

Skin autofluorescence is increased in systemic lupus erythematosus but is not reflected by elevated plasma levels of advanced glycation endproducts

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Objectives. To examine whether skin advanced glycation endproducts (AGEs) accumulation, plasma levels of AGEs—N^ε-carboxymethyllysine (CML) and N^ε-carboxyethyllysine (CEL)—and serum levels of soluble receptor for AGEs (sRAGE) are elevated in SLE patients compared with controls, and whether these parameters are related to disease activity and endothelial cell (EC) activation.

Methods. Ten SLE patients (9 women, age 34 ± 13 yrs, mean \pm s.d.) and 10 age- and sex-matched controls were included. Patients were analysed during inactive as well as active disease. Skin AGE accumulation was estimated using ultraviolet-A (UV-A) light for measurement of autofluorescence obtained by Excitation–Emission matrix Scanner (AF-EEMS). Levels of CML and CEL were determined by tandem mass spectrometry. Levels of sRAGE and of soluble vascular cell adhesion molecule-1 (sVCAM-1) were determined by ELISAs.

Results. Skin AF-EEMS was increased in SLE patients compared with controls ($P < 0.05$). Levels of CML and CEL were comparable between patients and controls and were not influenced by disease activity. sRAGE and sVCAM-1 levels were higher in quiescent SLE patients compared with controls ($P < 0.05$) and increased further during active disease ($P < 0.05$). In patients with quiescent disease and controls, sRAGE levels correlated to sVCAM-1 levels ($r = 0.579$, $P = 0.007$).

Conclusions. Skin AGEs and levels of sRAGE and sVCAM-1 were elevated in SLE patients, whereas levels of CML and CEL were comparable with controls. As sRAGE even further increased during endothelial activation, it might be hypothesized that sRAGE acts as a decoy receptor. Why this proposed mechanism is insufficient to prevent increased AGE accumulation in the skin of SLE patients has to be established.

KEY WORDS: Systemic lupus erythematosus, Advanced glycation endproducts, Receptor for advanced glycation endproducts, Endothelial activation.

Introduction

SLE is associated with an increased prevalence of cardiovascular disease (CVD) due to accelerated atherosclerosis [1–4]. This cannot be fully explained by increased prevalence of traditional cardiovascular risk factors. Therefore, non-traditional risk factors such as advanced glycation endproducts (AGEs) and disease-related factors seem to be involved.

AGEs are a class of compounds resulting from glycation of proteins, lipids or nucleic acids. Glycation is the non-enzymatic addition or insertion of saccharide derivatives to these molecules. This leads to the slow formation of intermediary Schiff bases and Amadori products and finally to irreversible AGEs [5]. In circumstances characterized by increased oxidative and carbonyl stress, AGEs can be formed more rapidly [6, 7]. This may explain increased AGE formation in inflammatory conditions such as RA and SLE [8, 9].

Accumulation of AGEs in the vessel wall has been related to the development of atherosclerosis [10, 11]. AGEs are implicated in the development of atherosclerosis by three general mechanisms. First, cross-linking of AGEs with proteins in the extracellular matrix results in a decrease of vessel elasticity [12]. Second, intracellular AGE formation may alter cellular function [13]. Third, AGEs may modulate the function of cells by interaction

with and activation of the receptor for AGEs (RAGE) and other receptors.

RAGE is a multi-ligand type I transmembrane glycoprotein belonging to the immunoglobulin (Ig) superfamily that engages diverse ligands relevant to the pathogenesis of atherosclerosis.

Expression of RAGE is induced by both inflammatory cytokines, such as TNF- α , and AGEs [14]. AGE-RAGE-dependent interaction results in cellular activation.

RAGE also exists in several soluble forms, termed soluble RAGE (sRAGE). Soluble RAGE is formed by alternative splicing or by carboxyterminal truncation of RAGE through metalloproteinases [15, 16]. Administration of sRAGE consisting of the extracellular ligand-binding domain, not only suppressed the development of atherosclerosis but also stabilized established atherosclerosis in diabetic apolipoprotein E-null mice [17, 18]. Based on these observations, it might be hypothesized that sRAGE acts as a decoy receptor preventing AGE–RAGE interaction.

In this study, we analysed skin autofluorescence as an estimate of tissue AGE accumulation, blood levels of AGEs and sRAGE in relation to disease activity and endothelial cell activation.

Methods

Subjects

Ten consecutive patients (nine females, one male) fulfilling the ACR criteria for SLE [19] who attended the outpatient clinic of the University Medical Center Groningen, were included (Table 1). Disease activity was assessed by the SLEDAI and active disease was defined as SLEDAI > 4 [20]. Active disease, pregnancy and comorbidity such as diabetes and renal failure were exclusion criteria. Stored serum and plasma samples were available from the same patients during active disease. Ten age- and sex-matched healthy subjects were recruited as controls. All patients and controls were Caucasians, except for one Asian patient. The local

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research ethics committee gave approval for the study and informed consent was obtained from each participant.

From all participants information was obtained regarding presence of CVD, defined as a history of ischaemic heart disease (ICD-9 classification 410–414), cerebrovascular accidents or peripheral vascular disease. All traditional cardiovascular risk factors were assessed. Blood glucose and plasma lipid concentrations (cholesterol, high-density lipoprotein, low-density lipoprotein and triglycerides) were measured by routine techniques. BMI, smoking status, diabetes and family history of CVD (considered positive if first-degree relatives suffered from CVD before 60 yrs of age) were recorded.

Creatinine clearance was estimated using the Cockcroft–Gault formula. The use of corticosteroid therapy, AZA, cyclophosphamide and HCQ was categorized as current and none, and daily dose was recorded.

Assessment of AGE accumulation

Tissue AGE accumulation was assessed as skin autofluorescence, following the principles of the AGE Reader, which is a validated and non-invasive technique [21]. Repeated measurements in controls and diabetic patients showed an intra-individual Altman error percentage of 5.0% on a single day and 5.9% for seasonal changes [22].

In the present study, skin autofluorescence was assessed using the Excitation–Emission Matrix Scanner (AF-EEMS), using ultraviolet-A (UV-A) radiation, as described before [23]. Measurements were performed at a skin site without lesions, to prevent influences by the presence of skin disease in SLE patients. Skin pigmentation is also known to influence autofluorescence by light absorption. Therefore, skin reflection should be >10% to perform an adequate measurement. In this study, skin reflection was >10% in all subjects and did not differ between patients and controls.

Blood analyses

Lipid, glucose and creatinine levels were measured by routine techniques. In patients, levels of C3 and C4 were measured by nephelometry, and levels of antibodies to double-stranded DNA (dsDNA) by ¹²⁵I Farr assay. The aCLs and lupus anticoagulant were measured as described [24]. The aPL status was considered positive if the levels of either IgG or IgM aCLs were ≥40 IgG phospholipids units or IgM phospholipids units, respectively, or if lupus anticoagulant was present on more than one occasion.

Serum levels of sRAGE were measured using a commercially available ELISA (R&D systems, Minneapolis, MN, USA) according to the manufacturer's protocol. The sVCAM levels were determined with a similar ELISA kit (R&D systems) and levels of CRP were determined using an in-house ELISA as described before [25]. All methods were performed with an inter- and intra-assay coefficient of variation (CV) of <10%.

TABLE 1. Cumulative ACR criteria in SLE patients (*n* = 10)

	Patients (<i>n</i>)
Malar rash	5
Discoid rash	3
Photosensitivity	4
Oral ulcers	1
Arthritis	6
Serositis	3
Renal disorder	5
Neurological disorder	2
Haematological disorder	9
Immunological disorder	10
ANA	10

Detection of CML and CEL

Plasma levels of N^ε-(carboxymethyl)lysine (CML) and N^ε-(carboxyethyl)lysine (CEL) were measured in the protein fraction by stable-isotope dilution tandem mass spectrometry [26]. Intra-assay CV was 2.7% for CML and 6.9% for CEL.

Statistical methods

Power analysis revealed that 10 subjects in each group had to be included to detect a difference in AF of 0.45 AU with an s.d. deviation of 0.33 at a significance level of 0.05 with a power of 80%.

Values are expressed as median (25th to 75th centile). Comparisons between quiescent and active patients and age- and sex-matched controls were made by Wilcoxon matched-pairs test.

Associations between CML, CEL, sRAGE, skin AF and other disease related factors were assessed using Spearman's correlation test. All analyses were performed using SPSS 14.0 (SPSS Inc., Chicago, Illinois, USA). A two-sided *P*-value <0.05 was considered to indicate statistical significance.

Results

Characteristics of patients and controls

Characteristics of patients and controls are presented in Tables 2 and 3. Patients and controls showed a comparable profile of traditional risk factors. Only levels of triglycerides were increased in quiescent patients compared with controls. Levels of high-density lipoprotein cholesterol and creatinine clearance were lowered during active disease. Three patients used angiotensin-converting enzyme inhibitors (ACEi). Concerning aPL status, two (20%) patients were positive for either IgG or IgM aCLs; none of the patients was positive for lupus anticoagulant. Furthermore, due to the design of the study patients were younger of age during active disease.

TABLE 2. Characteristics of patients and controls

	Controls (<i>n</i> = 10)	Patients (<i>n</i> = 10)	
		Quiescent	Active
Age (yrs)	34 (24–42)	32 (24–41)	29 (22–39) ^{††}
Sex	9 females	9 females	9 females
BMI (kg/m ²)	23.0 (21.8–24.0)	23.1 (21.1–30.2)	23.0 (21.4–26.6)
Blood pressure (mmHg)			
Systolic	126 (120–132)	120 (105–133)	131 (124–143)
Diastolic	80 (80–84)	80 (65–80)	80 (69–81)
Lipid levels (mmol/l)			
Cholesterol	4.56 (4.20–5.31)	4.96 (4.04–6.41)	4.25 (3.75–4.77)
LDL	2.68 (1.95–3.24)	2.98 (2.06–3.95)	2.53 (2.14–3.26)
HDL	1.78 (1.62–2.09)	1.50 (1.31–1.81)	0.91 (0.79–1.04) [†]
Triglycerides	0.80 (0.64–0.93)	1.54 (1.11–2.17) [*]	1.83 (1.50–2.31)
Smokers (<i>n</i>)	1	2	2
Family history CVD (<i>n</i>)	2	4	4
Manifest CVD (<i>n</i>)	0	1	1
Creatinine (μmol/ml)	73 (65–78)	77 (71–82)	80 (68–126)
CrCl (ml/min)	91 (76–111)	93 (67–108)	75 (47–100) [†]
Glucose (mmol/l)	4.45 (4.15–4.93)	4.40 (4.00–5.38)	4.55 (3.98–5.38)
Diabetes (<i>n</i>)	0	0	0
Statin use (<i>n</i>)	0	0	0
Anti-hypertensive use (<i>n</i>)			
β-Blockers	0	2	2
ACEi	0	3 [*]	3
Calcium antagonists	0	1	1
AT antagonists	0	0	0
Diuretics	0	1	1

Unless otherwise indicated, data are expressed as median (25th to 75th centile). LDL: low-density lipoprotein; HDL: high-density lipoprotein; CrCl: creatinine clearance; AT: angiotensin type 1 receptor. Quiescent patients vs controls: ^{*}*P* < 0.05. Quiescent patients vs active patients: [†]*P* < 0.05, ^{††}*P* < 0.01.

Autofluorescence

Skin AGE accumulation, as estimated by skin autofluorescence, was measured in controls and in patients with inactive disease. AGE accumulation was increased in patients (1.52 AU) compared with controls (1.09 AU, $P < 0.05$) (Table 4, Fig. 1). Univariate analysis performed on data of controls and quiescent patients revealed no significant correlations between skin AF-EEMS and traditional cardiovascular risk factors, disease-related factors as presented in Table 3, and levels of sRAGE, sVCAM-1, CRP, CML and CEL.

TABLE 3. Disease-related factors

	Patients (n = 10)	
	Quiescent	Active
SLEDAI	4 (2–4)	9 (8–10) ^{††}
Anti-dsDNA, Farr	24 (9–47)	65 (36–112) [†]
C3 (g/l)	0.96 (0.73–1.15)	0.64 (0.43–0.80) ^{††}
C4 (g/l)	0.13 (0.08–0.22)	0.07 (0.03–0.16)
Creatinine clearance (ml/min)	93 (67–108)	75 (47–100) [†]
Duration of disease (months)	93 (30–208)	68 (9–178) [†]
Prednisolone use (n)	8	3 [†]
Dose (mg/day)	8.75 (5.00–8.75)	10.00 (10.00–10.00)
HCQ use (n)	4	1
Dose (mg/day)	500 (400–500)	600
AZA use (n)	2	1
Dose (mg/day)	150 (150–150)	100
Mycophenolate mofetil use (n)	2	0
Dose (mg/day)	1500 (1000–1500)	–

Unless otherwise indicated, data are expressed as median (25th to 75th centile). Quiescent patients vs active patients: [†] $P < 0.05$, ^{††} $P < 0.01$.

TABLE 4. Autofluorescence and serological parameters

	Controls (n = 10)	Patients (n = 10)	
		Quiescent	Active
AF-EEMS (AU)	1.09 (0.77–1.50)	1.52 (1.31–1.79) [*]	ND
CML ($\mu\text{mol/l}$)	2.64 (2.41–3.19)	2.77 (2.16–2.93)	2.74 (2.30–2.95)
CEL ($\mu\text{mol/l}$)	0.80 (0.67–1.10)	0.77 (0.61–0.93)	0.83 (0.60–1.05)
sRAGE (pg/ml)	2107 (1771–2538)	2882 [*] (2363–3539)	3752 [†] (3107–5570)
sVCAM-1 (ng/ml)	335 (279–366)	407 [*] (359–452)	719 [†] (366–912)
CRP (mg/l)	0.70 (0.26–1.08)	4.65 (1.37–7.82) [*]	7.21 (1.17–34.51)

Data are expressed as median (25th to 75th centile). ND: not determined. Quiescent patients vs controls: ^{*} $P < 0.05$, or vs active patients: [†] $P < 0.05$.

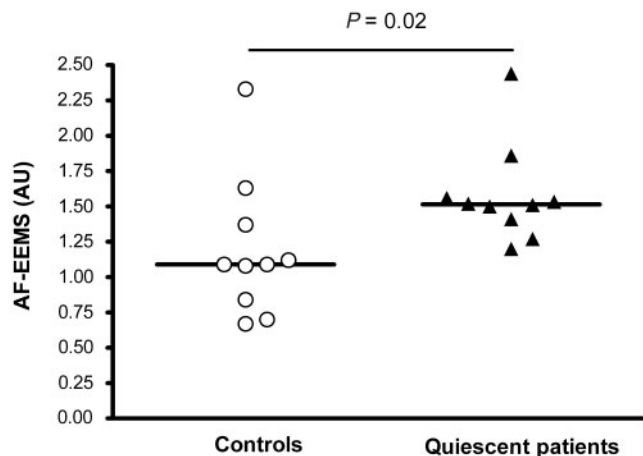


FIG. 1. Skin AGE accumulation. Open circles represent controls, closed triangles represent quiescent SLE patients. The median of autofluorescence (horizontal line) is significantly increased in quiescent SLE patients.

Blood analysis

Soluble RAGE and sVCAM-1 levels were higher in quiescent SLE patients compared with controls ($P < 0.05$) and further increased during active disease ($P < 0.05$) (Table 4, Fig. 2). Univariate analysis performed using data of quiescent patients and controls, revealed a negative correlation between sRAGE and age ($r = -0.469$, $P = 0.037$), a negative correlation between sRAGE and systolic blood pressure ($r = -0.486$, $P = 0.035$) and a positive correlation between sRAGE and sVCAM-1 ($r = 0.579$, $P = 0.007$). Univariate analysis performed using data of quiescent patients alone showed a negative correlation of sRAGE with complement C4 levels ($r = -0.705$, $P = 0.023$) and a positive correlation with anti-dsDNA antibody levels ($r = 0.697$, $P = 0.025$).

In active patients, no significant correlations were found between levels of sRAGE and traditional cardiovascular risk factors, disease-related factors, skin AF-EEMS, levels of CML, CEL, sVCAM-1 and CRP.

Levels of CML and CEL

Plasma levels of CML and CEL were comparable between patients (2.77 $\mu\text{mol/l}$ and 0.77 $\mu\text{mol/l}$, respectively) and controls (CML: 2.64 $\mu\text{mol/l}$ and CEL: 0.80 $\mu\text{mol/l}$). Disease activity did not alter CML and CEL levels significantly (CML: 2.74 $\mu\text{mol/l}$ and CEL: 0.83 $\mu\text{mol/l}$). Normalization of CML and CEL for protein concentration did not change results. No significant correlations were obtained between levels of CML and CEL and traditional cardiovascular risk factors, disease-related factors, skin AF-EEMS, levels of sRAGE, sVCAM-1 and CRP.

Discussion

In this study, we showed that skin autofluorescence used as an estimate of AGE deposition and serum levels of sRAGE were elevated in SLE patients, whereas plasma levels of two specific AGEs, CML and CEL, were comparable with controls.

Skin AGE was estimated by autofluorescence. The strong relations of autofluorescence with both fluorescent and non-fluorescent skin AGE levels in biopsy samples support the use of autofluorescence as a marker of AGE deposition [22]. As (glycated) immunoglobulin deposits might be present in the skin of SLE patients it can be speculated that this could lead to false positive results. This seems unlikely, as we also found increased skin autofluorescence in patients with WG (data not shown), an autoimmune disease characterized by absence of immune deposits. Furthermore, as the patients were not characterized by hyperglycaemia, increased AGE deposition in our patients might be the result of oxidative stress due to local and systemic inflammation as

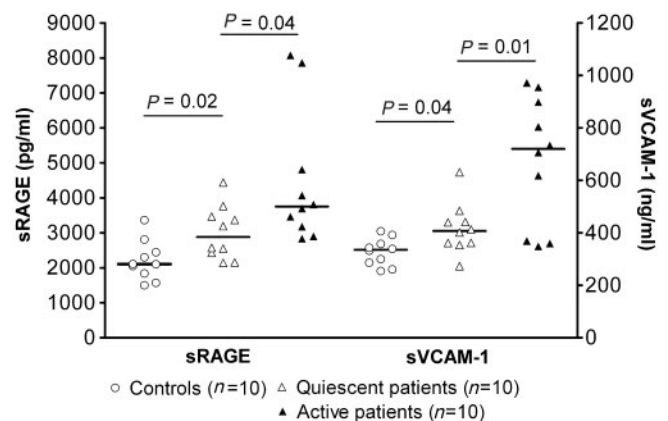


FIG. 2. Serum levels of sRAGE and sVCAM-1. Open circles represent controls, open triangles represent quiescent SLE patients and closed triangles represent active SLE patients. The median is indicated by the horizontal line.

demonstrated in patients with SLE [27–29]. This is supported by increased levels of CRP even during quiescent disease in our SLE patients and our previous finding that AGE accumulation in the skin correlates with disease duration of SLE [8]. Others have shown a relation between skin AGE levels and CRP or neopterin as inflammation markers [30, 31]. In our study, we did not find correlations between CRP, disease duration and skin AGE levels, probably due to the small number of subjects in each group.

Plasma levels of CML and CEL were also assessed. CML is one of the best characterized and most abundant AGEs, CEL is a homologue of CML. CML and CEL are two major non-enzymatic chemical modifications on proteins that are formed under conditions of oxidative stress, therefore these AGEs are most likely to be implicated in inflammatory diseases like SLE. Although AGE accumulation and levels of sRAGE were increased in SLE patients, plasma levels of these two specific AGEs were comparable with controls irrespective of disease activity. This suggests that AGEs primarily accumulate on proteins in tissue and intracellularly and not on plasma proteins with a short life-time. Also in patients with diabetes, increased levels of AGE-modified proteins were found in various tissues [11, 32] while plasma levels were normal or only slightly elevated [33–35]. Only in diabetic patients with renal failure extremely high serum AGE levels were found [36]. Furthermore, interaction of AGEs with endothelial RAGE might result in their removal from the plasma. Rapid removal of AGEs from the plasma through this mechanism has been shown in mice [37].

Interaction of AGEs with RAGE results in intracellular signalling, which leads to activation of the transcription factor nuclear factor- κ B resulting in enhanced expression of inflammatory mediators and adhesion molecules, such as VCAM-1 and expression of RAGE itself [38, 39].

Levels of sVCAM-1 and sRAGE were elevated in SLE patients and increased further during active disease indicating EC activation and enhanced RAGE expression in the vasculature. Some observations support the concept that levels of sRAGE reflect tissue RAGE expression [40]. As AGEs can induce RAGE expression, it can be hypothesized that sRAGE levels are elevated corresponding to AGE accumulation, but to what extent AGEs contribute to RAGE and sRAGE expression remains to be clarified. In a recent study by our group in persons with stable coronary artery disease, an independent positive relation between sRAGE and skin autofluorescence was indeed found [31].

The function of sRAGE is not fully understood but it has been suggested that the net effect of sRAGE and its ligand interactions can be either anti-inflammatory or pro-inflammatory depending on the inflammatory milieu. *In vitro* studies have shown that in the absence of ligands, sRAGE possesses pro-inflammatory properties through interaction with leucocyte β 2-integrin Mac-1. However, in the presence of HMGB-1 (one of the RAGE pro-inflammatory ligands) sRAGE suppressed inflammation by blocking the HMGB-1–RAGE interaction [41, 42]. Most studies support the concept that sRAGE has beneficial effects in a milieu rich in RAGE ligands such as AGEs. In a mouse model, the administration of recombinant sRAGE consisting of the extracellular ligand-binding domain, was shown not only to suppress the development of atherosclerosis but also to stabilize established atherosclerosis in diabetic apolipoprotein E-null mice [17, 18]. These observations suggest that exogenously administered sRAGE may capture and eliminate AGEs, thus protecting against AGE-elicited tissue damage by acting as a decoy receptor. Also in human studies, low levels of sRAGE were shown to be associated with increased risk for all-cause mortality [43] and were independently associated with the presence of coronary artery disease [44].

Based on these observations, we assume that increased levels of sRAGE in our patients are protective against AGE-elicited cellular activation, by facilitating the clearance of AGEs.

Apparently, this mechanism is insufficient to prevent increased AGE accumulation.

Use of medication might have influenced our results. Patients used more immunosuppressive drugs during quiescent disease compared with active disease, probably reducing AGE formation as these drugs suppress inflammation. Use of ACEi in three of the included patients might also have influenced our results. ACEi and angiotensin receptor blockers (ARBs) are known to reduce the formation and accumulation of AGEs [45, 46]. Furthermore, angiotensin II increases RAGE mRNA levels in endothelial cells and subsequently stimulates sRAGE formation. Treatment with telmisartan, an ARB, not only inhibits angiotensin II-elicited sRAGE generation by endothelial cells, but also decreases serum levels of sRAGE in patients with essential hypertension [47]. ACEi have been shown to increase sRAGE expression [48]. Exclusion of three patients using ACEi did, however, not significantly change our results (data not shown).

In blood and SF of RA patients autoantibodies specific for RAGE have been demonstrated. These antibodies can bind to sRAGE and form sRAGE/anti-RAGE antibody complexes and therefore might influence sRAGE levels [49]. Whether these antibodies are also present in the blood of SLE patients has not been established.

In summary, we observed increased AGE deposition in SLE patients, whereas plasma AGE levels were comparable with controls. Levels of sRAGE were elevated and increased further during active disease and endothelial activation. sRAGE might act as a decoy receptor, clearing AGEs from the circulation. Why this proposed mechanism is insufficient to prevent increased AGE accumulation in SLE patients has to be established.

Rheumatology key messages

- AGEs are a potential cardiovascular riskfactor in SLE.
- sRAGE might act as a decoy receptor, helping in the clearance of AGEs.

Disclosure statement: R.G. and A.J.S. are both founders and shareholders of DiagnOptics Technologies B.V., which produces the AGE Reader. All other authors have declared no conflicts of interest.

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