SPANDIDOS PUBLICATIONS

Advanced glycation end products regulate extracellular matrix protein and protease expression by human glomerular mesangial cells

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Abstract. Advanced glycation end products (AGEs) may play a role in the pathogenesis of diabetic nephropathy, by modulating extracellular matrix turnover. AGEs are known to activate specific membrane receptors, including the receptor for AGE (RAGE). In the present study, we analyzed the various receptors for AGEs expressed by human mesangial cells and we studied the effects of glycated albumin and of carboxymethyl lysine on matrix protein and remodelling enzyme synthesis. Membrane RAGE expression was confirmed by FACS analysis. Microarray methods, RT-PCR, and Northern blot analysis were used to detect and confirm specific gene induction. Zymographic analysis and ELISA were used to measure the induction of tPA and PAI-1. We show herein that cultured human mesangial cells express AGE receptor type 1, type 2 and type 3 and RAGE. AGEs (200 μg/ml) induced at least a 2-fold increase in mRNA for 10 genes involved in ECM remodelling, including tPA, PAI-1 and TIMP-3. The increase in tPA synthesis was confirmed by fibrin zymography. The stimulation of PAI-1 synthesis was confirmed by ELISA. AGEs increased PAI-1 mRNA through a signalling pathway involving reactive oxygen species, the MAP kinases ERK-1/ERK-2 and the nuclear transcription factor NF-κB, but not AP-1. Carboxymethyl lysine (CML, 5 μM), which is a RAGE ligand, also stimulated PAI-1 synthesis by mesangial cells. In addition, a blocking anti-RAGE antibody partially inhibited the AGE-stimulated gene expression and decreased the PAI-1 accumulation induced by AGEs and by CML. Inhibition of AGE receptors or neutralization of the protease inhibitors TIMP-3 and PAI-1 could represent an important new therapeutic strategy for diabetic nephropathy.

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Introduction

Diabetic nephropathy has become the most frequent cause of terminal renal failure in western countries, and is associated with severe cardiac and vascular complications (1). The natural course of diabetic nephropathy is well described and includes functional and structural changes in the kidney. More specifically, glomerular membrane thickening, mesangial matrix expansion, microvascular changes, arteriolar hyalinosis and tubular degeneration characterize the overt diabetic nephropathy. Both hyperglycemia and hypertension are known to participate in the formation of these lesions. However, the pathogenesis of diabetic nephropathy remains incompletely understood. Understanding the mechanisms of these degenerative complications is crucial, in view of the increase in the incidence of type 2 diabetes (1).

Extracellular matrix accumulation may result from an increased synthesis of matrix components and/or a decreased degradation. The proteolytic systems involved in matrix degradation are mainly the matrix metalloproteinase system (MMPs) and the plasminogen activating system (PAs), which are both controlled by potent inhibitors, tissue-inhibitors of metalloproteinases (TIMPs) and plasminogen activator inhibitors (PAIs) (2). TIMPs and PAI-1 have been suggested to play a role in the process of glomerulosclerosis (3).

Considerable evidence indicates that production of intracellular AGEs may play a major role in the pathogenesis of cellular and extracellular matrix changes that occur in diabetic nephropathy (4-6). AGEs, such as Nε-carboxymethyl lysine (CML) and pentosidine, which derive from the non-enzymatic coupling between amino-acid residues and oxidative derivatives of glucose or pentose, have been shown to accumulate during chronic hyperglycaemia and diabetes (7), and to either activate certain cell types, or to modify extracellular matrix proteins, which may become more resistant to degradation (8).

How cells respond to extracellular AGEs has been studied in different conditions. It appears that several membrane proteins may serve as receptors for AGEs: the macrophage

scavenger receptors class A, type II (MSR-AII) or class B type I (MSR-BI, CD36), the oligosaccharyl transferase - 48 termed AGE receptor 1 (AGE-R1), the 80 K-H phosphoprotein (AGE-R2) and galectin-3 (AGE-R3) and RAGE for receptor for AGEs (4). AGE accumulation in glomeruli (9), RAGE expression and diabetic changes have been demonstrated in the kidney of diabetic mice (10). Furthermore, it has been suggested that activation of RAGE could induce reactive oxygen species formation (11), NF-κB activation, TGF-ß synthesis, with subsequent ECM protein synthesis, and VEGF expression in podocytes leading to an increase in glomerular permeability (12-14).

The expression of AGEs receptors by human or murine glomerular cells has been demonstrated *in vitro* and *in vivo* (15-18). In particular, Yamagishi *et al* demonstrated that different types of AGEs induced apoptosis and overexpression of VEGF and MCP-1 in cultured human mesangial cells (19). Interestingly, these authors suggested that these effects were mediated by receptors other than RAGE.

In the present study, we explored the expression of AGE receptors by human mesangial cells in culture, and we analysed the effects of AGEs on gene expression, focusing on extracellular matrix proteins and proteolytic systems involved in matrix remodelling. We show herein that human mesangial cells express RAGE as well as AGE-R1, AGE-R2 and AGE-R3, and that AGEs and CML activate the expression of several proteins, including TIMP-3 and PAI-1, which may inhibit matrix remodelling and promote glomerulosclerosis.

Material and methods

Cell culture. Human glomerular mesangial and epithelial cells were isolated as described previously (20,21). Briefly, a preparation of glomeruli virtually free of non-glomerular contaminants was digested by collagenase IV for 30 min. The glomerular suspension was then sieved over a stainless mesh screen and isolated epithelial cells were recovered in the filtrate after repeated washings. The remaining glomerular fragments were explanted in Petri's dishes and incubated at 37˚C: smooth muscle-like mesangial cells appeared ~8-14 days following attachments. They appeared large and stellate. Immunofluorescence studies with specific antibodies raised against von Willebrand factor were negative, ruling out the presence of endothelial cells. To further eliminate any contamination, all experiments were performed between the third and fifth passages. Cells were grown in RPMI (Gibco, Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (50 µg/ml), L-glutamine (2 mM) and HEPES buffer. The cells were cultured in 100-mm culture plates.

Glycated (AGE and CML) proteins. Bovine serum albumin (Sigma, St. Quentin Fallavier, France) was incubated with glucose 0.5 M in phosphate buffer saline (PBS, Gibco) supplemented with aprotinin (Sigma) and antibiotics (penicillin, streptomycin-G) (Invitrogen) for 10 weeks in the dark. Control BSA was incubated in the same conditions but without glucose. After 10 weeks of incubation in the dark, the solutions were dialysed in PBS for 48 h.

The protein concentration was measured with the Bradford method and AGE-specific fluorescence was determined at 420 nm after excitation at 350 nm by using an Aminco SPF 500 ratio spectrofluorometer (Photon Technology International) as described previously (22).

To prepare CML-albumin, albumin and sodium cyanoborohydride (0.45 M) were dissolved in sodium phosphate buffer (0.2 M; pH 7.8) (13). Glycoxylic acid was then added, and the mixture incubated for 24 h at 37˚C. Control protein was prepared under the same conditions, except that glyoxylic acid was omitted. The preparation of CML-modified proteins was characterized by modifications in percentage, assessed via 2,4,6-trinitrobenzenesulfonic acid to determine the difference between lysine residues in modified versus unmodified pre-parations and also by gas chromatography-mass spectrometry (GC-MS). CML-albumin and control albumin were extensively dialyzed. Endotoxin levels evaluated by chromogenic Limulus assay (ACC, Falmouth, MA, USA) amounted to <0.002 EU/mg protein in the experimental preparations.

RAGE antibody preparation. For RAGE antibody production, rat RAGE was purified to homogeneity from rat lung powder by the methods previously described for bovine RAGE, and this corresponded to the amino-terminal two-thirds of the molecule (23). Purified rat RAGE was used to immunize rabbits according to previously described methods (24). This monospecific anti-RAGE IgG was purified by affinity chromatography with immobilized protein A.

Fluorescence activated cell sorter analysis. RAGE expression on the surface of human glomerular mesangial cells, mesothelial cells and glomerular epithelial cells were studied by flow cytometry as previously described (13). For each experiment, 2.10^5 cells were incubated for 30 min at 4˚C with anti-RAGE antibody. Antibody binding was evidenced by FITC-conjugated secondary antibodies (Immunotech, Marseille, France). Immunofluorescence intensity was quantified by FACS (FacScan, Beckton Dickinson, Mountain View, CA, USA). For each antibody, 5,000 events were recorded. The results are presented as the mean fluorescence intensity (MFI) expressed as arbitrary units of fluorescence (AUF). For negative control, primary antibody was omitted.

Gene expression analysis by microarray. Human glomerular mesangial cells from primary culture were grown in a 100-mm plate to ~80% confluence in their regular medium. Then the cells were deprived of serum during 24 h to prevent contamination by growth factors or other relevant proteins from FCS and also to synchronize the cells. Then, they were preincubated with BSA-AGE or control BSA during 4 h. The cells were then harvested in their medium using a rubber policeman, centrifuged 3 min at 1000 rpm and total RNA from the cell pellet were then isolated using the TRIzol (Invitrogen) single step reagent. The total RNA obtained was treated with DNase I to avoid genomic DNA contamination of reverse transcription reactions. Radioactive cDNA synthesis was carried out as described in the Atlas™ cDNA expression arrays user manual (Clontech, San Diego, CA, USA) as previously described (25). Equal amounts of ^{33}P-radiolabeled cDNAs (10^7 cpm) from BSA control and BSA-AGE treated glomerular mesangial cells RNA samples were hybridized in parallel to Atlas human cell interaction cDNA expression

italog number: 7746-1, Clontech) for 18 h at 68˚C. s were then washed 4 times in 2X SSC and 1% SDS for 30 min at 68˚C and twice in 0.1X SSC and 0.5% SDS at 68˚C, according to the manufacturer's protocol. Membranes were then exposed to Eastman Kodak Co. phosphor screens for 3 days. Hybridization signals were quantified with a Storm 840 phosphorimager using Image Quant software (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and normalized against glyceraldehydes-3-phosphate dehydrogenase mRNA levels in the same samples. Significant modulation of gene expression was set arbitrary to 2-fold.

Northern blot analysis. Total RNA samples (10 μg) prepared as described previously (26) of each cell condition were separated by electrophoresis through 0.9% agarose gels containing 20% of formaldehyde for 5 h at 60 V. The total RNA was then transferred overnight to nylon membrane (Geenscreen Plus hybridization transfer membrane, Perkin Elmer life Sciences Inc., Boston, MA, USA). Nucleic acids were immobilized by ultraviolet irradiation. The membrane were hybridized at 42˚C overnight in UltraHyb hybridization solution (Ambion Inc., Austin, TX, USA) containing cDNA probes labeled with α^{32}P-dCTP by random oligonucleotide priming (Boehringer-Mannheim Biochemicals Inc., Indianapolis, IN, USA), washed according to the Current Protocols in Molecular Biology (volume 1, section 4.9.2) and exposed for 4 h to autoradiography. The density of each band was quantified using the NIH image program (Bethesda, MD, USA). The results were adjusted for unequal RNA loading on the intensity of the the RNA band for a housekeeping gene (human GAPDH). Results were expressed in arbitrary densitometric units (AU).

The cDNA probes were human PAI-1 and Human GAPDH cDNAs generated by PCR using the following primers: PAI-1 sense: 5'-GAGAGAGCCAGATTCATCATC-3'; PAI-1 antisense: 5'-CAAGGTCTTGGAGACAGATCT-3'; GAPDH sense: 5'-CGATGCTGGCGCTGAGTAC-3'; GAPDH antisense: 5'-CGTTCAGCTCAGGGATGACC-3' as described (27).

Reverse transcription. The total RNA from glomerular mesangial cells stimulated with Control BSA or BSA-AGE (200 μg/ml) was first treated with DNase I (Sigma). DNase-treated total RNA (1 μg) was reverse-transcribed at 60˚C for 1 h in a reaction mixture containing a final concentration of 2.5 μM random hexamer primers, 1 mM of deoxynucleotide triphosphates (Promega, Charbonnieres, France), 1X PCR buffer II, 5 mM $MgCl_2$, 1 U/μl RNase inhibitor and 2.5 U/μl of Murine leukemia virus reverse transcriptase (Applied Biosystems, Rotkreuz, Switzerland). The cDNAs (50 ng/μl) were stored at -20˚C until real-time quantitative polymerase chain reaction (TaqMan PCR) or qualitative PCR was performed.

Polymerase chain reaction (PCR). One μl of cDNA (50 ng/μl) extracted from glomerular mesangial cells treated by BSA-AGE was mixed with 1 μl of dNTPs (25 mM), 3 μl of $MgCl_2$ (50 mM), 5 μl of 10X PCR buffer, 1 μl of primer sense (10 pmoles/μl) and 1 μl of primer antisense (10 pmoles/μl), 2.5 units of Taq polymerase (5 U/μl) in a 50 μl final volume. PCR was performed using a thermocycler (Bio-Tek Instruments Winooski, VT, USA). PCR primers were designed to amplify a 469-bp fragment located in the RAGE coding region: sense 5'-CCCTTCTCATTAGGCACCAG-3', antisense: 5-'CAGGAATGGAAAGGAGACCA-3' as previously described (13); a 221-bp fragment of the AGE R1 coding sequence: sense 5'-TTCAAGACCGCTGATGACC-3', antisense: 5'-AACTCAATCCCGCACTCAC-3', the 439-bp fragment of the AGE-R2 coding sequence: sense 5'-GTG GCGTCTGTCTGTGTGA-3', antisense 5'GGCCGTAAG GAGAGAGAGAGTC-3'; and the 513-bp fragment of the AGE-R3 coding sequence: sense 5'-CACCTGCACCTGGAG TCTAC-3', antisense 5'-GCACTGGTGAGGTCTATGTC-3'.

Quantitative RT-PCR. The PCR conditions were established according to the instructions of Applied Biosystems (Applied Biosystems). The respective cDNA was used in duplicate as a template for the real-time PCR analysis with the ABI PRISM 7000 sequence detection system (Applied Biosystems). The thermal cycle profile was: 95˚C for 15 sec, 60˚C for 1 min, repeated 45 times. To detect genomic DNA contaminations, all analyses included minus reverse transcriptase samples as negative controls.

The S18 subunit of ribosomal RNA (S18 rRNA) expression levels were analysed in all samples as a housekeeping gene to normalize expression between different samples and to monitor assay reproducibility. Relative quantification of all targets was calculated by the comparative cycle threshold method outlined by the manufacturer (User bulletin no. 2; Applied Biosystems), MMP-2, TIMP-3, t-PA, PAI-1, α2 macroglobulin, Collagen IVα1 vitronectin receptor, fibronectin 1, fibronectin receptor, L1-CAM and S18 rRNA. Assays-on-Demand gene expression products were used for each one as described in the manufacturer's protocol (Applied Biosystems). cDNA solution (1 μl) from 50 ng reverse transcribed total RNA was added to the PCR reaction mixtures of 24 μl. Reaction mixtures were composed as follows: TaqMan Universal PCR Master mix (Applied Biosystems; containing AmpliTaq Gold DNA polymerase) final concentration 1X, and Gene expression assay mix containing primers and probe, final concentration 1X.

Fibrin and gelatin zymography. Human glomerular mesangial cells from primary culture were grown in a 6-well plate to ~80% confluence in their regular medium. Then the cells were deprived of serum for 24 h and incubated with BSA-AGE or control BSA for 24, 48 and 72 h. The conditioned media were collected and was separated on a 10% SDS-polyacrylamide gel. Plasminogen activator activity was detected on a fibrin agar underlay (fibrinogen 10 mg/ml, thrombin 300 mU/ml, agarose 1%) containing plasminogen (20 μg/ml) as previously described (21). For the gelatin zymography, the supernatant of each condition was separated on a 10% SDS-polyacrylamide gel containing 1 mg/ml gelatin. After electrophoresis, the gel was soaked in Triton X100 2.5% for 1 h and incubated in Tris-HCl 50 mM pH 7.5, $CaCl_2$ 5 mM $ZnCl_2$ 1 μM overnight at 37˚C and stained with Coomassie Blue dye (21).

PAI-1 ELISA. Human glomerular mesangial cells from primary culture were grown and stimulated as described above. Cell

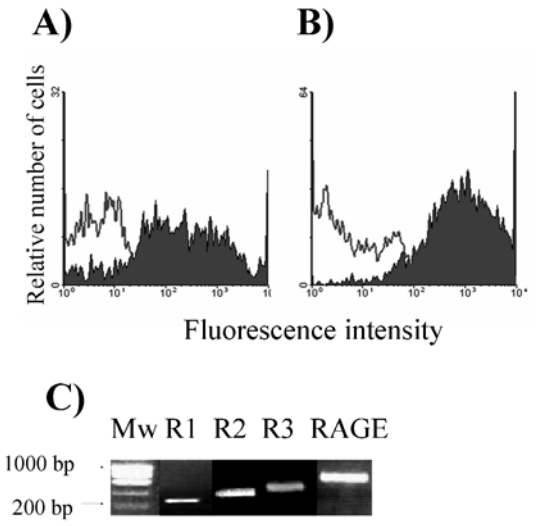

Figure 1. Detection of RAGE on human glomerular cells. By FACS analysis, RAGE was detected at the surface of cultured mesangial (A) and epithelial (B) glomerular cells. Non-immune IgG (white) did not detect any antigen, whereas anti-RAGE antibody (black) bound to the cell surface. By RT-PCR in human mesangial cells, AGE-R1, AGE-R2, AGE-R3 and RAGE mRNA could be detected (C).

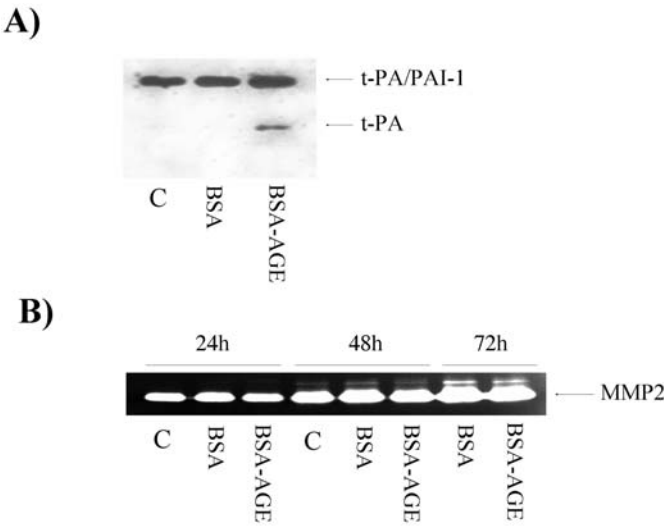

Figure 2. Effect of BSA-AGE on t-PA and MMP-2 synthesis. By fibrin zymography (2A), conditioned culture medium (C) of human mesangial cells was shown to contain a 110-120 kD form of plasminogen activator, which was previously identified as a t-PA/PAI-1 complex. Incubation with BSA did not change this pattern of synthesis, whereas after a 24-h incubation, BSA-AGE induced an increase in both complexed t-PA and free t-PA (arrows). Zymography on gelatin (2B) detected a 72 kD band corresponding to MMP-2, and no significant difference could be seen between control conditions (C), and incubation with BSA or BSA-AGE, at 24, 48 and 72 h.

supernatant was collected and the PAI-1 antigen was determined by ELISA (TintElize PAI-1, Biopool AB, Umeå, Sweden) according to the manufacturer's instructions (28).

Statistical analysis. Results are expressed as mean ± SEM, and were compared using Student t-test or one-way or two-way analysis of variance. $p<0.05$ was considered significant.

Table I. The genes upregulated by BSA-AGE in human mesangial cells.

	A) Microarray	B) Quantitative real-time RT-PCR
MMP-2	x2.0	x2.0
TIMP-3	x4.3	x1.8
t-PA	x3.9	x2.7
PAI-1	x2.0	x2.4
α2 macroglobulin	x3.3	x2.7
Collagen IV α1	x2.0	x2.6
Vitronectin receptor	x2.9	x2.0
Fibronectin	x2.0	x2.2
Fibronectin receptor	x3.2	x2.5
L1-CAM	x3.4	x2.3

Results

Human glomerular mesangial cells express membrane receptors for AGEs. As shown in Fig. 1, by FACS analysis using a specific polyclonal antibody directed against the human RAGE protein, we demonstrated that human mesangial cells express RAGE at their membrane: ~60-70% of the cell population do express RAGE at their membrane (Fig. 1A). In parallel experiments, we found that human glomerular epithelial cells, also known to express RAGE at their membrane *in vivo*, were positively stained with the RAGE antibody in FACS analysis (Fig. 1B). We used anti-PAR1 antibody as a positive control (data not shown). In addition, by RT-PCR we were able to amplify cDNA for RAGE from human mesangial cells in culture. Similarly CDNA for AGE-R1, -R2 and -R3 could also be amplified from human mesangial cells (Fig. 1C).

BSA-AGE stimulates the expression of several extracellular matrix genes. To evaluate the effect of AGEs on extracellular matrix remodelling in the mesangium, we analyzed gene expression profiles of human mesangial cells stimulated by BSA-AGE or by control BSA using the microarray method. To determine which genes were directly activated by AGEs, the cells were incubated for 4 h with a 200 μg/ml concentration of BSA-AGE or control BSA, and then the total RNA was extracted. For 10 genes, a ratio ≥2 was found in at least 3 different experiments (see Table I, A). The increased expression of the 10 genes was confirmed by the quantitative real-time RT-PCR method (Table I, B).

Among them, we found genes of ECM components such as α1 chain of collagen IV and fibronectin, but also genes involved in matrix remodelling such as MMP-2 and t-PA which was strongly induced. Interestingly, inhibitors of matrix metalloproteinases and of the plasminogen activator/plasmin system such as TIMP-3 and PAI-1, and α2 macroglobulin were also induced.

BSA-AGE stimulates the synthesis of t-PA and PAI-1 by human mesangial cells. In order to confirm that the increase in PAI-1 and t-PA mRNA was associated with an increased synthesis

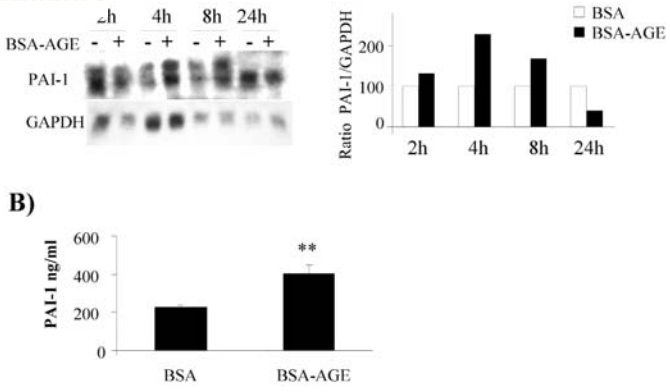

Figure 3. Effects of BSA-AGE on PAI-1 synthesis by human mesangial cells. By Northern blot, BSA-AGE increased PAI-1 mRNA as shown in a representative experiment in A (left panel). Quantitative assessment, compared to GAPDH mRNA, indicates that the maximum increase in PAI-1 mRNA was reached after 4 h of incubation (A, right panel). By ELISA, PAI-1 antigen was significantly increased after 24 h of incubation with 200 μg/ml BSA-AGE compared to BSA (B).

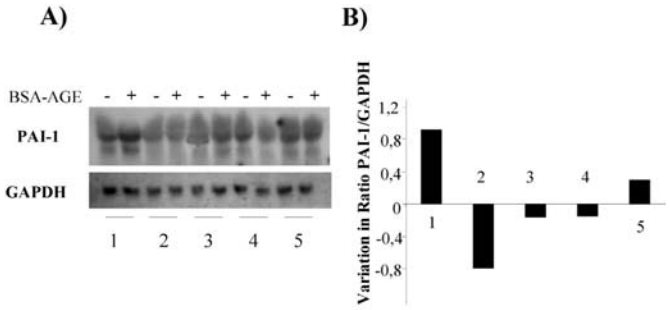

Figure 4. Intracellular pathways activated by AGEs in human mesangial cells. By Northern blot (A) and densitometric analysis (B), BSA-AGE increased PAI-1 mRNA at 4 h (lane 1); this effect was inhibited by 10 mM N-acetyl cysteine (lane 2), 50 μM PD-98059 (lane 3), and 5 μM norleucin (lane 4) but not by 20 μM curcumin (lane 5), suggesting that the AGE effect is mediated through an activation of reactive oxygen species, ERK1-ERK2, and NF-κB but not of AP-1.

of the corresponding proteins, we analyzed by zymography the culture medium of human mesangial cells incubated for 24 h with 200 μg/ml BSA-AGE or control BSA. As shown in Fig. 2A, by fibrin zymography, human mesangial cells in culture were shown to produce a 110-120 kD high molecular weight form of t-PA which was previously characterized by us and others as a complex between tPA and PAI-1. Incubation with control BSA did not change this expression, whereas BSA-AGE induced a significant increased in both t-PA/PAI-1 complexes and also in free t-PA, identified by its ability to activate plasminogen and its 70 kD molecular weight, comigrating with recombinant t-PA used as control.

In contrast, by gelatin zymography used to analyze the MMP synthesis, we were unable to detect a significant effect of AGEs on MMP-2 synthesis, after 24, 48 or even 72 h of incubation (Fig. 2B).

The effect of AGEs on PAI-1 synthesis was confirmed by the time course analysis of PAI-1 mRNA by Northern blot (Fig. 3A) and of the PAI-1 protein in the conditioned culture medium (Fig. 3B). An early and transient increase in PAI-1 mRNA was demonstrated after 4 h of incubation. It was associated with a 2-fold increase in PAI-1 protein at 24 h.

To determine which main signalling pathways were activated by AGEs to induce PAI-1 synthesis, we studied the effects of N acetyl cysteine, a blocker of oxygen radical derivatives, PD-98059, an inhibitor of the MAP kinases, norleucin, an inhibitor of the transcription factor NF-κB, and curcumin, an inhibitor of the AP-1 complex, on the PAI-1 induction by BSA-AGE. As shown by Northern blot (Fig. 4), the effect of BSA-AGE was blocked by NAC, PD-98059, and norleucin but not by curcumin.

Carboxymethyl-lysine, an AGE component, also activate human mesangial cells. To confirm that the effects of BSA-AGE were actually induced by AGE residues, we studied the effects of purified carboxymethyl-lysine and compared them to the effects of human serum albumin. We used quantitative real-time RT-PCR and found that CML induced a significant increase in PAI-1 mRNA, but its effects on other genes were less pronounced than those of BSA-AGE (Table II). In addition, PAI-1 protein was increased 3- to 4-fold in the conditioned culture medium when human mesangial cells were stimulated by CML for 24 h (Fig. 5). As a positive

Table II. The genes upregulated by CML in human mesangial cells.

	Quantitative real-time RT-PCR
MMP-2	x1.3
TIMP-3	x1.3
t-PA	x1.9
PAI-1	x1.9
α2 macroglobulin	x2.0
Collagen IV α1	x1.5
Vitronectin receptor	x1.5
Fibronectin	x1.3
Fibronectin receptor	x1.4
L1-CAM	x1.5

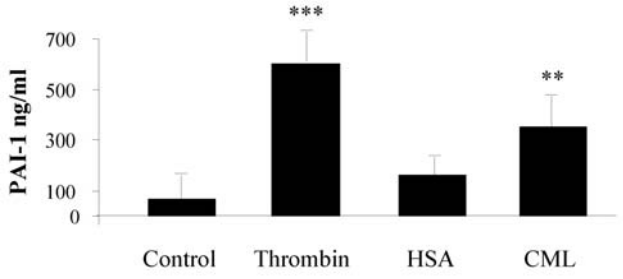

Figure 5. Effect of carboxymethyl-lysine on PAI-1 synthesis by human mesangial cells. Human mesangial cells were incubated with CML 5 μM, HSA 5 μM or thrombin 10 nM for 24 h and PAI-1 protein concentration was measured in the conditioned culture medium by ELISA. Thrombin, used as a positive control, induced a 6-fold increase in PAI-1 antigen concentration, whereas CML induced a 4-fold increase. HSA has no significant effect. Mean ± SEM are shown from 3 experiments ($^{**}p<0.05$, $^{***}p<0.01$).

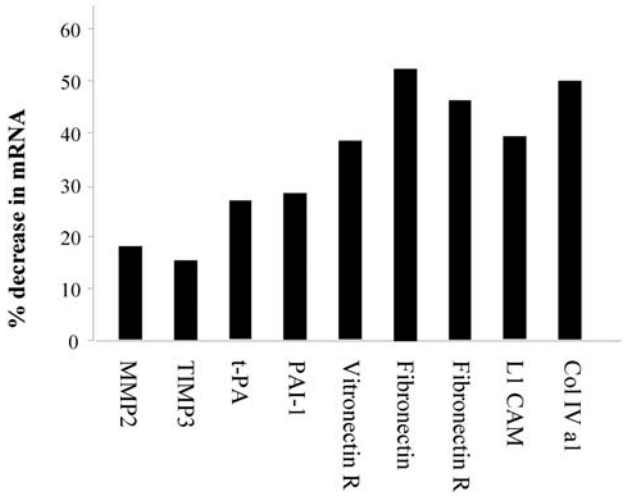

Figure 6. Effect of anti-RAGE antibody on BSA-AGE stimulated gene expression in human mesangial cells. Human mesangial cells were incubated with BSA-AGE or BSA, 200 μg/ml, for 4 h in the presence of non-specific rabbit IgG or rabbit anti-RAGE antibody. After mRNA extraction, specific mRNA were measured by quantitative real-time RT-PCR. The anti-RAGE antibody partially inhibited the effect of BSA-AGE on all the studied mRNA (one experiment made in duplicate).

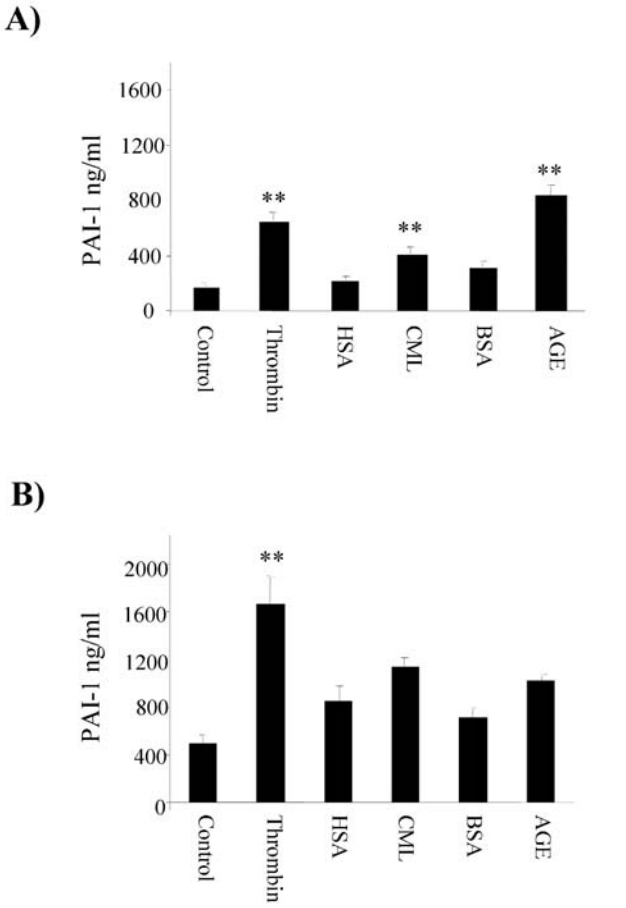

Figure 7. Effect of anti-RAGE antibody on PAI-1 synthesis by human mesangial cells. Human mesangial cells were incubated with thrombin 10 nM, HSA or CML 5 μM, or BSA or BSA-AGE 200 μg/ml for 24 h, in the presence of non-specific rabbit IgG or blocking anti-RAGE antibody. PAI-1 antigen concentration was determined by ELISA in the conditioned culture medium. Non-specific IgG did not inhibit the stimulating effect of thrombin, CML or BSA-AGE (A), whereas blockade of RAGE inhibited the effects of CML and of BSA-AGE but not that of thrombin (B) ($^{**}p<0.05$).

control, thrombin, known as a potent inducer of PAI-1 synthesis, induced a 6-fold increase in PAI-1 concentration.

RAGE mediates some of the effects of AGEs on human mesangial cells. It is known that CML is a ligand for RAGE (29). To evaluate the role of RAGE in the BSA-AGE and the CML effects, we used a blocking anti-RAGE antibody. As shown in Fig. 6, when compared to a non-specific rabbit IgG, the anti-RAGE antibody decreased significantly the gene activation induced by BSA-AGE. In addition, the increase in PAI-1 synthesis induced by thrombin, CML or BSA-AGE was not inhibited by a non-specific IgG (Fig. 7A), whereas the anti-RAGE antibody inhibited significantly the effects of CML and of BSA-AGE but not that of thrombin (Fig. 7B).

Discussion

The present study provides evidence that human mesangial cells express several membrane receptors for AGEs, including RAGE. It also shows that AGEs can induce the synthesis of various components of the extracellular matrix and modulate matrix remodelling by regulating the synthesis of MMP-2 and t-PA, and of inhibitors such as TIMP-3 and PAI-1.

This is one of the first analyses of AGE receptor expression in human mesangial cells. Some *in vivo* studies indicate that RAGE is expressed by both mesangial and epithelial glomerular cells (15), whereas others suggest that visceral epithelial cells, the podocytes, are the unique cell type expressing RAGE in the glomerulus (18). The reasons for these discrepancies are not clear, but may be related to technical differences and to the specificity of the antibody used to detect RAGE. Our study demonstrates that both human mesangial cells and podocytes expressed RAGE when they are inculture. In diseased conditions, upregulation of RAGE expression by the two cell types is likely, although podocytes seem the major source of RAGE expression in the normal or diseased kidney (15,18). Our results also show that human mesangial cells express specific mRNA for AGE-R1, R2 and R3, as do murine mesangial cells (4). The partial blocking effect of anti-RAGE antibodies that we observed suggest that RAGE is functional in human mesangial cells and induces NF-κB activation and matrix protein synthesis. However, the effects of CML, which is a quite specific ligand for RAGE (29), were smaller than those of BSA-AGE, as assessed by quantitative RT-PCR. This suggests that either the other receptors are also involved in the cellular effects of AGEs, or that RAGE was more activated by BSA-AGE than by CML.

We report herein that AGEs seem to activate the generation of oxygen radicals, the activation of MAP kinases, and the activation of NF-κB in human mesangial cells. Similar pathways have been implicated in the induction of cytokine adhesion molecules and growth factor synthesis by AGEs in various cell types (12,13,30). The pathways activated by the other receptors are less known, except for R3 which is involved in AGEs internalisation and clearance (31). Taken together, these results suggest that AGEs can activate human

l cells, mainly but not exclusively, through the) RAGE and its subsequent activation.

The changes in gene expression induced by AGEs were consistently observed whatever the techniques used to evaluate them. We focused on extracellular matrix components, since diabetic nephropathy is characterized by accumulation of ECM and development of glomerulosclerosis. In addition, we found that matrix remodelling enzymes were also regulated by AGEs. TIMP-3 induction, in the absence of upregulation of MMP-2 or MMP-9, may play a role as an inhibitor of matrix metalloproteinase, and promote matrix accumulation. Interestingly, Stricker *et al* previously reported that TIMP-3 was upregulated in mouse glomeruli after induction of diabetes by streptozotocine administration, and suggested that TIMP-3 is involved in the pathogenesis of diabetic nephropathy (32). AGEs seem to increase both PAI-1 and t-PA synthesis. PAI-1, which has been shown to be upregulated *in vivo* in diabetic kidneys, could promote glomerulosclerosis, by local inhibition of t-PA activity and plasmin generation, and thus inhibition of matrix degradation (3). However, our results indicate that t-PA is much more induced than PAI-1 by AGEs: on fibrin zymography, the net effect of the exposure of mesangial cells to BSA-AGE is revealed by the apparition of a tPA-induced lytic zone, suggesting that eventually, unopposed plasminogen activation prevails. More recent findings indicate that t-PA has a profibrogenic effect in certain circumstances, such as after unilateral ureteral obstruction (33). This paradoxical effect seems to be plasmin independent, but is related to a direct stimulating effect of t-PA on MMP-9 synthesis. In turn, MMP-9 could degrade basement membrane, and promote cell detachment leading to epithelial-mesenchymal transition (33). Whether a similar mechanism could be at play during diabetic nephropathy remains unknown. Another possibility for t-PA to promote matrix accumulation would be an increase in TGF-ß activation. TGF-ß has been shown to be strongly induced during diabetes, and to play a role in various form of glomerulosclerosis. If this is the case, PAI-1 would have a rather protective role, by inhibiting in part the large amounts of t-PA released in the presence of AGEs. This seems unlikely after the recent studies that PAI-1 knock-out mice have a lower proteinuria than their wild-type controls after induction of diabetes by streptozotocin (34-36).

AGEs accumulate during diabetes (8), but also during chronic renal failure and ageing. They may therefore be involved in the non-specific progression of chronic nephropathies, and in the progressive sclerosis of the kidney that occurs with age (37). Specific antagonists of RAGE would thus be beneficial in these various conditions, unless other receptors are also able to mediate similar effects when activated by AGEs (38).

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