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Renal fate of circulating advanced glycated end products (AGE): evidence for reabsorption and catabolism of AGE-peptides by renal proximal tubular cells

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Summary The presence of excessive amounts of advanced glycation end products (AGE) in tissues or in the circulation may critically affect the progression of diabetic nephropathy. Circulating AGE levels, mainly in the form of small peptides, increase in diabetic patients or in patients with end-stage renal disease. This rise correlates with the severity of the nephropathy. However, so far little is known about the fate of AGE-proteins and AGE-peptides in renal tissue, and in order to elucidate this issue we undertook the present study. AGE-bovine serum albumin (AGE-BSA) and AGE-peptides were prepared, characterized by spectrophotometry, spectrofluorometry, chromatography and SDS-PAGE. AGE-peptides reacted in vitro with LDL producing biochemical and ultrastructural modifications. Using colloidal gold post-embedding immunoelectron microscopy with an anti-AGE antibody generated in our laboratory, we followed, in a short-term kinetic study, the cellular and sub-cellular localisation of circulating AGE-products throughout the nephron. AGE-peptides or AGE-BSA were injected into otherwise normal rats and detected by protein A-gold immunocytochemistry after 15, 30 or 45 min of circulation. Most of the AGE- BSA was found in the lumen of capillary vessels and distributed along the endothelial side of the glomerular basement membrane. Presence on mesangial matrix was also apparent. AGE-peptides were easily filtered and actively reabsorbed by the proximal convoluted tubule. At 15 min, little labelling was found in the glomerular wall. Instead, the labelling was present in the urinary space and microvilli of epithelial cells. Early endosomes displayed intense labelling as well. At 45 min, late endosomes and lysosomes added to the pattern of labelling. The distal tubule epithelial cells were devoid of labelling for any of the intervals studied. AGE-peptides but not AGE-BSA could be detected in the urine of injected rats. These observations point to participation of the endo-lysosomal apparatus of the proximal convoluted tubule to the disposal of AGE-peptides, while giving an ultrastructural support for a key role of the kidney in AGE catabolism. [Diabetologia (1996) 39: 149–160]

Key words Diabetes mellitus, glycation, colloidal gold cytochemistry, glomerulosclerosis, LDL, clearance.

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Abbreviations: AGE, Advanced glycation end products; BSA, bovine serum albumin; BHT, butylated hydroxytoluene; DEAE, diethyl aminoethyl cellulose; GBM, glomerular basement membrane; PBS, phosphate buffered saline; PMSF, phenylmethylsulphonyl fluoride; TBS, Tris buffered saline.

The long-suspected but controversial issue of hyperglycaemia being a key step towards diabetic chronic complications is now resolved [1]. The recent publication of the Diabetes Control and Complications Trial (DCCT) unequivocally demonstrates the prevention of complications by good glycaemic control in insulin-dependent diabetic (IDDM) patients [2, 3]. One of the mechanisms by which sustained hyperglycaemia has an effect, is glycation and its ultimate consequence, advanced glycation [4–8]. Accelerated modification of proteins by advanced glycation is involved

in the pathogenesis of nephropathy, macro- and microangiopathy, and cataracts in diabetic patients [6, 9–11]. Advanced glycation end products (AGE) are produced in proteins as a consequence of the non-enzymatic formation of adducts between these molecules and reducing sugars. In this reaction, sugars react non-enzymatically with a wide range of proteins to form early glycation (Amadori) products. On long-lived proteins, a complex series of rearrangements and oxidative reactions follows leading to the formation of multiple, very reactive species collectively called AGE products. AGE products accumulate in vivo on vascular wall collagen and basement membranes as a function of age and levels of glycaemia [8, 12–18]. They are capable of producing cross-linking of proteins and have been shown to display diverse biological activities including increased endothelial cell permeability [19–21], binding to receptors on macrophages, endothelial and mesangial cells [19, 22–26], activation of macrophages with secretion of cytokines following AGE-ligand-receptor interaction [25–28], quenching of nitric oxide with the consequent inhibition of vascular dilatation [12], enhancing oxidative stress [21, 29, 30] and oxidation of LDL [31]. In fact, a growing body of evidence points to a causal relationship between AGE accumulation and the development of chronic diabetic complications. Diabetic nephropathy, leading to glomerulosclerosis, is one complication, and is responsible for kidney failure in 30–40 % of diabetic patients [32]. The development of new assays for AGE-products has shown that they not only accumulate in tissues but circulate as well [31, 33–37]. Particularly impressive are data showing a dramatic increase in the levels of circulating AGE-peptides in diabetic end-stage renal disease that correlate with the severity of the nephropathy [15, 34, 36]. Short-term administration of exogenous AGE-modified albumin to otherwise normal rats and rabbits leads to the covalent attachment of these adducts to the extracellular matrix in several tissues [16]. Moreover, following this modification, a range of vascular dysfunctions, resembling those observed in diabetes, occurs.

We have recently shown that AGE-bovine serum albumin (AGE-BSA) probes tagged with colloidal gold display great reactivity towards glomerular structures [38]. Similarly, chronic injections of AGE-BSA but not of BSA alone, produce the classical picture of diabetic glomerulopathy in otherwise euglycaemic animals [39]. It is apparent from these and other studies that small metabolic fragments of AGE proteins are implicated in these phenomena. In fact, it is this low molecular weight AGE-peptide fraction that accumulates preferentially in the blood of diabetic and uraemic patients, representing up to 80 % of AGE plasma immunoreactivity [15, 18, 32–34, 36, 40, 41]. Up to five times higher levels of AGE were demonstrated in diabetic patients undergoing haemodialysis as compared with euglycaemic subjects [15, 18, 34]. Circulating AGE-peptides are not easily removed by dialysis and some authors even suggest that they might be potential candidates for the *"uraemic toxins"* or *"middle molecules"* involved in complications related to dialysis [18, 33]. Kidney failure itself also leads to a dramatic accumulation of AGE even in the absence of diabetes [32].

In this regard, it is certain that the kidney plays an important role in the clearance of circulating AGE from the bloodstream. However, so far, little is known about the fate of AGE proteins and AGE-peptides in kidney tissue. In order to obtain further insight on this issue, we undertook the present study. AGE-BSA and AGE-peptides were prepared and injected into otherwise normal rats. Using immunoelectron microscopy with a newly generated anti-AGE antibody, we followed, in a short-term kinetic study, the cellular and subcellular localisation of circulating AGE throughout the nephron.

Materials and methods

Preparation and characterisation of BSA containing AGE. Crystallised and lyophilised BSA, fraction V (Sigma Chemicals, St. Louis, Mo., USA) was glycated in vitro by incubation at 37 °C for 120 days in the presence of 1 mol/l glucose in 0.5 mol/l sodium phosphate buffer containing 1 mmol/l EDTA, 1 mmol/l phenylmethylsulphonyl fluoride (PMSF), 1 mmol/l aprotinin and 1 mmol/l sodium azide. Protein concentration was 100 mg/ml and incubation was performed after sterilising the solution by passage through a 0.2-μm Millipore membrane (Millipore Canada Ltd, Mississauga, Ont., Canada). Presence of AGE in our probes was confirmed by fluorospectrophotometry using excitation at 370 nm and emission at 440 nm for total fluorescence AGE and excitation at 335 and emission at 385 nm for pentosidine-like fluorescence as previously described [42–44]. A Turner 430 spectrofluorometer (AMSCO Instruments, Carpinteria, Calif., USA) was employed for these measurements. Briefly, samples were adjusted to the same protein concentration (1 mg/ml) and an arbitrary value of 1 was assigned to fluorescence of control BSA. The fluorescence of modified BSA was reported to this reference value. The second criterion employed to measure AGE in BSA was their characteristic absorption spectrum [44] measured with a Shimadzu UV 160 U recording spectrophotometer (RPI Instruments Inc., Montreal, Quebec, Canada). Absorbance at 350 nm was reported to absorbance at 280 nm in order to correct for protein concentration [16].

Degree of modification of lysine residues was monitored by the trinitrobenzene sulphonic acid assay as previously described [45]. Briefly, 50-μl samples (1 mg/ml) were incubated with 1 ml 0.48 mmol/l $NaHCO_3$ and 50 μl 3.3 ml/l trinitrobenzene sulphonic acid for 1 h at 37 °C. Absorbance at 340 nm of control (non-modified) samples was measured and free amines were calculated from a standard plot performed with valine. The AGE-BSA probes were also characterized by polyacrylamide gel electrophoresis in non-denaturing conditions (SDS-PAGE). Electrophoresis was run according to the method of Laemmli [46–48] on 7.5 % acrylamide gels. Equipment employed was Mini Gel from BioRad (Bio Rad Laboratories, Mississauga, Ont., Canada). Gels were stained with Coomassie Brilliant Blue.

Preparation of AGE-peptides. AGE-peptides were prepared by proteolysis of AGE-BSA as previously described [31]. Briefly, 10 ml of AGE-BSA (50 mg/ml in 10 mmol/l phosphate buffered saline (PBS), pH 7.4) were incubated with 10 mg of proteinase K for 20 h at 37 °C. Low molecular weight peptides (< 10 kDa) were then isolated by centrifugation of the digested material through a Centripep-10 membrane (Amicon) for 3 h at 4000 × *g*. After 20 h proteolysis with proteinase K, over 80 % of AGE fluorescence was recovered in the less than 10 kDa fraction.

Determination of molecular weights of AGE-peptides. After separation by centrifugation of the digested AGE-BSA through a Centripep-10 membrane, 500 µl of 5 mg/ml AGE-peptides was applied onto a 40-cm Biogel P-6 gel filtration column (BioRad) equilibrated in 0.01 mol/l sodium phosphate buffer containing 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l PMSF, 1 mmol/l sodium azide, pH 7.4. Chromatography was run at 10 ml/min flow rate using the same solution for elution. One-ml fractions were collected and read at 280 nm for proteins, at 350 nm for browning products, and fluorescence at Excitation 370 nm and Emission 440 nm. The void volume, estimated as the exclusion volume for haemoglobin, was 19 ml. The BioGel column was calibrated with insulin (M_r 6000), bacitracin (M_r 1400), riboflavin (M_r 379) and glucose (M_r 180).

Reaction of AGE-peptides with LDL. LDL (d = 1.051–1.163 mg/ml) was isolated from blood of healthy volunteers as described previously [49]. After dialysis LDL (1 mg/ml) was incubated at 37 °C for different intervals up to 72 h in the presence or in the absence of 10 mg/ml AGE-peptides in 10 mmol/l sodium phosphate, 140 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l PMSF, and 1 mmol/l sodium azide. LDL was subsequently dialysed and examined under electron microscope by negative staining [50], and by spectral analysis.

Antibodies. Anti-AGE antibodies were obtained by immunization of rabbits with AGE-BSA essentially as described previously [34, 51, 52]. Standard immunization protocols were followed [53]. Briefly two New Zealand white rabbits received four i.m. injections of a total of 350 µg AGE-BSA emulsified with complete Freund's adjuvant. After 1 month the rabbits were subsequently given a booster of a half-dose of AGE-BSA emulsified with incomplete Freund's adjuvant, and two similar boosters were repeated every month. Ten days after each booster the animals were bled and their antibody titres checked. After the third booster, the animals were bled, anti-BSA antibodies were eliminated by adsorption of serum with BSA and an IgG fraction was prepared by ammonium sulphate precipitation and DEAE chromatography [54]. Specificity of the antibodies was checked by dot immuno blotting and double diffusion on agarose gels [54]. Advanced glycated Hb and IgG were prepared in a similar fashion as AGE-BSA. The three molecules as well as their non-glycated counterparts were employed to check the specificity of the antibody against AGE epitopes. Proteins were measured by the micro bicinconninic acid procedure [55] (Pierce, Chromatographic Specialties Inc, Brockville, Ont., Canada).

Circulation of exogenous AGE-BSA and AGE-peptides. Sprague-Dawley male rats weighing 100 g were employed. The animals were anaesthetized with i.p. injections of urethane. Two animals were employed for each probe at each indicated time. AGE-peptides or AGE-BSA (10 mg/animal) were injected in the inferior vena cava. These probe concentrations were chosen since they had provided good signals in pilot studies without producing any major increase in proteinaemia and did not significantly affect the overall glomerular filtration process. After 15, 30 or 45 min of circulation the animals were killed by terminal anaesthesia. The abdominal cavity was flooded with the fixative, 1 % glutaraldehyde to begin fixation. Such a rapid fixation in situ allows for the retention of circulating proteins in the blood vessels and reduces extraction of extracellular circulating proteins during processing of the tissue samples for electron microscopy. Small pieces of kidney were excised and further fixed by immersion in 1 % glutaraldehyde solution (0.1 mol/l phosphate buffer) for 2 h at 4 °C. The tissue fragments were then processed for electron microscopy by methanol dehydration and embedded in Lowicryl K4 M (IBEM Services, Inc, Saint Laurent, Québec, Canada) at –20°C as previously described [56]. Thin sections were cut, mounted on nickel grids coated with Parlodion and carbon films and processed through the labelling experiments.

Detection of AGE-peptides in urine of injected rats. AGE-peptides were detected in urine samples by dot immunoblotting [47]. Briefly, 2-µl aliquots were spotted onto nitrocellulose membranes and dried. Membranes were then blocked in 50 g/l (w/v) non-fat dry milk in Tris buffered saline (TBS) (10 mmol/l Tris HCl, 257 mmol/l NaCl, pH 7.5, TBS) for 2 h, washed three times (5 min each) in TBS, and then incubated at room temperature overnight with the anti-AGE antibody diluted at 1:500 in non-fat dry milk. After three additional rinses in TBS containing 0.01 % (w/v) Tween 20, blots were incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG at 1:500 dilution in non-fat dry milk for 2 h before developing with a nitroblue tetrazolium substrate (Sigma).

Cytochemical studies. Tissue sections were incubated for 60 min on a drop of 10 g/l glycine in 10 mmol/l PBS, pH 7.4. After washing in PBS the grids were transferred to a drop of 10 g/l ovalbumin and incubated for 30 min at room temperature. Next, grids were incubated overnight at 4 °C in a humidified chamber on a drop of the anti-AGE antibody. The grids were washed by three successive floatings on PBS while stirring, transferred to a drop of 10 g/l ovalbumin and incubated for 30 min at room temperature. Next, the grids were incubated with 10-nm protein A-gold complex for 30 min at room temperature [56]. Finally the grids were washed again by three successive floatings on PBS under stirring, then rinsed with distilled water and dried. Staining with uranyl acetate and lead citrate was performed before examination with a Philips 410 electron microscope (Saint Laurent, Québec, Canada).

Cytochemical controls. The specificity of the immunolabelling was assessed by several control experiments as described previously [56]: adsorption of the antibody by its specific antigen (AGE-BSA); use of protein A-gold alone and use of non-labelled protein A-gold prior to the use of protein A-gold complex.

Morphometrical evaluation of the labelling. Distribution of AGE-BSA over the glomerular basement membrane (GBM) was performed by a quantitative evaluation of the labelling intensity and expressed as a ratio: distance endothelium-gold particle/distance endothelium-epithelium (resulting in values between 0 and 1) using a Carl Zeiss Videoplan electronic digitiser (Carl Zeiss Inc., Toronto, Ontario, Canada) as described previously [57]. For each animal, ten microscopic fields were recorded on video at × 31,000 magnification and brought to a final magnification of × 105,000.

Table 1. Characterization of advanced glycated albumin (AGE-BSA)

	Free amines[a] (mmol/l)	Fluorescence[b] (Arbitrary units) Ex 370 nm/Em 440 nm	Fluorescence[c] (arbitrary units) Ex 350 nm/Em 385 nm	Chromogen products[d] A 350 nm/A 280 nm
BSA	0.86 ± 0.03	1.0 ± 0.0	1.0 ± 0.0	0.0
AGE-BSA	0.15 ± 0.02	42 ± 0.5	2.8 ± 0.0	0.56 ± 0.1

Data represent mean ± SD of two independent experiments run in duplicate. All the samples were adjusted to a protein concentration of 1.0 mg/ml.
[a] Measured by the TNBSA reaction calibrated with a valine standard.
[b] Fluorescence was measured at excitation maximum of 370 nm and emission maximum of 440 nm.
[c] Fluorescence was measured at excitation maximum of 335 nm and emission maximum of 385 nm.
[d] Measured as absorbance at 350 nm/absorbance at 280 nm

Results

In this work we set out to study the renal fate of exogenously administered AGE. For that purpose we generated a probe, AGE-BSA by long-term incubation of BSA in the presence of high glucose and phosphate concentrations. This probe was used to prepare small peptides by proteolysis. Either AGE-BSA or the resultant AGE-peptides were next injected into normal rats and their renal handling was followed by biochemistry and immunoelectron microscopy.

Biochemical characterisation of AGE-BSA and AGE-peptides. The AGE-BSA probe was checked for late glycation products by characteristic fluorescence and by spectrophotometric spectra. The number of lysine residues modified was quantitated by the trinitrobenzene sulphonic acid assay reaction. Table 1 summarises the biochemical characterisation of the AGE-BSA probe used in this work. AGE-BSA contained AGE producing the characteristic fluorescence and absorption spectra. Over 80 % of lysine residues in the AGE molecules have been modified as shown by direct measurement of still-reactive amino groups. As expected, extensive cross-linking of AGE-BSA was apparent from SDS-PAGE electrophoresis. On Figure 1 the band at 68 kDa characteristic for BSA has been substituted by large molecular weight cross-linked molecules with M_r over 200 kDa.

One part of AGE-BSA was next employed in the preparation of AGE-peptides. After ultrafiltration, AGE-peptides were further characterized by gel filtration chromatography. Figure 2a shows the elution profile of AGE-peptides on a BioGel P6 column. Over 90 % of AGE fluorescent products elute in peak I and display a M_r 3 kDa as calculated from the standard plot in 2b.

Reactivity of AGE-peptides towards LDL. To test in vitro the potential reactivity of our AGE-peptides we incubated them in the presence of LDL. In this way both binding to protein and lipids were assessed at the same time. AGE-peptides readily bind to LDL and produce drastic ultrastructural modifications. Even after only an overnight incubation in non-oxidative conditions (addition of EDTA, ascorbic acid and butylated hydroxytoluene) LDL showed major changes as illustrated in Figure 3b. Instead of the discrete 20-nm particles of LDL incubated in the absence of AGE (Fig. 3a), AGE-incubated LDL

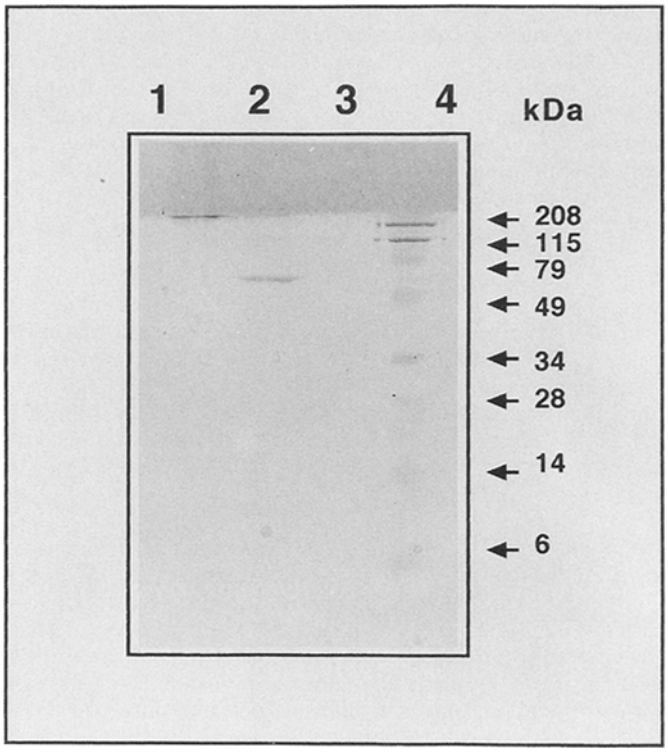

Fig. 1. SDS-PAGE of the AGE probes used in this study. AGE-BSA (line 1), BSA (line 2) and AGE-peptides (line 3) (15 µg each) were loaded onto a 7.5 % polyacrylamide gel with a stacking gel of 4 % polyacrylamide. Staining was performed with Coomassie Brilliant Blue. Line 4 contains molecular weight markers. Advanced glycation of BSA produced cross-linking of the molecule leading to the formation of high molecular weight adducts which scarcely enter the separation gel (line 1). Advanced glycated peptides produced by digestion of this AGE-BSA are not visible on the gel, which indicates that their M_r is inferior to 6 kDa (line 3) as judged by M_r markers on line 4

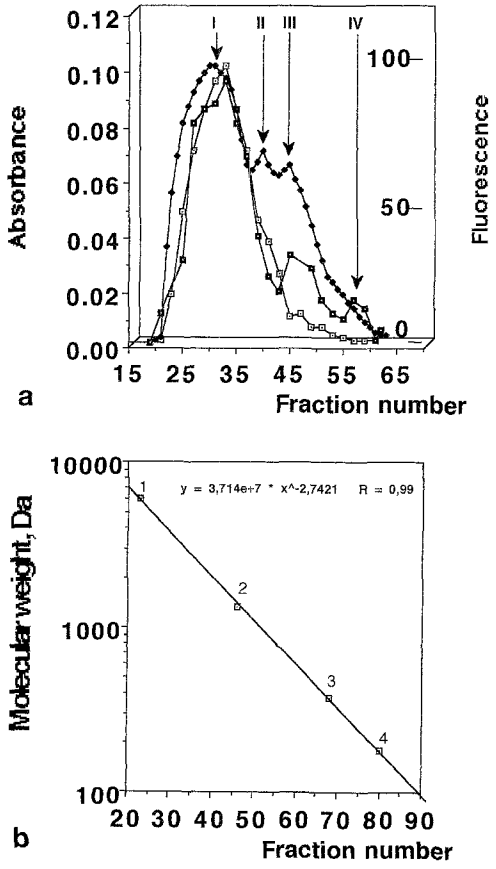

Fig. 2 a, b. Molecular weight determination of AGE-peptides by exclusion-diffusion chromatography. **a** Elution profile of chromatography of AGE-peptides on a Biogel P6 column. AGE-peptides (500 µl of 5 mg/ml) was applied onto a 40-cm Biogel P-6 exclusion-diffusion column equilibrated in 0.01 mol/l sodium phosphate buffer containing 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l PMSF, 1 mmol/l sodium azide, pH 7.4. Chromatography was run at 10 ml/min flow rate using the same solution for elution. One-ml fractions were collected and read at 280 nm (◆) for proteins, at 350 nm (■) for browning products, and fluorescence (□) at Excitation 370 nm and Emission 440 nm. The void volume, estimated as the exclusion volume for haemoglobin, was 19 ml. **b** Molecular weight standard plot. The BioGel column was calibrated with insulin (M_r 6000), bacitracin (M_r 1400), riboflavin (M_r 379) and glucose (M_r 180). Peak I = 3000 Da; Peak II = 2100 Da; Peak III = 1500 Da; Peak IV = 700. Over 90 % of AGE fluorescence elutes in Peak I which displays an M_r of 3 kDa. Most of the browned material also corresponds to this peak. It is noteworthy that some minor browned (Maillard reaction) peptides are not fluorescent

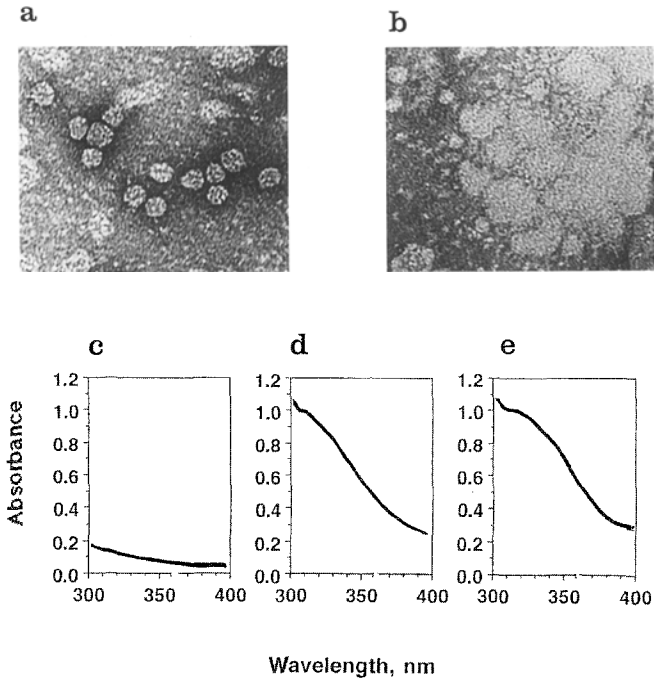

Fig. 3. a–d Reaction of AGE-peptides with LDL. Human LDL (d = 1.051–1.163 mg/ml) was incubated at 37 °C for 16 h in the presence or absence of 3 mmol/l AGE-peptides in 10 mmol/l sodium phosphate, 140 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l PMSF, and 1 mmol/l sodium azide. LDL was extensively dialysed and examined at the electron microscope by negative staining, and by spectrophotometry to detect attached AGE-peptides. *Ultrastructural modifications* (magnification 150 000 ×). **a**) LDL incubated in the absence of AGE-peptides. Typical monodispersed 20-nm LDL particles are apparent. **b**) LDL incubated in the presence of AGE-peptides. LDL particles appear coalescent. Reaction of AGE-peptides produced ultrastructural modifications of LDL particles which are not due to oxidation. *Biochemical modifications:* AGE products, the final result of the browning reaction display a characteristic absorption spectrum with maximum absorption at 350 nm. Absorption spectra of: **c**) LDL incubated in the absence of AGE-peptides. No peak is apparent. **d**) LDL incubated in the presence of AGE-peptides. A 350-nm peak of absorption, corresponding to browning products is apparent. LDL had been extensively dialysed after incubation with AGE-peptides and prior to this measurement. This indicates that AGE-peptides were bound to LDL particles and that binding produces the modifications depicted in (**b**). **e**) AGE-peptides

showed many coalescent particles as well as aggregation (Fig. 3 b). AGE brown products remained attached to LDL even after extensive dialysis as shown in spectra displayed in Figure 3 c–e.

Characterisation of antibodies. To carry out immunocytochemical follow-up of AGE-BSA and AGE-peptides on renal tissue we prepared suitable antibodies. Rabbits were immunised with extensively modified AGE-BSA and bled after the third booster. Antibodies to BSA epitopes were eliminated by two adsorption steps on BSA. The anti-AGE antibodies obtained reacted with AGE epitopes regardless of the protein on which these adducts reside. This was apparent from the fact that our antibodies recognize AGE-BSA, AGE-Hb and AGE IgG but not BSA, Hb or IgG in dot immuno blotting and double immunodiffusion tests (Fig. 4 a, b). AGE-peptides are also recognized (Fig. 4 a). Reaction with AGE-BSA could be abolished by pre-incubation with AGE-BSA, AGE-Hb or AGE IgG.

Cytochemical studies. In order to obtain further insight into the participation of different renal struc-

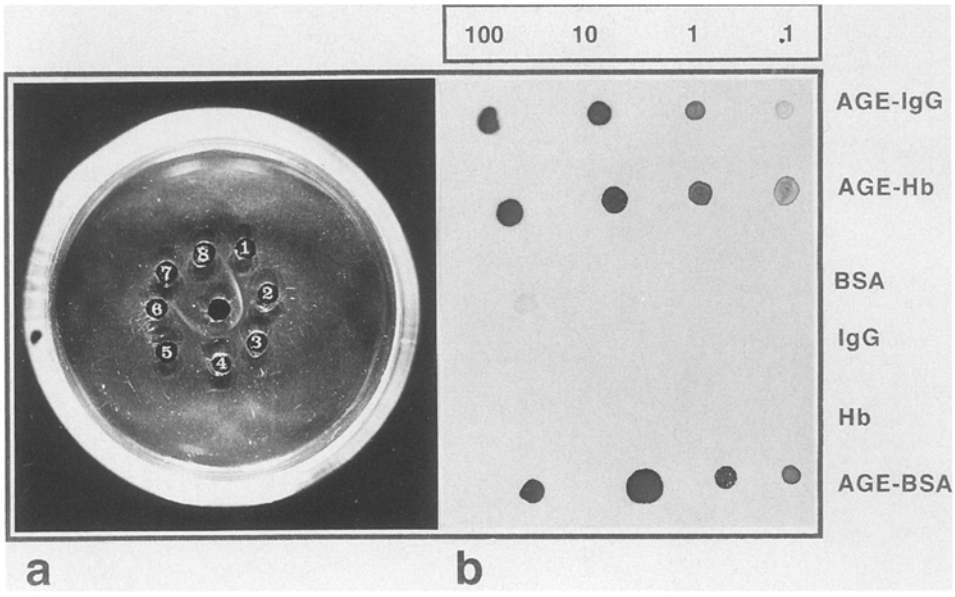

Fig. 4. Specificity of the AGE-antibody. The anti-AGE antibody generated in rabbits and purified as described in Methods was tested for specificity by double diffusion on agarose as well as by dot immunoblotting. The antibody not only recognises AGE adducts on AGE-BSA and AGE-peptides but on unrelated advanced glycated proteins such as AGE-Hb and AGE-IgG as well. No immunoreactivity towards the non glycated forms of these proteins is apparent. **a**) Double diffusion on agarose (1 % agarose in 50 mmol/l sodium barbital buffer, pH 8.6). The antibody (2 µl of a solution containing 7.5 g/l) was placed in the central well. 1, AGE-IgG; 2, AGE-BSA; 3, AGE-Hb; 4, Hb; 5, AGE-peptides; 6, AGE-peptides; 7, BSA; 8, IgG. **b**) Dot immunoblotting. Figures on top indicate ng of protein in each dot. Each protein was spotted onto nitrocellulose membranes and dried. Membranes were then blocked for 2 h, incubated at room temperature overnight with the anti-AGE antibody diluted at 1:500 in non-fat dry milk. Blots were incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG at 1:500 dilution in non-fat dry milk for 2 h before developing with a nitroblue tetrazolium substrate.

tures in the important clearance role of the kidney as regards the AGE-products and evidence the fate of AGE throughout the nephron we performed immunoelectron microscopy. AGE-BSA and AGE-peptides were prepared and injected into otherwise normal rats. Using colloidal gold post-embedding immunoelectron microscopy with our anti-AGE antibody we followed, in a short-term kinetic study, the cellular and subcellular localisation of circulating AGE throughout the nephron. AGE-peptides or AGE-BSA (10 mg/animal) were injected i.v. into Sprague Dawley rats. After 15, 30 or 45 min of circulation, renal tissue was sampled and processed for protein A-gold immunocytochemistry on thin tissue sections.

The distribution of AGE-BSA over the GBM is shown in Figure 5. When injected into normal rats most AGE-BSA was found in the lumen of capillary vessels and distributed along the endothelial side of the GBM. Presence of AGE-BSA in mesangial matrix was also apparent. A similar pattern was observed for all the time points studied, 15, 30 or 45 min (Fig. 5a, b and c, respectively). This suggests that, due to the size of AGE-BSA, during this period no significant elimination of AGE-BSA from the circulation by filtration takes place. A quantitative evaluation of the distribution of AGE-BSA on the glomerular wall confirmed its preferential location along the endothelial side of the GBM (Fig. 6).

A short-term follow-up of AGE-peptide localisation at the kidney level has shown that they are easily filtered and are actively reabsorbed by the proximal convoluted tubule. At 15 min, little labelling was found in the glomerular wall (Fig. 7). Instead, the labelling was present in the urinary space and associated with proximal microvilli of epithelial cells (Fig. 8a). Early and late endosomes of these proximal tubular cells also display significant labelling. At 30 min (Fig. 8b) and 45 min (Fig. 8c), lysosomes added to the pattern of labelling. The distal tubules were devoid of labelling in any of the intervals studied. Other cellular structures such as mitochondria and nuclei showed few gold particles. These are considered as background staining in view of the results obtained using pre-adsorbed antibodies. These observations point to participation of the endo-lysosomal apparatus of the proximal convoluted tubule in the disposal of AGE- peptides.

Cytochemical controls. All the labelling observed with the application of the anti-AGE-BSA antibody was specifically and significantly reduced by pre-incubation of the antibody with an excess of AGE-BSA but not with BSA (data not illustrated).

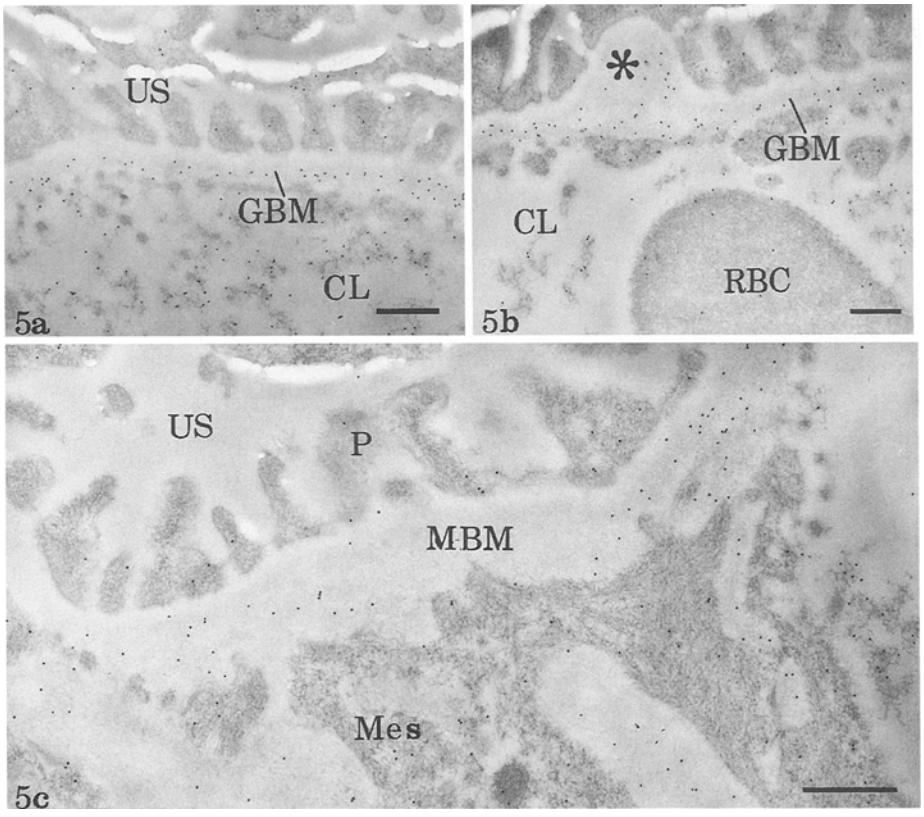

Fig. 5. a–c Localisation of injected AGE-BSA on the glomerular wall of rat kidney. Renal tissue sections from normal rats, injected with 10 mg of AGE-BSA. The probe was injected into the inferior vena cava and the animals were killed after 15 (**a**:), 30 (**b**:) or 45 (**c**:) min. The labelling by gold particles is distributed in the capillary lumen (CL), over the endothelial side of the glomerular basement membrane (GBM) and in the mesangium (MBM). The distribution was similar for all the time points studied. The urinary space (US) appears almost free of labelling. * focal accumulation of basement membrane labelled for AGE-BSA; Mes, mesangial cell; P, podocytes. Bars = 0.5 μm

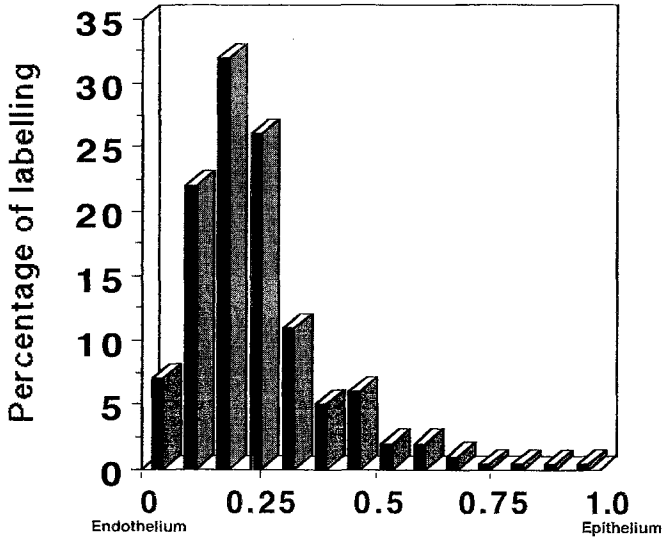

Fig. 6. Histogram of the distribution of AGE-BSA on the glomerular basement membrane. Renal tissue sections from normal rats, injected with 10 mg of AGE-BSA and killed 30 min later. The quantitative evaluation of the distribution of the labelling is expressed as the ratio: distance endothelium-gold particle/distance endothelium-epithelium, (resulting in values between 0 and 1). For each animal 10 microscopic fields were recorded on video at × 31,000 magnification and brought to a final magnification of × 105,000. A peak of distribution of the labelling at the endothelial side of the GBM shows the retention of AGE-BSA at this site.

Discussion

In the past few years several reports indicate that low molecular weight AGE-peptides accumulate in the blood of patients with kidney failure or diabetes mellitus [15, 32, 34, 36, 40]. These tissue-derived degradation products may be produced either by a specific macrophage AGE-receptor pathway [26] or by extracellular proteolytic systems. These are extremely reactive substances capable of modifying circulating or tissue proteins. LDL, for instance contains a fraction bearing AGE adducts, AGE-LDL. Given its short half-life it was difficult to conceive that this modification could be mediated by glucose. Instead, it has been demonstrated that AGE-peptides, both synthetic and extracted from patients, readily bind to LDL altering its metabolism [31]. Through this kind of evidence it is becoming clear that AGE-peptides are responsible for the modification of circulating and tissue resident proteins, leading to structural and functional changes. Thus, probably some molecular modifications previously attributed to glucose itself might be due to circulating AGE-peptides. More important, these peptides are active even when euglycaemia is achieved, so they are capable of perpetuating the hyperglycaemic insult through what becomes a vicious cycle. An effective clearance mechanism must be operative in the normal situation. Several lines of evidence show that AGE-peptides are eliminated by the kidney [34–36, 39, 41, 58]. They increase

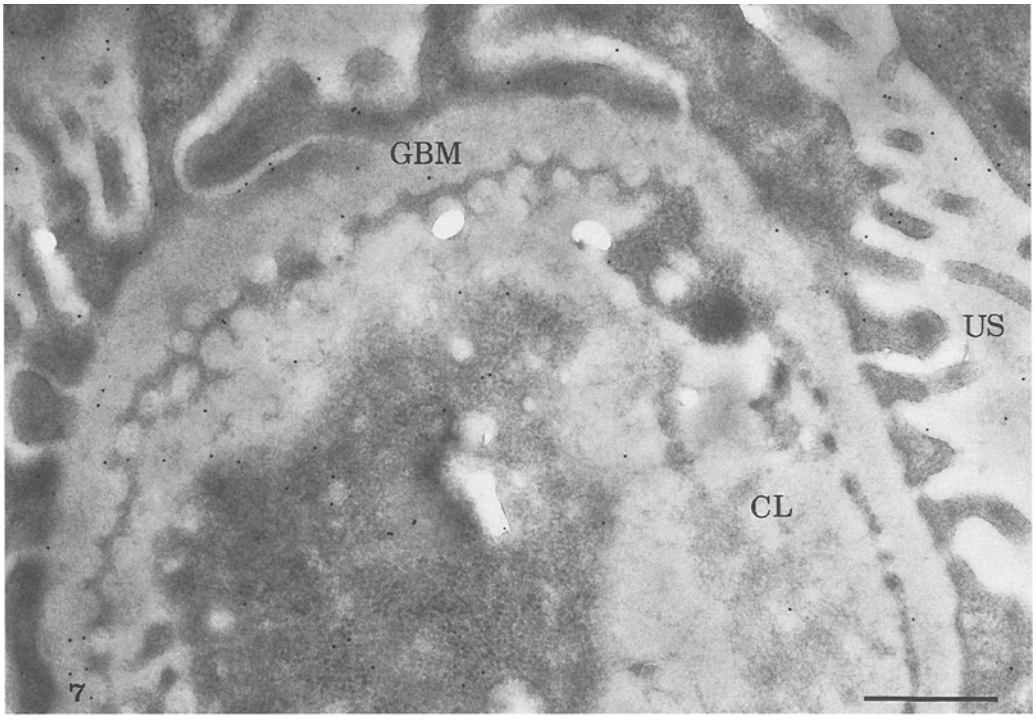

Fig. 7. Localisation of injected AGE-peptides on the glomerulus of rat kidney. Renal tissue sections from a normal rat, injected with 10 mg of AGE-peptides. The probe was injected into the inferior vena cava and the animal was killed 15 min later. The labelling by gold particles is distributed in the capillary lumen (CL) and in the urinary space (US). Little labelling is associated with the glomerular basement membrane (GBM). Bar = 0.5 μm

in renal failure even in the absence of diabetes, and up to sixfold in diabetic patients with renal impairment [34, 36]. They are only partly removed by dialysis and regain predialysis levels as soon as 3 h after the dialysis procedure. The superior survival rate of diabetic kidney transplant recipients suggests an additional benefit linked to the graft. One of the substances known to be better cleared by the kidney than by dialysis are circulating AGE-peptides [33]; they may account in part for the poor prognosis of diabetic patients with end-stage renal disease.

This study was designed to reveal the renal fate of AGE. We used AGE-BSA as a model AGE protein since it has been employed in multiple studies looking for the effects generated by AGE. Bucala et al. [8, 12] have demonstrated that AGE-BSA quenches nitric oxide and that formation of AGE-products in diabetic rats correlates with impaired vasodilatation, a characteristic feature of diabetes. Using a similar model (^{125}I-AGE- rabbit albumin and ^{125}I-AGE-rat albumin with detection in homogenates), Vlassara et al. [16] have demonstrated that AGE injected into otherwise healthy animals led to tissue accumulation and to changes in vascular tone paralleling those seen in diabetes. Using AGE-BSA tagged with colloidal gold particles, we have recently demonstrated the intrinsic reactivity of these adducts towards renal tissue structures [38]. AGE-BSA and AGE-peptides were then prepared in conditions previously reported and the fate of injected AGE-products in renal tissue of control rats was monitored by colloidal gold post-embedding immunoelectron microscopy.

Antibodies against AGE were prepared by injecting strongly modified AGE-BSA to rabbits. Antibodies to epitopes present both in BSA and in AGE-BSA were eliminated by a two-step adsorption with BSA. IgG was next purified by ammonium sulphate precipitation and DEAE chromatography. In keeping with previous results from other groups, these antibodies recognize AGE epitopes in several proteins but not in their non-glycated counterparts. In our case, they react with AGE-BSA, AGE-Hb and AGE-IgG but not against BSA, Hb and IgG. Furthermore, they are able to recognize epitopes on AGE-peptides.

Our short-term study on the renal handling of intact AGE-BSA showed a particular disposition of molecules along the endothelial side of the GBM. As reported previously [57], this reflects molecules effectively retained by the size and charge exclusion barrier of the GBM and not necessarily bound particles. However, our previous results [38], showing binding of AGE-BSA-gold but not BSA-gold to GBM, suggest that at least one portion of these molecules is effectively bound to GBM components. In fact, the combination of physical contact favoured by the intense haemodynamic pressure, linked to the extreme reactivity of AGE moieties should provide a particularly effective synergism for this binding. In

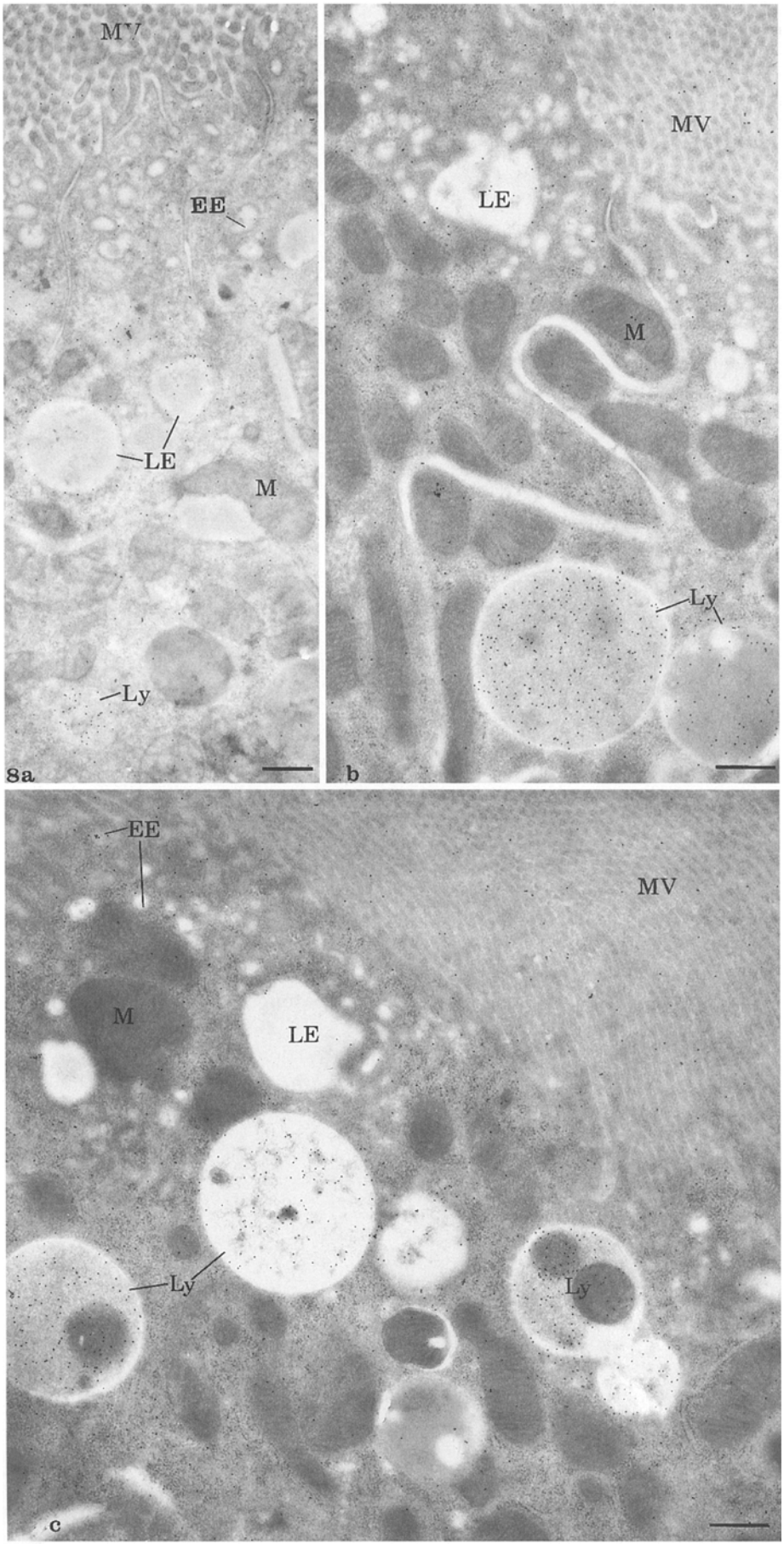

Fig. 8 a–c. Localisation of injected AGE-peptides on the proximal tubular epithelial cells of rat kidney. AGE-peptides were filtered by the glomeruli, reabsorbed and metabolised by the endo-lysosomal system of proximal convoluted tubular cells. Renal tissue sections from normal rats, injected with 10 mg of AGE-peptides. The probe was injected into the inferior vena cava and the animals were killed 15 (**a**), 30 (**b**) or 45 (**c**) min later. At 15 min (**a**) the labelling was present in the urinary space (US) and associated with microvilli of proximal epithelial cells (MV). Early (EE) and late endosomes (LE) of these proximal tubular cells display intense labelling as well. At 30 (**b**) and 45 (**c**) min, lysosomes (Ly) added to the pattern of labelling. Other cellular structures such as mitochondria and nuclei show few gold particles. Bars = 0.5 μm

keeping with this reasoning, recent results from Vlassara et al. [39] clearly demonstrate that chronic injection of AGE albumin into rats reproduces glomerular changes seen in glomerulosclerosis. Changes seen on these chronic administration experiments could be due not only to the direct cross-linking effect of AGE but to receptor-mediated cytokine effects as well.

The participation of these phenomena on the multifactorial pathogenesis of diabetic glomerulopathy is strongly suggested by these studies.

In this regard, we performed experiments to ascertain the renal handling of AGE-peptides. The AGE-peptides generated displayed the characteristics shown previously. Their M_r was about 3 kDa (about 30 amino acid residues) as shown by gel filtration chromatography and they exhibited the characteristic fluorescence and absorption spectra. These properties of our AGE-peptides closely resemble those reported for AGE-peptides isolated from plasma of diabetic and kidney failure patients [15, 32, 34, 36]. To test the reactivity of AGE-peptides in vitro, they were incubated in the presence of LDL. They proved to be extremely reactive towards LDL, producing similar changes as those recently reported for the interaction between AGE-BSA and LDL [31, 59]. Moreover, AGE-peptides seem to be somewhat more aggressive than AGE-BSA. The ultrastructural changes illustrated here were produced after 16 h incubation under strict non-oxidative conditions, while similar modifications with AGE-BSA needed concomitant incubation with copper ions as oxidants [59].

When injected into normal rats, a short-term follow-up of their localisation at the kidney level has shown that they are easily filtered and actively reabsorbed by the proximal convoluted tubule. At 15 min the labelling was found in the urinary space and on the microvilli. Endocytosis by the proximal tubular epithelial cells takes place as witnessed by the labelling of endocytic vesicles as well as early and late endosomal compartments. Further steps on the endocytic pathway are apparent at 30 and 45 min, when the labelling appeared to be particularly intense in late endosomes, while labelled lysosomes become predominant. Our data point to a participation of the endo-lysosomal apparatus of the proximal convoluted tubule to the disposal of AGE-peptides. We interpret these data in the light of recently reported values of AGE-peptide clearance in humans and rats [15, 39]. This clearance, being lower than the creatinine clearance, indicates that not all of circulating AGE-peptides are filtered; some process of reabsorption occurs. It is possible that both events take place simultaneously. The labelling we show here in the urinary space strongly suggests a very effective filtration. On the other hand, labelling of endosomal compartments reflects a great reabsorption process at the level of the proximal convoluted tubule and strongly suggests that this is the predominant elimination mechanism.

Reabsorption could represent an AGE-receptor-mediated mechanism since receptors for AGE adducts have been described in several cell types such as macrophages, endothelial and mesangial cells [19–21, 23, 29, 58]. Activation of these receptors triggers several cell responses including cytokine secretion and oxidation-enhancing reactions [7]. Following this reasoning, one might speculate that in diabetes, an increase in these processes could participate in the interstitial fibrosis reaction accompanying the characteristic glomerulosclerosis of end-stage renal disease. The final fate of AGE-peptides also remains to be determined since no mammalian enzyme has been discovered that could mediate the catabolism of AGE moieties once the lysosomal hydrolysis of peptide bonds has taken place. A slow secretory process of resulting AGE amino acids into urine could be suggested and could also account for the presence of AGE adducts such as pentosidine in the urine of diabetic patients [60]. In the long run, the increased tubular charge of AGE-peptides due to diabetes may overwhelm the whole process and lead to tubular disorders.

Finally, another feature of AGE-peptides is the ability to bind covalently to phospholipids as described previously [31]. It is reasonable to think that AGE-peptides react with membrane phospholipids if present in high local concentrations such as those shown here in lysosomes and if sufficient time is allowed. An accumulation of these adducts in tubular lysosomes might prove to be one further aggression to membranes and yet another process contributing to the overall toxicity.

In conclusion, our study confirms the key role played by the kidney in regard to AGE catabolism, gives ultrastructural evidence for the GBM and mesangial accumulation of AGE-BSA and demonstrates the important participation of resorption processes at the level of the proximal tubules in the clearance of circulating AGE-peptides.

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