Expression of Advanced Glycation End Products and Their Cellular Receptor RAGE in Diabetic Nephropathy and Nondiabetic Renal Disease

NOZOMU TANJI,* GLEN S. MARKOWITZ,* CAIFENG FU,† THOMAS KISLINGER,†
AKIHiko TAGUCHI, † MONIKA PISCHETSRIEDER, †
DAVID STERN, †‡ ANN MARIE SCHMIDT, ‡§ and VIVETTE D. D’AGATI*
Departments of *Pathology, † Physiology, ‡ Surgery, and § Medicine, Columbia University College of Physicians and Surgeons, New York, New York, and †Institut fur Pharmazie und Lebensmittelchemie, Universitat Erlangen-Nurnberg, Erlangen, Germany.

Abstract. Advanced glycation end products (AGE) contribute to diabetic tissue injury by two major mechanisms, i.e., the alteration of extracellular matrix architecture through nonenzymatic glycation, with formation of protein crosslinks, and the modulation of cellular functions through interactions with specific cell surface receptors, the best characterized of which is the receptor for AGE (RAGE). Recent evidence suggests that the AGE-RAGE interaction may also be promoted by inflammatory processes and oxidative cellular injury. To characterize the distributions of AGE and RAGE in diabetic kidneys and to determine their specificity for diabetic nephropathy, an immunohistochemical analysis of renal biopsies from patients with diabetic nephropathy (n = 28), hypertensive nephrosclerosis (n = 7), idiopathic focal segmental glomerulosclerosis (n = 11), focal sclerosis secondary to obesity (n = 7), and lupus nephritis (n = 11) and from normal control subjects (n = 2) was performed, using affinity-purified antibodies raised to RAGE and two subclasses of AGE, i.e., N-(carboxymethyl)-lysinine (CML) and pentosidine (PENT). AGE were detected equally in diffuse and nodular diabetic nephropathy. CML was the major AGE detected in diabetic mesangium (96%), glomerular basement membranes (GBM) (42%), tubular basement membranes (85%), and vessel walls (96%). In diabetic nephropathy, PENT was preferentially located in interstitial collagen (90%) and was less consistently observed in vessel walls (54%), mesangium (77%), GBM (4%), and tubular basement membranes (31%). RAGE was expressed on normal podocytes and was upregulated in diabetic nephropathy. The restriction of RAGE mRNA expression to glomeruli was confirmed by reverse transcription-PCR analysis of microdissected renal tissue compartments. The extent of mesangial and GBM immunoreactivity for CML, but not PENT, was correlated with the severity of diabetic glomerulosclerosis, as assessed pathologically. CML and PENT were also identified in areas of glomerulosclerosis and arteriosclerosis in idiopathic and secondary focal segmental glomerulosclerosis, hypertensive nephrosclerosis, and lupus nephritis. In active lupus nephritis, CML and PENT were detected in the proliferative glomerular tufts and crescents. In conclusion, CML is a major AGE in renal basement membranes in diabetic nephropathy, and its accumulation involves upregulation of RAGE on podocytes. AGE are also accumulated in acute inflammatory glomerulonephritis secondary to systemic lupus erythematosus, possibly via enzymatic oxidation of glomerular matrix proteins.

Advanced glycation end products (AGE) are generated by the sequential nonenzymatic glycation of protein amino groups and by oxidation reactions (1). AGE comprise several major molecular structures, such as N-(carboxymethyl)lysine (CML) and pentosidine (PENT). CML can be formed either by oxidative cleavage of fructoselysine or by reaction of protein with glyoxal, an auto-oxidation product of glucose or a Schiff base adduct (2). PENT can be formed from glycoxidation of Amadori products or oxidation of arabinose (3). The accumulation of these protein adducts in tissues alters the structure and function of matrix proteins (4).

The accumulation of AGE in kidneys and other tissues of patients with diabetes mellitus has been implicated in the development of diabetic nephropathy and vasculopathy (5,6). AGE may contribute to diabetic tissue injury by at least two major mechanisms. The first is receptor-independent alteration of the extracellular matrix architecture by nonenzymatic glycation and the formation of protein crosslinks. The second mechanism is receptor-dependent and consists of modulation of cellular functions through ligation of specific cell surface receptors, the best characterized of which is the receptor for AGE (RAGE), a member of the Ig superfamily (7,8).

AGE-modified proteins have been demonstrated to stimulate a number of cellular responses, such as synthesis of fibronectin and type IV collagen by glomerular mesangial cells (9,10). The
interaction of AGE with RAGE mediates monocyte migration and activation in response to AGE (11,12). The interaction of AGE with endothelial cell RAGE induces cellular oxidative stress, hyper-responsiveness to inflammatory cytokines, increased vascular permeability, and upregulation of cell adhesion molecules (13,14). Recent evidence suggests that enhanced interaction of AGE with RAGE may also be promoted by inflammatory processes and pro-oxidant states. In this context, AGE-RAGE interaction activates certain signaling pathways that are linked to altered gene expression, including those involving p21<sup>ras</sup>, erk1/2 kinases, and NF-κB (13,15). Although engagement of AGE with specific receptors on vascular endothelial cells and macrophages has been demonstrated to play a role in the pathogenesis of diabetic macrovascular disease (16), it is not known how AGE-RAGE interactions might contribute to the development of diabetic nephropathy.

Little is known regarding the distribution of AGE and RAGE in the kidneys of patients with diffuse or nodular diabetic glomerulosclerosis. To address this issue, we performed an immunohistochemical analysis of AGE and RAGE expression at various stages in the progression of diabetic nephropathy and correlated these findings with clinical and morphologic markers of disease severity. To determine the specificity of these findings for diabetic nephropathy, the results for diabetic nephropathy were compared with those for a variety of nondiabetic renal diseases of sclerosing or inflammatory nature.

**Materials and Methods**

**Patients**

Renal biopsies from patients with diabetic nephropathy (n = 26), hypertensive nephrosclerosis (n = 7), idiopathic focal segmental glomerulosclerosis (FSGS) (n = 11), secondary FSGS attributable to obesity-induced hyperfiltration (n = 7), and lupus nephritis (n = 11) and from normal control subjects (n = 2) were studied by immunohistochemical analysis, using affinity-purified polyclonal antibodies raised to RAGE and two subclasses of AGE, i.e., CML and PENT. The mean ages of the patients in each diagnostic category were 58.7 ± 5.1 yr for hypertensive nephrosclerosis, 29.0 ± 5.0 yr for idiopathic FSGS, 39.0 ± 3.1 yr for secondary FSGS, 26.0 ± 2.4 yr for lupus nephritis, and 20.5 ± 4.5 yr for the normal control subjects.

**Table 1. Diabetic nephropathy: pathologic and immunohistochemical findings**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Light Microscopy</th>
<th>Electron Microscopy</th>
<th>Immunostaining</th>
<th>Vessels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type</td>
<td>Severity</td>
<td>GBM Thickening</td>
<td>Sclerotic Glomeruli</td>
</tr>
<tr>
<td>1</td>
<td>Diff</td>
<td>Mod</td>
<td>Severe</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>Diff</td>
<td>Mod</td>
<td>Severe</td>
<td>NA</td>
</tr>
<tr>
<td>3</td>
<td>Diff</td>
<td>Mod</td>
<td>Severe</td>
<td>3+</td>
</tr>
<tr>
<td>4</td>
<td>Nod</td>
<td>Severe</td>
<td>Mod</td>
<td>2+</td>
</tr>
<tr>
<td>5</td>
<td>Diff</td>
<td>Mod</td>
<td>Severe</td>
<td>NA</td>
</tr>
<tr>
<td>6</td>
<td>Diff</td>
<td>Mod</td>
<td>Mod</td>
<td>3+</td>
</tr>
<tr>
<td>7</td>
<td>Diff</td>
<td>Mild</td>
<td>Nod</td>
<td>NA</td>
</tr>
<tr>
<td>8</td>
<td>Nod</td>
<td>Severe</td>
<td>Mod</td>
<td>2+</td>
</tr>
<tr>
<td>9</td>
<td>Nod</td>
<td>Mod</td>
<td>Mod</td>
<td>3+</td>
</tr>
<tr>
<td>10</td>
<td>Diff</td>
<td>Mod</td>
<td>Mod</td>
<td>3+</td>
</tr>
<tr>
<td>11</td>
<td>Nod</td>
<td>Severe</td>
<td>Severe</td>
<td>3+</td>
</tr>
<tr>
<td>12</td>
<td>Hypertrophic</td>
<td>Mild</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>13</td>
<td>Diff</td>
<td>Severe</td>
<td>Severe</td>
<td>3+</td>
</tr>
<tr>
<td>14</td>
<td>Diff</td>
<td>Mod</td>
<td>Mild</td>
<td>1+</td>
</tr>
<tr>
<td>15</td>
<td>Nod</td>
<td>Severe</td>
<td>Mod</td>
<td>2+</td>
</tr>
<tr>
<td>16</td>
<td>Diff</td>
<td>Severe</td>
<td>Severe</td>
<td>2+</td>
</tr>
<tr>
<td>17</td>
<td>Diff</td>
<td>Mod</td>
<td>Mod</td>
<td>NA</td>
</tr>
<tr>
<td>18</td>
<td>Nod</td>
<td>Severe</td>
<td>Severe</td>
<td>1+</td>
</tr>
<tr>
<td>19</td>
<td>Nod</td>
<td>Mod</td>
<td>Mod</td>
<td>2+</td>
</tr>
<tr>
<td>20</td>
<td>Diff</td>
<td>Mild</td>
<td>Mod</td>
<td>3+</td>
</tr>
<tr>
<td>21</td>
<td>Nod</td>
<td>Mod</td>
<td>Nod</td>
<td>1+</td>
</tr>
<tr>
<td>22</td>
<td>Diff</td>
<td>Mod</td>
<td>Mod</td>
<td>NA</td>
</tr>
<tr>
<td>23</td>
<td>Nod</td>
<td>Severe</td>
<td>Mod</td>
<td>3+</td>
</tr>
<tr>
<td>24</td>
<td>Diff</td>
<td>Mod</td>
<td>Mod</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>Nod</td>
<td>Mod</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>26</td>
<td>Nod</td>
<td>Severe</td>
<td>Severe</td>
<td>3+</td>
</tr>
</tbody>
</table>

*Diff, diffuse type; Nod, nodular type; Mod, moderate; NA, not applicable; CML, N<sup>-</sup>carboxymethyllysine; PENT, pentosidines; GBM, glomerular basement membranes; TBM, tubular basement membranes.
The group with diabetic nephropathy consisted of 26 patients with adult-onset diabetes mellitus (18 male and 8 female patients, of whom 11 were Caucasian, 11 were African American, 2 were Hispanic, and 2 were Asian American), with a mean age of 59.7 ± 2.2 yr. Cases were chosen retrospectively from the files of the Nephropathology Laboratory of Columbia University to represent a range of severity of diabetic nephropathy, from mild to advanced. This spectrum is reflected in the wide range of serum creatinine levels, from 0.8 to 11.4 mg/dl (mean, 2.9 ± 0.4 mg/dl), and in the broad range of urinary protein excretion values, from 0.3 to 19.5 g/d (mean, 6.7 ± 0.2 g/d). All renal biopsies were performed between August 1997 and March 1998, and specimens were studied with light microscopy, immunofluorescence microscopy, and electron microscopy, using standard techniques. Diagnoses of diabetic nephropathy were based on the characteristic findings of glomerular basement membrane (GBM) thickening and mesangial sclerosis, as detected using light and electron microscopy (17). Cases included 10 examples of nodular diabetic glomerulosclerosis, 15 cases of diffuse diabetic glomerulosclerosis, and 1 example of the hypertrophic phase of diabetic nephropathy with no recognizable mesangial sclerosis at the light microscopic level. All cases exhibited linear staining of GBM and tubular basement membranes (TBM) with antisera to IgG and albumin. The light and electron microscopic features are listed in Table 1.

**Generation of Specific Antibodies to RAGE, CML, and PENT**

Monospecific polyclonal antibodies were raised against human soluble RAGE. Human soluble RAGE was prepared in a baculovirus expression system, purified to homogeneity, subjected to amino-terminal sequence analysis, and used to immunize New Zealand White rabbits. IgG from immunized rabbits was purified and tested for recognition of RAGE in both enzyme-linked immunosorbent assay (ELISA) and immunoblotting studies, as described previously (7,8). Antibody specificity was confirmed by Western blotting performed with homogenates of normal human kidney (data not shown).

Affinity-purified antibodies recognizing CML adducts of proteins or PENT-modified adducts were prepared and characterized according to previously published procedures (18,19). CML- or PENT-modified keyhole limpet hemocyanin (KLH) (Sigma Chemical Co., St. Louis, MO) was used to immunize rabbits. Antibodies specific for the KLH backbone were removed by passage of the immune IgG through KLH-preadsorbed Affi-gel resin (Bio-Rad, Hercules, CA). The material not adhering to this column was then chromatographed on Affi-gel resin bearing CML- or PENT-modified bovine serum albumin (BSA) and was eluted with 1 M NaCl. Specificity was tested using ELISA and immunoblotting assays, according to previously published procedures (18,19).

**Immunohistochemical Analysis of Renal Biopsy Specimens**

Formalin-fixed, paraffin-embedded tissues were cut at 3 μm on 3-aminopropyltriethoxysilane (Sigma)-coated slides, deparaffinized, and rehydrated in graded alcohols. Sections to be stained with the antibody to RAGE were heated in a microwave for 25 min before immunostaining. Sections to be stained with the antibodies to CML or PENT were pretreated with trypsin for 90 min. After blocking with 10% normal goat serum (Vector Laboratories, Burlingame, CA), serial sections were stained with the antibodies to CML (0.7 μg/ml), PENT (10 μg/ml), or RAGE (4 μg/ml) and were incubated overnight at 4°C in a humidified chamber. After washing with phosphate-buffered saline (PBS), sections were stained with biotinylated secondary goat anti-rabbit antibody (1:100; Vector Laboratories). Sections were washed with PBS and incubated with avidin-biotin complex (Vector Laboratories) for 30 min, followed by 3,3′-diaminobenzidine solution containing 0.003% H₂O₂. Sections were counterstained with hematoxylin and mounted. Negative controls consisted of serial sections stained with equivalent concentrations of preimmune IgG in place of the primary antibody. To prove the podocytic distribution of RAGE immunostaining, serial sections were stained with a monoclonal antibody to synaptopodin (Maine Biotechnology, Portland, ME), a podocyte-specific, actin-associated protein. After microwave heating, sections were overlaid sequentially with 10% normal horse serum (Vector Laboratories)
and 10 individual vessels were captured sequentially on separate polymer
vation. For each specimen, 60 individual glomeruli, 60 individual tubules,
glomeruli, proximal tubules, and vessels under direct microscopic obser-
size and beam intensity were adjusted to microdissect pure populations of
Mountain View, CA) was used for laser microdissection. The laser spot
air-dried for 20 min. The PixCell I LCM system (Arcturus Engineering,
alcohols diluted with DEPC-treated water, immersed in xylene, and
20 s, rinsed briefly in DEPC-treated water for 5 s, dehydrated in graded

treated water. The sections were stained with Weigert's hematoxylin for
briefly in graded alcohols diluted with diethyl pyrocarbonate (DEPC)-

AGE Immunolocalization in diabetic nephropathy versus nondiabetic renal diseases

<table>
<thead>
<tr>
<th></th>
<th>Sclerotic Glomeruli</th>
<th>GBM</th>
<th>Mesangium</th>
<th>TBM</th>
<th>Interstitium</th>
<th>Vessels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CML</td>
<td>PENT</td>
<td>CML</td>
<td>PENT</td>
<td>CML</td>
<td>PENT</td>
</tr>
<tr>
<td>Diabetic nephropathy</td>
<td>94.4</td>
<td>87.8</td>
<td>42.3</td>
<td>3.9</td>
<td>62.1</td>
<td>76.9</td>
</tr>
<tr>
<td>Primary FSGS</td>
<td>71.4</td>
<td>100.0</td>
<td>50.0</td>
<td>10.0</td>
<td>20.0</td>
<td>40.0</td>
</tr>
<tr>
<td>Secondary FSGS</td>
<td>40.0</td>
<td>60.0</td>
<td>0.0</td>
<td>0.0</td>
<td>14.3</td>
<td>85.7</td>
</tr>
<tr>
<td>Hypertensive nephrosclerosis</td>
<td>100.0</td>
<td>71.4</td>
<td>42.9</td>
<td>0.0</td>
<td>28.6</td>
<td>28.6</td>
</tr>
<tr>
<td>Lupus nephritis</td>
<td>80.0</td>
<td>60.0</td>
<td>54.5</td>
<td>45.5</td>
<td>27.3</td>
<td>63.6</td>
</tr>
</tbody>
</table>

Diabetic nephropathy     | 77.8                | 55.6                 | 18.2      | 3.9                  | 30.8         | 30.8    | 53.8    | 11.5   | 3.9    | 61.5   | 80.8    | 19.2    |
| Primary FSGS             | 14.3                | 100.0                | 0.0       | 0.0                  | 0.0          | 0.0     | 10.0    | 30.0   | 10.0   | 30.0   | 30.0    | 10.0    |
| Secondary FSGS           | 20.0                | 40.0                 | 0.0       | 0.0                  | 0.0          | 0.0     | 0.0     | 0.0    | 0.0    | 0.0    | 0.0     | 0.0     |
| Hypertensive nephrosclerosis | 57.1               | 71.4                 | 0.0       | 0.0                  | 0.0          | 0.0     | 28.6    | 28.6   | 0.0    | 28.6   | 100.0   | 28.6    |
| Lupus nephritis          | 60.0                | 40.0                 | 9.1       | 27.3                 | 9.1          | 18.2    | 0.0     | 18.2   | 0.0    | 27.3   | 18.2    | 9.1     |

*AGE, advanced glycation end products; FSGS, focal segmental glomerulosclerosis.

b Expressed as percentage of cases with any positive immunostaining.

c Expressed as percentage of cases with 2+ or 3+ immunostaining.

Laboratories), synaptophysin-specific antibody (1:1), and biotinyl-
ated horse anti-mouse antibody (1:100; Vector Laboratories). The
combined intensity and distribution of immunostaining were deter-
determined on a scale of 0 to 3+ (0, absent; 1+, low intensity and
<25% of structures stained; 2+, moderate intensity and <50% of
structures stained; 3+, high intensity and >50% of structures stained) for the GBM, mesangium, TBM, interstitium, and vessels.
Correlations between the prevalence of CML and PENT immuno-
staining and severity of proteinuria and serum creatinine levels were
performed using t tests. Correlations between the grade of immuno-
staining (0 to 3+) for CML and PENT and the severity of diabetic
glomerulosclerosis (assessed histologically) were performed using a
doubly ordered contingency table (Jonckheere-Terpstra test) and the
line-by-linear association test.

**Laser Capture Microdissection**

For the studies of RAGE mRNA expression, snap-frozen, archival, renal
biopsy tissue (which had been stored at −80°C) from patients with
diabetic nephropathy and from normal control subjects was used. The
frozen tissue was cut at 8 μm onto noncoated slides and was rehydrated
briefly in graded alcohols diluted with diethyl pyrocarbonate (DEPC)-
treated water. The sections were stained with Weigert's hematoxylin for
briefly in graded alcohols diluted with diethyl pyrocarbonate (DEPC)-

**RNA Extraction and Reverse Transcription (RT)-PCR**

Total RNA was extracted using TRI Reagent (Sigma), according to the
single-step method reported by Chomczynski and Sacchi (20). The
RNA was redissolved in 4 μl of DEPC-treated water, and RT was
performed at 60°C for 30 min, using a GeneAmp EZ rTth RNA PCR
kit (Perkin Elmer, Foster City, CA), in a final volume of 50 μl, with 2
μl of total RNA solution, 0.3 mM levels of each dNTP, 2.5 mM
manganese diacetate, 0.45 μM levels of each primer, and 5 U of rTth
dna polymerase. The sense primer for RAGE corresponded to bp
1801 to 1820 (5'-GCCCTCCAGTACTACTCTCG-3'), and the anti-
sense primer corresponded to bp 2043 to 2062 (5'-TGTTGTGCCAC-
CCATTCCAG-3'). The sense and antisense primers for glyceralde-
hyde-3-phosphate dehydrogenase corresponded to bp 204 to 226
(5'-CAATGGGAAATCCCATCCCATCT-3') and bp 908 to 930 (5'-
AATGACCGTCAGAAGGTGTCG-3'), respectively. The entire RT reaction product was used for PCR amplification. Forty cycles of
amplification were performed with a thermal programmer (Perkin
Elmer), as follows: denaturation at 94°C for 15 s and annealing and
extension at 60°C for 30 s. The PCR products were then electropho-
resed on a 1.5% agarose gel stained with ethidium bromide.

**Results**

**Characterization of Antibodies to CML and PENT**

Characterization of the antibodies was performed by ELISA,
using affinity-purified anti-CML IgG and affinity-purified anti-
PENT IgG. In the former case, CML-BSA or native BSA (both

<table>
<thead>
<tr>
<th></th>
<th>Sclerotic Glomeruli</th>
<th>GBM</th>
<th>Mesangium</th>
<th>TBM</th>
<th>Interstitium</th>
<th>Vessels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CML</td>
<td>PENT</td>
<td>CML</td>
<td>PENT</td>
<td>CML</td>
<td>PENT</td>
</tr>
<tr>
<td>Diabetic nephropathy</td>
<td>77.8</td>
<td>55.6</td>
<td>18.2</td>
<td>3.9</td>
<td>30.8</td>
<td>30.8</td>
</tr>
<tr>
<td>Primary FSGS</td>
<td>14.3</td>
<td>100.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Secondary FSGS</td>
<td>20.0</td>
<td>40.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Hypertensive nephrosclerosis</td>
<td>57.1</td>
<td>71.4</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Lupus nephritis</td>
<td>60.0</td>
<td>40.0</td>
<td>9.1</td>
<td>27.3</td>
<td>9.1</td>
<td>18.2</td>
</tr>
</tbody>
</table>
at 5 μg total protein/well) was coated onto wells of Maxisorp plates (Nunc, Naperville, IL) in buffer (pH 9.6). After overnight incubation, wells were washed with PBS (0.02 M, pH 7.4), and unoccupied sites were blocked with PBS containing 1% BSA. ELISA was then performed using affinity-purified anti-CML IgG (0.88 μg/ml), followed by peroxidase-conjugated goat anti-rabbit IgG. Results in OD₄₉₀ nm were as follows: native BSA, 0.048; CML-BSA, 1.781. In other studies, immunoreactivity to CML-ovalbumin was 1.742, that for CML-ribonuclease was 1.090, and that for PENT-BSA was 0.051. Similarly, ELISA was performed using affinity-purified anti-PENT IgG (1 μg/ml), as described above. Results in OD₄₉₀ nm were as follows: native BSA, 0.042; PENT-BSA, 0.542; CML-ovalbumin, 0.047; CML-BSA, 0.047; CML-ribonuclease, 0.042.

**Immunohistochemical Detection of AGE and RAGE in Normal Control Samples**

With the exception of small amounts of CML and PENT in the intima of medium-sized and large arteries, normal control samples exhibited negative immunostaining for CML and PENT in the glomerular, tubular, and interstitial compartments (Figure 1, A and B). Diffuse low-level expression of RAGE was restricted to the podocytes of normal control subjects (Figure 1C).

**Immunohistochemical Detection of AGE and RAGE in Diabetic Nephropathy**

The immunohistochemical staining results for diabetic nephropathy are itemized in Table 1 and summarized in Table 2. CML was the major AGE identified in the GBM (42% of cases), mesangium (96%), TBM (85%) of both atrophic and nonatrophic tubules, and vessels (96%) (Table 2; Figure 2). Although PENT was identified in the mesangium in 77% of cases, it was rarely identified in the GBM (4%) (Figure 3A). Both CML and PENT were readily identified in mesangial nodules and in areas of glomerular hyalinosis (“fibrin cap lesions”). In contrast, PENT was the major AGE identified in areas of subcapsular fibrosis (65%) and interstitial fibrosis (92%) (Figure 3, B and C). PENT was less frequently identified in TBM (31%) and vessel walls (54%), compared with CML. Thus, in diabetic nephropathy, there was differential localization of PENT to interstitial collagen as well as mesangium and vascular...
basement membranes, whereas CML was more restricted to renal basement membranes known to contain collagen IV. Weak focal tubular epithelial staining for CML and PENT was identified in patients with moderate or severe diabetic nephropathy. There was marked uniform upregulation of RAGE expression in the podocytes of diabetic nephropathy (Figure 4A). The distribution of RAGE at the base of the podocytes (Figure 4B) was confirmed in serial sections

Figure 3. Diabetic glomerulosclerosis. (A) Severe diabetic glomerulosclerosis. PENT immunostaining is seen in the outer layers of a mesangial nodule. Magnification, ×550. (B) Severe diabetic glomerulosclerosis. Immunoreactivity for PENT is largely confined to the areas of subcapsular fibrosis, with only focal mesangial positivity. Magnification, ×420. (C) Moderate diabetic glomerulosclerosis. Prominent staining for PENT is identified in areas of interstitial fibrosis and glomerular subcapsular fibrosis. Magnification, ×250.

Figure 4. Diabetic glomerulosclerosis. (A) In mild diabetic glomerulosclerosis, RAGE immunoreactivity is markedly upregulated in the podocytes. Magnification, ×550. (B) With high-power magnification, staining for RAGE in a diabetic glomerulus has a punctate distribution at the base of the podocytes, above the GBM. Magnification, ×700. (C) The same field as in B reveals an almost identical distribution of synaptopodin immunoreactivity at the base of the podocytes. Magnification, ×700.
stained for the podocyte-specific marker synaptopodin (Figure 4C). No RAGE expression was identified in other renal cell types.

Correlations between the severity of diabetic nephropathy and the semiquantitative assessments of CML immunostaining in mesangium \((P = 0.004)\), GBM \((P < 0.05)\), and TBM \((P = 0.003)\) were observed. There was no correlation between the severity of diabetic nephropathy and CML immunostaining in globally sclerotic glomeruli, interstitium, or vessel walls \((P = NS)\). In contrast, there were significant correlations between the severity of diabetic nephropathy and the semiquantitative assessments of PENT staining in TBM \((P = 0.035)\) and interstitium \((P = 0.037)\) but not globally sclerotic glomeruli, GBM, mesangium, or vessel walls \((P = NS)\). Among the patients with diabetic nephropathy, no correlation was found between the immunohistochemical quantification of AGE deposition and the severity of proteinuria or renal insufficiency.

**RAGE mRNA Expression**

The results of RT-PCR for RAGE are shown in Figure 5. RAGE mRNA expression was seen in glomeruli, but not tubules or vessels, in renal biopsy tissue from patients with diabetic nephropathy. No band was identified in glomeruli, tubules, or vessels from normal control kidney samples (data not shown). No expression was observed in the negative control samples.

![Figure 5. Reverse transcription-PCR results for microdissected glomeruli, tubules, and vessels from a diabetic glomerulosclerosis specimen. A band corresponding to the predicted RAGE PCR product (262 bp) is identified in the microdissected glomeruli from a diabetic nephropathy specimen but not in the diabetic tubular or vascular compartments. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as an internal positive control (727 bp). No expression is identified in the negative control sample. The molecular marker used was a 100-bp ladder.](image)

**Immunohistochemical Detection of AGE and RAGE in Nondiabetic Renal Diseases**

In primary and secondary forms of FSGS and in hypertensive nephrosclerosis, CML and PENT were commonly detected in the distribution of partially or globally sclerotic glomeruli (Figure 6, A and B). However, in contrast to their distribution in diabetic nephropathy, these AGE were infrequently identified in the mesangium or GBM of nonsclerotic glomeruli (Table 2). CML and PENT were also present in the TBM of atrophic tubules but were rarely identified in nonatrophic tubules. In all of these conditions, CML and PENT staining was common in the vessel walls, particularly the intima (Figure 6C). CML was universally identified (100%) in the arteries of hypertensive nephrosclerosis samples, compared with the 86% prevalence of PENT in vessel walls. PENT, but not CML, was typically identified in the fibrotic interstitium. In all three of these sclerosing conditions, the levels of podocyte RAGE were indistinguishable from those of normal control samples (Figure 6D).

The differences between diabetic nephropathy and primary and secondary forms of FSGS and hypertensive nephrosclerosis were best observed when these categories were dichotomized into subgroups of \(\geq 1\) or greater immunostaining versus \(\leq 1\) or no immunostaining. As shown in Table 2, no patient with primary or secondary FSGS or hypertensive nephrosclerosis exhibited \(\geq 1\) or greater immunostaining for either CML or PENT in the mesangium or GBM of nonsclerotic glomeruli. In contrast, 18 and 31% of diabetic nephropathy biopsies exhibited this level of CML or PENT immunostaining in the GBM and mesangium, respectively, of nonsclerotic glomeruli.

In lupus nephritis, PENT and, to a lesser extent, CML were found in active glomerular necrotizing and inflammatory lesions, including cellular crescents (Figure 7, A to C). However, the uniform distribution of mesangial and GBM staining characteristic of diabetic nephropathy was not observed. The intensity of staining in these active lesions was particularly high, often exceeding that observed for scarred sclerotic glomeruli. Podocyte RAGE expression was focally upregulated in the glomeruli of patients with active lupus nephritis (World Health Organization class IV) (Figure 7D). As shown in Table 2, lupus nephritis was the only category of nondiabetic renal disease studied in which CML or PENT staining of \(\geq 2\) or more was identified in the mesangium or GBM of nonsclerotic glomeruli. This pattern of staining was most frequently observed in the distribution of active glomerular lesions with endocapillary hypercellularity, infiltrating neutrophils, and necrotizing features.

**Discussion**

This study was designed to investigate the distribution and specificity of AGE formation in human diabetic nephropathy and to correlate these findings with the disease severity and renal expression of RAGE. We have demonstrated that the extent of AGE formation in the glomerular and tubulointerstitial compartments correlates with the severity of diabetic nephropathy. In the glomeruli, this correlation was much stronger for CML than for PENT, suggesting that CML is an important
AGE in the pathogenesis of diabetic glomerulosclerosis. In contrast, PENT was the major AGE identified in the interstitium, with less consistent mesangial and extremely rare GBM involvement. These findings are consistent with the report by Horie et al. (19) of renal AGE formation in the distribution of glomerular mesangial and basement membrane collagens (IV, V, and VI), as well as interstitial collagen (predominantly III) and renal arterial collagens (III, IV, V, and VI). However, our findings differ from theirs (19,21) in that we found a much more significant correlation between the severity of diabetic nephropathy and glomerular immunostaining for CML, compared with PENT. These observations are of interest in light of the recent demonstration that CML adducts are the most physiologically relevant ligands for RAGE in the activation of cell signaling pathways that are operative in diabetic rodents with accelerated vascular disease (22). We attribute the distinctive distribution of CML in diabetic nephropathy to a disease effect, rather than a nonspecific aging effect, because our cases of hypertensive nephrosclerosis, which involved patients of comparable mean age, exhibited markedly different staining distributions in nonsclerotic glomeruli. Although some groups described tubular staining for AGE that suggested tubular reabsorption of filtered AGE (19,21), we attribute this weak tubular positivity to background staining, because similar tubular staining was observed in serial sections stained with preimmune serum.

An unexpected and intriguing finding was the normal distribution of RAGE at the base of the podocytes and its upregulation in diabetic nephropathy but not other sclerosing renal conditions. This podocytic distribution was confirmed by labeling of serial sections with an antibody to the podocyte-specific marker synaptopodin. The exclusively glomerular distribution of RAGE immunostaining and its upregulation in diabetic glomeruli was corroborated by the RT-PCR results in microdissected diabetic glomeruli. The failure to detect RAGE mRNA in normal control specimens may be explained by the long-lived nature of the protein in terminally differentiated, quiescent podocytes. Glomerular localization of RAGE was also described by Soulis et al. (23), although the precise cellular localization within glomeruli was not characterized. Although RAGE expression has been reported in vascular endothelial cells of large arteries (6,16), in mononuclear phagocytes (24), and in vascular smooth muscle cells (25), we did not identify RAGE expression, by immunohistochemical

Figure 6. Nondiabetic glomerulosclerosis. (A) Primary focal segmental glomerulosclerosis (FSGS). CML immunoreactivity is identified in an area of segmental glomerulosclerosis. Magnification, ×380. (B) Primary FSGS. The same glomerulus as in A also shows positive staining for PENT in the area of segmental sclerosis. Magnification, ×380. (C) Primary FSGS. There is no upregulation of podocyte RAGE in the same glomerulus. Magnification, ×380. (D) Hypertensive nephrosclerosis. CML staining is seen in globally sclerotic glomeruli, arterial walls, and the basement membranes of atrophic tubules. Magnification, ×380.
analysis, in the renal endothelium, mesangium, or vascular smooth muscle of normal control subjects or patients with diabetic nephropathy. The reason for these discrepancies may be related to the different antibodies and methods used, such as the use of anti-bovine versus anti-human RAGE, the use of frozen versus fixed tissues, and our introduction of microwave treatment for antigen retrieval.

The presence of RAGE on developing neurons of the central nervous system and the recent identification of RAGE as a receptor for amphoterin, which promotes the outgrowth of neuritic processes, support the hypothesis that RAGE is a receptor for ligands other than AGE under normal physiologic conditions (26). In fact, other members of the Ig superfamily have the capacity to bind to more than one biologically relevant ligand in vivo. Podocytes share many structural similarities with telencephalic dendrites, including a complex cytoarchitecture characterized by long cellular processes that are endowed with a highly organized actin cytoskeleton (27). Synaptopodin, a podocyte-specific renal protein that is involved in the organization of the actin cytoskeleton at the junction between the primary processes and the foot processes, is also expressed in telencephalic neurons of developing brain tissue (28). These striking structural similarities between neurons and podocytes raise the question of whether podocyte RAGE may act as a receptor for a currently unidentified renal ligand involved in process formation.

The fact that podocyte RAGE is upregulated in diabetic nephropathy suggests a potential role for the engagement of podocyte RAGE with AGE formed in the underlying GBM. AGE-RAGE interactions in other cellular systems have been reported to promote oxidative stress through modulation of cellular processes (14). Once activated, podocytes are capable of undergoing an oxidative respiratory burst, with release of reactive oxygen species into the GBM; this cellular mechanism has been shown to mediate proteinuria in Heymann nephritis (29). In diabetic nephropathy, it remains to be determined whether ligand-specific activation of podocyte RAGE could promote similar cellular activation, leading to oxidative injury and lipid peroxidation of GBM.

AGE have been reported to form in human tissues in the course of normal aging processes (18). Therefore, the abundant immunostaining of obsolescent glomeruli and arteriosclerotic vessels observed in hypertensive nephrosclerosis and other sclerosing renal conditions is not surprising. These findings
support a generalized role for AGE-mediated crosslinking of matrix proteins in the course of irreversible glomerular scarring and progressive arteriosclerosis of intraparenchymal renal vessels.

The generalized accumulation of AGE in obsolescent glomeruli cannot account for the marked deposition of AGE observed in the glomeruli of patients with active lupus nephritis. The formation of AGE in euglycemic inflammatory conditions through enzymatic oxidation of extracellular matrix proteins is a potential explanation for this observation. Lupus nephritis is characterized by neutrophil-mediated tissue injury, including release of reactive oxygen species and proteases. In addition to nonenzymatic mechanisms, CML formation is promoted by enzymatic catalysis by such neutrophil enzymes as myeloperoxidase (30). Activation of the myeloperoxidase-hydrogen peroxide-chloride system converts hydroxy-amino acids into glycoaldehyde, a precursor of CML (30). In lupus nephritis, the formation of AGE appears to be rapid, occurring in the acute phase of glomerular injury before glomerular scarring supervenes. Moreover, the upregulation of podocyte RAGE in this condition suggests a potential role for receptor-dependent cellular effects and correlates with the reported upregulation of RAGE in inflammatory vasculitides (31). The limitations of our study design do not allow us to address the mechanisms mediating this RAGE upregulation or its cellular consequences.

In summary, our findings indicate that CML constitutes a major AGE in renal basement membranes in diabetic nephropathy and is associated with upregulation of podocyte RAGE. The distinctive pattern of mesangial and GBM accumulation of CML observed in diabetic nephropathy differs from the more nonspecific AGE accumulation observed in progressive sclerosing renal conditions characterized by irreversible glomerular scarring and arteriosclerosis. The unexpected finding of AGE in active lupus nephritis suggests that AGE may also play a role in glomerular injury in acute inflammatory glomerulonephritis, probably through oxidative effects on glomerular matrix proteins. These observations in human renal disease provide the foundation for future design of functional studies to address differences in the mechanisms of AGE accumulation in these disorders and the cellular consequences of RAGE upregulation.

References


