Enhanced expression of receptor for advanced glycation end-products is associated with low circulating soluble isoforms of the receptor in Type 2 diabetes

Xystus H. L. TAM*, Sammy W. M. SHIU*, Lin LENG†, Richard BUCALA†, D. John BETTERIDGE‡ and Kathryn C. B. TAN*

*Department of Medicine, University of Hong Kong, Hong Kong, People's Republic of China, †Departments of Medicine and Pathology, Yale University School of Medicine, New Haven, CT 06520-8031, U.S.A., and ‡Department of Medicine, Royal Free and University College London Medical School, London, U.K.

ABSTRACT

The sRAGE [soluble RAGE (receptor for advanced glycation end-products)] lack the transmembrane and cytoplasmic domain of the full-length receptor and can function as a decoy for RAGE ligands. Recent evidence suggests that sRAGE may be a potential biomarker of RAGE-mediated pathology. The present study aimed to examine the relationship between RAGE expression in peripheral blood monocytes and circulating sRAGE and esRAGE (endogenous sRAGE, a splice variant of sRAGE) in Type 2 diabetes. Protein expression of RAGE and esRAGE in monocyte cell lysate was determined by Western blot in 53 diabetic patients and 52 controls. Monocyte cell-surface-bound full-length RAGE expression was measured using flow cytometry. Serum sRAGE, esRAGE and AGE (advanced glycation end products) were assayed by ELISA. The mean HbA1c (glycated haemoglobin) of the diabetic patients was 9.74% and serum AGES was increased. Monocyte full-length RAGE expression was significantly higher in diabetic patients whereas esRAGE expression was reduced, and serum AGES concentration was an independent determinant of monocyte cell surface full-length RAGE expression. Serum levels of sRAGE [573.3 (375.7–754.3) compared with 608.1 (405.3–940.8) pg/ml, P < 0.05] and esRAGE [241.8 (154.6–356.6) compared with 286.5 (202.6–390.0) pg/ml, P < 0.05; values are medians (interquartile range)] were decreased. There was an inverse association between monocyte RAGE expression and log(serum sRAGE) (r = −0.34, P = 0.01) but not with esRAGE. In conclusion, despite an increase in full-length RAGE expression, esRAGE expression was down-regulated in the diabetic patients, and serum sRAGE and esRAGE was also reduced. Hence increased full-length RAGE levels are not associated with a similar increase in sRAGE isoforms levels.

Key words: advanced glycation end-product (AGE), biomarker, soluble receptor for advanced glycation end-product (sRAGE), Type 2 diabetes mellitus.

Abbreviations: ACEI, angiotensin-converting enzyme inhibitor; ADAM, a disintegrin and metalloproteinase; AGE, advanced glycation end-product; ARB, angiotensin receptor blocker; BMI, body mass index; CRP, C-reactive protein; HbA1c, glycated haemoglobin; MDA, malondialdehyde; MFI, mean fluorescence intensity; PBMC, peripheral blood mononuclear cell; RAGE, receptor for AGES; sRAGE, soluble RAGE; esRAGE, endogenous sRAGE; TBST, Tris-buffered saline containing Tween 20.

Correspondence: Professor Kathryn C.B. Tan (email kcbtan@hkucc.hku.hk).
INTRODUCTION

Long-term hyperglycaemia accelerates the formation and accumulation of AGEs (advanced glycation end-products) and leads to the over-expression of their cellular receptors in diabetes [1]. The RAGE (receptor for AGEs), a multi-ligand member of the immunoglobulin superfamily of transmembrane cell surface molecules, is the best characterized receptor for AGEs [2], and activation of the RAGE by AGEs plays a major role in the pathogenesis of diabetic vascular complications [3]. A number of RAGE isoforms have been found in both human and murine models recently. Among them, three major mRNA variants of RAGE have been identified, encoding the full-length RAGE which has full signalling and AGE-binding potential; N-truncated RAGE which is a membrane-bound isoform that contains no AGE-binding domain; and C-truncated sRAGE (soluble RAGE) which has AGE-binding properties in the absence of a signalling cascade [4]. The soluble forms of the receptor found in the circulation can act as a decoy for RAGE ligands and blockage of RAGE using sRAGE ameliorates diabetic vascular complications in animal models [5–7]. Hence modulating sRAGE is a novel means to protect tissues against the toxic effects of AGEs [8]. In addition to being a potential therapeutic target, recent clinical evidence suggests that sRAGE may also be a biomarker for disease [9]. A number of studies have investigated human circulating sRAGE levels and their association with a variety of pathophysiological conditions including diabetes. There are conflicting data with some studies suggesting that sRAGE levels are lower in subjects with vascular diseases and more advanced clinical syndromes [10,11], whereas others have shown the contrary or no associations [12–15].

sRAGE comprises a heterogeneous population of RAGE-related proteins and sRAGE in the circulation can be formed by different mechanisms. sRAGE can be generated by ectodomain shedding of the membrane-associated receptor, and ADAM10 (a disintegrin and metalloproteinase 10) has been identified as one of the main membrane proteases that elicits the proteolytic cleavage of full-length RAGE [16]. Alternatively, sRAGE can be formed by alternative splicing of RAGE pre-mRNA transcripts [17]. A number of soluble splice variants have been identified. Most of the soluble splice variants of RAGE are degraded at the mRNA level and esRAGE (endogenous sRAGE, also named RAGEv1) is the main splice variant that is secreted from cells [18]. Relatively little is known about factors that regulate sRAGE and esRAGE levels in human subjects and whether circulating levels reflect tissue RAGE expression and activity is unclear. The majority of the studies investigating the roles of RAGE and sRAGE in disease to date have been based on experiments performed in murine models. However, there are differences in the biology of RAGE between humans and mice in that although there is a comparable amount of soluble splice variants in murine tissues at the transcript level, plasma levels of sRAGE are hardly detectable [19,20]. Hence, in the present study, we have performed a cross-sectional study of Type 2 diabetic patients with poor glycaemic control to determine (i) the expression level of RAGE and esRAGE in monocytes and the relationship with serum levels of sRAGE and esRAGE, and (ii) whether the degree of glycaemic control and metabolic parameters were important determinants of the levels of full-length RAGE and sRAGE in vivo.

MATERIALS AND METHODS

Type 2 diabetic patients with inadequate glycaemic control [HbA1c (glycated haemoglobin) >7%] referred to the Diabetes Centre at Queen Mary Hospital, Hong Kong, were invited to participate. Exclusion criteria included a history of cardiovascular disease, proteinuria and/or impaired renal function. Patients on thiazolidinediones and/or lipid lowering agents were also excluded. Fifty-two healthy age-matched non-diabetic control subjects were recruited from the community. In all subjects, fasting blood samples were taken for the measurement of monocyte RAGE, serum sRAGE, esRAGE, AGEs, CRP (C-reactive protein), glucose, HbA1c, and lipids. The study protocol was approved by the Institutional Review Board of the University of Hong Kong and written informed consent was obtained from all subjects.

Western blot analysis was performed to measure protein expression of RAGE, esRAGE and ADAM10 in PBMCs (peripheral blood mononuclear cells) isolated from 20 ml of fresh blood in a random subgroup of ten controls and ten patients using the Ficoll–Paque gradient method. Samples were first separated by SDS/PAGE (7.5% gel) and the gel was subsequently electrotransferred on to polyvinylidene difluoride membranes (Millipore). The membranes were then blocked in TBST (Tris-buffered saline containing Tween 20) with 10% powdered skimmed milk. The blocked membrane was probed with primary antibody diluted in TBST with 1% powdered skimmed milk and then with HRP (horseradish peroxidase)-conjugated secondary antibodies. Bandings were visualized by an enhanced chemiluminescence advanced detection system (Amersham-GE Healthcare) and then exposed to X-ray film (Kodak). The mouse monoclonal antibody against human RAGE MAb55413 (R&D Systems) was raised against N-terminal Gln54–Ala134 of the extracellular domain of RAGE and therefore recognized all isoforms of RAGE (full-length RAGE, N-truncated RAGE that contains no AGE-binding domain and C-truncated soluble RAGE including esRAGE) in cell lysate. To detect esRAGE specifically,
anti-esRAGE antibody (Sigma) was raised according to the method described by Yonekura et al. [4]. Briefly, the anti-human esRAGE antibody was raised in rabbit against the unique C-terminal 16-amino-acid peptide (amino acids 332–347, EGFDKVREAEQSPQHM) of esRAGE and then purified using an immune-affinity column which contained the antigen peptide. The mouse monoclonal antibody raised against human β-actin A5316 (Sigma) was used to detect β-actin in PBMCs. The rabbit polyclonal antibody raised against amino acids 732–748 of human ADAM10 AB19026 (Millipore) was used to detect the ADAM10 protein expression level in the samples.

Since measurement of RAGE protein expression in cell lysate includes all isoforms of RAGE, flow cytometry was used to quantify specifically the expression of cell surface bound full-length RAGE receptor in PBMCs in all subjects. The full-length cell surface receptor is functional and has ligand-binding and signalling potential. Anticoagulated whole blood (100 μl) freshly collected from participants was incubated for 30 min on ice with 1:50 diluted rabbit anti-RAGE IgG ab37647 (Abcam) (raised against amino acids 39–58 of the ligand-binding domain of RAGE and hence recognized only the membrane-bound full-length RAGE) or FITC-conjugated rabbit IgG isoyme control ab37406 (Abcam). Primary antibodies were detected with 1:50 diluted FITC-conjugated goat anti-rabbit IgG ab6717 (Abcam). Monocytes were differentiated from other leucocytes by staining with PC5-conjugated anti-CD14 IgG (Abcam). Erythrocytes were lysed by adding VersaLyse lysis solution (Beckman Coulter) after all staining steps were finished. The samples were analysed with an EPICS X flow cytometer (Beckman Coulter). A fixed number of 5000 monocytes were analysed for each sample. MFI (mean fluorescence intensity) was analysed with FAScan, and the results are presented as the magnitude increase in MFI (MFI units of tested antibody staining/MFI units of control antibody staining).

Serum sRAGE levels were determined using ELISA (Quantikine; R&D Systems) according to the manufacturer’s protocol. This assay measures the total serum sRAGE level. Briefly, a monoclonal antibody raised against the N-terminal extracellular domain of human RAGE consisting of amino acids Gln24–Ala344 was used to capture sRAGE from serum. Captured sRAGE was detected with a polyclonal antihuman sRAGE antibody raised against the same immunogen. After washing, plates were incubated with streptavidin–HRP, developed with appropriate substrate and the absorbance at 450 nm was determined using an ELISA plate reader. The intra- and inter-assay coefficients of variation were 1.9% and 5.5% respectively. Serum esRAGE levels were measured directly using a different ELISA (B-Bridge International). The capture antibody in the ELISA assay for esRAGE uses the monoclonal antibody 278–13G4 and the detection antibody is a rabbit esRAGE-specific polyclonal antibody raised against the unique C-terminal 16-amino-acid peptide (amino acids 332–347) of esRAGE. The intra- and inter-assay coefficients of variation were 3.7% and 5.4% respectively. Serum AGEs was measured by a competitive ELISA developed in-house using a well-characterized polyclonal rabbit antiserum raised against AGE-RNase as previously described [21]. The polyclonal anti-AGEs antibody recognizes Nε-(carboxymethyl)lysine as well as the major non-fluorescent, AGE-cross-link arginine–lysine imidazole and therefore detects pathologically relevant AGEs [22]. The intra- and inter-assay coefficients of variation were 2.5% and 7.4% respectively.

Plasma total cholesterol and triacylglycerol were determined enzymatically (Boehringer Mannheim) on a Hitachi 717 analyser (Boehringer Mannheim). HDL-C (high-density lipoprotein–cholesterol) was measured by the same method after precipitation of apoB (apolipoprotein B)-containing lipoproteins with PEG [poly(ethylene glycol)] 6000. LDL-C (low-density lipoprotein–cholesterol) was calculated by the Friedewald equation. HbA1c was measured in whole blood using ion-exchange HPLC using a Bio-Rad Laboratories Variant Analyser System. Serum high-sensitivity CRP was measured by a sandwich ELISA (Antibody & Immunoassay Services, The University of Hong Kong, Hong Kong). MDA (malondialdehyde), a marker of lipid peroxidation and an equivalent of thiobarbituric acid reactive substances in serum was measured using an OXI-TEK TBARS assay kit (Alexis Biochemicals).

Results are expressed as means ± S.E.M. or medians and interquartile range if data were not normally distributed. All clinical data were tested for normality using the Kolmogorov–Smirnov test. Comparisons between two different groups were done using an independent sample Student’s t test, and skewed data were logarithmically transformed before analysis. Pearson’s correlations were used to test the relationship among variables. General linear model univariate analysis was used to simultaneously assess the relation between RAGE isoforms and various variables.

RESULTS

The clinical characteristics of controls and diabetic subjects are shown in Table 1. Fifty-three Type 2 diabetic patients with HbA1c >7% and 52 age-matched normal controls were recruited. Forty-three percent of the diabetic subjects were treated with a combination of metformin and sulfonylurea, 47% were on a combination of metformin and insulin and 10% were on insulin only. Forty-nine percent of the patients were receiving either an ACEI (angiotensin-converting enzyme inhibitor) or ARB (angiotensin receptor blocker). The mean duration
Table 1 Clinical characteristics of controls and Type 2 diabetic patients

Values are expressed as means ± S.E.M. or medians (interquartile range). ∗P < 0.05 and ∗∗P < 0.01 when compared with controls, as determined using a Student’s t test. BP, blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Controls</th>
<th>Diabetic patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (n) (male/female)</td>
<td>25/27</td>
<td>23/30</td>
</tr>
<tr>
<td>Age (years)</td>
<td>51.6 ± 0.9</td>
<td>52.6 ± 1.3</td>
</tr>
<tr>
<td>Duration of diabetes (years)</td>
<td>—</td>
<td>13 (7–16.5)</td>
</tr>
<tr>
<td>Smoker (%)</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>ACEI or ARB (%)</td>
<td>—</td>
<td>49</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.4 ± 0.5</td>
<td>27.6 ± 0.7**</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>81.9 ± 1.4</td>
<td>91.1 ± 1.7**</td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>5.43 ± 0.07</td>
<td>9.34 ± 0.45**</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>—</td>
<td>9.74 ± 0.25</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>120.1 ± 1.9</td>
<td>126.4 ± 2.3*</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>75.5 ± 2.5</td>
<td>77.2 ± 1.4</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.11 ± 0.11</td>
<td>4.96 ± 0.12</td>
</tr>
<tr>
<td>Triacylglycerol (mmol/l)</td>
<td>1.05 (0.73–1.38)</td>
<td>1.40 (1.11–2.21)**</td>
</tr>
<tr>
<td>LDL (mmol/l)</td>
<td>3.15 ± 0.11</td>
<td>3.02 ± 0.10</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>1.34 ± 0.06</td>
<td>1.19 ± 0.05*</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>0.69 (0.44–1.64)</td>
<td>1.58 (0.66–2.24)**</td>
</tr>
</tbody>
</table>

†Skewed data are logarithmically transformed before analysis.

of diabetes was 13 years and 38% of the patients had diabetic retinopathy. Diabetic patients have a significantly higher fasting glucose level. HbA1c level was only measured in diabetic subjects and mean HbA1c in these poorly controlled diabetic patients was 9.74%. Serum level of CRP was elevated in the diabetic patients compared with controls (P < 0.01).

Western blot analysis of cell lysate from PBMCs showed that the protein expression level of RAGE was significantly higher in diabetic patients than in controls (Figure 1A). Despite the increased expression level of RAGE, expression of esRAGE was significantly reduced in the diabetic patients (Figure 1B). Since ADAM10 was involved in the shedding of sRAGE, we have also measured the expression level of ADAM10 and there was no significant difference between controls and diabetic patients (Figure 1C). Consistent with the increased level of RAGE in monocyte cell lysate, we found that membrane bound full-length RAGE present on the cell surface of monocytes in peripheral blood was also significantly higher in diabetic patients than in controls (P < 0.01) (Table 2). Serum level of AGEs was increased in diabetic patients. However, serum sRAGE and esRAGE of diabetic patients were significantly lower than normal controls (P < 0.05), and this was in keeping with the reduced level of esRAGE expression in monocytes. To determine whether increased ligation of RAGE due to the high level of full-length RAGE expression resulted in changes in redox status in diabetic subjects, serum level of MDA was measured as a marker of oxidative stress. Diabetic patients had higher MDA level than controls (9.79 ± 0.40 compared with 6.91 ± 0.34 nmol/ml respectively, P < 0.01). Monocyte cell surface RAGE expression level was associated with serum MDA
Table 2. Monocyte cell-surface RAGE, serum total sRAGE and esRAGE in controls and Type 2 diabetic patients

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Controls</th>
<th>Diabetic patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGEs (unit/ml)</td>
<td>4.2 ± 0.2</td>
<td>5.2 ± 0.2**</td>
</tr>
<tr>
<td>sRAGE (pg/ml)†</td>
<td>608.1 (405.3–940.8)</td>
<td>573.3 (375.7–754.3)*</td>
</tr>
<tr>
<td>esRAGE (pg/ml)†</td>
<td>286.5 (202.6–390.0)</td>
<td>241.8 (154.6–356.6)*</td>
</tr>
<tr>
<td>Monocyte cell-surface RAGE (MFI)</td>
<td>2.06 ± 0.07</td>
<td>3.12 ± 0.15**</td>
</tr>
</tbody>
</table>

†Skewed data are logarithmically transformed before analysis.

Table 3. General linear model univariate analysis with monocyte membrane-bound RAGE as the dependent variable

<table>
<thead>
<tr>
<th>Regression coefficient</th>
<th>S.E. of regression coefficient</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>0.097</td>
<td>0.909</td>
</tr>
<tr>
<td>Age</td>
<td>−0.001</td>
<td>0.011</td>
</tr>
<tr>
<td>Gender</td>
<td>0.190</td>
<td>0.211</td>
</tr>
<tr>
<td>BMI</td>
<td>0.026</td>
<td>0.021</td>
</tr>
<tr>
<td>Smoker (yes/no)</td>
<td>0.190</td>
<td>0.183</td>
</tr>
<tr>
<td>ACEI/ARB (yes/no)</td>
<td>−0.397</td>
<td>0.221</td>
</tr>
<tr>
<td>AGEs</td>
<td>0.299</td>
<td>0.075</td>
</tr>
</tbody>
</table>

Figure 2. Relationship between monocyte membrane-bound RAGE and serum sRAGE in diabetic subjects (A) and controls (B)

Monocyte cell-surface RAGE, serum total sRAGE and esRAGE in controls and Type 2 diabetic patients. Values are expressed as means ± S.E.M. or medians (interquartile range). "P < 0.05 and ""P < 0.01 when compared with controls as determined using a Student’s t test.

levels ($r = 0.31, P < 0.01$) in all subjects and a trend was observed when diabetic subjects were analysed on their own.

Correlation analysis was performed to investigate the relationship between monocyte cell surface RAGE expression level and serum concentrations of soluble forms of RAGE. There was an inverse correlation between monocyte cell surface full-length RAGE expression and serum sRAGE level in the diabetic patients ($r = −0.34, P = 0.01$, Figure 2A), but no correlation was observed in normal controls (Figure 2B). In both diabetic patients and controls, no association between monocyte cell surface full-length RAGE expression and serum esRAGE was found.

Further analysis was performed to evaluate which clinical and metabolic parameters are the determinants of monocyte cell surface full-length RAGE expression and circulating sRAGE and esRAGE. There was a significant association between monocyte cell surface full-length RAGE and serum levels of AGEs in both diabetic patient and controls (Figures 3A and 3B respectively). These results were in keeping with the notion that the expression of RAGE can be induced by its ligands. There was also a correlation between HbA$_1c$ and monocyte cell surface full-length RAGE in the diabetic patients ($r = 0.31, P = 0.03$). No significant correlations between monocyte cell surface full-length RAGE were found with fasting glucose, anthropometric parameters like age, BMI (body mass index), waist circumference or with CRP.

To determine whether serum level of AGEs was an independent determinant of monocyte cell surface full-length RAGE expression, general linear model univariate analysis was performed. Age, gender, BMI, smoking status, the use of ACEI/ARB and AGEs were entered into the model. Since the relationship between AGEs and monocyte cell surface full-length RAGE was similar in the diabetic and control subjects, the two groups were combined in the analysis. Serum AGEs remained an independent determinant of monocyte cell surface full-length RAGE (Table 3), accounting for 20% of the variance on regression analysis. Forcing log(sRAGE) or log(esRAGE) into the model did not change our results.

In contrast, there was no correlation between serum sRAGE and esRAGE with AGEs, HbA$_1c$ or fasting glucose levels in either the diabetic patients or control subjects. However, BMI showed an inverse association with serum sRAGE ($r = −0.32, P = 0.02$) and esRAGE ($r = −0.32, P = 0.02$) in control subjects, whereas a
A weak trend was observed in the diabetic subjects (sRAGE: \( r = -0.27, P = 0.06 \); esRAGE: \( r = -0.22, P = 0.10 \)). Similar relationships were found with waist circumference in controls (sRAGE: \( r = -0.33, P = 0.02 \); esRAGE: \( r = -0.33, P = 0.02 \)). The association between waist circumference and sRAGE (\( r = -0.23 \)) and esRAGE (\( r = -0.19 \)) was not significant in diabetic patients. Neither sRAGE nor esRAGE was related to CRP levels in the two groups of subjects. To investigate which clinical parameters influence serum sRAGE or esRAGE levels, age, gender, BMI, smoking status, the use of ACEI/ARB and the level of monocyte cell surface full-length RAGE expression was included in the model as sRAGE might be partially derived from cleavage of full-length RAGE. The circulating AGE level was also forced into the model as there was previous evidence to suggest that AGE concentration was associated with sRAGE, although we failed to show a correlation between AGES and soluble forms of RAGE in our present study. Our analysis showed that serum sRAGE and esRAGE levels were mainly related to BMI (Table 4). On regression analysis, BMI accounted for 11% of the variation in sRAGE and 10% in esRAGE.

**DISCUSSION**

It has been suggested that sRAGE may be a useful biomarker of RAGE-mediated pathology as sRAGE may reflect RAGE expression [23]. However, data directly supporting this hypothesis in humans is lacking. This is the first study investigating the relationship between RAGE and esRAGE expression in monocytes and serum soluble RAGE levels in Type 2 diabetic patients. We have shown that Type 2 diabetic patients had higher levels of expression of cell surface-bound full length RAGE receptor in their monocytes than non-diabetic control, but the expression of the splice variant esRAGE was, on the other hand, significantly reduced. Serum levels of both esRAGE and sRAGE were also decreased. There was no relationship between RAGE expression in monocytes and serum esRAGE, and only a weak inverse relationship was seen with serum sRAGE. Our study therefore provides important human data showing that an increase in full-length RAGE gene expression is associated with down-regulation of other RAGE variants like esRAGE, and serum levels of soluble RAGE might not directly reflect RAGE expression in human diabetes.
In the present study, we have used monocytes because these cells are easily accessible and our findings suggest that expression of full-length RAGE and other splice variants of RAGE may be differentially regulated. RAGE expression in peripheral blood monocytes can be induced when ligands for RAGE accumulate and, consistent with previous studies showing that RAGE expression is inducible by its ligands [24, 25], we have shown that serum AGEs were an independent determinant of monocyte cell surface RAGE expression. Despite the up-regulation of full length RAGE expression in monocytes, esRAGE expression was reduced. Much less is known about the regulation of esRAGE expression. The significance of alternative splicing of RAGE pre-mRNA is increasingly being recognised and it has been proposed that the pre-mRNA of RAGE is subjected to regulation by alternative splicing activated by extracellular cues of yet unknown cellular signalling pathways [26]. Increasing soluble RAGE variant expression may potentially be beneficial. Forbes et al. [27] have shown that treatment with ACEI increases the expression of the splice variant C-truncated soluble RAGE in streptozotocin-induced diabetic rats and their data suggest that ACE inhibition reduces the accumulation of AGE in diabetes partly by increasing the production and secretion of sRAGE into plasma.

Serum levels of esRAGE and sRAGE were decreased in our diabetic patients. esRAGE is the main splice variant secreted into the circulation by cells. Reduced expression of esRAGE in our diabetic patients would account for the lower serum level of esRAGE, and we have shown that serum esRAGE level does not reflect full-length RAGE receptor expression. esRAGE constitutes only part but not all of the human sRAGE in serum and the esRAGE level is approximately 2- to 3-fold less than the total sRAGE level [28]. Serum levels of soluble RAGE can also be derived by ectodomain shedding of the membrane-associated receptor [29]. The reduction in serum total sRAGE in our diabetic patients might be partly due to a decrease in esRAGE, but whether there was any reduction in sRAGE formed by shedding cannot be determined as there are no assays that can specifically measure shedded sRAGE in the circulation. With an increase in cell surface RAGE receptor expression in our diabetic patients, one would expect an increase in sRAGE formed by shedding. However, we in fact found a weak inverse correlation between monocyte cell surface-bound full-length RAGE and serum sRAGE in our diabetic patients. Shedding of cell surface proteins can be constitutive and/or inducible [29, 30] but what factor(s) influence the rate of cleavage in vivo is unclear. Variation in enzyme level of ADAM10 was unlikely to contribute to this as we did not find any significant differences in the level of expression of ADAM10 between controls and diabetic patients. However, we have not measured the activity of ADAM10. Shedding of cell surface RAGE may potentially be a protective mechanism against the activation of RAGE signalling, as cleavage of RAGE not only reduces the number of receptors available for activation but also generates sRAGE which can act as a decoy for RAGE ligands. The regulation of ectodomain shedding of RAGE in humans is poorly understood. Since we have observed an inverse correlation between monocyte cell surface-bound full-length RAGE and serum sRAGE in our diabetic subjects, we speculate that this process may be attenuated in poorly controlled Type 2 diabetic patients and this certainly warrants further investigations.

We also observed an inverse correlation between serum sRAGE and esRAGE with BMI and waist circumference in our healthy controls, and the correlation was less strong in the diabetic subjects. An inverse correlation between serum sRAGE, BMI and waist circumference has been reported in Japanese [31] and Italian general populations [32] as well as in Japanese non-diabetic hypertensive patients [33]. Koyama et al. [34] have shown that BMI or insulin resistance index was the major factor determining plasma esRAGE in both non-diabetic and diabetic population. The majority of these studies have shown that BMI is an independent predictor of sRAGE. Whether the relationship is causal and what the underlying mechanism(s) are is unclear. Ueno et al. [35] have recently demonstrated that RAGE is expressed in adipocytes as well as in endothelial cells in epididymal adipose tissue in mice. Hence, it would be of interest to determine whether obesity may potentially influence the expression of RAGE isoforms.

Our study has a number of limitations. Since our study is cross-sectional in design, we can only demonstrate associations and not causal relationships. Monocytes are not the only source of sRAGE and esRAGE. RAGE can be found in a variety of cell types including endothelial, mesangial, monocyteic, neuronal and smooth muscle cells [36] and it is not clear at present from which tissue or cell type serum sRAGE or esRAGE mainly originates in humans. We have studied poorly controlled diabetic patients as they were likely to have high levels of RAGE ligands. We attempted to reduce potential confounding factors that can influence full-length RAGE expression and sRAGE levels in our study by excluding patients with cardiovascular disease, proteinuria and/or impaired renal function. We also have excluded patients on medication that can potentially influence sRAGE and esRAGE, such as thiazolidinediones and lipid-lowering agents [37, 38]. We did not exclude patients on ACEI or ARB as this would make recruitment very difficult. We therefore adjusted for the use of ACEI/ARB in our analysis. All our subjects are of Chinese descent, and ethnic differences in sRAGE have been reported. The serum level of sRAGE in our present study was lower than that reported in Caucasians [32, 39], but similar to levels found in Japanese subjects [15, 31].
In conclusion, poorly controlled Type 2 diabetic patients have increased expression of full-length RAGE but reduced esRAGE expression in their monocytes. Circulating levels of both sRAGE and esRAGE were reduced. This would suggest that up-regulation of full-length RAGE expression was not associated with a similar increase in serum sRAGE and esRAGE in human diabetes.

**AUTHOR CONTRIBUTION**

Xystus Tam and Sammy Shiu performed the experiments and analysed the results; Lin Leng and Richard Bucala were involved in the AGE assay and provided valuable technical advice for the study; John Betteridge was involved in the study design and reviewed the manuscript; Kathryn Tan designed the study, recruited the subjects and acquired the funding for the project.

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