of 1-3) for glomerular sclerosis, fibrous crescents, tubular atrophy, and interstitial fibrosis.

- The renal cores were evaluated by routine light microscopic evaluation, electron microscopic examination and finally immunohistochemical (IHC) staining for immunoglobulins using IgG, IgM, IgA and C3 antibodies.
  - Light microscopic examination of Hx and E (or PAs) stained; previously formalin fixed and paraffin embedded 4-5µm thick sections. Sections examined contained at least six glomeruli.
  - Electron microscopic examination of 60-80 nm thick sections double stained with uranylactate and lead citrate “previously examined by L/M of (1µm semithin) toluidine-blue stained sections.
  - Immunohistochemical staining (IHC):

  Avidin-biotin peroxidase complex method was used for IHC staining of coated slides from the paraffin sections. Deparaffinized sections were pretreated for antigen retrieval by incubation in an oven for 24 hours at 37°C. Sections were incubated in peroxidase block after excess buffer tapping for 5 minutes after which incubation with primary antibody “monoclonal mouse antihuman IgG, IgM, IgA and C3”: Life Trade Code No. N1508, N1507, N1509, A00b2 respectively or “the negative control reagent” for 60 minutes takes place. Then a labeled polymer is added for another 20 minutes. Enough amount of prepared liquid DAB and substrate chromogen solution was added for 10 minutes (Supersensitive immuno-detection system, Biogenix, Cat. No. QD0005L a horse radish peroxidase conjugated streptavidin and DAB chromogen/substrate mixture). Lastly hematoxylin counter stain (DAKO Code No. 53309) and mounting by aqueous based mounting medium (Code. No. C0563).

  Scoring of the IHC staining for the immunoglobulin is based according to the percentage of positive immunostaining in the glomeruli,
tubulointerstitium and the vascular wall ranging from +1 (<10%) up to +4 (>75%).

**Statistical Analysis:**

Data were expressed as mean and standard deviation. Student’s t test was used for comparison of the means. Correlation coefficients were used to detect association between two quantitative data. In all tests if p value > 0.05 it is considered not significant, if p value < 0.05 it is considered significant and if p value < 0.001 it is considered highly significant.

**RESULTS**

Twenty SLE patients (19 females and 1 male) were included in this study in addition to 10 normal healthy subjects as a control group matched for both age and sex. The age of the control group ranged from (19-30 years) with (mean 22.5 ± 3.6 years). As regards patient group their age ranged from (19-32 years) with (mean 22.1 ± 4.1 years). The disease duration ranged from (2-60 months) with (mean 20.3 ± 16.7 months). Renal biopsy was done for all patients within 3 months of onset of proteinuria or hematuria. Biopsy specimens were examined with light microscopy and showed that 10 patients were in class IV, 3 patients were in class V, 3 patients were in class III and 1 patient was in class II according to the WHO histopathological classification, and 3 patients their renal biopsies did not show any specific kidney disease (Figure 3-6).

- **Comparison between patients and control groups:**

  There was a highly significant differences as regards ESR, osteopontin, Hb% and RBCs count with higher ESR and osteopontin and lower Hb% and RBCs count in the patients than the control group (p<0.001).

  There was a significant difference as regards creatinine serum level in patients than the control group (p<0.05).

  There was no significant differences as regards age and leucocytic count (p>0.05) (Table 1).

- **Correlation between osteopontin and clinical, laboratory and histopathological parameters:**

  There was a significant positive correlation between osteopontin and creatinine, SLEDAI and activity index of renal biopsies (Fig 1, 2).

  There was a significant negative correlation between osteopontin and Hb% and RBCs count.
There was no significant correlation between osteopontin and age, disease duration, ESR, WBCs count, platelet count and chronicity index of renal biopsies (Table 2) (Figure 2).

- Comparing patients with and without renal affection:

There were a highly significant differences as regards osteopontin and total leucocytic count, with higher osteopontin and lower total leucocytic count in patients with than those without renal affection (p<0.001).

There were a significant differences as regards age, Hb%, RBCs count, creatinine and SLEDAI with lower Hb% and RBCs in patients with than those without renal affection (p<0.05).

There were no significant differences as regards disease duration, ESR and platelet count in patients with than those without renal affection (p>0.05) (Table 3).

Table (1): Comparison between SLE patients and control groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Patients mean±SD n=20</th>
<th>Control mean±SD n=10</th>
<th>t</th>
<th>p</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age/years</td>
<td>22.1 ± 4.1</td>
<td>22.5 ± 3.6</td>
<td>-0.30</td>
<td>0.777</td>
<td>(NS)</td>
</tr>
<tr>
<td>ESR mm/hr</td>
<td>64.4 ± 31.1</td>
<td>9.2 ± 4.5</td>
<td>5.5</td>
<td>0.000</td>
<td>(HS)</td>
</tr>
<tr>
<td>Hb% gm%</td>
<td>9.6 ±1.9</td>
<td>13.9 ± 0.9</td>
<td>-6.6</td>
<td>0.000</td>
<td>(HS)</td>
</tr>
<tr>
<td>RBCs 10⁹/mm³</td>
<td>3.5 ± 0.5</td>
<td>4.8 ± 0.4</td>
<td>-7.1</td>
<td>0.000</td>
<td>(HS)</td>
</tr>
<tr>
<td>WBCs 10³/mm³</td>
<td>5.8 ± 2.8</td>
<td>6.6 ± 1.1</td>
<td>-0.85</td>
<td>0.402</td>
<td>(NS)</td>
</tr>
<tr>
<td>Plat 10⁹/mm³</td>
<td>252.9 ± 77.9</td>
<td>264.1 ± 127.2</td>
<td>-0.25</td>
<td>0.802</td>
<td>(NS)</td>
</tr>
<tr>
<td>Creatinine mg/dl</td>
<td>2.1 ± 1.9</td>
<td>0.5 ± 0.2</td>
<td>2.2</td>
<td>0.033</td>
<td>(S)</td>
</tr>
<tr>
<td>Osteopontin ng/ml</td>
<td>355.0 ± 52.9</td>
<td>127.0 ± 23.1</td>
<td>12.9</td>
<td>0.000</td>
<td>(HS)</td>
</tr>
</tbody>
</table>

Table (2): Correlation between osteopontin and clinical, laboratory and histopathological parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>R</th>
<th>p</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>-0.385</td>
<td>0.203</td>
<td>(NS)</td>
</tr>
<tr>
<td>Disease duration</td>
<td>-0.361</td>
<td>0.118</td>
<td>(NS)</td>
</tr>
<tr>
<td>ESR</td>
<td>0.205</td>
<td>0.385</td>
<td>(NS)</td>
</tr>
<tr>
<td>Hb%</td>
<td>-0.482</td>
<td>0.031</td>
<td>(S)</td>
</tr>
<tr>
<td>RBCs count</td>
<td>-0.471</td>
<td>0.048</td>
<td>(S)</td>
</tr>
<tr>
<td>WBCs count</td>
<td>-0.314</td>
<td>0.178</td>
<td>(NS)</td>
</tr>
<tr>
<td>Platelet count</td>
<td>0.074</td>
<td>0.756</td>
<td>(NS)</td>
</tr>
<tr>
<td>Creatinine</td>
<td>.504</td>
<td>0.023</td>
<td>(S)</td>
</tr>
<tr>
<td>SLEDAI</td>
<td>0.807</td>
<td>0.000</td>
<td>(HS)</td>
</tr>
<tr>
<td>Activity index</td>
<td>0.533</td>
<td>0.028</td>
<td>(S)</td>
</tr>
<tr>
<td>Chronicity index</td>
<td>0.214</td>
<td>0.410</td>
<td>(NS)</td>
</tr>
</tbody>
</table>
Table (3): Comparison between SLE patients with and without renal affection groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Patients with renal affection mean±SD n=17</th>
<th>Patients without renal affection mean±SD n=3</th>
<th>t</th>
<th>p</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age/years</td>
<td>27.0 ± 5.6</td>
<td>21.2 ± 3.2</td>
<td>-2.6</td>
<td>0.019</td>
<td>(S)</td>
</tr>
<tr>
<td>Disease duration (months)</td>
<td>36.7 ± 30.6</td>
<td>17.4 ± 12.2</td>
<td>-2.00</td>
<td>0.060</td>
<td>(NS)</td>
</tr>
<tr>
<td>ESR mm/hr</td>
<td>69.1 ± 30.3</td>
<td>37.7 ± 23.7</td>
<td>1.7</td>
<td>0.108</td>
<td>(NS)</td>
</tr>
<tr>
<td>Hb% gm%</td>
<td>9.3 ± 1.8</td>
<td>11.6 ± 1.2</td>
<td>-2.2</td>
<td>0.043</td>
<td>(S)</td>
</tr>
<tr>
<td>RBCs 10^6/mm^3</td>
<td>3.4 ± 0.5</td>
<td>4.0 ± 0.3</td>
<td>-2.2</td>
<td>0.040</td>
<td>(S)</td>
</tr>
<tr>
<td>WBCs 10^3/mm^3</td>
<td>4.9 ± 1.9</td>
<td>10.6 ± 1.5</td>
<td>-4.8</td>
<td>0.000</td>
<td>(HS)</td>
</tr>
<tr>
<td>Plat 10^3/mm^3</td>
<td>266.8 ± 138.4</td>
<td>248.7 ± 13.6</td>
<td>0.21</td>
<td>0.827</td>
<td>(NS)</td>
</tr>
<tr>
<td>Creatinine mg/dl</td>
<td>3.3 ± 2.5</td>
<td>0.7 ± 0.3</td>
<td>2.7</td>
<td>0.017</td>
<td>(S)</td>
</tr>
<tr>
<td>SLEDAI</td>
<td>13.5 ± 4.5</td>
<td>4.0 ± 0.1</td>
<td>3.6</td>
<td>0.002</td>
<td>(S)</td>
</tr>
<tr>
<td>Osteopontin ng/ml</td>
<td>371.2 ± 38.1</td>
<td>363.3 ± 15.3</td>
<td>4.7</td>
<td>0.000</td>
<td>(HS)</td>
</tr>
</tbody>
</table>

Fig. (1): Linear regression curves showing a positive significant correlation between osteopontin and both SLEDAI and S. creatinine.
Systemic Lupus Erythematosus (SLE), the prototype human systemic autoimmune disease, is characterized by the production of pathogenic autoantibodies and tissue deposition of immune complexes, which result in multiple organ damage (Klinman & Steinberg, 1995).

It has been speculated that Th2 cells may play a pivotal role in the development of autoantibody-mediated autoimmune disease such as SLE (Goldman et al., 1991).

Akahashi et al. (1999) identified a shift in cytokine expression in DPLN with a predominance of IFN-γ producing T-cells. These data suggest an important role for cell-mediated immunity in the pathogenesis of DPLN. Osteopontin (ops, early T-lymphocyte activation protein 1, Eta-1) plays various biological roles for host defense, bone formation, osteoclast activation and wound healing (Ashkar et al., 2000). Its cytokine activities include the stimulation of macrophage and T-cell migration, protection against herpes viruses and bacterial infection through the activation of Th1 cell mediated autoimmunity.
Fig. 3: (A) Class III lupus nephritis with segmental endocapillary proliferation stained with PSA (PSA X200).
(B) Class III lupus nephritis: This shows the constituents of a cellular crescent. Bowmen capsule is seen in the upper right with two parietal epithelial cells in between, which an apoptotic body is seen (equivalent to hematoxylin body by light microscopy). (EM, Neg., Mag 4600).

Figure 4: (A) Class IV lupus nephritis with global glomerular hypercellularity, fibrocellular crescent and periglomerular fibrosis. There is extensive interstitial inflammation, epithelial cells in the tubular lumens, focal tubular atrophy and thick walled small arteriole. (H&E X 100).
(B) Class IV lupus nephritis: one of the glomerular capillaries shows extensive subendothelial electron dense deposits (SED) consistent with wire loop configuration seen by LM. Extensive SED and a hyaline thrombus occlude the other capillary in the lower side. Both capillaries showed few scattered sub-epithelial deposits. Glomerular basement membrane shows splitting and stratification. There is wide fusion of epithelial foot processes. (EM, Neg., Mag 2800).
Fig. 5: (A): Class V lupus nephritis, with almost normal mesangial hypercellularity and mild to moderate diffuse thickening of glomerular capillary walls (H& E X200). (B): Class V nephritis; the glomerular basement membrane is irregularly thickened with spikes formation around multiple sub-epithelial electron dense deposits. Focal intra-membranous deposits are seen. Parietal cell is swollen with wide fusion of its foot processes. (EM, Neg., Mag., 4600).

Fig. 6: (A) Class IV LN showing positive granular capillary wall and tubular basement membrane staining for IgG (IgG x 200) 
B) Class IV LN showing positive strong (3+) granular mesangial, and faint capillary wall staining (1+) for IgM (IgM x 200)
It is expressed in mineralized tissue (bone and teeth) and damaged renal tissue (Chabas et al., 2001). However, the circulating level of OPN in SLE patients and its correlation with disease activity and histopathological parameters has not been well defined.

The aim of this work was to measure the plasma concentration of osteopontin and to correlate these levels with clinical, laboratory, disease activity and histopathological parameters in SLE patients.

Lupus nephritis is an immune complex mediated glomerulonephritis. The pattern of immune complex localization in the glomeruli is the major factor in histopathological changes in lupus nephritis. The immune complexes may deposit preferentially in mesangial, subendothelial or subepithelial sites, depending on their site, charge, avidity, concentration and local formation of immune complexes by planting of antigen on glomerular basement membrane (Aruora & Piette, 2003).

In our study, the results of renal biopsies showed that 10 patients were in class IV, 3 patients were in class V, 3 patients were in class III and 1 patient was in class II. In human SLE with renal manifestations, marked heterogeneity is observed which can be explained by genetic, pathogenetic and environmental diversities (Klinman & Steinberg, 1995).

In our study there was a highly significant increase in plasma concentration of osteopontin in the patients group than the control group (p<0.001). Our results in agreement with that of (Wong et al., 2005). They found that osteopontin was significantly increased in SLE patients compared with controls, and these suggested that elevation of osteopontin is associated with inflammatory process and development of SLE.

Our results revealed that osteopontin was positively correlated with SLEDAI, again these in agreement with that of (Wong et al., 2005). Osteopontin can enhance the Th1 mediated inflammatory process, activation of NK, T cells and macrophage migration in the exacerbation of SLE (Masutani et al., 2001).

On comparing patients with and without renal affection, there was a highly significant increase in osteopontin plasma concentration in patients with than those without renal affection (p<0.001).

Osteopontin is a highly acid phosphoprotein that has a number of diverse biological functions such as cell adhesion and migration (Denhardt & Guo, 1993). Acting together with other proinflammatory cytokines including IL-1 and TNF-α, osteopontin may be an important cytokine for initiating and perpetuating Th1 immune response and renal derangement.
(Masutani et al., 2001 and Wuthrich et al., 1998) reported that osteopontin by tubular cells is a prominent feature of murine lupus nephritis and might be promoted by proinflammatory cytokine environment in MRL-Fas (1pr). The chronic upregulation of osteopontin could participate in the recruitment of monocyte in kidney of MRL-Fas (1pr) mice, thereby contributing to the pathogenesis of autoimmune renal disease.

In human and various animal models of lupus upregulated expression of osteopontin supports the functional role of this protein in glomerular macrophage infiltration and crescent formation (Hudkins et al., 2000). In fact osteopontin has been shown at least partly to account for SLE nephritis probably through the predominance of Th1 type response in both peripheral and renal tissue (Masutani et al., 2001).

Another intriguing result of our study was the correlation between osteopontin plasma concentration and the histologic activity index but not with chronicity index. Since activity index is a significant prognostic factor in lupus nephritis, measurement of osteopontin in plasma may be a useful parameter for estimating local histologic activity and the response to treatment, in addition to its prognostic value.

Conclusion:

Osteopontin has been shown at least partly to account for SLE nephritis probably through predominance of Th1-type response in both peripheral and renal tissue.

Recommendation:

Immunohistochemical analysis and staining of renal biopsies for osteopontin with its correlation with other histopathological parameters to confirm its role as a cytokine in early T1-mediated cellular immunity and renal derangement.

Further investigation of this mechanism in lupus nephritis may allow the design of new therapeutic strategies of lupus nephritis such as manipulation of Th1/Th2 and down-regulation of Th1 response.

REFERENCES


