Advanced Glycation Endproducts Stimulate Renal Epithelial Cells to Release Chemokines That Recruit Macrophages, Leading to Renal Fibrosis

Yosuke Sasai, Kousei Iwakawa, Kanako Yanagida, Yan Shen, Takashi Hosono, Toyohiko Ariga, and Taichiro Seki

Laboratory of Nutrition and Physiology, Department of Chemistry and Life Science, Nihon University College of Bioresource Sciences, 1866 Kameino, Fujisawa, Kanagawa 252-0880, Japan

Received May 7, 2012; Accepted June 9, 2012; Online Publication, September 7, 2012

[doi:10.1271/bbb.120347]

Diabetic nephropathy is a major complication of diabetes and tubulointerstitial fibrosis is one of its manifestations. This study aimed to clarify the pathogenicity of advanced glycation endproducts (AGEs) toward NRK-52E, a tubular epithelial cell line. The AGE-exposed cells significantly increased gene expression of transforming growth factor beta, plasminogen activator inhibitor-1, and tissue transglutaminase, and a medium conditioned by them showed strong potential to recruit macrophages, partly through a chemokine, monocyte chemoattractant protein-1. Albumin denatured at 37°C for 120 d exhibited similar activities, but they were lower than those of the AGES. Thus, AGES generated in diabetic patients might exacerbate fibrosis in the kidneys directly through renal epithelial cell stimulation, and indirectly by recruitment of macrophages.

Key words: advanced glycation endproducts; monocyte chemoattractant protein-1; diabetic nephropathy; renal fibrosis; plasminogen activator inhibitor-1

The reaction between reducing sugars and free-amino groups of proteins is known to proceed nonenzymatically via the formation of Schiff-base intermediates and Amadori rearrangement products. These reactions are known to occur also in patients with diabetes, in whom persistent hyperglycemia permits the reaction and generates undissociable heterogeneous molecules, the so-called advanced glycation endproducts (AGEs). Although the detailed structure-activity relationship of AGEs has not been clarified yet, it is believed that these compounds act as one of the pathogenic factors that lead to retinopathy as well as nephropathy in diabetic patients. Diabetic nephropathy, a major complication in patients with diabetes mellitus, is characterized by an increased content of collagen, protein accumulation, and that these organs also have an increased content of collagen.

Monocyte chemoattractant protein-1 (MCP-1) is a potent chemokine that plays pivotal roles in both monocyte/macrophage migration and monocyte activation. MCP-1 has been found to play an important role under various inflammatory conditions. Administration of anti-MCP-1 antibody has been reported to ameliorate both glomerulosclerosis and renal dysfunction and creatinine clearance. Also, the degree of diabetic nephropathy experimentally generated by injecting streptozotocin is known to be lower in MCP-1-deficient mice than in wild-type mice similarly treated, in which nephropathy is severe.

Plasminogen activator inhibitor-1 (PAI-1) is a member of serine protease inhibitor superfamily. It serves as a major physiological inhibitor against both plasminogen activators, viz., tissue-type plasminogen activator and urokinase-type plasminogen activator. These convert the zymogen plasminogen to its active enzyme, plasmin,(9) which is involved not only in fibrinolysis, but also in the transformation of pro-matrix metalloproteinases (pro-MMPs) into active MMPs. Thus PAI-1 plays important roles in the regulation of ECM degradation through its inhibitory potential toward plasminogen activation. It has been reported that PAI-1 expression is upregulated in tubular epithelial cells of both type-1 and type-2 diabetic model rats, and that in PAI-1-deficient mice, renal fibrosis is retarded. Transforming growth factor beta (TGF-β) induces the production of these ECM proteins, tissue inhibitor of metalloproteinase (TIMP)12 and PAI-1, via the Smad signaling pathway. Tissue transglutaminase (tTG) forms γ-(γ-glutamyl) lysine-isopeptide bonding between glutamine and lysine residues in proteins, and is thought to stabilize the structure of ECMs. It has also been reported that tTG is involved in the pathogenesis of tubular interstitial fibrosis.

This study was aimed to clarify the effect of AGES and related products on the expression of the above-described fibrogenic genes including TGFβ, PAI-1, and tTG, in tubular epithelial cells, and to elucidate the pathogenic mechanisms causing nephropathy in diabetes.
mellitus. AGEs, but not denatured albumin, acted as a potent stimulator of renal epithelial cells, which then recruit macrophages by producing MCP-1 and other attractants, leading to the interstitial fibrosis observed in diabetic nephropathy.

Materials and Methods

Preparation of AGEs. AGEs were prepared as described previously. Briefly, a solution containing 50 mg/mL of bovine serum albumin (BSA; fatty-acid free, low endotoxin, Sigma-Aldrich, St. Louis, MO) and 500 mM glucose was prepared in 400 mM sodium phosphate buffer (pH 7.4). After it was sterilized through a membrane filter (pore size 0.22 μm, Millipore, Bedford, MA), the solution was incubated at 37 °C in the dark for up to 120 d, and then dialyzed extensively against phosphate-buffered saline (PBS; 5 L × 5 changes) at 4 °C for 18 h using dialysis membrane (type C20-32-100, molecular mass cutoff 14 kDa, Sanko Pure Chemicals, Tokyo). The AGE preparation was tested for endotoxin by the Endospecy ES-20S system (Seikagaku Kogyo, Tokyo), and was confirmed to have an endotoxin level of less than 2.0 ng/mg of protein. Two other solutions, 50 mg/mL of BSA and 500 mM glucose, both in 400 mM sodium phosphate buffer (pH 7.4), were also prepared and incubated for the same period of time, 120 d, and were designated incubated BSA (iBSA) and incubated glucose (iGlucose) respectively. The BSA, iBSA, and AGEs thus prepared were examined for changes in molecules by SDS–PAGE using a 8% gel and Coomassie Blue staining.

Cell culture. NRK-52E cells, an epithelial cell line derived from the rat kidney (cell no. IF050480, Health Science Research Resources Bank, Tokyo), were grown and maintained in Dulbecco’s Modified Eagle’s Medium (DMEM, Nissui Pharmaceutical, Tokyo) supplemented with 10% FBS (Biowest, Rue de la Caille, Nuaille, France) at 37 °C in 95% air and 5% CO2. After the cells reached confluence, the conditioned medium was replaced with serum-free fresh medium, and the cells were then cultured for a further 72 h. Then a sample of AGEs or another stimulant was added to the cultures. After stimulation for 3 or 6 h, the conditioned medium was analyzed for cellular responses involving chemokine production.

RNA isolation and quantitative real-time PCR. Total RNA was extracted from NRK-52E cells with Isogen (Nippon Gene, Tokyo) following to the manufacturer’s instructions. Real-time PCR was performed using Light-Cycler, a rapid thermal cycler system (Roche Diagnostics, Lewes, UK), as described previously. The primers used were as follows: Rat MCP-1 primers, sense 5'-AAC GAC CCT GAG AAG AAG GAC-3', antisense 5'-GGC ACC AAC CAC TAC TCA GCA GCA C-3'; PAI-1 primers, sense 5'-GAC AAT GGA AGA GCA ACA TG-3', antisense 5'-ACC TCG ATC TTG ACC TTT TG-3'; tTG primers, sense 5'-GTC AGC TAC ACC CCC GGC GTG-3', antisense 5'-GCC ACC CGC TGT ACT TCT-3'; TGFβ1, sense 5'-TGC TTC ACC TCC ACA GAG AA-3', antisense 5'-TGT GTT GTG TGT AGA GGG CA-3'; and GAPDH, sense 5'-AAC GAC CCC TCC TTC AGT GAC-3', antisense 5'-TCC ACG ACA TAC TCA GCA C-3'.

Chemotaxis assay. Peritoneal macrophages were harvested from 30-week-old ICR mice on the 3rd day after injection of an elicitor, 4% thioglycolate medium (Nissui Pharmaceutical, Tokyo), washed with phosphate-buffered saline, and resuspended in RPMI 1640 medium (Gibco, Carlsbad, CA). All animal experiments were performed in accordance with the Guidelines for Animal Experiments, College of Bioresource Sciences, Nihon University. Cell migration assays were performed by a method using chemotaxis chambers, the so-called Boyden Chamber, with minor modifications. A 24-well cell culture plate (Sumitomo Bakelite, Tokyo) and chemotaxis chambers were used. A sample (0.6 mL), AGEs or conditioned medium harvested from NRK-52E cultures, was placed in the lower wells of the chamber. A sample (0.6 mL), AGEs or another stimulant was added to the cultures. After stimulation for 3 or 6 h, the conditioned medium was analyzed for changes in molecules by SDS–PAGE using a 8% gel and Coomassie Blue staining.

Results

AGEs upregulated the expression of fibrogenic genes in the NRK-52E cells

Infiltration of inflammatory cells into renal tissues and upregulation of fibrogenic components have often been observed in diabetic nephropathy. In this study, we focused on the role of AGEs in the pathogenesis of renal dysfunction due to diabetic nephropathy.

As Fig. 1 shows the AGEs appeared as a smear of larger molecules (see lane a, panel A), whereas iBSA (lane b) and native BSA (lane c) showed tighter bands. Likewise, as shown in panel B, AGEs were determined to have marked fluorescent emission at 425 nm, as compared with much weaker emission for iBSA (b) and the native BSA (c). These data suggest highly modified and polymerized albumin molecules were present in the AGE preparation.

First, we examined the effect of AGEs, iBSA, and iGlucose on genes closely related to renal inflammation and fibrosis in a tubular epithelial cell line, NRK-52E (Fig. 2). The AGE-exposed cells showed significant increases in the mRNA levels of all the genes examined, those for PAI-1, tTG, MCP-1, and TGFβ1. As compared with iBSA and iGlucose, the AGEs caused a 4–6 times greater increase in these levels. Although iBSA upregulated MCP-1 mRNA expression, it did not affect the other genes examined. Similarly, iGlucose was ineffective on these fibrogenic genes.

As shown in Fig. 3, AGEs dose-dependently upregulated MCP-1 gene expression in NRK-52E cells. On the other hand, at the concentrations used for the AGE, iBSA did not show any stimulatory effect on the gene. Also, both the AGE and the iBSA preparation failed to show cytotoxicity as assessed by lactate dehydrogenase activity.

![Fig. 1. Characterization of the AGE Prepared in This Study.](image)
activity, at the concentrations used in this study (data not shown).

**AGEs stimulate macrophage chemotaxis**

The finding that AGEs upregulated the mRNA level of MCP-1 in NRK-52E cells, as shown in Fig. 2A, suggests that the inflammation caused in the diabetic kidney by AGEs might be partly mediated by MCP-1 secreted by renal epithelial cells. Hence we examined to determine whether conditioned medium from AGE-stimulated cultures of NRK-52E cells would exhibit macrophage chemoattractant activity. In this experiment, NRK-52E cells were exposed to AGEs or iBSA, at 2.5 mg/mL, for 24 h. Then, the conditioned media were harvested and subjected to checkerboard analysis using a Boyden Chamber. As shown in Fig. 4, the conditioned medium from the AGE-exposed NRK-52E cells increased the number of macrophages that migrated by about 3 times over the control, when 100% of the conditioned medium was placed in the lower chamber. The chemoattractant activity induced by the AGEs or and even by iBSA was proportional to the amount of MCP-1 mRNA expressed by the NRK-52E cells exposed to each of the various albumin materials (see Figs. 2A and 3).
AGE-stimulated chemotaxis was suppressed by the anti-MCP-1 antibody

To clarify the participation of MCP-1 in enhanced macrophage chemotaxis with AGEs, we introduced anti-MCP-1 antibody into the lower chamber which contained the stimulatory conditioned medium. The level of macrophage migration increase due to iBSA and to AGEs was clearly suppressed by the antibody. Especially, the number of cells that migrated when exposed to the medium conditioned by the AGE-treated cells was significantly decreased by the antibody as compared with that obtained with exposure to the unimmunized control IgG (Fig. 5).

As shown by the striped bar on right in Fig. 5, the effect of the antibody was no stronger when the antibody concentration was doubled to 10 µg/mL. These results indicate that the observed AGE-induced chemotaxis was at least partly mediated by MCP-1 as elaborated by renal epithelial cells.

Discussion

It has been reported that under conditions of chronic hyperglycemia in patients with diabetes mellitus glucose autooxidation occurs, leading to the generation of AGEs via binding between the oxidized glucose and amino groups of plasma proteins, especially those of albumin, the most abundant of these proteins.1) AGEs are known to act as pathogenic substances that cause the activation of NFκB in tubular epithelial cells and TGFβ-Smad signaling in mesangial cells.19,20) It is also known that the stimulative activity of AGEs is mediated by their specific receptor, RAGE.21,22) On the other hand, because AGEs are detected at the loci of the diabetic glomeruli, most notably in their nodular lesions, they might possibly impair the ordinary assembly of matrix proteins, causing deteriorated renal structures with an imperfect meshwork.23–25) Based on this background information, we hypothesized that intrarenal AGEs are involved in the pathogenesis of diabetic nephropathy, tubulointerstitial fibrosis.25) It is known that increased amounts of diabetic products passing through the kidney from circulation or by filtration affect renal cells by acting as a stimulant causing these cells to produce cytokines, in addition to causing hyperplasia of tubulointerstitial areas.18,26–28) However, the pathogenic mechanism by which glycation endproducts initiate and exacerbate renal fibrosis is not fully understood.

To address this issue, first we prepared three compounds by long-term (120 d) incubation, and obtained AGES, iBSA, and iGlucose, viz., highly-modified AGES, denatured albumin, and autoxidized glucose respectively. The electrophoretic patterns and fluorescence absorption clearly suggested the formation of AGES larger than the unincubated native albumin. There was little change in the SDS–PAGE pattern of the iBSA preparation as compared to that of the native one, although detailed structural changes (e.g., intermolecular glutamic acid-cysteine bonding) must have occurred.29) We confirmed that reactivity with anti-carboxymethyllysine antibody was dramatically increased in the AGE preparation, but not at all in the iBSA preparation. Of the compounds, only AGES significantly elevated the expression of the fibrogenic genes examined, MCP-1, PAI-1, tTG, and TGFβ1, in the NRK-52E tubular epithelial cells. Therefore, the newly generated structures in AGES molecules, such as Nε-(carboxymethyl) lysine or Nε-(carboxyethyl) lysine, must have been responsible for the stimulation.30)

In the present study, we found that AGES can induce the expression of both PAI-1 and tTG mRNAs in tubular epithelial cells. The elevated level of PAI-1 mRNA in the cells treated with AGES confirms our previous observation that in a type 2 diabetic rat model this gene was upregulated in the same type of cells in the kidney.11,13) The marked increase in tTG, a cross-linking enzyme, in the AGE-exposed epithelial cells might also explain the pathogenetic role of these compounds as a fibrotic stimulant. Glucose is known to induce tTG expression in proximal tubular epithelial cells in vitro,15) but we could not confirm such activity with iGlucose when NRK-52E cells were exposed to it. Thus the tTG-elevating activity of glucose, if any, must be far weaker than that of AGES. Induction of TGFβ1 and of MCP-1 genes by albumin was reported by Eddy et al.18) and Takaya et al.22) respectively. A significant increase in MCP-1 mRNA due to iBSA was observed even in the present study (Figs. 2A and 3). BSA activated extracellular signal-regulated kinase (ERK1/2) and p38 MAPK, inducing MCP-1 expression in the tubular cells.22) We also found that AGES more strongly and dose dependently upregulated MCP-1 mRNA production than iBSA (Fig. 2). Then we found for the first time that the conditioned medium of the AGE-exposed NRK-52E cells exhibited a positive chemotactic effect on macrophages (Fig. 3). This activity of AGES must due mainly to their MCP-1-inducing potential, but it should be noted that AGES can directly and potently enhance macrophage migration. By checkerboard analysis,17) AGE-treated NRK-52E conditioned medium caused chemotaxis, but AGE alone caused macrophage migration, so-called chemokines (data not shown). In exhibiting these activities, the carboxymethyllysine in AGES, formed oxidatively from lysine residue in albumin molecules, is perhaps required as a ligand for...
the scavenger receptors of the macrophages.\textsuperscript{31}) To evaluate the degree of contribution of MCP-1 to the chemoattractant potential of the medium conditioned by renal epithelial cells, we used an antibody specific for MCP-1. We found that it significantly reduced the total activity in the conditioned medium from AGE-exposed cultures by only 30%, and also reduced that in the case of the iBSA-exposed cells, though the reduction was not significant. Thus AGEs would appear to have multiple means in addition to the MCP-1-releasing activity of realizing inflammatory chemoattractant potential of renal epithelial cells.

In conclusion, glycation endproducts produced in patients with diabetes mellitus may be the main factor that brings about renal failure in both direct and indirect ways. That is, these products are effective stimulants of renal epithelial cells, AGEs also caused increased expression of PAI-1 and tTG in these cells, may help further the understanding of renal fibrosis, since these gene products are expected to participate positively to the hyperplasia of the renal tubular system seen in some diabetic patients.

Acknowledgments

We thank Dr. Ryoji Nagai (Tokai University) for helpful discussion and for providing anti-AGE antibodies for our project. This work was supported by grants from the following sources: i) Ministry of Education, Culture, Sports, Science, and Technology of Japan; Academic Frontier Project (to T.S.), and ii) A Grant from the Nihon University College of Bioresource Sciences (to T.S.). Y.S. was supported by the Mantani Memorial Scholarship Trust.

References