Advanced Glycation End Products Inhibit Tubulogenesis and Migration of Kidney Epithelial Cells in an Ezrin-Dependent Manner

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Nonenzymatic glycation of proteins to form advanced glycation end products (AGE) is implicated in diabetic complications, including nephropathy. It was shown recently that AGE bind to the ERM (ezrin, radixin, and moesin) family of membrane-cytoskeletal linker proteins in renal homogenates. Herein is reported the effects of AGE-BSA on ezrin-dependent LLC-PK1 kidney epithelial cellular functions: migration and hepatocyte growth factor (HGF)-induced tubulogenesis. LLC-PK1 cells were stably transfected with cDNA for ezrin sense, ezrin antisense, and N-ezrin. Transfection of LLC-PK1 cells with ezrin antisense and dominant negative N-ezrin decreased basal tubulogenesis and migration relative to vector-only transfection, establishing the ezrin dependency of these processes. AGE-BSA (20 or 40 μM) significantly decreased HGF-induced tubulogenesis and basal migration in two vector control lines relative to BSA-treated cells. However, AGE-BSA inhibition of both HGF-induced tubulogenesis and migration was overcome by overexpressing ezrin. These results demonstrate that the AGE–ezrin interaction significantly alters cellular function. These changes may be relevant to detrimental renal consequences as a result of diabetes.


Nonenzymatic glycation of proteins, lipids, and nucleic acids resulting in the accumulation of advanced glycation end products (AGE) is a prominent feature of diabetes. AGE levels correlate with the development of chronic diabetic complications such as retinopathy, nephropathy, neuropathy, and vasculopathy (1–3). In the normal rat, injection of AGE leads to renal changes similar to those of diabetic nephropathy, whereas interference with AGE formation reduces diabetic complications in various animal models (4–6). The importance of AGE as a pathogenic mechanism in diabetic nephropathy is suggested further by the finding that AGE formation/accumulation precedes diabetic renal disease (2). Together with a cellular receptor RAGE, AGE accumulate in diabetic renal tissue (2,7,8). However, the precise cellular mechanism(s) whereby AGE promote the development of diabetic complications remains unknown.

We recently described the novel binding of AGE to the ERM (ezrin, radixin, and moesin) family of proteins (9). ERM proteins are critical regulators of interactions between the cell membrane and the cytoskeleton (10). As members of the erythrocyte protein 4.1 superfamily, ERM proteins are characterized by a conserved N-domain (FERM domain), which associates with the cytoplasmic domain of specific membrane-associated proteins such as the hyaluronan receptor CD44, CD43, and CD95 and the intercellular adhesion molecules-1, -2, and -3. The C-terminal domain of ERM binds to F-actin in vitro and in vivo (11,12). However, the F-actin binding site and binding sites for membrane proteins are masked by intramolecular association between the N- and C-domains in the inactive cytoplasmic molecule (13). Tyrosine phosphorylation by receptors for EGF and hepatocyte growth factor (HGF) activate ezrin by unmasking the F-actin binding site (14,15). In LLC-PK1, a proximal tubule cell line, tyrosine phosphorylation of ezrin increases cell survival (16).

Tubular injury features prominently in the development of renal dysfunction in diabetes (17,18). In vivo, epithelial tubulogenesis requires the migration and repopulation of the tubular conduit with proliferating cells of the renal proximal tubular epithelium during kidney development or after renal injury. In vitro, ezrin is a mediator of HGF-induced LLC-PK1 tubulogenesis because dominant negative N-ezrin expression and ezrin overexpression inhibit and enhance this process, respectively (15). In this model, tyrosine phosphorylation of ezrin is essential, and this is also the case with respect to HGF-induced migration of these cells. As with tubulogenesis, ezrin overexpression in LLC-PK1 cells enhances cell migration, whereas N-ezrin expression impairs HGF-induced migration (15). We recently showed that AGE-BSA inhibited LLC-PK1 tubulogenesis and ezrin tyrosine phosphorylation (9).

In this article, we describe the effect of AGE-BSA on LLC-PK1
migration and tubulogenesis. We show that inhibition of ezrin activity or expression decreased migration and tubulogenesis. We also show that ezrin overexpression overcame the inhibitory effect of AGE-BSA on migration and tubulogenesis, suggesting that AGE-BSA inhibited these processes by suppressing ezrin function.

**Materials and Methods**

**Materials**

Tissue culture plastics and reagents were purchased from Nunc (Roskilde, Denmark) and Trace Biosciences (Melbourne, Australia), respectively. All other laboratory reagents were purchased from Sigma Corp. (St. Louis, MO) unless otherwise specified.

**AGE Preparation**

AGE-BSA and AGE-RNase were prepared as described previously with modifications (19). Briefly, BSA or RNase (10 mg/ml) was incubated at 37°C for 12 or 9 wk, respectively, with n-glucose (90 g/L) in 0.4 M phosphate buffer. Control BSA or RNase was prepared by incubation without glucose. Preparations were lyophilized, resuspended in water, and dialyzed against PBS to remove free glucose. The characteristic glycation fluorescence (excitation 370 nm, emission 440 nm) of AGE-BSA was increased seven- to 28-fold compared with BSA and for AGE-RNase was increased four-fold compared with RNase.

**Cell Culture**

LLC-PK1, a polarized porcine kidney proximal tubule epithelial cell line, was obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in growth medium (GM) that consisted of DMEM that contained 4.5 g/L glucose, 2 mM L-glutamine, 5000 l/g penicillin, 5 mg/L streptomycin, 125 U/L Fungizone, and 2.2 g/L sodium bicarbonate and supplemented with 10% FCS in a 5% CO2 incubator. For experiments under serum-free conditions, cells were cultured in serum-free medium that consisted of DMEM with antibiotics and glutamine as above supplemented with 0.5 g/L BSA.

**Ezrin Antisense, N-Ezrin, and Ezrin Transfection**

The mammalian expression vector pCR3 (Invitrogen, Carlsbad, CA) with cloned inserts encoding human N-ezrin–green fluorescent protein (GFP) or ezrin antisense were prepared (20). Full-length human ezrin was subcloned into mammalian expression vector pMV7 (21). Subconfluent LLC-PK1 cells were incubated in serum-free medium that contained DNA vector construct with insert (1 lg/ml) and lipofectamine (12 lg/ml; Life Technologies, Gaithersburg, MD) for 6 h. Control transfections were done using vector alone. Cells were incubated for an additional 72 h in GM before passage and selection with Genetecin (900 lg/ml; Life Technologies) in GM. Surviving cell colonies were subcloned over a period of 2 to 3 mo. Clones were assessed for ezrin under- and overexpression by Western blotting using an anti-human N-ezrin antiserum and for N-ezrin expression by N-ezrin ELISA and GFP immunofluorescence and subcloned further. The presence of relevant DNA inserts in subclones was confirmed by real-time reverse transcription–PCR (RT-PCR) as described below.

**Western Blotting**

Cell lysates were prepared from parental and transfected cells in 50 mM HEPES, 0.05 M NaCl, 0.05% Tween 20, and 1% Triton X-100 and centrifuged at 12,500 rpm for 1 min, and supernatants were stored at −20°C until used. Proteins (100 lg/sample) were separated by nonreducing SDS-PAGE and transferred to nitrocellulose membranes. All washes were performed in and antibodies were diluted in 5% skim milk/PBS. Membranes were blocked with 5% skim milk/PBS and probed with a rabbit polyclonal antiserum raised against human N-ezrin at a 1:5,000 dilution and incubated overnight at 4°C. The blot was washed and then incubated with biotinylated anti-rabbit antiserum diluted 1:5000 for 1 h at room temperature, washed, and incubated with streptavidin-labeled peroxidase at a 1:5,000 dilution for 1 h at room temperature. Signal was detected using enhanced chemiluminescence (Supersignal West Pico Chemiluminescent Substrate; Pierce, Rockford, IL) and exposure to x-ray film for 5 min. Blots were quantified using Image J (National Institutes of Health, Bethesda, MD).

**RT-PCR**

Total RNA (5 lg) that was extracted from transfected LLC-PK1 cell lines was used to synthesize cDNA with the Superscript First Strand Synthesis system for reverse transcription (Life Technologies Invitrogen, Carlsbad, CA). Gene expression was analyzed by real-time quantitative PCR performed with the TaqMan system. Fluorescence for each cycle was analyzed by an ABI Prism 7700 Sequence Detection System (PE, Perkin-Elmer, Biosystems, Foster City, CA). For controlling for variation in the amount of DNA that was available for PCR in each reaction, gene expression of the target sequence was normalized for the amount of an endogenous control, 18S ribosomal RNA (18S rRNA reagent kit; PE Biosystems). Primers and TaqMan probe for human ezrin (forward primer CTGAGACTGCCGTCTGCTT, reverse primer GTAAGTTTGTGCTGTCACCATC, probe 6FAM-CACAAGTCTGGTACCTC-TAMRA) were constructed with the help of Primer Express (ABI Prism 7700). Amplification was performed as follows: 50°C for 2 min, 95°C for 10 min, then 40 cycles at 94°C for 20 s and 60°C for 1 min. A standard curve was generated using a human ezrin cDNA standard (1 ng to 0.01 fg), and levels of ezrin cDNA in samples were converted to ezrin copy number (×10⁵/μg RNA). Samples were measured in duplicate.

**Immunofluorescence**

Cells that were transfected with N-ezrin–GFP were grown on glass coverslips. After fixation in 3.7% paraformaldehyde/PBS for 10 min, cells were washed in PBS and then water before mounting in Permafluor (Beckman-Coulter, Marseille, France). Cells were viewed by confocal microscopy using a Leica fluorescence microscope at an excitation wavelength of 488 nm and emission wavelength of 568 nm.

**Tubulogenesis**

Tubulogenesis experiments were performed as described previously (9). Briefly, LLC-PK1 cells were cultured in 24-well plates in 10% FCS/DMEM. Conditioned medium (CM) from 3T3 cells that were grown in 10% FCS/DMEM was used as a source of HGF (22). LLC-PK1 cells were washed once in 10% FCS/DMEM and incubated with 0.5 ml/well 3T3 CM diluted 1:2 in GM. Alternatively, 100 ng/ml recombinant HGF (Sigma) was used in some experiments as indicated. BSA or AGE-BSA was added to a final concentration of 20 and 40 μM with the equivalent volume of PBS added to control wells. Collagen type I (6.5 μl/well of 3.66 mg/ml stock; Beckman-Coulter) was added, and cells were incubated for 48 h, after which tubule numbers were counted in each well.

**Migration**

Parental or transfected LLC-PK1 cells were grown to confluence in six-well plates. Four wounds per well were made with a pipette tip, and cells were washed twice in 1% FCS/DMEM before adding 1 ml/well 1% FCS/DMEM and 1 μg/ml Mitomycin C to inhibit cell proliferation.
BSA or AGE-BSA was added to a final concentration of 20 µM with the equivalent volume of PBS added to control wells. Photographs of wounds were taken at the start of the experiment and after 24 h of incubation. Wound areas were calculated using Image J (National Institutes of Health). The distance of cell migration for each treatment was calculated by subtracting the wound area at T = 24 h from that at T = 0 h and expressing this as a percentage of PBS-treated control.

Statistical Analyses
Tubulogenesis and ezrin antisense data were analyzed by ANOVA. Migration data were analyzed by repeated measures ANOVA after logarithmic transformation to stabilize variance. Correction for multiple comparisons was performed using Fisher PLSD test. P < 0.05 was considered significant. Results are expressed as the mean ± SEM. Experiments were repeated between two and nine times as indicated.

Results
Characterization of N-Ezrin–GFP–, Ezrin Antisense–, and Ezrin-Overexpressing LLC-PK1 Cell Lines
Clones were initially screened for N-ezrin–GFP by N-ezrin ELISA (results not shown). N-ezrin–GFP–transfected LLC-PK1 cell clones then were subcloned and selected on the basis of the presence of GFP as determined by confocal microscopy (Figure 1A). GFP–N-ezrin localized to the cell periphery, and punctate fluorescence staining of the cytoplasm was also noted.

Ezrin antisense–transfected LLC-PK1 cells were screened by Western blotting using a rabbit anti-human N-ezrin antibody. Clones were chosen on the basis of reduced expression of ezrin compared with parental LLC-PK1 cells (Figure 1, B and C). Compared with the parent LLC-PK1 cell line, ezrin antisense lines AS1 and AS2 showed 66% and 88% reductions, respectively, in ezrin expression. Ezrin-overexpressing LLC-PK1 cells were also screened by Western blot and shown to produce more ezrin than parental LLC-PK1 cells (Figure 1D). Transfected cell lines were also screened by real-time RT-PCR for human ezrin mRNA (Figure 1E). Minimal levels of human ezrin (0.096 × 10^9 copies/µg RNA) were detected in the vector control cell line as a result of cross-reactivity with pig ezrin cDNA, whereas up to 1000-fold more ezrin and N-ezrin mRNA (128 and 4.4 × 10^9 copies/µg RNA, respectively) was measured in the ezrin- and N-ezrin–transfected cell lines, respectively.

LLC-PK1 Tubulogenesis Is Ezrin-Dependent
We showed previously that LLC-PK1 cells, when treated with HGF in the presence of collagen I, form tubules between domes and that treatment with AGE-BSA inhibited tubulogenesis (8,9). This model has been reported previously to be ezrin-dependent (15). This was confirmed in our study, in which tubule formation was significantly lower in ezrin antisense– and N-ezrin–transfected cell lines compared with vector control cells (P < 0.05 or P < 0.01; Figure 2A). Inhibition of tubulogenesis by N-ezrin confirms a dominant negative effect of N-ezrin on endogenous ezrin function (15). The cell viability of transfected cell lines during the tubulogenesis assay was unaffected as shown in Figure 2B. HGF signaling in transfected ezrin antisense–, N-ezrin–, and ezrin-overexpressing cell lines was shown to be intact as shown by phosphorylation of Akt in response to HGF (results not shown).
Ezrin Overexpression Overcomes Inhibition of Tubulogenesis by AGE-BSA

When vector control LLC-PK1 cells were incubated in the presence of 40 μM AGE-BSA, tubulogenesis was inhibited compared with BSA-treated cells (Figure 3A). AGE-BSA significantly inhibited tubulogenesis in vector controls to 68 ± 6% and 58 ± 13% of vehicle control, respectively, whereas BSA had no effect (100 ± 3% and 126 ± 15%, respectively; P < 0.05 and P < 0.001, respectively). For ezrin-overexpressing LLC-PK1 cell lines, AGE-BSA (40 μM) also significantly inhibited tubulogenesis to 47 ± 4% and 43 ± 19% of vehicle control, respectively, whereas BSA had no significant effect (121 ± 25% and 116 ± 8% of vehicle control, respectively; P < 0.01 for both). However, a reduced concentration of AGE-BSA (20 μM) continued to inhibit tubulogenesis in vector control but not ezrin-overexpressing lines (Figure 3B). AGE-BSA significantly inhibited tubulogenesis in vector control lines to 51 ± 9% and 43 ± 3% of vehicle control, respectively, compared with BSA-treated cells (103% ± 15 and 75 ± 6% of vehicle control, respectively; P < 0.01 and P < 0.001, respectively). In contrast, AGE-BSA treatment (20 μM) did not inhibit tubulogenesis in ezrin-overexpressing cell lines. Ezrin overexpression thus overcame the inhibitory effect of AGE-BSA in this model of kidney epithelial cell tubulogenesis.

LLC-PK1 Migration Is Inhibited by AGE-BSA

A wound migration model was also established in LLC-PK1 cells (Figure 4A). BSA had no effect on migration (107 ± 6% of control), whereas migration in the presence of AGE-BSA was significantly lower than that of BSA and control (86 ± 7%, P < 0.05 versus control; P < 0.001 versus BSA; Figure 4B). These
experiments were repeated with RNAse, an unrelated protein. Unglycated RNAse significantly increased migration compared with vehicle control (129/11006 10%; P<0.05; Figure 4C). Glycation of RNAse produced similar inhibitory effects on LLC-PK1 migration compared with unglycated RNAse (93/11006 5% versus 129/11006 10%; P<0.01; Figure 4C).

LLC-PK1 Migration Is Ezrin-Dependent

LLC-PK1 migration was found to be ezrin-dependent as shown in Figure 5, where the basal rate of migration was reduced in N-ezrin–transfected (P<0.05 for both compared with V1 and V2) and ezrin antisense-transfected LLC-PK1 cell lines (P<0.05 for both compared with V1 and V2).

Ezrin Overexpression Overcomes Inhibition of Migration by AGE-BSA

The same concentration of AGE-BSA (20 µM) that inhibited tubulogenesis in vector-control but not ezrin-overexpressing LLC-PK1 cell lines had a similar effect on migration (Figure 6). For the vector control clone V1, AGE-BSA treatment (70 ± 5%) significantly decreased migration compared with vehicle control (P<0.01) and BSA (92 ± 6%; P<0.05). For the vector control clone V2, AGE-BSA (88 ± 7%) significantly inhibited migration compared with BSA (116 ± 16%; P<0.05) but not vehicle control. However, AGE-BSA treatment did not significantly inhibit migration in either ezrin-overexpressing cell line. As with parent LLC-PK1 cells, BSA did not significantly stimulate migration in any clone.

Discussion

We showed recently that advanced glycated proteins bind to the N-terminal domain of the ERM family of proteins isolated
Inhibitory effect of AGE-BSA on tubulogenesis and migration in LLC-PK1 cells involves interference of ezrin function by the glycated moiety, presumably as a consequence of binding to the N-domain of ezrin.

The effect of AGE on migration was tested using two unrelated proteins: BSA and RNAse. Unglycated BSA did not significantly affect migration of LLC-PK1 cells, but RNAse stimulated migration. A similar increase in migration induced by an unglycated protein, when compared with control and glycated protein, was observed by Morita et al. (23). These results indicate the importance of comparing the effects of glycated proteins with their unglycated counterparts to avoid possible confounding as a result of nonspecific effects of the proteins. Our results clearly show that the effects observed with the glycated proteins are due to the glycated moiety.

Ezrin plays a significant role in proximal tubule cell migration and tubulogenesis. Ablation of ezrin impairs membrane ruffling and migration in fibroblasts (24). Crepaldi et al. (15) showed increased LLC-PK1 migration with ezrin overexpression. Ezrin phosphorylation is essential for its activation and transfection of LLC-PK1 cells, while ezrin mutated at its tyrosine phosphorylation sites inhibited HGF-induced cell migration and tubulogenesis (15). We previously found that AGE-BSA inhibited tyrosine phosphorylation of ezrin by the EGF receptor (9). Therefore, inhibition of phosphorylation of ezrin by AGE-BSA could explain the dramatic inhibition of tubulogenesis and migration seen in these studies.

Ezrin exerts its cellular actions by using a number of downstream intracellular signaling pathways. One of these is the phosphatidylinositol (PI) (3) kinase/Akt pathway (16,25). Ezrin activates PI (3) kinase during tubulogenesis by binding to its p85 regulatory subunit (16,26). It is therefore possible that AGE may prevent PI (3) kinase activation by inhibiting its interaction with p85. However, we confirmed that HGF stimulates Akt phosphorylation, a downstream target of PI (3) kinase, in LLC-PK1 cells in this study but that AGE-BSA did not inhibit HGF-stimulated Akt phosphorylation in vector control or ezrin-overexpressing cells (results not shown). It is therefore unlikely that the inhibitory effects of AGE on tubulogenesis and migration are mediated by this pathway.

HGF activates ERK and ERM proteins have been implicated in this process (27). HGF-induced ERK activation is important for the initiation of tubulogenesis, so we examined the possibility that AGE-induced inhibition of this pathway may underlie its effects in LLC-PK1 cells (28). However, although HGF increased ERK phosphorylation in vector control and ezrin-overexpressing cells, AGE-BSA had no effect in either cell type (results not shown), suggesting that this pathway is not involved in the inhibitory effect of AGE.

ERM actions may also involve a number of other signaling pathways. Ezrin overexpression in LLC-PK1 cells activates focal adhesion kinase, which is involved in cell motility (29). ERM proteins may also affect cell motility through their interactions with Rho family proteins (30). Rho GTPases, which include the Cdc2, RhoA, and Rac subfamilies, are critical for actin cytoskeletal re-organization in epithelial cell morphogenesis (31). Rho GDI is an inhibitory regulator of all of the Rho family proteins.
members. The N-terminal domain of ERM interacts with Rho GDI and decreases its inhibitory action, initiating activation of Rho family members (32). However, there is also evidence that ERM proteins may inhibit Rho activity in some systems (30). ERM proteins are also implicated in other signaling pathways, including those involving CD44 or protein kinase C (25,27,33,34). The inhibitory effects of AGE on ezrin function could be mediated by one or more of these pathways. Although beyond the scope of this study, exploring these possibilities is an important direction for future studies.

Actin reorganization and changes in cell shape and adhesion are required for ERM-dependent tubulogenesis and migration. Actin disassembly, cell shape alterations, and reduced adhesion have been described in diabetes (35–37). We therefore postulate that the high levels of AGE in diabetes and renal failure may contribute to the development of diabetic nephropathy by inhibiting ERM actions, resulting in actin disorganization and possibly disruption of signal transduction pathways such as those described above.

Our studies provide evidence of the cellular consequences of AGE binding to ERM proteins in proximal tubule cells. The ezrin-dependent processes of migration and tubulogenesis are inhibited by AGE-BSA, and ezrin overexpression overcame these effects. These results suggest that AGE may induce significant pathologic effects on proximal tubule cell function during the course of diabetes by inhibiting ERM function. Because the formation of AGE is a prominent cause of diabetic complications and ERM have important membrane-cytoskeletal functions, the AGE–ERM interaction may be a novel target for therapies aimed at reducing diabetic nephropathy.

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References


