

Differential expression of renal AGE-receptor genes in NOD mice: Possible role in nonobese diabetic renal disease

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Differential expression of renal AGE-receptor genes in NOD mice: Possible role in nonobese diabetic renal disease.

Background. Nonobese diabetic mice (NOD) are prone to glomerular pathology, which is accelerated with the onset of diabetes. Advanced glycation end product (AGE) interactions with AGE-receptors (AGE-Rs) in kidneys can contribute to glomerular injury and diabetic nephropathy (DN). The significant elevation in kidney AGE deposits noted in prediabetic NOD mice suggested that delayed AGE turnover in this model may contribute to its propensity toward DN.

Methods. To explore whether excess tissue AGE was linked to altered AGE-R status in the kidney, mRNA/protein expression, and of several AGE-Rs [AGE-R1, AGE-R2, AGE-R3, scavenger receptor II (ScR-II), and receptor for AGE (RAGE)], was determined in renal cortex and in mesangial cells (MCs) isolated from ND-, D-NOD, and ILE mice ($N = 20$ per group). Ligand binding, receptor site number, and affinity were determined in MCs from the same mouse groups.

Results. Prediabetic NOD kidney AGE-R1 mRNA and protein level were threefold lower than that of ILE mice ($P < 0.01$), while AGE-R3 mRNA was enhanced by twofold ($P < 0.05$) and AGE-R2, RAGE, and ScR-II mRNA remained close to normal (ILE). The onset of diabetes in NOD mice, while enhancing AGE-R1 mRNA expression by approximately twofold, failed to raise it above the normal (ILE) level, despite increases in tissue, and serum AGE. The latter was associated with higher elevation in AGE-R3 (sixfold, $P < 0.05$), RAGE (twofold, $P = \text{NS}$), and ScR-II mRNA (2.8-fold, $P = \text{NS}$) above control. MCs from prediabetic NOD mice showed a threefold lower level of AGE-R1 mRNA ($P < 0.02$ vs. ILE) and AGE-R1-protein, and AGE-binding activity (<40% of control ILE). In contrast, AGE-R3 mRNA was enhanced (twofold), while AGE-R2 showed no change. Cultured ND-NOD MCs displayed only one fourth of the AGE-binding sites/cell present on ILE MCs (1.6×10^6 vs. 6.6×10^6 , $P < 0.05$), which after the onset of diabetes rose to the normal range (7.0×10^6 /cell), but failed to exceed it.

Conclusions. Reduced AGE-R1 gene expression in this

strain may contribute to delayed AGE removal from and early AGE deposition in kidney tissues. This may act as a trigger for those AGE-R genes involved in growth-promoting changes, leading to DN in this strain.

A substantial subset of diabetic patients develops severe complications, regardless of glycemic control and disease duration [1, 2], emphasizing that genetic susceptibility is an important factor in the host response to the diabetic environment. Several genes have been implicated in the development of diabetic complications, including those related to hypertension [1], angiotensin-converting enzyme (ACE), or angiotensin II receptors [1, 2]. Although no major gene loci have been established as yet, it is presumed that such genes are principally linked to kidney disease and are inducible by diabetes.

Mounting evidence indicates that glucose-derived glycoxidation derivatives or advanced glycation end products (AGEs) and AGE receptors (AGE-R) play a major role in normal tissue homeostasis, aging, and the development of diabetic complications [3–8]. In the kidney, AGE-Rs of mesangial cells (MCs) mediate ingestion and degradation of AGEs [9] and regulate cytokine, growth factor, and extracellular matrix (ECM) synthesis [6, 10, 11], all major contributors to renal disease [12]. Presumably, impaired function of this system can occur at multiple points, causing delayed cellular AGE processing and/or enhanced AGE toxicity; however, no receptor-based defects have been reported. We have identified three MC-associated molecules that interact with AGEs: (1) AGE-R1, a 48 kD AGE-binding and endocytosis-mediating protein [13, 14], initially copurified with the oligosaccharyl-transferase (OST) complex; (2) AGE-R2, an 80 to 87 kD AGE-inducible tyrosine-phosphorylated protein kinase C substrate, copurified with AGE-R1 [13, 15–17]; (3) and AGE-R3 [18], a high-affinity AGE-binding peptide [18], also known as galectin-3, linked to cellular activation, anti-apoptotic events, and cellular anti-oxidant factors, such as bcl-2 [19, 20]. Human and murine

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cDNA sequences and chromosomal location of all three molecules are identified and found to be present on renal MCs and other cells (unpublished data) [13, 17, 19]. Other known AGE-binding molecules with diverse specificities not limited to AGEs and with no clear role in MC function include scavenger receptor II (ScR-II) and receptor for AGE (RAGE) [21–24]. Reactive AGE precursors include substances that have cell-activating, pro-oxidant and/or cross-linking properties, while their final elimination in animals and humans depends on normal renal function [25–27]. Thus, impaired AGE clearance, as in renal failure, can lead to significant retention of AGEs in blood and tissues and can promote tissue injury, notably within the glomerulus [10, 11].

Among the various animal models of spontaneous IDDM, over 85% of the female nonobese diabetic (D-NOD) mice develop an autoimmune type I diabetes (IDDM), appearing within the first three months of life and associated with evidence of early kidney lesions [28, 29]. In addition, nondiabetic NOD (ND-NOD) mice exhibit a propensity to develop mesangial ECM deposits and increased cellularity, which progresses to glomerulosclerosis after the onset of diabetes [29]. We have reported that NOD mice exhibit disproportionately high kidney tissue AGE levels, transforming growth factor- β 1 (TGF- β 1), and insulin-like growth factor-1 (IGF-1), together with evident glomerular hypertrophy at a young age, compared with other strains, for example, Swiss Jackson Laboratory (SJL) mice [30]. The accumulation of AGEs even in ND-NOD kidneys turned our attention to impaired tissue turnover of these substances, possibly AGE-R related. This could trigger renal cell dysfunction prior to the onset but be exacerbated by diabetes in this strain.

In this report, we focus on the expression and AGE-binding activity of several molecules known to interact with AGEs in renal cortical tissue, as well as in isolated NOD MCs prior to and after the development of IDDM. The findings revealed a selective decrease in AGE-R1 gene expression in the kidney associated with reduced AGE-R binding capacity despite the significant rise in other AGE-R components. These changes, when combined, may account in part for abnormal AGE processing and in part for growth effects that contribute to the glomerulopathy specific to NOD mice.

METHODS

Animals

Diabetic-NOD, ND-NOD (3 to 6 months old), and age-matched ILE (diabetes resistant, genetically related to NOD) mice ($N = 20$ per group) were obtained from Dr. Hattori (Joslin Diabetic Center, Boston, MA, USA). Age-matched SJL mice were from Jackson Laboratory (Bar Harbor, ME, USA). After sacrifice by decapitation,

blood was collected, and mice were perfused extensively through the aorta with sterile phosphate-buffered saline (PBS) containing RNase inhibitor (Boehringer Mannheim, Indianapolis, IN, USA). For total RNA membrane and total protein extraction, kidney cortices were snap frozen in liquid nitrogen immediately after removal or were gradually frozen in OCT compound (Miles Inc., Elkhart, IN, USA) for immunostaining [30, 31].

Mesangial cell culture

Glomerular MCs from each animal group were isolated and characterized, as described [9–11]. Two different cell preparations from each mouse group were cultured in complete medium [2/3 Dulbecco's modified Eagle's medium (DMEM):1/3 F12, supplemented with 1 mmol/L glutamine, penicillin at 100 U/mL, streptomycin at 100 μ g/mL, and 10% fetal bovine serum; GIBCO, Gaithersburg, MD, USA]. Cells were passaged weekly (for 8 to 10 passages).

Antibodies, ligands, and assays

Rabbit polyclonal antisera and the respective affinity-purified IgG raised against recombinant human AGE-R1 (rhAGE-R1) and AGE-R2 (rhAGE-R2) have previously exhibited excellent immunoreactivity against human or murine tissues and cells [13, 17, 32]. In the present mouse studies, anti-AGE-R2, although adequate for Western analysis, exhibited weak cross-reactivity against mouse kidney tissue. Rat monoclonal antibody (mAb) specific to murine AGE-R3 was purified from culture supernatants of hybridoma M3/38 (ATCC TIB166), using a protein G-sepharose column (Boehringer Mannheim Biochemicals) and used together with an isotypic rat IgG (Sigma Chemical Co., St. Louis, MO, USA) [18, 32]; anti-ScR-II and anti-RAGE reagents were not available for these studies.

Low-endotoxin bovine serum albumin (BSA; Sigma Chemical Co.) passed over an affi-Gel Blue column (Bio-Rad, Hercules, CA, USA), a heparin-Sepharose CL6B column (Pharmacia, Uppsala, Sweden), and an endotoxin-binding affinity column (Pierce, Rockford, IL, USA) to remove various contaminants were incubated with or without 0.5 mol/L D-glucose in 0.2 mol/L phosphate buffer (pH 7.4) at 37°C for eight weeks under sterile conditions. Low molecular weight reactants and free glucose were removed by dialysis against PBS. AGE levels, based on an AGE enzyme-linked immunosorbent assay (ELISA) [33] were AGE-BSA, 250 U/mg protein, and unmodified BSA, 0.9 U/mg protein. Aliquots of each preparation were iodinated using the Iodo-Beads (Pierce) and after trichloroacetic acid (TCA; 20%) precipitation, approximately 95% of 125 I was protein bound (specific activity of approximately 0.8 to 1.0 $\times 10^3$ /ng of protein) [32].

Serum and kidney tissue AGE determination

Kidney tissues were homogenized and digested as described [9, 26]. The supernatant of kidney homogenates and serum samples was used for AGE determination by an AGE-ELISA [33]. Urine creatinine (Stanbio Laboratory, San Antonio, TX, USA) and albumin levels were measured accordingly (by an anti-mouse antibody-based ELISA) [29, 30].

AGE receptor immunolocalization

Coronal 4 mm thick sections were cut from frozen kidney samples and fixed in Carnoy's fixative. The sections, after preincubation with goat serum for 10 minutes, were coated with rabbit anti-human AGE-R1 (50 to 100 $\mu\text{g}/\text{mL}$) and rat anti-human AGE-R3 IgG (10 to 20 $\mu\text{g}/\text{mL}$) [18] at room temperature (RT) for one hour followed by biotin-conjugated goat anti-rabbit or anti-rat antibody (20 to 50 $\mu\text{g}/\text{mL}$; Amersham Life Science Inc., Cleveland, OH, USA) and streptavidin-conjugated FITC (Zymed Laboratories, Inc., San Francisco, CA, USA). Anti-AGE-R2 IgG did not exhibit sufficient staining on renal tissue sections. The sections were examined and coded, and the fluorescence intensity was graded in a blinded fashion on a 0 to 4+ scale.

AGE binding to intact NOD mesangial cells

Confluent MCs from each group of mice were grown in six-well culture dishes and were incubated in binding medium consisting of complete DMEM and 1% BSA [9, 11, 31] for 30 minutes at 4°C. ^{125}I -AGE-BSA or ^{125}I -BSA was added at different concentrations in the presence or absence of 100-fold excess of unlabeled ligand (AGE-BSA, BSA) for four hours at 4°C or for one hour at RT. Cells were washed extensively with cold PBS and then lysed using 0.1 mol/L NaOH. The radioactivity and protein content (BCA-protein analysis; Pierce) in each sample were measured. Competitive inhibition assays were also performed by incubating cells with ^{125}I -AGE-BSA ($\sim 3 \times 10^6$ cpm/well) and increasing amounts of cold ligand. All experiments were carried out in triplicate, and data were evaluated by Scatchard analysis. In separate experiments, the effects of excess glucose (40 mmol/L), insulin (1 to 100 U/mL), and IGF-1 (1 to 10 $\mu\text{g}/\text{mL}$) on ligand binding were evaluated after incubation of NOD MC to these reagents for up to 72 hours.

Preparation of membrane fractions from mesangial cell and kidneys

Mesangial cell monolayers were harvested by gentle scraping and washing in PBS and were centrifuged at $500 \times g$ for five minutes and disrupted with a tight Dounce homogenizer in a solution of PBS containing 1 mmol/L ethylenediaminetetraacetic acid (EDTA) and a protease inhibitor cocktail of 1 mmol/L benzamide, 5

ng/mL pepstatin, 2 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 10 mg/mL aprotinin [11, 31]. Sections of kidney cortices were homogenized with a Dounce homogenizer in the presence of protease inhibitors, as described previously in this article, and washed with ice-cold PBS to eliminate blood contamination and nondissolved fragments. Nuclei and cell debris were removed after centrifugation at 1100 r.p.m. for 10 minutes. The crude membrane fraction was separated from the cytosolic fraction by centrifugation at 15,000 r.p.m. for 20 minutes at 4°C, and the resuspended pellet was centrifuged at 27,000 r.p.m. for one hour at 4°C. The pelleted partially purified membrane extract was resuspended in PBS, and the protein content was determined using the BCA protein assay method (Pierce).

Ligand blot and Western blot analyses

Ligand blotting was carried out as described previously [13]. Briefly, 10 to 20 μg cell or tissue membrane fractions in Laemli sample buffer containing 2% β -mercaptoethanol were heated at 95°C for five minutes and were electrophoresed [12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)]. Gels were transferred to nitrocellulose (NC) membranes (0.2 mm pore size) using a mini-gel transfer apparatus (Bio-Rad). The NC membranes were blocked with 2% BSA in PBS for one hour, washed twice with PBS containing 0.05% Tween-20, and probed with 4×10^6 cpm ^{125}I -AGE-BSA for one hour in the presence or absence of cold AGE-BSA ($\times 100$ excess). The blots were washed extensively with PBS/Tween-20, air dried, and exposed to XAR film (Kodak, Rochester, NY, USA) at -80°C . The radioactivity associated was quantitated by a phosphorimager (Packard, Downers Grove, IL, USA) and expressed as the percentage of cpm of specific protein species/total cpm of respective lane. This procedure was repeated at least three times using membrane extracts from different cell preparations.

For Western blotting, samples of membrane fractions were electrophoresed and transferred to NC membranes, as described previously in this article. The membranes were blocked with 2% dry milk in PBS/Tween-20 for one hour, washed twice, and incubated with the indicated amount of receptor primary antibody for one hour. The appropriate peroxidase-conjugated secondary antibody was added at a final dilution of 1:2000 for one hour. The bands were detected using the electrochemiluminescence method (ECL; Amersham). Equal sample loading onto membranes was checked using Amido black staining subsequent to Western or ligand blotting.

RNA isolation and reverse transcription-polymerase chain reaction

Total RNA was extracted from kidney cortex or cultured MCs using RNazol solution (TET-TEST Inc.,

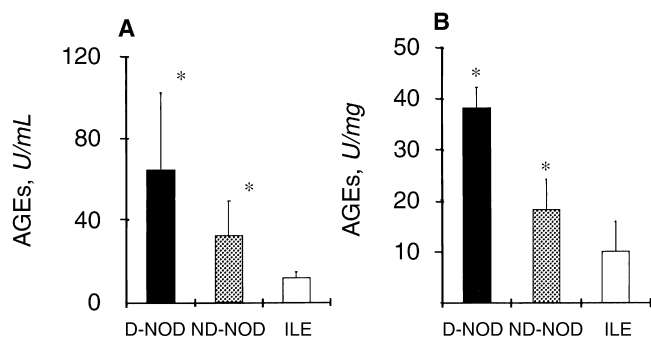


Fig. 1. Elevated advanced glycation end product (AGE) levels in serum (A) and kidneys (B) of prediabetic (ND-NOD) or newly diabetic (D-NOD) nonobese diabetic mice versus nondiabetic ILE. Serum and renal cortical sections from each strain of mice (age of 3 months, $N = 20$ per group) were processed for AGE determination by an AGE-specific ELISA [27, 35]. Data are expressed as the means \pm SD AGE U/mL of serum or U/mg tissue protein, with each sample tested in triplicate. * P values < 0.05 vs. ILE control.

Friendswood, TX, USA) for assessing AGE-R1, AGE-R2, AGE-R3, ScR-II, and RAGE mRNA expression. One microgram of total RNA was reverse transcribed to cDNA. PCR was performed as described [30, 31] using a Perkin Cetus kit. The following primers were used: AGE-R1: sense, GCT, CTT, CCA, CTC, CTT, ACT, CCT, and antisense, CCA, GAC, AGG, CAA, CTA, TGA, AC; AGE-R2: sense, TGG, TGT, GGT, GGC, TAT, TGA, CCT, TTG, C, and antisense, CGC, AGT, AGT, CGT, CGT, TCA, CCT, GAT, C; AGE-R3: sense, CAC, CTG, CAC, CTG, GAG, TCT, AC, and antisense, GCA, CTG, GTG, AGG, TCT, ATG, TC; ScR-II: sense, AGA, CCT, TCT, GCT, GTT, CCC, CT, and antisense, CCC, TGG, GAC, AGT, GTT, CTC, TGG, TT; and RAGE: sense, CCT, GGG, AAG, CCA, GAA, ATT, and antisense, ACA, CAG, GTC, AAG, GTC, ACA. The mutants were made by creating a deletion in the original PCR product [24], and competitive PCR was performed by adding a different amount of mutant to each cDNA amplification tube. After electrophoresis of PCR products, each band was quantitated by computer-assisted densitometric analysis. The ratio of unknown cDNA/mutant was measured for each molecule. A competitive PCR for β -actin (sense, GTG, GGC, CGC, TCT, AGG, CAC, CA; antisense, TGG, CCT, TAG, GGT, GCA, GGG, GG) was also performed for each sample, and the data were expressed as the ratio of AGE-R to β -actin mRNA level.

RESULTS

AGE levels in kidneys and sera from NOD mice

Prediabetic ND-NOD mice (3 to 4 months old) exhibited approximately twofold to threefold greater levels of AGE in serum ($P < 0.025$; Fig. 1A) and in kidneys ($P <$

0.005; Fig. 1B) compared with the genetically related, ILE (Fig. 1), or SJL controls (data not shown) [30]. Age-matched diabetic (~1 month of diabetes) NOD mice exhibited greater AGE levels than the ILE controls (serum: $>$ sixfold, $P < 0.005$, and kidney approximately fourfold, $P < 0.01$; Fig. 1). AGE levels in sera and kidney tissue continued to rise as a function of age in all groups (up to 6 months, data not shown).

AGE-receptor mRNA and protein expression in NOD kidney cortex membrane

To test the level of renal tissue expression of the different receptor genes, quantitative reverse transcription-PCR (RT-PCR) was used [30, 31]. ND-NOD renal tissue AGE-R1 mRNA level was approximately threefold lower than in ILE mice ($P < 0.01$; Fig. 2), as was AGE-R1 protein, by Western blotting (Fig. 3). In contrast, AGE-R2 and AGE-R3 mRNA (Fig. 2) and protein level (Fig. 3) were either unchanged or moderately elevated in both ND-NOD (-R2, $P = NS$, -R3, $P < 0.01$) and D-NOD (-R2, $P = NS$, -R3 $P < 0.005$) compared with ILE mice (Figs. 3 and 4). To test the relative expression level of other AGE-recognizing gene products in prediabetic renal cortex, mRNA expression of ScR-II and RAGE was evaluated by RT-PCR: Neither of them differed significantly from the control ILE tissue extracts; however, after the onset of hyperglycemia (within 4 weeks), a notable rise was observed in both (RAGE, 2-fold, $P = NS$; ScR-II, 2.5-fold, $P = NS$; Fig. 2 A, B), a pattern that resembled that of AGE-R3 expression.

AGE-receptor immunostaining of NOD kidney tissue sections

Based on the previously mentioned findings, we examined the relative immunoreactivity (IR) of AGE-R1 on renal cortex from NOD mice. Compared with kidney sections from ILE controls, which displayed a distinct glomerular AGE-R1 staining pattern (Fig. 4B), AGE-R1 IR was nearly undetectable in prediabetic NOD mice (Fig. 4C) but was comparable to normal after onset of diabetes (Fig. 4D). In contrast, AGE-R3 staining, which was positive in ND-NOD (Fig. 4G), was considerably more pronounced after diabetes (Fig. 4H); preimmune rabbit IgG or isotopic rat IgG control was negative (Fig. 4 A, E). Anti-R2 IgG did not exhibit sufficient sensitivity on tissue sections (data not shown).

AGE-receptor mRNA and protein levels in NOD mesangial cells

To assess whether the differences in receptor expression seen in whole renal tissue was also reflected on MCs, MCs from each strain were isolated [9, 30, 31] and evaluated with a focus on AGE-R1, AGE-R2, and AGE-R3 mRNA and protein expression by Western analyses. AGE-R1 mRNA levels were markedly attenuated in

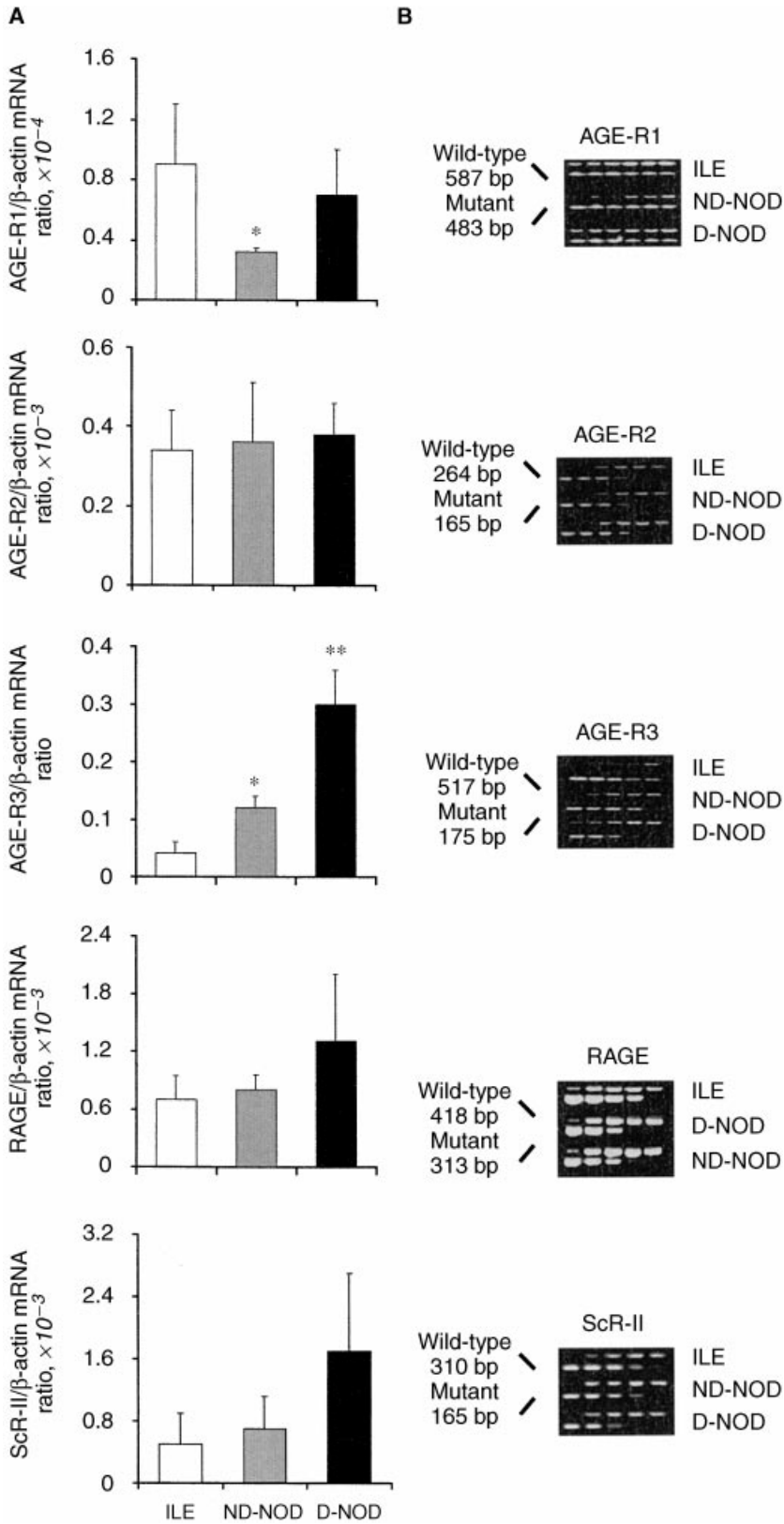


Fig. 2. Low AGE-R1 mRNA levels in prediabetic ND-NOD and D-NOD versus control ILE mouse kidney cortex. (A) Densitometric analysis of competitive RT-PCR data on AGE-R1, AGE-R2, AGE-R3, RAGE, and ScR-II expression from total RNA extracts of kidney cortex from NOD and ILE mice as per Figure 1 ($N = 8$ per group). (B) Representative competitive RT-PCR data on each molecule tested(A). Data are expressed as the mean ratio \pm SD of each component/mutant to β -actin/mutant mRNA. * $P < 0.01$, AGE-R1 in ND-NOD vs. ILE; AGE-R3 in ND-NOD vs. ILE, ** $P < 0.05$, AGE-R3 in D-NOD vs. ILE and ND-NOD.

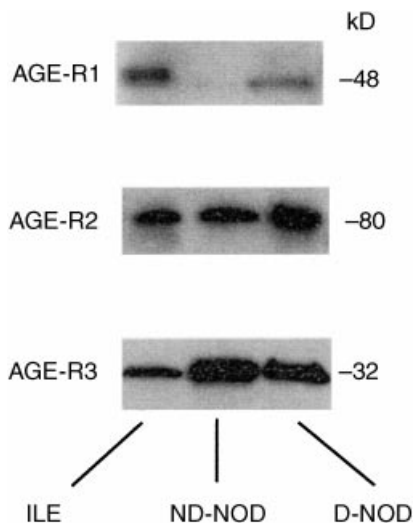


Fig. 3. AGE-R1 protein level is low in prediabetic ND-NOD compared with nondiabetic control ILE mouse kidney cortex. AGE-R1, AGE-R2, and AGE-R3 levels were determined by Western blot analysis on cortical membrane extracts from ND-NOD, D-NOD, and ILE mice ($N = 5$ per group). Samples were separated on 12% polyacrylamide gels, transferred, immunoblotted with the respective antibody, and then visualized by ECL (anti-AGE-R1, 50 $\mu\text{g}/\text{mL}$, anti-AGE-R2, 50 $\mu\text{g}/\text{mL}$, AGE-R3, 10 $\mu\text{g}/\text{mL}$). Molecular weights are indicated on the right side of the panel. The protein concentration used was 20 $\mu\text{g}/\text{lane}$. Data are representative of five identical experiments.

ND-NOD MCs compared with ILE and D-NOD MCs (Fig. 5A), while AGE-R2 and AGE-R3 mRNA were enhanced (Fig. 5A). Consistent with the mRNA pattern, AGE-R1 protein was nearly undetectable in ND-NOD MC membrane fractions, in contrast to AGE-R2 and AGE-R3, which were either unchanged or enhanced in relationship to control (Fig. 5B).

AGE-receptor sites and binding affinity on NOD mesangial cells

To link the variable AGE-R gene expression to the AGE-specific ligand binding activity exerted by all AGE-R components present on MCs from NOD mice, radioligand-binding assays were performed on MCs isolated from each NOD strain. AGE-binding activity on MCs from prediabetic ND-NOD mice was markedly suppressed compared with D-NOD or ILE (by $>60\%$, $P < 0.05$ in either comparison), consistent with an estimated 1.6×10^6 binding sites per cell from ND-NOD versus 6.6×10^6 and 7.0×10^6 sites per cell from ILE and D-NOD mice ($P < 0.002$ and $P < 0.005$, respectively; Fig. 6B), while the binding affinity did not vary significantly (K_a , 160 to 300 nmol/L in all three strains; Fig. 6B) from that reported previously for mouse strains [9, 11]. Ligand blot analysis on membrane extracts from the same cell preparations demonstrated low AGE-binding activity on ND-NOD MCs, compared with ILE, associated with a single approximately 48 to 50 kD protein species (Fig.

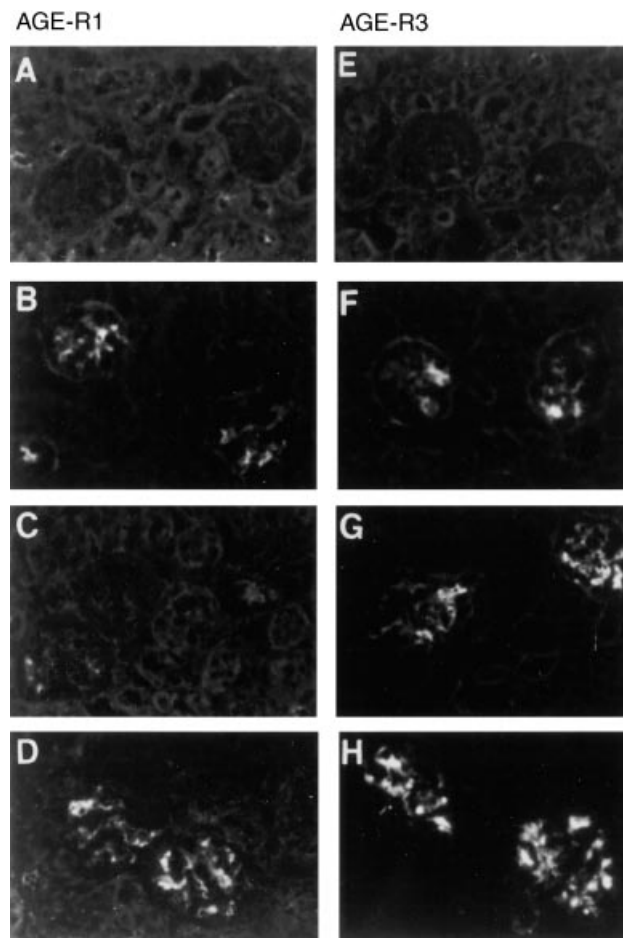


Fig. 4. AGE-R1 immunofluorescence is reduced in prediabetic ND-NOD versus nondiabetic ILE mouse kidney cortex. Frozen cortical kidney sections from ND-NOD, D-NOD, and ILE mice ($N = 20$ per group) were incubated with rabbit anti-human recombinant AGE-R1 IgG (50 $\mu\text{g}/\text{mL}$) or rat anti-mouse AGE-R3 monoclonal antibody (10 $\mu\text{g}/\text{mL}$) followed by goat anti-rabbit or anti-rat IgG biotin-conjugated antibodies and streptavidine-conjugated FITC. (A–D) AGE-R1. (E–H) AGE-R3. (A and E) Nonimmune IgG. (B and F) ILE mice. (C and G) ND-NOD mice. (D and H) D-NOD mice. Magnification $\times 200$.

6C), presumably corresponding to AGE-R1 (as binding was blocked by anti-AGE-R1 IgG); AGE binding by D-NOD MC membrane was comparable to and not exceeding that of control ILE (Fig. 6C). Additional experiments were performed on cultured NOD MCs and were incubated with excess concentrations of D-glucose, insulin, or IGF-1 for varying time periods. No effects were observed on subsequent AGE ligand-binding studies (data not shown).

DISCUSSION

The previously mentioned studies present the first evidence, to our knowledge, of altered *in vivo* expression and binding activity of kidney-specific AGE-R in connection with premature AGE deposition in NOD mice,

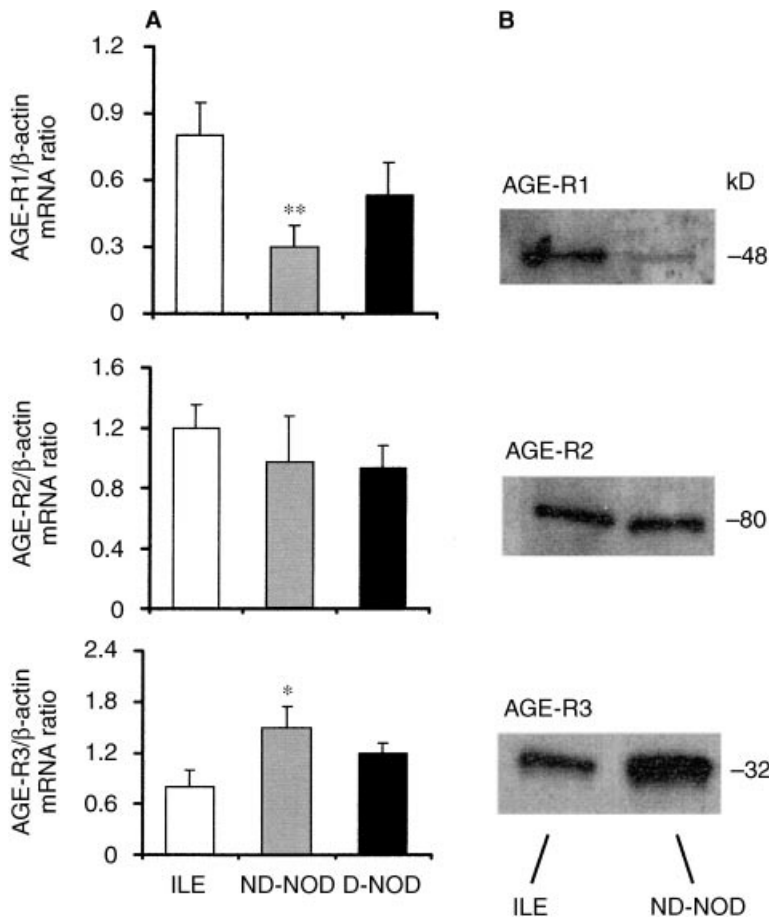


Fig. 5. AGE-R1 mRNA and membrane protein levels are low in MCs isolated from prediabetic ND-NOD mice versus MCs from nondiabetic ILE MCs. (A) RT-PCR: Total RNA was extracted from mouse MCs ($N = 5$ mice/group) and analysis was performed as in Figure 2. Densitometric data of five identical tests performed on five mice/group are shown ($*P < 0.01$, ND-NOD AGE-R1 vs. ILE, $*P < 0.05$ ND-NOD AGE-R3 vs. ILE). (B) Western blot analysis was performed on ND-NOD and ILE mouse MC membrane, as in Figure 3. After transfer, 10 μ g of MC membrane protein were incubated with the respective anti-AGE-R IgG and visualized by ECL. Data are representative of five independent experiments.

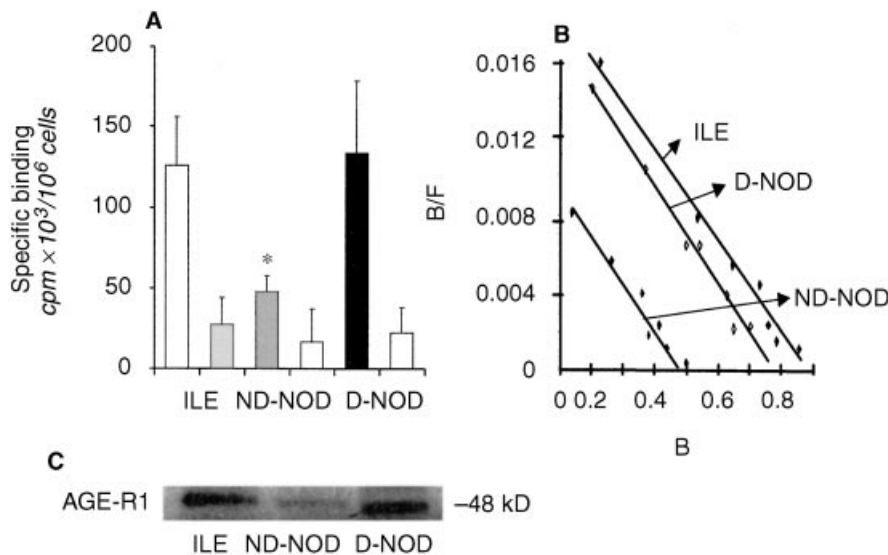


Fig. 6. Mesangial cells (MCs) from prediabetic ND-NOD mice exhibit low number of AGE-specific binding sites compared to nondiabetic ILE MC. (A) Specific 125 I-AGE-BSA-binding: Cultured MCs from age-matched ND-NOD, D-NOD, and ILE mice ($N = 3$ mice per group) were incubated with 125 I-AGE-BSA alone (25 μ g/mL) plus a 100-fold excess amount of cold AGE-BSA for four hours at 4°C or 125 I-BSA (25 μ g/mL; hatched bars) or 125 I-AGE-BSA. Data are expressed as the means \pm SD of specific binding (total minus - nonspecific) cpm/ 10^6 cells from three independent measurements, each done in triplicate wells. (B) Scatchard analysis of specific binding data from each strain are indicated by arrows. (C) Ligand blot: 125 I-AGE-BSA binding by membrane preparation of MC used in studies shown (A and B) and revealed by autoradiography. Note the marked reduction of ligand binding by a 48 kD species on ND-NOD membrane.

a model of insulin-dependent diabetes (T1D) that is also prone to nephropathy [28–30]. Since abnormal AGE deposits appear prior to the development of hyperglycemia, a delay in the physiologic turnover of tissue AGEs

emerged as a possible mechanistic explanation for the early renal lesions in this model. After the onset of diabetes, impeded AGE removal could act together with hyperglycemia-mediated mechanisms to precipitate dia-

betic nephropathy (DN). The findings support a possible association between impaired AGE processing, differential expression of AGE-R genes, and susceptibility to nephropathy in this model [30]. Since a significant subpopulation (>30%) of diabetic humans are prone to severe DN [1, 2], the findings may carry certain implications for the human diabetic renal disease, even though no animal model develops lesions identical to those of human diabetic kidneys.

A two-step process is considered to lead to the elimination of AGE-modified macromolecules in the absence of hyperglycemia: (1) a peripheral or systemic step, by which tissue AGEs once formed are degraded locally via receptor-dependent [26] or nonspecific proteolytic mechanisms; and (2) an excretory step, involving the clearance of AGE degradation products by the urinary system [25, 27]. Receptor-dependent AGE binding and degradation has been found enhanced in response to excess AGE *in vitro* [24] or in insulin-deficient diabetic animal models [34], a self-regulatory property that is key to tissue AGE homeostasis [35]. The animals used in this study were of an age not associated with renal dysfunction, pointing to an alteration in the systemic phase, possibly at the AGE-R level.

Among the cellular components of the kidney, MCs display AGE-Rs that are active in the uptake, processing, and degradation of AGEs [9, 10, 13]. Because of the abundance of MCs in the glomerulus, these cells could be important in the processing of glomerular AGEs [3, 8]. While some receptors serve in the removal of AGEs, others, such as RAGE [23, 24], ScR-II [21, 22], and AGE-R3 [18–20], have been implicated in the regulation of cell growth or cell–matrix interactions, regardless of etiology [12]. Extracellular and intracellular AGEs are known enhancers of oxidative stress [23, 36] and cell activation, including the up-regulation of several AGE-receptor (AGE-R) genes, for example, AGE-R2, AGE-R3, and RAGE [23, 24, 32, 35], which can then trigger inflammatory events [4, 7, 8, 24]. It is thus evident that a loss or negative balance in AGE-removing receptors could lead to excessive AGE, which could in turn act as a stimulus for the proinflammatory type of AGE-Rs. The actual players in the unexplained abundance of tissue AGE in the NOD mice are not identified, but could include a defective AGE-removal pathway.

Of the five AGE-related receptor molecules tested in NOD renal tissue, AGE-R1 was the only component found to exhibit a consistent pattern of underexpression, compared with the wild type, at the transcriptional and translational levels, as well as in AGE-binding activity. This was most evident at the prediabetic stage, and even after four months of hyperglycemia, it never exceeded the nondiabetic level of the wild-type control. The NOD mouse is thus far the only strain in which the expression and activity of an AGE-binding molecule are signifi-

cantly below the level of the corresponding wild-type mouse, a trend directionally countered to the distinct rise in tissue AGE and independent of hyperglycemia. AGE-R1, a 48 kD AGE-binding molecule [13, 14], exhibits distinct extracellular, transmembrane, and cytoplasmic domains, a structure that is typically thought to facilitate ligand binding and endocytosis. The protein is found on both cell surface and intracellular RE organelle membranes; its reported physical association to oligosaccharide transferases is suggestive of a role in their stability and possibly in intracellular AGE detoxification.

To confirm that AGE-R1 underexpression occurred in cells native to the glomerulus, native MCs from these mice were tested in culture. AGE-R1 expression and AGE ligand binding were both suppressed in MCs from prediabetic NOD mice. Here again, AGE-binding capacity of MC taken after the onset of diabetes failed to rise above the nondiabetic ILE. Indeed, whether using complex kidney tissue or a single cell type (MC), a consistent pattern of underexpression and/or low responsiveness to AGE and/or diabetes were found limited to one receptor component, AGE-R1. In addition, this was linked to reduced AGE-R sites on prediabetic cell surface, despite the rise in expression levels of AGE-R3 and other receptor molecules, suggesting that AGE-R1-mediated AGE turnover is important to normal homeostasis. Impaired AGE-R1 gene function may be naturally shared by other renal or extrarenal cells involved in the disposal of altered macromolecules, namely macrophages [3, 4, 35]. The latter was indeed indicated in studies of NOD mouse peritoneal macrophages (unpublished data). These findings stand in contrast to the reported enhanced AGE binding and degradation in different diabetic models, for example, alloxan-induced C57BL6 or db/db KsJ mice [34], further pointing to a NOD-specific receptor defect. Moreover, since all isolates of MCs were cultured under identical conditions, the sustained phenotype of subnormal AGE-R1 expression and activity during culture suggest a genetic regulatory or mutational defect unrelated to the diabetic environment. Whether such a defect is primary or secondary is not clear at this point.

Young NOD kidneys overexpress growth factors (TGF- β 1) and ECM components [30, 31]. Although of unclear mechanism, this finding could be in part attributed to premature kidney AGE accumulation, inducing those AGE-R known to trigger activation-dependent events, namely AGE-R3, RAGE, or ScR-II. Among these, AGE-R3 induction was the most prominent, even in prediabetic NOD. AGE-R3 or galectin-3 has been known to promote growth and anti-apoptotic events [18–20, 22, 32]. Induction of this gene by high tissue AGE levels leads to cell-surface AGE-R3 overexpression and enhanced ligand-binding activity [18]. However, the process of endocytosis and degradation of AGEs may be

dictated by a combination of factors. For instance, a threshold of AGE-R1 synthesis and dislocation to the cell surface may be required, below which AGE-R3 cannot form thiol ester-dependent AGE-binding complexes for high-efficiency ligand internalization [18]. Indeed, the AGE-binding capacity of diabetic MCs was clearly subnormal despite the pronounced increase in AGE-R3 expression.

Overexpression of growth factors (TGF- β 1) and ECM components in diabetic NOD mouse glomeruli [30] could also be contributed to by the signal-promoting RAGE [23, 24], as well as ScR-II [21, 22], based on the enhanced expression of these genes after the onset of diabetes. Although these molecules are implicated in several chronic inflammatory states, including diabetic and non-diabetic vascular diseases, their specific roles in renal or MC function are not yet defined. Indeed, no inference can be made about their role in AGE retention or in lesion development in diabetic NOD [7]. Similarly, no clear association can be drawn here between excessive AGE and AGE-R2 expression. One explanation for this finding might be that the AGE-R2 gene is not involved in events specific to the kidney. Alternatively, it may be that AGE-R2 modulation is dependent on a minimum of AGE-R1 expression not achieved in the NOD model. The latter is supported by studies on normal human endothelial cells in which AGE-driven upregulation of R1 was linked to AGE-R2 increases and to enhanced phosphorylation of this tyrosine kinase [17]. The specific function of this molecule is still undetermined and needs further study.

Other diabetes-related metabolites are also likely to influence receptor expression in these mice, although attempted fluctuations in glucose, insulin, or IGF-1 were found ineffective in cultured NOD MCs (data not shown).

In summary, we present evidence of differential regulation of AGE-R genes and their products against a background of elevated AGE in blood and kidneys in a diabetic animal model susceptible to glomerulosclerosis. Whether of genetic or epigenetic etiology, ineffective receptor-dependent AGE removal could prime renal tissues for injury following long-term hyperglycemia in this model.

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APPENDIX

Abbreviations used in this article are: ACE, angiotensin converting enzyme; AGE, advanced glycation end product; AGE-R, advanced glycation end product-receptor; BSA, bovine serum albumin; D-NOD, nonobese diabetic mice; ECM, extracellular matrix; IDDM, autoimmune type 1 diabetes; MC, mesangial cell; ND, nondiabetic; NOD, nonobese diabetic mice; OST, oligosaccharyl-transferase; PBS, phosphate-buffered saline; PMSF, phenylmethylsulphonyl fluoride; RAGE, receptor for advanced glycation end product; RT, room temperature; ScR-II, scavenger receptor (class II).

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