Role of Megalin in Endocytosis of Advanced Glycation End Products: Implications for a Novel Protein Binding to Both Megalin and Advanced Glycation End Products

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Abstract. Advanced glycation end products (AGE) are filtered by glomeruli and reabsorbed and metabolized by proximal tubule cells (PTC). In renal failure, decreased renal AGE metabolism likely accounts for the accumulation in serum that is related to uremic complications. In diabetes, AGE generation is increased, and the handling mechanisms in PTC are likely associated with the pathogenesis of diabetic nephropathy. To this end, this study focused on the molecular analysis of AGE metabolism to develop a strategy for removing AGE in uremia and to elucidate the pathogenesis of diabetic nephropathy. It is important to clarify the mechanisms of the AGE metabolism to develop a strategy for removing AGE in uremia and to elucidate the pathogenesis of diabetic nephropathy. Megalin was originally identified as a target antigen of rat Heymann nephritis, an experimental model of membranous nephropathy (1). It is a large (approximately 600 kD) glycoprotein belonging to the LDL receptor gene family (2). Megalin is located at the clathrin-coated pits, internalizes the ligands into the endocytic compartments, and is recycled to the cell surface (3,4). It is expressed abundantly at the apical membranes of proximal tubule cells (PTC) that normally reabsorb and metabolize low–molecular weight proteins (LMWP) filtered by glomeruli (3). Megalin is known to serve as a major receptor for endocytosis of multiple LMWP, including transcobalamin-B12 (5), vitamin D-binding protein (6), retinol-binding protein (7), parathyroid hormone (8), insulin, β2-microglobulin (β2-m), epidermal growth factor, prolactin, lysozyme, cytokine (9), α1-microglobulin, PAP-1, odorant-binding protein (10), and transthyretin (11). In renal failure, LMWP accumulate in serum and tissues; some of them, such as β2-m causing dialysis-related amyloidosis (DRA) (12), act as uremic toxins associated with complications in patients. Parathyroid hormone is also recognized as a uremic toxin (13). Megalin thus appears to be a useful therapeutic molecular tool to remove such uremic toxins in uremia, and we recently developed a novel cell therapy model by subcutaneous implantation of megalin-expressing cells to metabolize β2-m in renal failure (14).

Advanced glycation end products (AGE), involved in the pathogenesis of diabetic complications (15), are also filtered by glomeruli and reabsorbed and metabolized by PTC (16,17). In renal failure, AGE increase in serum and are associated with the pathophysiology of uremic complications such as DRA (18) and atherosclerosis (19), suggesting that they also represent a uremic toxin (20,21). Decreased renal metabolism of AGE is likely to be associated with the serum accumulation in uremia. Elucidation of the mechanisms of the cellular endocyt-
tosis of AGE is also useful to establish a strategy for removing AGE in uremia, as we suggested for metabolizing $\beta_2$-m using megalin-expressing cells (14). We therefore designed this study to investigate the role of megalin in the endocytosis of AGE.

AGE are involved in the pathogenesis of diabetic glomerulopathy (22,23) as well as tubulopathy (24–27). Recently, AGE were demonstrated to mediate proximal tubule epithelial-myofibroblast transdifferentiation via the receptor for AGE (RAGE) (28), a cell-surface signaling receptor (29). However, the molecular mechanisms of AGE endocytosis by PTC, which should regulate cellular AGE uptake and determine the amounts of AGE available for stimulation of the cell surface receptor, have not been elucidated. Including RAGE, several AGE-binding proteins have been identified, such as galectin-3 (30), macrophage scavenger receptor class A types I and II (SR-A) (31,32), CD36 (33), and scavenger receptor class B type I (SR-B1) (34), although they have not been reported to be expressed by PTC.

In this study, we used a rat yolk sac-derived L2 cell culture system, which has been well characterized for studies of endocytic functions of megalin (4,35,36). We show that the cells specifically take up and degrade AGE and that megalin is involved in these processes. In addition, we demonstrate that the cells express novel AGE-binding proteins and that one of them binds to megalin.

Materials and Methods

Materials

Na $^{125}$I (IODINE-125, 3.7 GBq/ml), reduvix Pro-mix L-[35S] in vitro cell labeling mix (530 MBq/ml), CNBr-activated Sepharose 4B, and Hyperfilm MP were obtained from Amersham Pharmacia Biotech UK Limited (Little Chalfont, Buckinghamshire, England). Iodo-Beads were purchased from Pierce (Rockford, IL). Dulbecco modified Eagle medium (DMEM) (high glucose) and fetal calf serum (FCS) were obtained from Life Technologies BRL (Rockville, MD). Bovine Eagle medium (DMEM) (high glucose) and fetal calf serum (FCS) were obtained from Sigma (St. Louis, MO). Centriplus-100 was purchased from Amersham Pharmacia Biotech UK Limited (Little Chalfont, Buckinghamshire, England). Iodo-Beads were purchased from Pierce (Rockford, IL). Dulbecco modified Eagle medium (DMEM) (high glucose) and fetal calf serum (FCS) were obtained from Life Technologies BRL (Rockville, MD). Bovine serum albumin (BSA, Fraction V), chloroquine, and leupeptin were from Sigma (St. Louis, MO). Centriplus-100 was purchased from Millipore (Bedford, MA). Ready Gel J, Immun-Blot polyvinylidene difluoride (PVDF) membrane, Coomassie Stain Solution, and Coomassie R-250 Destain Solution were obtained from Bio-Rad Laboratories (Hercules, CA).

Protein Purification

AGE-BSA was prepared as described previously (31–34,37). Recombinant rat receptor–associated protein (RAP) was prepared using a prokaryotic expression system as a fusion protein with glutathione S-transferase (GST) as described previously (38). Rat megalin was prepared from renal microvillar membranes by affinity chromatography using monoclonal antibody 20B as described previously (39). Anti-rat megalin rabbit sera were raised as described previously (35), and protein A-purified IgG was prepared as described previously (40). Nonimmune rabbit IgG was also prepared.

Radioiodination

Proteins (100 µg) were radioiodinated using 1 mCi Na $^{125}$I and one Iodo-Bead according to the manufacturer’s instructions. The specific activities of $^{125}$I-AGE-BSA, $^{125}$I-RAP, and $^{125}$I-megalin were $2.9 \times 10^3$, $4.0 \times 10^3$, and $4.5 \times 10^3$ cpm/ng protein, respectively.

Ligand Uptake Analysis

Rat yolk sac tumor-derived L2 cells (41) were grown (37°C, 5% CO$_2$) to confluence (1 × 10$^6$ cells/well) in DMEM supplemented with 10% FCS on 12-well tissue culture plates coated with 0.1% gelatin. The cells were washed with DMEM and incubated in DMEM containing 2% BSA with $^{125}$I-AGE-BSA (1.5 µg/ml). The cell incubation was carried out at 37°C in 5% CO$_2$ in the absence or presence of competitors: unlabelled AGE-BSA, anti-rat megalin rabbit IgG, rabbit nonimmune IgG, RAP, and GST. The cells were also incubated in the presence of chloroquine or leupeptin, inhibitors of lysosomal enzyme activity (42). The media containing $^{125}$I-AGE-BSA were also incubated on cell-free gelatin-uncouated plates to measure the spontaneous degradation of the radiolabeled protein. After incubation, the culture media were precipitated with 15% TCA in the presence of 1% BSA and the radioactivity levels of the degradation products in the supernatants were counted. Cell-mediated degradation of $^{125}$I-AGE-BSA was determined by subtracting degradation in the absence of cells. The cells were washed with ice-cold PBS, and cell-associated radioactivity was measured following solubilization of the cells with 1 N NaOH. Statistical analyses were carried out using the unpaired t test.

Cell Surface Ligand Binding Analysis

L2 cells were grown to confluence as above on 12-well tissue culture plates coated with 0.1% gelatin. The cells were washed twice with the binding buffer (10 mM HEPES, 150 mM NaCl, 2 mM CaCl$_2$, pH 7.4, at 25°C) to confluence (1 × 10$^6$ cells/well) and incubated in the buffer containing 2% BSA at 37°C in 5% CO$_2$ for 20 min. The cells were then washed twice with the ice-cold binding buffer containing 2% BSA. The cells were incubated with 1.5 µg/ml $^{125}$I-AGE-BSA or $^{125}$I-RAP at 4°C for 4 h in the buffer containing 2% BSA with or without anti-rat megalin rabbit IgG (200 µg/ml) or rabbit nonimmune IgG (200 µg/ml). After incubation, the cells were washed twice with the ice-cold binding buffer and solubilized with 1 N NaOH for radioactivity counting. Statistical analyses were carried out using the unpaired t test.

Preparation of Membrane and Cytosolic Proteins of L2 Cells

Cultured L2 cells were homogenized in ice-cold binding buffer containing 0.2 mM phenylmethylsulfonyl fluoride and 10 µg/ml leupeptin, and centrifuged at 600 × g for 5 min to prepare postnuclear supernatants (PNS) (43). The PNS were centrifuged at 100,000 × g to prepare membrane (pellet) and cytosolic (supernatant) fractions. The membrane proteins were solubilized with the binding buffer containing 1% Triton X-100. $^{35}$S-labeled L2 cell membrane and cytosolic proteins were prepared using the cells cultured in the medium containing 2.1 MBq/ml reduvix Pro-mix L-[35S] in vitro cell labeling mix.

AGE-BSA-Sepharose Affinity Chromatography

CNBr-activated Sepharose 4B was used to conjugate BSA-AGE according to the manufacturer’s protocol. Fifty microliters of the AGE-BSA Sepharose 4B was washed in an Eppendorf tube twice with 1 ml of the binding buffer containing 1% Triton X-100. The Sepharose was used for incubation with the $^{35}$S-labeled L2 cell membrane and cytosolic proteins (5 mg/ml each) separately in the same Sepharose/protein ratio in the binding buffer containing 1% Triton X-100 and 2% BSA at 4°C for 14 h with gentle shaking. The Sepharose was washed twice with the binding buffer containing 1% Triton X-100 and equally aliquoted in Eppendorf tubes. An aliquot of the Sepharose was
incubated in 2 × Laemmlli SDS-PAGE sample buffer containing 4% β-mercaptoethanol to elute the proteins bound to the Sepharose both specifically and nonspecifically. The elution was mixed with the same volume of the binding buffer before heating at 95°C for 5 min and applying onto SDS-PAGE. The other aliquots were used for elution with 0.5 mg/ml AGE-BSA, 0.5 mg/ml BSA, respectively, in the binding buffer or the binding buffer containing 20 mM EDTA. The eluted samples were mixed with the same volume of 2 × Laemmlli SDS-PAGE sample buffer containing 4% β-mercaptoethanol, heated at 95°C for 5 min, and used for SDS-PAGE.

Preparation of the AGE-Binding Proteins
To prepare the AGE-binding proteins, the PNS of L2 cells was used for binding to the AGE-BSA Sepharose 4B at 4°C for 14 h in the binding buffer containing 1% Triton X-100 and 2% BSA. The proteins bound to the Sepharose were eluted with 100 mM Tris-HCl and 20 mM EDTA (pH 7.4 at 25°C). The eluted solution was concentrated using Centriplus-100 (Millipore, Bedford, MA). The Sepharose was washed with the binding buffer and stored at 4°C in the presence of 0.02% NaN3.

Ligand Blot Analysis
Megalun (10 μg) was prepared in Llaemmlli sample buffer containing no β-mercaptoethanol and resolved by 4% SDS-PAGE. The protein was then transferred to PVDF membranes. Nonspecific sites on membranes were blocked by incubation in the binding buffer containing 0.2% Tween 20 (buffer A) and 3% BSA. The membranes were then incubated with 125I-AGE-BSA or 125I-RAP (1 × 106 cpm/ml) in buffer A with 3% BSA for 2 h at 25°C, washed with buffer A (4 times, 15 min each), air dried, and exposed to Hyper film at −80°C with an intensifier screen.

The proteins (30 μg) specifically eluted from AGE-BSA Sepharose 4B with EDTA were also dissolved in the Laemmlli sample buffer supplemented with 4% β-mercaptoethanol, heated at 95°C for 5 min, resolved by 4% SDS-PAGE, and transferred to PVDF membranes. The protein blotting was confirmed by staining the membranes with Coomassie Stain Solution and washing with Coomassie R-250 Destain Solution. The membranes were then incubated with 125I-AGE-BSA (1 × 106 cpm/ml) in the presence or absence of unlabeled AGE-BSA (35 μg/ml) or EDTA (20 mM), washed, and subjected to autoradiography as described above. Also, the membranes were used for ligand blot analysis by incubation with 125I-megalin (1 × 106 cpm/ml) in the presence or absence of RAP (300 nM) or EDTA (20 mM).

Results
Megalin-Dependent Cellular Uptake and Metabolism of AGE
In this study using megalin-expressing L2 cells, we used nonspecifically modified AGE-BSA for the following reasons. First, BSA per se is not significantly taken up by L2 cells (data not shown). Therefore, BSA-AGE is an appropriate tool to determine the specific effect of AGE-modification on the cellular uptake and metabolism in the cells. Second, nonspecifically modified AGE-BSA is the most general material available for cellular AGE receptor analysis.

To investigate whether megalin, a multi-ligand endocytic receptor, is involved in the AGE uptake and metabolism in the cells, we performed 125I-AGE-BSA uptake assays using antimegalin IgG as a specific competitor (Figure 1, A and B). Cell association and degradation of 125I-AGE-BSA were significantly suppressed by the addition of anti-megalin IgG to the culture medium compared with the addition of nonimmune IgG, demonstrating that megalin is involved in the uptake and metabolism of AGE in L2 cells. However, the addition of recombinant RAP, an inhibitor of megalin’s binding to its ligands, decreased the degradation but increased the cell association, compared with the addition of GST (Figure 1, A and B). This result suggests that blocking megalin with RAP may inhibit the cellular internalization or intracellular metabolism of AGE. It also suggests that the involvement of megalin in the cellular uptake and metabolism of AGE differs from the processes for the ligands that directly bind to megalin at its RAP-binding sites.

Receptor-mediated endocytosis and lysosomal degradation of AGE-BSA in L2 cells were tested by the ligand uptake analysis using chloroquine, a membrane-diffusible base which raises pH in intracellular compartments, and leupeptin, a membrane-diffusible proteinase inhibitor (42) (Figure 1C). Both reagents significantly suppressed 125I-AGE-BSA degradation, while only the latter increased the cell association. These results are consistent with the previous report (42), and they confirm the endocytosis and lysosomal degradation of AGE-BSA because chloroquine is supposed to inhibit endosomal and lysosomal activities and leupeptin to accumulate endocytosed, undegraded proteins in lysosomes.

Megalin Is Not Identified as a Direct Cell Surface Receptor for AGE-BSA
To investigate whether megalin is a direct AGE receptor, cell surface ligand binding analysis was carried out using L2 cells incubated at 4°C with 125I-AGE-BSA in the presence of 2% BSA (Figure 2A). First, the addition of unlabeled AGE-BSA to the incubation buffer was shown to significantly suppress the 125I-AGE-BSA cell surface binding, indicating the presence of a specific L2 cell surface receptor for AGE. However, the addition of anti-megalin IgG to the incubation buffer failed to suppress the specific binding. As a control, 125I-RAP was shown to specifically bind to L2 cells at 4°C, and the binding was significantly suppressed by the addition of anti-megalin IgG (Figure 2B). In addition, the ligand blot analysis revealed that 125I-AGE-BSA did not bind to megalin, whereas 125I-RAP bound to it under the same conditions (Figure 3). We also performed surface plasmon resonance analysis to investigate whether megalin binds to AGE-BSA, but we did not find evidence of direct binding (data not shown). These findings indicate that megalin is not identified as a direct cell surface receptor for AGE-BSA.

Identification of Ca2+-Dependent AGE-Binding Proteins in L2 Cells
To identify the AGE-binding receptors in L2 cells that may cooperate with megalin, affinity chromatography was carried out using AGE-BSA-conjugated Sepharose 4B to find L2 cell proteins that bind to AGE (Figure 4A). The Sepharose was incubated with 35S-labeled L2 cell membrane and cytosolic proteins for 14 h at 4°C in the binding buffer (10 mM HEPES,
150 mM NaCl, 2 mM CaCl$_2$, pH 7.4, at 25°C) containing 1% Triton X-100 and 2% BSA. After washing the Sepharose with the buffer, AGE-binding proteins were specifically eluted from the Sepharose with AGE-BSA but not with BSA. The eluted proteins included three large-molecular weight ones (190, 200, and 400 kD, respectively, shown under the reducing condition in 4% SDS-PAGE). These proteins were also eluted from the Sepharose with EDTA, indicating that the AGE binding is Ca$^{2+}$-dependent. In non-reducing 4% SDS-PAGE, the three proteins were stacked at the gel top (Figure 4B), suggesting that they may form a complex.

To examine whether each of the three proteins directly binds to AGE, we performed ligand blot analysis (Figure 5A). The proteins were reduced with 4% β-mercaptoethanol, separated by SDS-PAGE, and blotted to PVDF membranes. After blocking with the binding buffer containing 0.2% Tween 20 and 3% BSA, the membranes were incubated with $^{125}$I-AGE-BSA in the same buffer in the presence or absence of unlabeled AGE-BSA or EDTA. The ligand blot analysis showed that $^{125}$I-AGE-BSA specifically bound to the 200-kD and 400-kD proteins but not to the 190-kD protein, and the binding was blocked by EDTA, indicating that the AGE-binding is Ca$^{2+}$-dependent. The 190-kD protein did not appear to be directly bound by AGE, suggesting that it may be associated with either the 200-kD or 400-kD proteins. The 200-kD and 400-kD proteins are very likely novel AGE-binding proteins; to our knowledge, there have been no reports of AGE-binding proteins with such molecular weights and properties.

Ca$^{2+}$-Dependent Binding of Megalin to the 200-kD AGE-Binding Protein

To examine whether the AGE-binding proteins interact with megalin, ligand blot analysis was carried out using $^{125}$I-megalin (Figure 5B). The 190-kD, 200-kD, and 400-kD proteins were separated by 4% SDS-PAGE and blotted to the PVDF membranes. The addition of anti-megalin IgG to the culture medium inhibited degradation and cell association of $^{125}$I-AGE-BSA, compared with the addition of nonimmune IgG, indicating that megalin is involved in the cellular uptake and metabolism of advanced glycation end products (AGE). The addition of receptor-associated protein (RAP; 300 μg/ml) suppressed degradation but increased cell association, compared with the addition of glutathione S-transferase (GST), suggesting that RAP inhibits the cellular internalization or intracellular metabolism of AGE. $^*P < 0.01$. (C) The cells were incubated as well with $^{125}$I-AGE-BSA (1.5 μg/ml) for 4 h in the presence of chloroquine or leupeptin (100 μM each), inhibitors of lysosomal activities. Both chloroquine and leupeptin suppressed the AGE degradation (■) while the latter increased the cell association (□) ($^*P < 0.01$ versus control), confirming that the degraded products were the results of receptor-mediated endocytosis and lysosomal degradation. Values (means ± SD, $n = 4$) are expressed relative to the control.
membranes. The analysis revealed that 125I-megalin bound to
the 200-kD protein in the same conditions as described above.
The binding was blocked by EDTA, indicating that the reaction
is Ca\(^{2+}\)-dependent. Also, the binding was blocked by RAP,
suggesting that megalin may interact with the 200-kD protein
at the site for binding with RAP.

Discussion
Using the L2 cell line system, we found that AGE are
specifically internalized via receptor-mediated endocytosis
and degraded in lysosomes. In the cell system, we identified
megalin, a multi-ligand endocytic receptor, as being involved
in endocytosis of AGE, but not the direct binding receptor. We
also found that the cells have novel 200-kD and 400-kD
proteins that bind AGE in a Ca\(^{2+}\)-dependent manner. Either of
the proteins is likely to form a complex with a 190-kD protein
that is co-purified with the AGE-binding proteins using AGE-
BSA-bound Sepharose 4B chromatography. The proteins ap-
pear to be present both in the membrane and cytosolic fractions
of L2 cells. Ligand blot analysis revealed that the 200-kD
protein binds to megalin, suggesting an interactive function of
the proteins. The binding is also Ca\(^{2+}\)-dependent, and it is
blocked by RAP, suggesting that megalin binds to the 200-kD
protein at the site for binding with RAP.

Megalin is known to be involved in the endocytosis of
multiple ligands in PTC by directly binding them or by coop-
erating with cubilin, another cell-surface receptor that binds its
specific ligands (44). Cubilin, also known as the intestinal
intrinsic factor-cobalamin receptor, is a 460-kD glycoprotein
with no transmembrane domain and no known signal for en-
docytosis. It binds multiple ligands, including albumin, Ig light chain, HDL, and apolipoprotein A-I, and most likely is mediated for internalization by megalin at the clathrin coated-pits (45,46). Cubilin is reported to be expressed in cultured yolk sac epithelial cells (47), but our mass spectrometry analysis in progress for identifying the AGE-binding proteins has found that the proteinase-digested fragments do not match those deduced from the amino acid sequence of rat cubilin (data not shown).

Like the indirect role of megalin involved in the endocytosis of cubilin’s ligands, we speculate that a similar mechanism exists for the endocytosis of AGE in L2 cells, in which a putative AGE receptor is present at the cell surface and is mediated by megalin for internalization. The addition of RAP, an inhibitor of megalin’s binding to its ligands, to the L2 cell culture system decreased AGE-BSA degradation but increased the cell-assocation. This result suggests that blocking megalin with RAP may inhibit the cellular internalization of the com-
plex of AGE and the cell-surface AGE receptor or suppress the intracellular metabolism of endocytosed AGE. The RAP-binding sites of megalin are likely involved in the molecular mechanisms of interaction or cooperation with the putative AGE receptor.

In this study, highly glucose-modified AGE-BSA was used as a ligand for binding and uptake assays. We found that mildly modified AGE-BSA, prepared by incubating 50 mg/ml BSA with 50 mM D-glucose at 37°C for 4 wk, was not specifically taken up by the cells in our assay system (data not shown). It suggests that mildly modified AGE is not be recognized by the cells or that the sensitivity of our assays is insufficient to evaluate the cellular uptake. Highly glucose-modified proteins contain various AGE structures, and it should be determined which structures are recognized by the megalin-mediated endocytosis system. We have evidence that glycoaldehyde-modified AGE structures, which are reactive intermediates of the Maillard reaction (48), may be recognized by the system (Saito et al., unpublished observation).

The mechanism of AGE metabolism associated with megalin has an important aspect regarding the pathogenesis and treatment of uremic complications. Various uremic toxin proteins are known to accumulate in serum and tissues of patients with end-stage renal disease and cause serious complications (49). β₂-m is a well-established 12-kD uremic toxin protein that causes DRA characterized by osteoarthropathy and failure of various organs due to the deposition of β₂-m-derived amyloid proteins (50). Despite the development of high-flux membrane hemodialysis devices and a direct absorbent column, β₂-m accumulation is inevitable in afflicted patients because the therapeutic effects are transient and insufficient. Megalin appears to be an effective molecular tool to remove LMWP in the therapeutic effects are transient and insufficient. Megalin and developing a strategy for its treatment.

In the development of diabetic nephropathy, tubulointerstitial injury is thought to be as significant as glomerulopathy (26). A number of studies have indicated that AGE are associated with PTC injury (24,25,27). From our current work, it is very likely that megalin is also involved in the endocytosis of AGE in PTC and could thus be a therapeutic molecular target for preventing AGE accumulation in the cells. Recently, PTC were found to express RAGE (28), a cell surface AGE-binding receptor that functions for AGE signal transduction (51). It remains to be determined whether or not RAGE is also involved in the endocytosis through cooperation with endocytic receptors such as megalin. Further characterization of megalin-mediated endocytosis of AGE would be useful for elucidating the molecular mechanisms of diabetic tubulointerstitial injury and developing a strategy for its treatment.

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References


