Adrenomedullin—a potential disease activity marker and suppressor of nephritis activity in systemic lupus erythematous

A. Mak, B. M. Y. Cheung, C. C. Mok\(^1\), R. Leung and C. S. Lau

Objectives. To investigate whether plasma adrenomedullin (AM) level is elevated in lupus nephritis and to examine if plasma AM level is correlated with systemic lupus erythematous (SLE) disease activity and severity of lupus nephritis after multivariate adjustment.

Methods. Consecutive SLE patients and healthy volunteers of age ≥16 were recruited from the rheumatology clinics of two hospitals in Hong Kong. SLE patients with nephritis fulfilled the American College of Rheumatology criteria for renal involvement and had percutaneous renal biopsy performed. Subjects were divided into three groups: (i) SLE patients with nephritis, (ii) SLE patients without nephritis and (iii) normal controls. The demographic and clinical variables were compared between these groups of patients and plasma AM level was determined by radioimmunoassay. Factors associated with plasma AM level were explored by regression analysis with adjustment of confounding factors.

Results. Sixty SLE patients (39 with nephritis and 21 without) and 23 normal subjects were studied. The plasma AM level of SLE patients was significantly higher than that of normal controls. SLE patients with nephritis had significantly higher plasma AM level than those without nephritis and normal controls \((P<0.001)\). In regression analysis, proteinuria was negatively associated with plasma AM level \((P=0.006)\) whereas SLE disease activity index was positively associated with plasma AM level after multivariate adjustment \((P=0.002)\).

Conclusions. Plasma AM is elevated in lupus nephritis, which correlates with lupus disease activity. It is negatively associated with urine protein excretion although it is unrelated to the type of renal pathology per se. Plasma AM may play a role to suppress the activity of lupus nephritis.

KEY WORDS: Adrenomedullin, Lupus, Nephritis, Disease activity, Proteinuria.

Introduction

Adrenomedullin (AM), a novel peptide consisting of 52 amino acids, was first identified in human phaeochromocytoma in 1993 [1]. While AM is rich in phaeochromocytoma, the peptide is also abundant in normal tissues such as adrenal medulla, lungs, kidneys and the heart [1, 2]. Besides having confirmed that plasma AM is elevated in certain disease conditions such as essential hypertension, chronic renal failure, chronic idiopathic glomerulonephritis and IgA nephropathy [3-8], AM has an intimate relationship with the immune system. Using radioimmunoassay (RIA) and RNA blot analysis, proinflammatory cytokines including interleukin (IL)-1\(\alpha\), IL-1\(\beta\), tumour necrosis factor (TNF)-\(\alpha\) and TNF-\(\beta\) effectively stimulated AM production in cultured rat vascular smooth muscle cells (VSMCs) [9]. Furthermore, interferon-gamma (IFN-\(\gamma\)) is capable of stimulating AM production in cultured macrophages in a dose- and time-dependent manner [10]. Since systemic lupus erythematous (SLE) is an inflammatory autoimmune condition characterized by a dysregulated milieu of pro-inflammatory cytokines, it is tempting to investigate whether plasma AM is elevated in SLE.

A cross-sectional study of 47 consecutive SLE patients showed that the plasma reactivity of AM was elevated in patients with SLE when compared with healthy controls and plasma AM level correlated with SLE disease activity [11]. In patients with active lupus, the plasma AM levels tended to be lower for those who were on immunosuppressive therapy than those who were not [11]. While plasma AM level is elevated in SLE, expression of AM in peripheral blood mononuclear cells (PBMCs) is suppressed in SLE patients. Using real-time quantitative PCR, it was demonstrated that patients with SLE had depressed AM messenger RNA (mRNA) level in PBMCs [12]. The AM mRNA expression in PBMCs was negatively correlated with SLE disease activity index (SLEDAI), anti-double-stranded (ds)DNA titre, blood urea nitrogen, serum creatinine (Cr), 50% haemolytic unit of complement and 24 h protein excretion in urine [12].

Knowledge of the relationship between AM and glomerulonephritis accumulated through experimental studies in cultured rat mesangial cells (MCs) and clinical studies of IgA nephropathy. Early experimental work demonstrated that AM augmented the cyclic adenosine monophosphate (cAMP) content in cultured rat mesangial cells [14]. In a subsequent study performed by Chi et al. [15], the presence of AM was confirmed immunohisto logically in mesangial and microvascular areas of rat glomeruli. Furthermore, AM was able to suppress the generation of reactive...
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Assessment of disease activity of SLE

The overall disease activity of lupus patients was measured by the SLEDAI [19]. The SLEDAI is a validated tool and has been shown to be reliable even when used by inexperienced physicians [20, 21]. The SLEDAI has also been shown to be sensitive to change in disease activity [22, 23].

Renal histopathology

All patients with renal involvement had percutaneous renal biopsy performed by nephrologists after obtaining their written informed consents. Renal biopsy samples were immediately transferred to the pathology departments of the respective hospitals. The biopsy specimens were fixed, sectioned and examined under high-power light microscopy after haematoxylin and eosin staining (with or without electron microscopy). The renal biopsy specimens were examined by the same histopathologists of the respective hospitals who were blinded for the clinical information of the patients. Renal histopathology was classified according to the World Health Organization (WHO) criteria for lupus nephritis [24].

Plasma adrenomedullin assay

All patients with SLE and normal subjects had plasma AM determined at the time of recruitment. Venous blood of 5 ml were sampled from the antecubital veins of all subjects and the blood sample of each was decanted to an edetate (EDTA) and a plain gel bottle immediately after venepuncture. All the samples were immediately transferred to the respective biochemistry departments and were centrifuged and stored at −70°C until being retrieved for analysis.

Plasma immunoreactivity of AM was determined using ELISA and was analysed by a commercial radioimmunoassay kit (Peninsula Laboratories, Belmont, CA, USA). Plasma samples of 3 ml were acidified with 0.75 ml of 2 mol/l hydrochloric acid and centrifuged at 1500g for 10 min. The supernatants were loaded onto Sep-Pak C18 cartridges (Waters Associate, Milford, MA, USA), which were activated with 100% methyl alcohol and double-distilled deionized water. The cartridges were subsequently washed twice with 5 ml of 0.1% trifluoroacetic acid (TFA) and eluted with 60% acetonitrile in 0.1% TFA. The eluates were then dried under vacuum overnight and resuspended in 250 ml of radioimmunoassay buffer. Standard AM or assay sample of 100 ml were incubated overnight at 4°C with 100 μl of rabbit anti-AM antiserum. One hundred micro litres of 125I-AM (18 000 cpm) was added to each tube and incubated for another 24 h. Using a goat antirabbit antiserum, antibody-bound AM was precipitated and counted in a gamma counter. A standard curve was constructed using serial dilutions of freshly reconstituted synthetic human AM. The detection limit was 1 pg/tube or 0.14 pmol/l AM in plasma. All samples were above the limit of detection. The intra-assay coefficient of variation was 11%. The plasma level of AM was expressed as picomole per litre.

Statistical analysis

Unless otherwise specified, values were expressed as mean ± s.d. Comparison of categorical data was made by the chi-square test. Fisher’s exact test was used instead when the expected frequency was small. Comparison of continuous data was made by using the Student’s t-test and the one way analysis of variance when analysis was made between two groups and more than two groups, respectively. Mann-Whitney U-test and Kruskal-Wallis test were used where appropriate if the data did not follow a normal distribution or if equal variance could not be assumed.
Associations between clinical and laboratory variables and the plasma AM level were studied using simple and multiple linear regression. The following covariates were tested for their relationship with plasma AM level: age, sex, duration of SLE, presence of nephritis, class IV nephritis, SLEDAI, serum C3 level, Hb level, proteinuria, serum Cr, serum albumin, anti-dsDNA level and prednisolone dose. For multivariate analysis, a stepwise procedure was adopted on the basis of a likelihood ratio test with $P > 0.1$ for removal and $P < 0.05$ for entry of variables. Analysis of residuals was employed to evaluate the validity of the regression equation [25].

Statistical significance was defined as a $P$-value of $< 0.05$, two-tailed. All analyses were performed using the SPSS program, version 11.5 (SPSS, Chicago, IL, USA) for Windows XP Professional.

This study was approved by the local regional Medical Ethics Review Committee and a written informed consent was obtained from all participating subjects.

## Results

### Demographic data of SLE patients and normal subjects

Sixty patients with SLE and 23 normal subjects were studied. The mean age of the SLE patients and control subjects were 36.8 ± 10.0 and 35.7 ± 10.7 yrs, respectively. The mean disease duration of SLE patients was 8.7 ± 6.4 yrs. The proportion of female subjects was 56/60 (93%) and 11/23 (48%), respectively, in SLE patients and the control group. Amongst the SLE patients, 39 (65%) had renal involvement at diagnosis or during the subsequent course of the disease (Table 1).

### Comparison of disease activity, corticosteroid use and laboratory data between groups

Subjects were divided into three groups: (i) SLE patients without renal involvement ($n = 21$); (ii) SLE patients with a history of renal involvement ($n = 39$) and (iii) normal controls ($n = 23$). Comparison of demographic, clinical and laboratory variables were made between these three groups of patients (Table 2). The mean age was similar between the three groups ($P = 0.489$) and the mean disease duration was similar between SLE patients with and without nephritis ($P = 0.417$). At the time of measurement of plasma AM level, patients with lupus nephritis demonstrated a significantly higher SLE disease activity as reflected by higher SLEDAI scores ($5.3 ± 3.9$ vs $2.9 ± 2.9$ units, $P = 0.013$) and more severe proteinuria ($2.2 ± 1.9$ vs $0.3 ± 0.1$ gm/day, $P < 0.001$) than those without nephritis. Disease activity as reflected by anti-dsDNA level tended to be higher in patients with nephritis than those without ($P = 0.077$). SLE patients with renal disease received a higher daily dose of oral prednisolone to control disease activity than those without lupus nephritis although it did not reach a statistical significance ($P = 0.327$). The serum C3 and Cr levels were similar between SLE patients with and without renal involvement, although the latter appeared to be higher in patients with nephritis ($94.6 ± 61.0$ vs $76.0 ± 11.4$ μmol/l, $P = 0.165$).

## Table 1. Demographic characteristics of SLE patients and healthy controls

<table>
<thead>
<tr>
<th></th>
<th>SLE patients ($n = 60$)</th>
<th>Normal controls ($n = 23$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± s.d.; Number (%)</td>
<td></td>
</tr>
<tr>
<td>Age, yrs</td>
<td>36.8 ± 10.0</td>
<td>7 ± 10.7</td>
</tr>
<tr>
<td>Female</td>
<td>56 (93%)</td>
<td>11 (48%)</td>
</tr>
<tr>
<td>Duration of SLE, yrs</td>
<td>8.7 ± 6.4</td>
<td>NA</td>
</tr>
<tr>
<td>Renal involvement</td>
<td>39 (65%)</td>
<td>NA</td>
</tr>
</tbody>
</table>

SLE, systemic lupus erythematosus; NA, not applicable.

## Table 2. Comparison of demographic, clinical and laboratory variables between groups mean ± s.d.

<table>
<thead>
<tr>
<th></th>
<th>SLE without GN ($n = 21$)</th>
<th>SLE with GN ($n = 39$)</th>
<th>Normal control ($n = 23$)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yrs</td>
<td>38.7 ± 9.4</td>
<td>35.7 ± 10.0</td>
<td>35.7 ± 10.7</td>
<td>0.489</td>
</tr>
<tr>
<td>Duration of SLE, yrs</td>
<td>7.6 ± 5.1</td>
<td>9.3 ± 7.0</td>
<td>NA</td>
<td>0.417</td>
</tr>
<tr>
<td>SLEDAI</td>
<td>2.9 ± 2.9</td>
<td>5.3 ± 3.9</td>
<td>NA</td>
<td>0.013</td>
</tr>
<tr>
<td>Plasma AM level, pmol/l</td>
<td>11.4 ± 5.9</td>
<td>20.9 ± 11.0</td>
<td>11.1 ± 5.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Proteinuria, gm/day</td>
<td>0.3 ± 0.1</td>
<td>2.2 ± 1.9</td>
<td>NA</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Prednisolone, mg/day</td>
<td>2.7 ± 3.3</td>
<td>11.0 ± 11.6</td>
<td>NA</td>
<td>0.327</td>
</tr>
<tr>
<td>Serum C3</td>
<td>71.0 ± 22.6</td>
<td>59.0 ± 35.9</td>
<td>NA</td>
<td>0.172</td>
</tr>
<tr>
<td>Serum Cr</td>
<td>76.0 ± 11.4</td>
<td>94.6 ± 61.0</td>
<td>NA</td>
<td>0.165</td>
</tr>
<tr>
<td>Anti-dsDNA</td>
<td>65.2 ± 80.7</td>
<td>117.0 ± 139.4</td>
<td>NA</td>
<td>0.077</td>
</tr>
</tbody>
</table>

GN, glomerulonephritis; SLEDAI, systemic lupus erythematosus disease activity index; AM, adrenomedullin; C3, serum complement of C3 fraction; Cr, creatinine; NA, not applicable.
AM, adrenomedullin; SLEDAI, systemic lupus erythematosus disease activity index; Cr, creatinine; C3, serum complement of C3 fraction.

Table 4. Determinants of plasma AM level by regression analysis

<table>
<thead>
<tr>
<th></th>
<th>Univariate</th>
<th>Multivariate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slope (SE)</td>
<td>R</td>
<td>P</td>
</tr>
<tr>
<td>Age, yrs</td>
<td>−0.184 (0.143)</td>
<td>−0.174</td>
</tr>
<tr>
<td>Sex, female</td>
<td>−3.910 (6.201)</td>
<td>−0.084</td>
</tr>
<tr>
<td>Serum Cr, μmol/l</td>
<td>0.001 (0.027)</td>
<td>0.034</td>
</tr>
<tr>
<td>Proteinuria, gm/day</td>
<td>0.319 (1.176)</td>
<td>0.054</td>
</tr>
<tr>
<td>Renal involvement of SLE</td>
<td>9.554 (2.537)</td>
<td>0.449</td>
</tr>
<tr>
<td>SLEDAI</td>
<td>1.065 (0.347)</td>
<td>0.385</td>
</tr>
<tr>
<td>Serum albumin, gm/l</td>
<td>−0.406 (0.228)</td>
<td>−0.238</td>
</tr>
<tr>
<td>Serum C3, gm/l</td>
<td>−0.074 (0.041)</td>
<td>−0.240</td>
</tr>
<tr>
<td>Anti-dsDNA level</td>
<td>0.028 (0.011)</td>
<td>0.329</td>
</tr>
<tr>
<td>Haemoglobin, g/dl</td>
<td>−1.635 (0.786)</td>
<td>−0.275</td>
</tr>
<tr>
<td>Prednisolone, mg/day</td>
<td>0.109 (0.161)</td>
<td>0.127</td>
</tr>
<tr>
<td>Class IV nephritis</td>
<td>6.249 (2.743)</td>
<td>0.291</td>
</tr>
</tbody>
</table>

SE, standard error; R, correlation coefficient; Cr, creatinine; SLE, systemic lupus erythematosus; SLEDAI, systemic lupus erythematosus disease activity index; C3, serum complement of C3 fraction.

Identification of associations of plasma AM levels and clinical parameters in regression analyses

Table 4 shows the results of regression analyses of variables that were associated with plasma AM level in SLE patients. Factors significantly associated with plasma AM level in univariate analysis were renal involvement of SLE, SLEDAI, Hb level, anti-dsDNA level and class IV nephritis. Daily prednisolone dose tended to be positively related to plasma AM level whereas serum albumin and C3 level tended to be associated with lower plasma AM level. Sex, age and duration of SLE were not associated with the plasma AM level.

In multiple regression analysis, proteinuria became negatively associated with plasma AM level ($R = -0.781; P = 0.006$) whereas SLEDAI was positively associated with plasma AM level independently ($R = 0.919; P = 0.002$). Class IV nephritis, renal involvement of SLE and prednisolone dose were not independently associated with plasma AM level after multivariate adjustment.

Discussion

Our study confirms that plasma AM level is elevated in patients with SLE compared with healthy controls. Since patients with disease conditions, known to affect plasma AM levels; namely cardiopulmonary disorders, diabetes mellitus, renal diseases, liver dysfunction and pregnancy [3–8], were excluded in the present study, the elevation of plasma AM level appears to be genuinely related to SLE itself. People with hypertension are known to have higher levels of plasma AM [8]. This is partly related to left ventricular hypertrophy and renal dysfunction [26]. Plasma AM level correlates poorly with blood pressure [27]. However, treatment with calcium channel blockers did not appear to reduce the elevated plasma AM level in patients [28]. Therefore, any antihypertensive agents taken by our patients were unlikely to be the cause of the increased plasma AM level. Undoubtedly, very subtle degrees of cardiac and renal dysfunction which were not clinically apparent could not be totally ruled out as contributing factors to the increase in plasma AM in those patients. Though we agree that such conditions may elevate the plasma AM level and render an elevation of AM level not clinically useful for diagnosis or monitoring lupus disease activity, the mechanism of AM elevation in SLE patients and more interestingly, the exact role of AM in the immunopathogenesis of SLE warrant further elucidation. Gathering from previous experimental studies, it appears that proinflammatory cytokines especially TNF-α, IL-6 and IL-1, which are pivotal in mediating the inflammatory process of SLE [29], stimulate the production in AM in various cell types in SLE. Macrophages, one of the key immune cell types actively involved in the immunopathological process of SLE [29], has been shown to be an important cell population account for the elevation of plasma AM [10]. Immune complex deposition with subsequent endothelial damage is also a key pathological phenomenon of SLE [29]. Endothelial cells are capable of producing AM [1] and upon oxidative stress stimulation, the endothelial cells exaggerate the production of AM to counteract the vasoconstrictive endothelin [30]. Thus, these mechanisms possibly explain the possible reason for, in SLE patients with active disease or when vasculitis is evident, the elevation of plasma AM level.

A positive association was found between SLEDAI and plasma AM level in multivariate analysis. This confirms the previous finding by Cheung et al. [11] who demonstrated a significant correlation between plasma AM level and SLEDAI by
glomerular cells is the same regardless of the histological class between plasma AM level, class IV nephritis and renal involvement. No sex difference was found [27]. Similar to a study of plasma AM level in the general population, between normal and female SLE patients would remain significant. If only male and female patients with SLE although the number of subjects was not large enough to detect a small difference. If only female controls were used, the difference in plasma AM level of lupus nephritis was shown.

The suppressed expression of AM mRNA in PBMC in patients with lupus nephritis and IgA nephropathy as shown in previous studies may signify an important underlining feedback mechanism [12, 16]. In IgA nephropathy, AM mRNA expression in PMBC was suppressed and the degree of suppression was more prominent in patients with active IgA nephropathy [16]. As IgA nephropathy somehow shares similar pathological features with lupus nephritis [32], the similar results regarding suppression of AM mRNA in PBMC in both types of nephritis underscore a pathologically pertinent relationship between AM and nephritis.

Though direct comparison is not feasible, AM mRNA expression in PBMCs in patients with lupus nephritis was also negatively associated with urine protein excretion [11]. When lupus patients were subclassified into those with active and inactive nephritis based on the activity index of the renal histopathology, it was found that in patients with active nephritis, AM mRNA expression was significantly lower than those with inactive nephritis [11].

As experimental studies showed that AM could suppress the proliferation of rat glomerular MCs [14] and the degree of proteinuria indicates the activity of nephritis (unless in chronic damaged kidneys, which did not happen in our patients), the finding of the inverse relationship between plasma AM and proteinuria supports the possible role of AM as an endogenous anti-proliferative peptide of mesangial and glomerular cells.

WHO class IV nephritis is the most severe type of lupus nephritis which carries the worst prognosis [31]. Comparison between WHO class IV and non-WHO class IV nephritis in our SLE patients failed to demonstrate a significant difference in the plasma AM level. Nevertheless, trends of higher plasma AM levels, SLEDAI, proteinuria and anti-dsDNA levels towards patients with class IV nephritis were shown.

Our study revealed no effect of gender towards plasma AM levels. Male and female controls were included because a percentage of our SLE patients were male. There was no statistically significant difference between the AM levels in our male and female patients with SLE although the number of subjects was not large enough to detect a small difference. If only female controls were used, the difference in plasma AM level between normal and female SLE patients would remain significant. Similar to a study of plasma AM level in the general population, no sex difference was found [27].

Our regression analysis showed no evidence of any association between plasma AM level, class IV nephritis and renal involvement of SLE. It implies that the behaviour of AM towards MCs and glomerular cells is the same regardless of the histological class of lupus nephritis. Higher plasma AM level in patients with class IV nephritis than those with non-class IV nephritis in univariate analysis may be explained by higher SLEDAI of the former.

Though corticosteroid administration is known to stimulate AM secretion in vitro [9], our regression analysis did not reveal a significant relationship between corticosteroid use and plasma AM level. In SLE patients, the effect of corticosteroids on plasma AM levels is likely to be complex. While corticosteroids may have a direct stimulatory effect on AM production, any suppression of disease activity might result in decreased stimulation of AM secretion. It was previously reported that in patients with more severe disease, those who had immunosuppressive therapy including the use of prednisolone had lower levels of AM than those who did not have any immunosuppression [11].

Gathering all the, as yet known, information regarding AM and SLE in the current multiple regression model and the previous experimental and clinical studies, we postulate the way AM is involved in the immunopathogenesis of SLE (Fig. 1). This model may hopefully guide the future direction of research in elucidating the exact role of AM in the pathogenesis of SLE and lupus nephritis.

There are several limitations with the current study. First, not all renal biopsies were taken during the period of plasma AM level measurement since they were performed for clinical indications rather than for research purposes. It was therefore invalid to assess
and correlate the renal activity index with plasma AM levels. Second, it is a cross-sectional study and we were unable to correlate the plasma AM level with various clinical variables, before and after effective immunosuppressive treatment. Third, if it is of interest to seek whether plasma AM level is correlated with disease activity of particular organ systems, correlation with the British Isles Lupus Assessment Group (BILAG) index may be more practical than with SLEDAI. The BILAG index is a computerized index based on the principle of the physician’s intention to treat, for measuring clinical disease activity in different organ systems [33]. Finally, enlargement of the sample size both in the SLE patients and normal controls will undoubtedly augment the fidelity of the results.

**Conclusion**

In conclusion, it is confirmed that plasma level of AM is elevated in patients with active SLE and plasma AM level is correlated with the disease activity of SLE. Higher level of plasma AM in SLE patients with nephritis may be related to more active disease in those with renal involvement. A negative association between plasma AM concentration and urine protein excretion in patients with lupus nephritis was hitherto found. Though the mechanism by which the elevation of plasma AM level in SLE patients remains unanswered, AM undoubtedly plays a pathologically important and significant role in the immunology of SLE. Plasma AM level is a potential candidate of disease activity marker for SLE patients and it probably provides a protective role in reducing the severity of lupus nephritis. With the accumulation of knowledge regarding the exact pathogenic role of AM, hopefully molecules targeting AM may become one of the potential therapeutic strategies in the management of SLE.

**Key messages**

- Plasma adrenomedullin level correlates with lupus disease activity.
- Adrenomedullin probably confers a protective role in alleviating lupus nephritis.
- Adrenomedullin may play an important role in the immunology of SLE.

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**References**