Serum angiopoietin-2: vascular growth factor looking for a role in lupus nephritis in children with systemic lupus erythematosus

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Objective Several lines of evidence demonstrate excessive endothelial activation in systemic lupus erythematosus (SLE). Angiopoietin-2 (Ang-2) is an antagonistic ligand of the endothelial-specific Tie2 receptor. It is a biomarker of endothelial cell activation that may be clinically useful in SLE and lupus nephritis (LN).

Results Ang-2 concentration was significantly increased in patients with SLE in comparison with controls (P<0.001). However, the increase was significantly higher in patients with LN than in patients without LN. Ang-2 was significantly positively correlated with urinary proteins, C-reactive protein, serum creatinine, and platelets.

Conclusion Ang-2 may be used as a biomarker for renal involvement in SLE patients. However, a larger sample size study is needed to confirm this result. Med Res J 14:12–17 © 2015 Medical Research Journal.

Keywords: angiopoeitin-2, endothelial activation, lupus nephritis, systemic lupus erythematosus

Background Systemic lupus erythematosus (SLE) is one of the autoimmune diseases characterized by multisystem involvement associated with autoantibody and immune complex vasculitis along with endothelial cell damage [1]. Evidence is accumulating for endothelial cell dysfunction as one of the main factors initiating vessel wall damage in SLE [2]. In SLE, nearly every organ may be affected; however, the most commonly involved are the skin, joints, kidneys, blood vessels, and central nervous system. Lupus nephritis (LN) is one of the most serious manifestations of SLE; its clinical manifestation is highly variable, ranging from mild asymptomatic proteinuria and/or hematuria to rapidly progressive glomerulonephritis [3].

Histological evidence of LN is present in most patients with SLE, even when they do not yet have clinical manifestations [4]. SLE nephritis bears considerable morbidity and ~10% overall will progress to dialysis or transplantation, which represents a major threat to long-term quality of life and survival [5].

Angiogenesis plays a critical role in several pathological conditions. It has been reported that inflammation precedes and accompanies pathological angiogenesis, as evidenced by increased vascular permeability as well as monocyte, macrophage, and neutrophil recruitment at angiogenic sites [6].

During inflammatory processes, newly formed vessels supply inflamed tissue with oxygen and nutrients and facilitate the transport of inflammatory cells. In this process, the activation state of the endothelial layer is a major determinant for the initiation, localization, extent, and propagation of inflammatory damage [7]. The process of angiogenesis and microvascular endothelial injury has been considered in the pathogenesis of SLE [8]. Because angiogenesis and inflammation are two tightly linked processes, the search for factors that modify the inflammatory response among angiogenic growth factors has been conducted [9,10].

angiopoietins are a class of angiogenic growth factors that act selectively on the endothelial cells [11]. The angiopoietin family includes four ligands [angiopoietin-1 (Ang-1), Ang-2, Ang-3, and Ang-4], three of which are expressed in humans, namely, Ang-1, Ang-2, and Ang-4. All of these act on two corresponding tyrosine kinase receptors (Tie1 and Tie2). Angiopoietins, Ang-1 and Ang-2, are antagonistic ligands [12]. Suppression of plasma leakage, inhibition of vascular inflammation, and prevention of endothelial death are the main accepted vascular
Ang-2 was described initially as a natural endogenous Tie2 antagonist, thereby destabilizing existing vessels [14]. However, under certain circumstances, Ang-2 may induce Tie2 phosphorylation, and biological activities such as endothelial cell migration, platelet activation, neutrophil activation, and vascular permeability [15]. Glomerular Ang-2 expression is markedly upregulated in animal models of diabetic nephropathy and glomerulonephritis, and it has been hypothesized that Ang-2 might have significant roles in the pathogenesis of glomerular diseases [16].

The aim of this study is to assess the Ang-2 as a marker of renal involvement in patients with SLE and its correlation with disease activity.

**Patients and methods**

A total of 30 patients aged from 10 to 24 years, of whom 21 were female and nine were male were recruited in this study. They were chosen from the Rheumatology Clinic, Abu El Rish Hospital, Cairo University. They were diagnosed as having juvenile SLE, according to the American College of Rheumatology 1997 Revised Classification Criteria for SLE.

Patients were compared with 22 healthy age-matched and sex-matched controls. These controls were confirmed to have no medical illness, family history of SLE, or any other collagen vascular disease.

Our patients were submitted to full history taking, physical examination, laboratory investigations, abdominal ultrasonography, and renal biopsy to exclude nephritis. Of our patients, 16 (53%) were confirmed by renal biopsy to have nephritis of different grades, whereas the other 14 (47%) were free of nephritis. Written informed consents were obtained from all patients as well as an institutional board approval before starting the study.

**Sampling**

Seven milliliters of venous blood samples after fasting for 6–8 h were drawn from each child participating in the study. Two milliliters were drawn into tube containing EDTA for determination of complete blood picture on coulter counter T890 (coulter counter; Beckman Coulter Ltd., London, UK). In another tube containing 0.4 ml of citrate 1.6 ml of blood were put for determination of erythrocyte sedimentation rate using Westergren's method. The rest of the blood was drawn into a plain tube and left to clot, and the serum was separated by centrifugation at 3000g for 5 min. C-reactive protein (CRP) was determined immediately by rapid latex agglutination [17], and the rest of the serum was stored at −20 °C for determination of anti-DNA, anti-ANA, C3, kidney function tests (urea and creatinine), and Ang-2.

Our patients were submitted to:

1. Anti-DNA determined using indirect immunofluorescence assay on mouse kidney and stomach slides (Immuno-Diagnostics Inc., New York, USA). The slides were analyzed with Nikon epifluorescence microscope (Nikon Inc., Melville, New York, USA).
2. Complement 3 (C3) determined by single radial immune diffusion plates (Diffuplates; Biocientifica, New Delhi, India).
3. Complete urine analysis performed for the presence of red blood cells, pus, albumin, and urinary casts.
4. The determination of serum Ang-2 performed using quantitative sandwich enzyme immunoassay technique [18]. The enzyme-linked immunosorbent assay kit was supplied from R&D Systems Inc., Minneapolis, USA.

**Statistical analysis**

Data were statistically described in terms of mean ± SD, median and range, or frequencies (number of cases) and percentages when appropriate. Comparison of numerical variables between the study groups was made using Student t-test for independent samples in comparing two groups when normally distributed and Mann–Whitney U-test for independent samples when not normally distributed. Comparison of angiopoietin levels was made using one-way analysis of variance. For comparing categorical data, $\chi^2$-test was conducted. Fisher exact test was used instead when the expected frequency is less than 5. Correlation between various variables was done using Pearson correlation equation for quantitative normal variables and linear relation and Spearman rank correlation equation for qualitative data. $P$-value less than 0.05 was considered statistically significant. All statistical calculations were done using computer programs SPSS version 15 for Microsoft Windows (Statistical Package for the Social Science; SPSS Inc., Chicago, Illinois, USA).

**Results**

Table 1 shows demographic and clinical features of SLE patients with biopsy-proven LN in comparison with those without nephritis. The SLE patients with nephritis had similar mean age to those without nephritis. The duration of disease was slightly longer in the non-nephritic group with no significant difference. There is female predominance in both groups, but the difference is not statistically significant. With regard to the clinical features, there were no significant differences between SLE patients with and without nephritis except for serositis.

Table 2 shows laboratory features of SLE patients with and without nephritis, which revealed significantly higher prevalence of proteinuria in the nephritic group compared with those without nephritis.

Table 3 shows the serum level of Ang-2 in three groups: Controls, SLE without nephritis, and SLE with nephritis. The serum level of Ang-2 was analyzed in the control and systemic lupus patients without and with nephritis using analysis of variance test. It revealed a high significant difference in the serum level of Ang-2 between the three groups ($F = 197.930$ and $P < 0.0005$).
Table 1  Demographic and clinical features of systemic lupus erythematosus patients with and without lupus nephritis

<table>
<thead>
<tr>
<th>Variables</th>
<th>N = 16 patients</th>
<th>N (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean ± SD) (years)</td>
<td>16.9 ± 4.5</td>
<td>15.4 ± 4.7</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Duration (mean ± SD) (years)</td>
<td>5.6 ± 2.7</td>
<td>6.2 ± 2.7</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>9 (56)</td>
<td>12 (86)</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Male</td>
<td>7 (44)</td>
<td>2 (14)</td>
<td></td>
</tr>
<tr>
<td>Photosensitivity</td>
<td>3 (19)</td>
<td>2 (14)</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Nephrotic</td>
<td>2 (12.5)</td>
<td>0 (0.0)</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Epistaxis</td>
<td>1 (6)</td>
<td>1 (7)</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Alopeia</td>
<td>16 (100)</td>
<td>13 (93)</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Cerebritis</td>
<td>4 (25)</td>
<td>2 (14)</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Oral ulcers</td>
<td>3 (19)</td>
<td>2 (14)</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Hepatomegaly</td>
<td>1 (6)</td>
<td>1 (7)</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Splenomegaly</td>
<td>1 (6)</td>
<td>1 (7)</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Serositis</td>
<td>5 (31)</td>
<td>0 (0.0)</td>
<td>&lt; 0.05*</td>
</tr>
<tr>
<td>Lymphadenopathy</td>
<td>1 (6)</td>
<td>0 (0.0)</td>
<td>&gt; 0.05</td>
</tr>
</tbody>
</table>

*P<0.05, significant.
P>0.05, nonsignificant.

Table 2  Laboratory features of systemic lupus erythematosus patients with and without nephritis

<table>
<thead>
<tr>
<th>Variables</th>
<th>N = 16 patients</th>
<th>N (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBCs (mean ± SD) (106/m3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBCs (mean ± SD) (103/m3)</td>
<td>6.5 ± 2.9</td>
<td>6.9 ± 2.5</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Platelets (mean ± SD) (103/m3)</td>
<td>313.5 ± 101.7</td>
<td>234.6 ± 111.5</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Urea (mean ± SD) (mg/dl)</td>
<td>24 ± 16</td>
<td>25.3 ± 9.9</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Creatinine (mean ± SD) (mg/dl)</td>
<td>0.9 ± 0.6</td>
<td>0.6 ± 0.2</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>C3 (mean ± SD)</td>
<td>67.4 ± 49.2</td>
<td>66.9 ± 38.6</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>ESR (mean ± SD) (mm/h)</td>
<td>46.5 ± 22.6</td>
<td>25.1 ± 21.7</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Anti-DNA (U/ml) [n (%)]</td>
<td>11 (69)</td>
<td>7 (50)</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>ANA [n (%)]</td>
<td>14 (88)</td>
<td>9 (64)</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Coomb’s test [n (%)]</td>
<td>3 (19)</td>
<td>2 (14)</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>CRP (mg/l) [n (%)]</td>
<td>7 (44)</td>
<td>6 (43)</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Proteinuria (g/24 h) [n (%)]</td>
<td>11 (69)</td>
<td>3 (21)</td>
<td>&lt; 0.05*</td>
</tr>
<tr>
<td>Cast [n (%)]</td>
<td>3 (19)</td>
<td>0 (0.0)</td>
<td>&gt; 0.05</td>
</tr>
</tbody>
</table>

ANA, antinuclear antibody; C3, complement 3; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; RBC, red blood cell; WBC, white blood cell.

*P<0.05, significant.

Table 3  Mean ± SD and analysis of variance of serum level of Ang-2 in the three studied groups

<table>
<thead>
<tr>
<th>Variables</th>
<th>Controls</th>
<th>SLE without nephritis</th>
<th>SLE with nephritis</th>
<th>F</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ang-2 (mean ± SD) (ng/ml)</td>
<td>2.45 ± 0.67</td>
<td>6.9 ± 1.6</td>
<td>14.3 ± 2.8</td>
<td>197.930</td>
<td>&lt; 0.0005*</td>
</tr>
</tbody>
</table>

SLE, systemic lupus erythematosus.
*P<0.05, significant.

Table 4 shows correlation between some SLE clinical and laboratory parameters and the serum level of Ang-2. It demonstrates a significant positive correlation between Ang-2 levels with the platelets, CRP, serum creatinine, and urinary proteins.

**Discussion**

SLE is an autoimmune disease in which immunologically mediated vascular endothelial cell activation is regarded as a potential pathophysiological mechanism of systemic organ damage [19]. Endothelial cell activation and injury occur through a variety of stimuli and mechanisms, such as immunologically mediated vascular endothelial cell injury including cytokines, autoantibodies, and complement [20]. LN is one of the most serious manifestations of SLE, with increased risk of end-stage renal disease [4]. Early diagnosis of LN remains an important clinical challenge.

In the present work, the mean levels of serum Ang-2 serum levels were elevated in all SLE patients (with and without nephritis), compared with healthy controls which agreed with those described previously [9,21], they found that Ang-2 was significantly elevated in SLE patients. Moreover the current work showed that, serum Ang-2 levels were significantly higher in patients with LN than in those without nephritis. This is in accordance with the
endothelial cells and increases vascular inflammation. The angiogenic peptide Ang-2 activates Tie2 signaling and facilitates endothelial inflammation, and prevents recruitment and transmigration of leukocytes [13,29]. In contrast, binding of Ang-2 disrupts protective Ang-1/Tie2 signaling and facilitates endothelial inflammation [6,30]. The angiogenic peptide Ang-2 activates endothelial cells and increases vascular inflammation.

In this study, serum Ang-2 was correlated positively with serum creatinine that was also reported by David et al. [24] who reported that Ang-2 level increases with deterioration of renal function.

The Ang–Tie2 ligand–receptor system has a key regulatory role in regulating vascular integrity and quiescence, besides its role in angiogenesis; it is an important regulator of inflammation [7]. Upregulation of the angiopeptin system has been reported in chronic inflammatory disorders [27]. Ang-1 and Ang-2 are antagonistic ligands that bind with similar affinity to the extracellular domain of the tyrosine kinase with Ig-like and epidermal growth factor-like domains (Tie2) receptor, which is almost exclusively expressed by the endothelial cells [28].

Ang-1/Tie2 signaling maintains vessel integrity, inhibits vascular leakage, suppresses inflammatory gene expression, and prevents recruitment and transmigration of leukocytes [13,29].

In contrast, binding of Ang-2 disrupts protective Ang-1/Tie2 signaling and facilitates endothelial inflammation [6,30]. The angiogenic peptide Ang-2 activates endothelial cells and increases vascular inflammation.

Endothelial activation is characterized by increased vascular permeability, leukocyte adhesion, and transmigration [31].

Therefore, the Ang-2 has been regarded as the dynamic regulator within the Tie2 system, as it constitutes a Weibel-Palade body-stored molecule, which is rapidly released and induced upon endothelial stimulation [32].

It is noteworthy to mention that Ang-2 could be released by vascular endothelial cells, and is an important cytokine that participates in physiological activities and pathological events of endothelial cells [33]. Therefore, one of the mechanisms for the increase in circulating Ang-2 in SLE children could be the lack of endothelial nitric oxide that may predispose to a release of Weibel-Palade bodies that would theoretically increase serum Ang-2 level [34]. Moreover, another mechanism by which Ang-2 could increase vascular inflammation is by upregulating the response of endothelial cells to tumor necrosis factor-α and exerting a chemotactic effect on peripheral blood mononuclear cells that favors their migration, and thus the tissue infiltration by inflammatory cells [35].

Moreover, in this study, about 53% of our patients were found to have clinical evidence of LN, which is confirmed by renal biopsy. This result is comparable to that performed by Levy and Kamphuis [36] who estimated the same results, whereas Gilliam et al. [37] found a lesser percentage of patient with Juvenile Lupus Erythematosus suffering from renal involvement (37.5%).

In this study, when the patients with biopsy-proven nephritis were compared with those without nephritis with regard to all demographic features, only the duration of the disease and female sex showed a nonsignificant increase in those not suffering from nephritis. When we compare the clinical and laboratory features in SLE cases with and without nephritis, only serositis and proteinuria were found to be significantly higher in the nephritic than in the non-nephritic groups (< 0.05). This ensures that proteinuria interprets the occurrence of active LN [38].

In this study, a significant positive correlation was found between Ang-2 and proteinuria in SLE patients. This finding coincides with the results of other investigators [9,22] who found that Ang-2 level was upregulated in the glomeruli of patients with LN, and it has also significant positive correlation with proteinuria; therefore, the current study denotes that there is a close relation between circulating Ang-2 and vascular barrier function, presented by proteinuria as a marker for glomerular endothelial permeability. These findings are in line with increased glomerular expression of Ang-2 in the preclinical models of glomerulonephritis [39].

Davis et al. [40] provide evidence that the slit diaphragm protein nephrin, an essential component of the glomerular permselectivity barrier, is downregulated in Ang-2 overexpression mice; as such, Ang-2 is a candidate growth factor that might play a role in destabilizing glomerular endothelium, causing a breakdown of glomerular permselectivity in proteinuric renal diseases.
Furthermore, in this study, we found a positive correlation between Ang-2 level and platelet count. This might be explained by the important role of platelets in angiogenesis, which may be related to the released growth factors such as vascular endothelial growth factor and Ang-2 from platelets [41].

The lack of a significant correlation between anti-DNA antibodies and Ang-2 in the present work could be explained by the fact that all serum samples were taken after beginning steroids and/or other immunosuppressive treatments, and anti-DNA values fall very rapidly after treatment [42].

Conclusion
From these results, it could be concluded that serum Ang-2 may be used as a predictive biomarker of renal involvement in SLE patients. Furthermore, we suggest that Ang-2 could be introduced into routine investigations in the follow-up of SLE.

Acknowledgements
Conflicts of interest
There are no conflicts of interest.

References
الملخص العربي

دور عامل نمو الأوعية الدموية: الأنجيوبيوتين في حدوث التهاب الكلى عند الأطفال المصابين بمرض الذببة الحمراء

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مرض الذببة الحمراء عند الأطفال هو أحد أمراض المناعة الذاتية ويتميز بزيادة في نشاط الغشاء المبطن للأوعية الدموية. ووجد أن الخلل في عمل خلايا هذا الغشاء هو أحد الأسباب الرئيسية لحدوث التهاب الأوعية الدموية في هذا المرض. أن تأثير الكلى هو أحد أعراض الخطرة لمرض الذببة الحمراء. الأنجيوبيوتين2 هو عامل بيوولوجي يسبب زيادة في نشاط خلايا الغشاء المبطن للأوعية الدموية ويحفز عوامل التهاب في هذا الغشاء وخصوصاً في الكلى. الهدف من هذا البحث هو معرفة دور الانجوبيوتين2 في تأثير الكلى عند مرضى الذببة الحمراء كعامل مؤثر على الغشاء المبطن للأوعية الدموية ووسب التهاب الكلى. ودراسة العلاقة بين الانجوبيوتين2 وبعض المؤثرات في مرض الذببة الحمراء وقد قمنا في هذه الدراسة بقياس تركيز الانجوبيوتين2 في الدم عند 30 طفلاً منهم يعانون من مرض الذببة الحمراء في زعم 16 حالة مصابون بالتهاب الكلى. كما تم قياس نسبة الانجوبيوتين2 في الدم عند 22 حالة من الأطفال الإصحاء وقد وجدنا ان تركيز الانجوبيوتين2 قد زاد بشكل كبير عند مرضى الذببة الحمراء مقارنة بتركيزه عند الأطفال الإصحاء. كم ان هذه الزيادة كانت أعلى في المرضى المصابون بالالتهاب الكلى ووجدنا علاقة مطردة بين تركيز الانجوبيوتين2 في الدم مع نسبة البروتين في البول، الكرياتينين في الدم، عدد الصفائح الدموية ونسبة البرونتينات المتقلبة. ويتضح من هذا انه من الممكن الاستفادة بعامل الانجوبيوتين2 ككلامة بيوولوجية لتأثير الكلى عند مرضى الذببة الحمراء.