Differential effect of plasma or erythrocyte AGE-ligands of RAGE on expression of transcripts for receptor isoforms

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Abstract

Aim. – Binding of advanced glycation end-products (AGEs) to the receptor for AGEs (RAGE) contributes to diabetic vascular complications. RAGE transcript splicing generates membrane-bound proteins [full-length (FL) and N-truncated (Nt)] and a soluble protein [endogenous secretory (esRAGE)] that may act as a decoy. We tested the effect of AGE-ligands on the regulation of RAGE isoforms and the consequences on red blood cell (RBC) adhesion.

Methods. – RAGE isoforms were measured by real-time RT-PCR assay, using a LightCycler System, in human umbilical vein endothelial cells (HUVECs), incubated with either characterized AGEs [Nε-(carboxymethyl)lysine human serum albumin (CML-HSA) and methylglyoxal-modified HSA (MG-HSA)] or with RBCs from diabetic patients (DRBCs). Inhibition of RAGE access was achieved by using blocking either anti-RAGE antibodies or recombinant RAGE. Adhesion of DRBCs to endothelium was measured under flow conditions using HUVECs stimulated with MG-HSA or CML-HSA. Antibodies directed to RBC membrane proteins were tested for blocking DRBC adhesion in static conditions.

Results. – MG-HSA stimulated the expression of membrane-bound RAGE (FL + Nt) and esRAGE transcripts to similar extents, while CML-HSA and DRBC more selectively induced mRNA for FL and Nt-RAGE. Anti-RAGE antibody inhibited the effect of glycated proteins. Stimulation of HUVECs with CML-HSA enhanced DRBC adhesion, while MG-HSA had no effect. CD233 (band 3) was glycated in DRBC membrane, and anti-CD233 antibodies inhibited the adhesion of DRBCs, as did the anti-RAGE and anti-AGE antibodies.

Conclusions. – Receptor engagement by distinct AGEs differentially enhances expression of RAGE isoforms and DRBC adhesion. The CML-adduct, by facilitating adhesion, has more deleterious effects than MG-derived AGEs.

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Keywords: Diabetes; Advanced glycation endproducts; AGE; RAGE; Red blood cell; Adhesion

Résumé

Modulation de l’expression des isoformes du RAGE par les AGE circulants ou érythrocytaires.

Introduction. – L’activation par les produits de glycation avancée (AGE) du récepteur des AGE (RAGE) est un événement précoce dans le développement des complications vasculaires du diabète. Le RAGE est soumis à un épissage alternatif qui génère des protéines membranaires (full-length [FL], N-truncated [Nt]) et une protéine soluble (endogenous secretory [esRAGE]) qui pourrait capter les AGE. Nous avons étudié l’effet de deux types d’AGE sur la régulation des isoformes du RAGE et les conséquences sur l’adhésion des globules rouges (GR) à l’endothélium.

Méthodes. – Les isoformes du RAGE ont été mesurées par RT-PCR en temps réel au LightCycler dans des cellules endothéliales humaines de la veine du cordon ombilical (HUVEC). Les cellules ont été incubées en présence de deux AGE caractérisés (la Nε-carboxymethyllysine-albumine humaine [CML-HSA] et les AGE dérivés du méthylglyoxal [MG-HSA]) ou avec des GR de patients diabétiques (GRD). L’accès au RAGE a été

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1. Introduction

Binding of advanced glycation endproducts (AGEs) to the receptor for AGEs (RAGE) has been suggested to contribute to the pathogenesis of diabetic vascular complications [1]. RAGE belongs to the immunoglobulin superfamily and is present on multiple cell types, including macrophages and endothelial cells. The RAGE transcript is the target of alternative splicing that generates three isoforms: full-length (FL-RAGE); N-truncated (Nt-RAGE) and a soluble form of RAGE: endogenous secreted RAGE (esRAGE) (Fig. 1A) [2]. Other than by splicing, soluble RAGE (sRAGE) can also be produced consequentially to FL-RAGE proteolysis [3], and may act as a decoy, preventing RAGE engagement of ligands. Expression of RAGE is up-regulated in human tissues, including those involved in the long-term complications of diabetes such as podocytes and endothelial cells.

AGEs were initially identified as products of non-enzymatic glycoxidation resulting from the oxidative reactions between free amino groups on proteins and carbohydrates. The biochemistry of glycoxidation products is now better characterized, and some products formed from the degradation of glucose, such as glyoxal or methylglyoxal (MG), are also recognized to generate AGEs following interaction with the appropriate substrates [4,5]. Such glycoxidation may occur in the extracellular compartment, but also takes place in a more rapid and extensive manner intracellularly. Nε-(carboxymethyl)lysine (CML) and MG-derived AGEs are major glycated proteins in diabetes mellitus and their blood levels are correlated to diabetic angiopathy [6–8]. It has been previously demonstrated that red blood cells (RBCs) from diabetic patients (DRBCs) display increased adhesion to vascular endothelium, and that the extent of adhesion is significantly correlated to the severity of vascular complications [9].

As RAGE isoforms are competitors for AGE binding, we measured the expression of membrane-bound isoforms (FL + Nt) vs esRAGE. In the present study, we have tested the ability of DRBCs and two characterized AGEs generated in vitro–MG-modified human serum albumin (MG-HSA) and CML-HSA—to modulate RAGE isoform expression in human umbilical vein endothelial cells (HUVECs), and assessed the subsequent modulation of DRBC adhesion.

2. Methods

2.1. Patients

RBCs were obtained from type 2 diabetes patients already included in a previous study [10]. DRBCs were subcategorized in patients with controlled diabetes (HbA1c 6.7 ± 0.1%; n = 12) and with poorly controlled diabetes (HbA1c 9.9 ± 1.1%; n = 14). RBCs from euglycaemic/non-diabetic subjects (NRBCs) were used as controls.

The present study protocol was approved by the internal review boards of the participating institutions, and the patients’ informed consent was obtained in accordance with the Declaration of Helsinki.

2.2. Cell culture

HUVECs were isolated from human umbilical cords and cultured in M199 and 20% fetal calf serum (Dominique Dutscher, Brumath, France) [9].

2.3. Semi-quantitative real-time RT-PCR of RAGE isoforms

Only three isoforms of RAGE mRNA were detected in endothelial cells, corresponding to Nt-, FL- and esRAGE. Quantification of mRNA encoding RAGE isoforms was performed using LightCycler technology (Roche Diagnostics). For optimal quantification by the LightCycler System, the PCR (polymerase chain reaction) products need to be as short as possible (< 500 b; the optimal size is approximately 100 b). In addition, to ensure high specificity in the detection of RAGE isoforms and to reach high conformity in PCR kinetics, a common reverse primer and a common fluorescent hydrolyzation probe were used. As RAGE splicing also conserves introns that are more than 1 kb away, we measured FL and Nt-RAGE isoforms together. The content of the amplification mix was set according to the manufacturer’s instructions. The common reverse primer (ex11/Re: 5′-AGGTTCCTCCGACTGATCAGTTC-3′) and the common fluorescent hydrolyzation RAGE probe (5′-AGGCCCCAGAAACCCAGGAGG AAGAGGAGGA-3′) were both located in exon 11 (Fig. 1B). Amplification of RAGE isoforms was performed with forward primers in exon 10 (ex10/Fo: 5′-AAGGCGGCAACGCGGAGGAGA-3′)
Fig. 1. (A) RAGE isoforms; (B) position of primers and probe on the 3’ ends of RAGE isoform cDNA; (C) determination of PCR efficiencies by LightCycler for detection of RAGE isoforms (FL: full-length; Nt: N-truncated; esRAGE: endogenous secretory RAGE); and (D) mass spectrometry analysis of glycated proteins.

for FL-RAGE (Genbank AB036432) and Nt-RAGE (Genbank AB061669) simultaneously, and in intron 9 (in9/Fo: 5’-GGGGATGG TCAACAAGAAAGG-3’) for esRAGE (Genbank AB061668). β2-Microglobulin (B2 M) served as the reference gene [11]. All primers and probes were from MWG Biotech (Ebersberg, Germany). Amplification conditions for LightCycler involved an initial 20-min reverse-transcription step at 61 °C, followed by a denaturation step for 90 s at 95 °C, 40 cycles of denaturation at 95 °C for 5 s and annealing at 55 °C for 15 s, with extension at 70 °C for 25 s.

The relative changes in the RAGE/B2 M mRNA ratio were determined by the ΔΔCt (crossing threshold) procedure where:

\[ \text{Ratio} = 2^{-\Delta \Delta Ct} \]

\[ \Delta \Delta Ct = (Ct_{\text{RAGE}} - Ct_{\text{B2 M}})_{\text{treated cells}} - (Ct_{\text{RAGE}} - Ct_{\text{B2 M}})_{\text{non-treated cells}} \]

All efficiency curves for RAGE cDNA showed linearity over the quantification range with correlation coefficients > 0.99,
indicating a log-linear relationship. PCR efficiencies were $2.01 \pm 0.2$ for FL+Nt-RAGE and $1.99 \pm 0.3$ for esRAGE (Fig. 1C), as determined by amplification of consecutive dilutions of random samples.

2.4. Preparation of glycated proteins

Methylglyoxal-modified human serum albumin (MG-HSA) was prepared by incubating HSA (30 mg/mL) for 5 h at 37 °C with methylglyoxal (150 mM, Sigma) in the presence of sodium cyanoborohydride (100 mM) in a sodium-bicarbonate buffer (0.1 M; pH 10.0) [12]. CML-HSA was obtained by incubating HSA (30 mg/mL) and glyoxalic acid (135 mM) in a sodium-phosphate buffer (0.2 M; pH 7.8) containing sodium cyanoborohydride (450 mM) for 16 h [12]. Endotoxin levels were < 0.002 endotoxin units (EU)/mg protein in all of the study preparations. The degree of chemical modification of HSA was measured by mass spectrometry, using a 4800 Plus MALDI TOF/TOF Analyzer (Applied Biosystems, Foster City, CA). The masses of MG-HSA and CML-HSA were 68,830 Da and 67,819 Da, respectively (Fig. 1D). The extent of chemical modification was also determined by 2,4,6-trinitrobenzenesulphonic acid as the difference in amino groups found in modified and unmodified protein preparations, as described elsewhere [13]. The extent of lysine modification with MG-HSA and CML-HSA is identical (750 pmol/µg HSA). Although glycated CML-HSA in vitro is modified to a greater extent than in vivo, the amount of glycated protein added in our experiments (3.7–10 nmol modified lysine/mL) resulted in concentrations of modified protein comparable to those found in the blood of diabetic patients (range: 0.7–7.5, mean: 2.8 nmol modified lysine/mL).

2.5. Antibodies and soluble proteins

Anti-RAGE antibody was obtained with purified recombinant rat RAGE (used to immunize rabbits) at a final concentration of 100 µg/mL to block endothelial RAGE [14]. Non-immune isotype-matched immunoglobulin served as a control. The blocking peptides–recombinant rat RAGE (r-RAGE) and recombinant rat vascular cell adhesion molecule-1 (r-VCAM-1)–were a gift from M. Nagashima. Anti-CD233 (BRIC6, anti-band 3) and anti-CD235a (BRIC256, anti-glycophorin A) antibodies were from the International Blood Group Reference Laboratory (IBGRL; Bristol, UK). Rabbit anti-AGE and isotype-matched immunoglobulin were a gift from A.M. Schmidt.

2.6. Red blood cell adhesion to HUVECs

The technique designed to measure RBC adhesion to endothelium in static condition has been previously described in Fig. 2. MG-HSA, CML-HSA and DRBCs have different stimulatory effects on RAGE isoform expression. HUVECs were incubated for 6 h with MG-HSA (10 nmol/mL), CML-HSA (10 nmol/mL) and RBCs (haematocrit 25%) from normal subjects (NRBC) and diabetic patients (DRBC), who were either controlled (HbA₁c 6.7 ± 0.1%) or poorly controlled (HbA₁c 9.9 ± 1.1%). HUVECs were pre-incubated for 30 min with either anti-RAGE antibody or isotype-matched IgG (100 µg/mL). The ratio of expression was calculated by the ΔΔCt procedure, using β2-microglobulin normalization, and compared with HUVECs incubated with control HSA (NRBC). Results are expressed as the means ± SEM of two experiments performed in triplicate. *P < 0.05, **P < 0.01, ***P < 0.001.
RBC adhesion to HUVECs was also measured under flow conditions: an RBC suspension was perfused through a microslide at a flow rate equivalent to a wall shear stress of 0.02 Pa for 5 min, followed by washout of non-adherent cells for 5 min. The wall shear stress was then increased stepwise (from 0.003 to 0.4 Pa). Adherent RBCs were counted using a computed image-analysis system (Optimas, Media Cybernetics, Silver Spring, MD; R&D Vision, Paris, France). Counts were averaged per field and expressed as the number of adherent RBCs per square millimetre [16].

### 2.7. Analysis of AGE-proteins in RBC membrane

RBC ghosts were prepared by incubating RBC in phosphate buffer (pH 7.4) for 30 min at 4 °C, then lysed in hypotonic phosphate buffer. Membrane RBC proteins (30 μg) were transferred to a 5% polyacrylamide gel, and their reactivity to anti-AGE or control immunoglobulin was assayed. The optical density plot was measured with a laser densitometer (LKB Ultrascan XL, Pharmacia).

### 2.8. Statistical analysis

Results are presented as means ± standard error of mean (SEM). Statistical significance was determined using one-way analysis of variance (ANOVA) followed by the parametric Dunnett’s test.

### 3. Results

#### 3.1. Glycated HSA and DRBCs stimulate expression of RAGE mRNA

Tumour necrosis factor-α (TNF-α) has been described as an inducer of RAGE expression in endothelial cells [17]. To validate the relationship between mRNA and protein expression, we measured the levels of RAGE mRNA and proteins after TNF-α stimulation, and found that RAGE mRNA synthesis is associated with protein increase (Supplemental file 1).

Compared with control HSA, MG-HSA (10 nmol/mL) significantly increased expression of transcripts for both membrane-bound RAGE (FL + Nt-RAGE: 1.96 ± 0.15; P < 0.01) and esRAGE (1.54 ± 0.18; P < 0.05) (Fig. 2). CML-HSA (10 nmol/mL) also enhanced mRNA for membrane-bound RAGE (FL + Nt-RAGE) by 1.90 ± 0.23 (P < 0.01), but had no apparent stimulatory effect on esRAGE transcripts. At a lower concentration (3.7 nmol/mL), neither MG-HSA nor CML-HSA had any effect on RAGE expression. The effect of MG-HSA and CML-HSA on RAGE mRNA expression was blocked by the incubation of HUVECs with anti-RAGE antibody. Incubation of endothelial cells with DRBCs, which contain glycated protein within the membrane in vivo, caused an increase in membrane-bound RAGE transcripts, but not in esRAGE mRNA. The degree of DBRC glycation, likely to be reflected by the HbA1c levels of the patients from whom the DRBCs were harvested, appeared to parallel the effect of DRBCs on RAGE mRNA expression. Thus, NRBCs had no effect, whereas DRBCs from patients with either controlled (HbA1c <7%) or poorly controlled (HbA1c >7%) diabetes displayed increased stimulation of RAGE mRNA. These results indicate that the engagement of cell-surface RAGE by AGE-modified ligands up-regulates the expression of receptor mRNA in an isoform-specific pattern.

In further studies to explore RAGE-mediated signalling induced by MG-HSA and CML-HSA, our findings were consistent with the data previously reported elsewhere [10,18]: both forms of glycated HSA activated nuclear factor (NF)-κB and generated reactive oxygen species (ROS) (data not shown). The differences in the affinities of each AGE-ligand for RAGE (KdCML-HSA 218 ± 78 nM and KdMG-HSA 664 ± 118 nM), calculated using the Biacore system in unpublished data, did not explain the variation in RAGE splicing.

#### 3.2. Modulation of DRBC adhesion by AGE

MG-HSA, which enhanced endothelial expression of transcripts for FL, Nt and esRAGE, did not affect DRBC adhesion at any shear stress compared with native HSA (Fig. 3). In contrast, CML-HSA augmented endothelial adhesion of DRBCs at shear stresses up to 0.4 Pa. This may be related to the differential modulation of RAGE isoform expression by MG-HSA, which up-regulated esRAGE, whereas CML-HSA did not.

![Fig. 3. Modulation of adhesion of RBCs from diabetic patients (DRBC) to endothelial cells. HUVECs were stimulated by MG-HSA and CML-HSA for 6 h, after which DRBC adhesion was assessed. These results represent the DRBCs still adherent under increasing flow conditions (up to 0.4 Pa). Stimulation by CML-HSA (5 μM) potentiated DRBC adhesion, while MG-HSA had no such effect. ***P < 0.001 vs HSA.](image-url)
3.3. Blockade of DRBC adhesion to endothelium

Membrane proteins of DRBCs were tested for their reactivity to rabbit anti-AGE antibody (Fig. 4A). Analysis of the intensity plot showed that, compared with NRBCs, α and β chains of spectrin and CD233 (band 3) from DRBCs display the largest increase in binding of the anti-AGE antibody, consistent with the highest level of AGE modification. CD233, a transmembrane protein, contrary to spectrin chains that are intracellular membrane-bound proteins, making CD233 a good candidate for DRBC adhesion. Other peaks corresponded to adducin, ankyrins or protein 4.1.

Compared with RBCs from euglycaemic/non-diabetic patients, DRBCs had an enhanced adhesion to endothelium, and the adhesion was increasing according to the HbA1c levels (Fig. 4B). Blockade of endothelial RAGE normalized the adhesion of DRBCs to the level of NRBCs ($P < 0.01$). Similarly, incubation of rR-RAGE or anti-AGE antibody with DRBC resulted in reduced adhesion (Fig. 4C). These data confirm that endothelial RAGE is responsible for DRBC adhesion.

Anti-CD233 antibody also inhibited the adhesion of DRBC by 63 ± 6%, while anti-CD235a (anti-glycophorin A) had no such effect. Taken altogether, the results confirm that endothelial RAGE binds DRBCs, and demonstrate that glycated CD233 is the ligand of RAGE in RBC membrane.

4. Discussion

In the present study, we analyzed the consequences of two characterized AGEs on the induction of changes in the expression of isoform transcripts of the AGE receptor in endothelial cells. A recent publication summarizing the work of many laboratories showed that extensive splicing of RAGE transcripts led to as many as 20 splice variants [19]. In endothelial cells, only three isoforms of RAGE were detected at significant levels: Nt-RAGE, FL-RAGE and esRAGE. An unexpected finding was that MG-HSA and CML-HSA, two major AGEs present in vivo and binding to the same receptor, differentially regulated the expression of RAGE isoform transcripts. MG-HSA stimulated expression of mRNA for all three isoforms of RAGE.
found in endothelial cells, whereas CML-HSA only stimulated transcripts for FL- and Nt-RAGE isoforms, without affecting esRAGE mRNA expression levels. In both cases, MG-HSA and CML-HSA stimulated RAGE expression by interacting with RAGE itself. Other RAGE downstream-signalling pathways, including those leading to generation of reactive oxygen species and NF-κB activation, did not respond differently to RAGE engagement by either MG-HSA or CML-HSA. However, MG-HSA enhanced esRAGE expression, potentially implicating a negative feedback loop, because soluble RAGE thus generated may act as a decoy intercepting the interaction of ligands with cell surface RAGE and, thereby limiting RAGE-mediated cellular activation. Factors involved in the regulation of RAGE isoform expression could be important in rendering a particular vascular bed more or less vulnerable to the effect of RAGE ligands.

The expression of RAGE transcripts was also increased by DRBCs adherent to the endothelium, and this effect could also be correlated to HbA1c levels (which presumably reflect the level of glycation of plasma/RBC surface proteins). Glycation of erythrocyte membrane proteins in vivo appears to primarily reflect ketoamine linkage between glucose and the free amino group of lysine residues [20]. We confirm that endothelial membrane-bound RAGE supports DRBC adhesion. CD233 (band 3) is one of the most heavily glycated RBC membrane proteins in diabetic patients and, when glycated, appears to be a counter-receptor for RAGE on endothelium. The inhibition of DRBC adhesion by recombinant sRAGE points to the potential role of endogenous sRAGE in limiting such adhesion and to the contribution of the up-regulation of such AGE decoy. Adhesion of diabetic erythrocytes drives the generation of oxidative stress through NADPH-oxidase activation [10]. The risk of ischaemia due to RBC adhesion in microvessels is increased by CML-HSA via enhancement of RAGE expression that is not balanced by up-regulation of esRAGE. These data suggest the potential enhanced toxicity of CML-HSA (compared with MG-HSA) in terms of vascular perturbations mediated by RAGE. In diabetic patients, high levels of CML-modified proteins have been shown to correlate to microvascular complications [7]. On the other hand, low levels of circulating sRAGE have been associated with an increased risk of cardiovascular mortality [21]. Similarly, diabetic patients with reduced levels of sRAGE displayed increased incidence of microvascular complications (retinopathy and nephropathy) [22]. The measurement of RAGE mRNA isoforms in diabetic patients could be of great interest to understand the relative role of RAGE isoforms in diabetic complication outcomes.

In conclusion, we provide evidence that a receptor–ligand interaction modulates RAGE isoform expression differentially according to AGE type, resulting in modulation of DRBC adhesion. Stimulation of RAGE expression by CML-HSA facilitated RBC adhesion to endothelium and, thus, may lead to an increased risk of vascular occlusion and thrombosis. These data support the idea that the nature of AGE and RAGE isoform modulation could be important factors underlying diabetic microvascular complications.

Conflicts of interest

Dr Stern is a consultant for TransTech Pharma (High Point Pharmaceuticals) in High Point, NC, USA.

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Appendix A. Supplementary data


References


