ABSTRACT

In the kidney, the receptor for advanced glycation end products (RAGE) is principally expressed in the podocyte at low levels, but is upregulated in both human and mouse glomerular diseases. Because podocyte injury is central to proteinuric states, such as the nephrotic syndrome, the murine adriamycin nephrosis model was used to explore the role of RAGE in podocyte damage. In this model, administration of the anthracycline antibiotic adriamycin provokes severe podocyte stress and glomerulosclerosis. In contrast to wild-type animals, adriamycin-treated RAGE-null mice were significantly protected from effacement of the podocyte foot processes, albuminuria, and glomerulosclerosis. Administration of adriamycin induced rapid generation of RAGE ligands, and treatment with soluble RAGE protected against podocyte injury and glomerulosclerosis. In vitro, incubation of RAGE-expressing murine podocytes with adriamycin stimulated AGE formation, and treatment with RAGE ligands rapidly activated nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase, via p44/p42 MAP kinase signaling, and upregulated pro-fibrotic growth factors. These data suggest that RAGE may contribute to the pathogenesis of podocyte injury in sclerosing glomerulopathies such as focal segmental glomerulosclerosis.


RESULTS

RAGE Null Mice Are Protected From ADR-induced Podocyte Injury and Glomerulosclerosis

We performed laser-capture microdissection of mouse glomeruli to monitor RAGE transcript levels in response to ADR. At 72 h, 7 d, and 14 d, compared with normal saline (NS), ADR resulted in in-
Figure 1. Homozygous RAGE null mice display decreased podocyte stress, albuminuria, and glomerulosclerosis consequent to ADR. (a-d) ADR upregulates RAGE. (a) ADR, 10.5 mg/kg, was administered to male BALB/cJ mice. Mice were treated with sRAGE or vehicle, PBS, immediately after ADR and continued once daily until death. Laser capture microdissection of glomeruli was performed, RNA prepared, and quantitative real-time PCR performed for detection of murine RAGE transcripts and reported as fold change versus sham.
creases in RAGE transcripts of approximately 2.1-, 1.7-, and 1.7-fold, respectively (P < 0.001; Figure 1a). On day 3, by confocal microscopy, RAGE was expressed in the podocyte, as illustrated by colocalization with synaptopodin epitopes (Figure 1, b through d).

To address the role of RAGE in ADR injury using homozygous RAGE null mice, we first genotyped three informative microsatellite markers (D16Mit165, D16Mit34, D16Mit156) spanning the DOXNPH susceptibility locus on chromosome 16.11,12 We confirmed that RAGE null mice, backcrossed more than 12 generations into BALB/cJ, were homozygous for BALB/cJ susceptibility alleles at the DOXNPH locus.

Upon administration of ADR, RAGE null mice displayed significantly less excretion of urinary albumin/creatinine versus wild-type mice at 2 wk, 276.5 ± 164.6 and 1228.3 ± 440.9 μg/mg, and at 6 wk, 170.0 ± 119.3 and 1111.9 ± 405.7 μg/mg, respectively (P < 0.01; Figure 1e). We were unable to detect any albumin in the urine of RAGE null mice at baseline, suggesting that the RAGE null mouse did not display a phenotype at baseline, and that it was partially protected from ADR.

Based on the significant reduction in albuminuria observed in RAGE null mice after ADR, we examined pathology and glomerular gene expression. Two weeks after ADR, marked effacement of podocyte foot processes was observed in wild-type but not in RAGE null mice (Figure 1, f through h). RAGE null mice displayed significantly less glomerulosclerosis and cast formation versus wild-type mice (Figure 1, i and k versus Figure 1, j and l). Mean mesangial matrix/total glomerular area and numbers of sclerotic/total glomeruli and tubular casts area were significantly higher in wild-type versus RAGE null mice (P < 0.01; Figure 1, m through o). Pre-ADR, RAGE null mice displayed no detectable sclerotic glomeruli or tubular casts, indicating that the genetic deletion of RAGE resulted in partial protection from ADR.

ADR induces glomerulosclerosis; thus, we performed quantitative real-time PCR on laser capture-microdissected glomeruli to detect TGF-β1 and connective tissue growth factor (CTGF) transcripts. Both transcripts were significantly higher in wild-type mice 3 d post-ADR versus NS (~2.1-fold and ~2.9-fold, respectively; P < 0.004; Figure 1, p and q) and were significantly lower in RAGE null mice glomeruli (P < 0.01).

**ADR Generates RAGE Ligands**
To establish RAGE-dependent mechanisms in ADR injury, we tested whether ADR bound RAGE. Anti-ADR IgG-immobilized AffiGel 15 was incubated with ADR followed by sRAGE; Figure 2b reveals that ADR was eluted, but not RAGE (RAGE was assessed by Western blot; Figure 2d, lane 1). When AffiGel 15 beads were coated with anti-RAGE IgG and incubated with sRAGE followed by ADR, ADR was not detected in the eluate (Figure 2c), but eluate contained sRAGE (Western blot; Figure 2d, lane 3). Figure 2a is a typical ADR elution profile, and in Figure 2d, lane 2 is standard sRAGE control detected by anti-RAGE IgG.

Because ADR did not bind RAGE, we then hypothesized that ADR generated RAGE ligands. On day 3, approximately 1.8-fold higher AGE epitopes were identified in the cortex of wild-type mice versus NS and were significantly lower in RAGE null cortex (P < 0.05). The primary site of AGE after ADR was the glomerulus, and especially the podocyte, based on colocalization with anti-synaptopodin IgG (Figure 2, f through h).

Previous work linked ADR to reactive oxygen species (ROS) generation as a mediator of injury, but did not identify the specific mechanisms by which ROS were generated.13,14 Compared with wild-type mice receiving NS, mice receiving ADR displayed an approximately 3.1-fold increase in renal cortex nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase activity on day 3 (P < 0.000001); this increase was significantly less in RAGE null mice (P < 0.05; Figure 2i). Malondialdehyde, a lipid peroxidation product, was significantly higher in the cortex of wild-type mice treated with ADR versus RAGE null mice at 3 and 7 d (P < 0.01; Figure 2j).

Additional sources of ROS were assessed; compared with NS, animals treated with ADR revealed approximately 2.5-fold increase in iNOS glomerular transcripts on day 3, whereas no significant increase in iNOS mRNA was observed in RAGE null mice (Figure 2k). Levels of total nitrite and nitrate and nitrotyrosine epitopes were significantly higher in wild-type versus RAGE null mice cortex (Figure 2, n and o).
Figure 2. ADR generates RAGE ligands. (a-d) ADR does not bind RAGE. In panel b, anti-ADR IgG-immobilized AffiGel 15 was incubated first with ADR followed by sRAGE, ADR was eluted, but not RAGE (Western blot) (d, lane 1). When AffiGel 15 beads were coated with anti-RAGE IgG and incubated with sRAGE followed by ADR, ADR was not detected in the eluate (c), but eluate contained sRAGE (Western blot) (d, lane 3). In panel d, lane 2 is standard sRAGE control detected by anti-RAGE IgG. Panel a is a typical ADR elution profile. (e-p) ADR induces generation of AGE and ROS. (e-h) ADR generates AGEs. On day 3 post-ADR, kidney cortices were retrieved and ELISA for detection of AGE epitopes performed (e). In f through h, confocal microscopy using anti-AGE (f) or anti-synaptopodin IgG (g) was performed and localized AGE epitopes to the podocyte as indicated by merged studies in panel h (original magnification ×200). (i-p) Wild-type and RAGE null mice were injected with ADR. (i) NADPH oxidase activity at 3 d post-ADR was determined in kidney cortex. (j) Renal cortex malondialdehyde levels were determined on days 3 and 7 after ADR. (k-l) Three days post-ADR, glomeruli were laser capture microdissected and real-time PCR was performed to determine iNOS transcripts (fold change...
Administration of Soluble RAGE Suppresses ADR-mediated Injury in BALB/cJ Mice

As ADR generated RAGE ligands in vivo, it was logical to test the effect of the ligand decoy. Soluble RAGE (sRAGE), administered intraperitoneally immediately after ADR, was continued daily until death. Administration of sRAGE blocked upregulation of RAGE transcripts in glomeruli versus ADR/ phosphate-buffered saline (PBS) at 72 h, 7 and 14 d (P < 0.01; Figure 1a).

Compared with ADR/PBS-treated mice, where significant podocyte foot process effacement was noted (Figure 3, a, c, and e), sRAGE-treated animals significantly decreased effacement (Figure 3, b, d, f, and g) at 2 and 6 wk (P < 0.01; Figure 2r), in part in podocytes, based on colocalization with anti-synaptopodin IgG (Figure 2, q through s).

Effects of ADR and RAGE Ligands on Murine Podocytes: AGE Generation and Upregulation of Growth Factors

Our findings suggested that ADR stimulated RAGE ligand generation in podocytes in vivo. To further probe this concept, we incubated cultured murine podocytes with ADR and found increased production of AGEs (Figure 5a). These findings led us to test the role of RAGE on podocyte stress; we used RAGE ligand S-100b, as S-100/calgranulin was increased by ADR in vivo.2 Compared with sham, incubation of podocytes with S-100b (10 μg/ml) resulted in significantly increased transcripts for TGF-β1 (P < 0.05; Figure 5b). Pretreatment with anti-RAGE IgG, but not nonimmune IgG, significantly attenuated S-100b-stimulated TGF-β1 transcripts (P < 0.0002; Figure 5c).

To probe the requirement for RAGE-mediated signal transduction, we prepared adenoviral vectors expressing dominant negative (DN) RAGE, cytoplasmic domain-deleted RAGE.1–3 Whereas S-100b/Ad vector revealed significant increases in TGF-β1 transcripts versus sham, a marked suppression of S-100b-stimulated upregulation of TGF-β1 transcripts was noted in the presence of Ad DN RAGE (Figure 5d). Incubation of podocytes with S-100b resulted in a significant approximately 1.9-, 2.4-, 1.8-, and 1.8-fold increase in CTGF transcripts at 2, 4, 6, and 8 h, respectively, versus sham (P < 0.05; Figure 5e).

Administration of sRAGE Suppresses Oxidative Stress

AGE epitopes were higher in ADR- versus NS-treated mice, and mice receiving ADR and sRAGE demonstrated significantly less AGEs in the cortex (P < 0.05; Figure 4a). On days 3 and 7, administration of ADR/PBS resulted in a significant increase in NADPH oxidase activity in kidney cortex versus NS/PBS (~2.8-fold and ~2.7-fold, respectively; P < 0.01; Figure 4b), which was significantly decreased by sRAGE (P < 0.01; Figure 4b). Levels of malondialdehyde in cortex of sRAGE-treated mice were significantly lower at 6 and 72 h after ADR (P < 0.01; Figure 4c). Compared with ADR/PBS-treated mice, glomerular iNOS transcripts in sRAGE-treated mice were lower at 24 h, 3 d, and 7 d by approximately 1.4-, 2.1-, and 2.4-fold versus PBS, respectively (P < 0.01; Figure 4d). Levels of total nitrite and nitrate were significantly higher in ADR/ PBS-treated mice versus sRAGE-treated animals at 3 h and 3 d post-ADR (P < 0.05; Figure 4e). Three days after ADR, sRAGE-treated mice displayed significantly lower nitrotyrosine epitopes in cortex (P < 0.01; Figure 4f).

Compared with wild-type non-ADR controls (k). In l, total nitrite and nitrate levels were determined in kidney cortex. In m-p Nitrotyrosine epitopes in kidney cortex were studied by ELISA on days 3 and 7 after ADR; in panels n through p, confocal microscopy using either anti-nitrotyrosine (n) or anti-synaptopodin IgG (o) was performed and localized sites of nitrotyrosine epitopes to the podocyte as indicated by merged studies in (p) (original magnification ×200). In e, i, j, k, l, and m, n = at least 7 mice/group. (q-v) ADR stimulates S-100/calgranulin generation. Confocal microscopy. Sections from NS (Con) or ADR-treated mice on day 3 were subjected to confocal microscopy for detection of S-100 (r, u) and synaptopodin (q, t) epitopes. Merged images are illustrated in panels s and v. Scale bar (r-w) = 10 μm.
Figure 3. Administration of soluble RAGE suppresses ADR-mediated podocyte stress, albuminuria, and glomerulosclerosis. Wild-type mice were treated with ADR and sRAGE, 100 μg/d, or PBS. (a–g) Effect of ADR on foot process effacement and podocyte loss; 2 and 6 wk post-ADR, mice were killed and electron microscopy performed on kidney sections. Quantification of foot process effacement is shown in panel g. (h) Effect of ADR on albuminuria; 24-h urine was collected from wild-type mice treated with sRAGE or PBS at 2 and 6 wk after ADR; urinary albumin/creatinine ratio is shown. n = at least 10 mice/group. (i–l) Impact of ADR on glomerulosclerosis: effect of sRAGE. At 6 wk after ADR, kidneys were retrieved and subjected to PAS staining (i–j). Quantification was performed to establish mesangial/total glomerular area (k) and sclerotic/total glomeruli (l). n = at least 10 mice/group. Scale bar: i–j 50 μm. (m–p) Real-time PCR for detection of TGF-β1 and CTGF transcripts. On days 3 and 7 after ADR, mice were killed, and laser capture microdissection of glomeruli was performed; RNA was prepared and real-time PCR performed for detection of transcripts for TGF-β1 (m, o) and CTGF (n, p). Results are reported as fold change compared with wild-type sham-treated controls. n = at least 10 mice/group.
Figure 4. Administration of soluble RAGE suppresses AGE generation and ROS in ADR-treated mice. Wild-type mice were treated with ADR and sRAGE or PBS. (a) ADR generates AGEs: effect of sRAGE. On day 3 post-ADR, kidney cortices were retrieved, and ELISA for detection of AGE epitopes was performed. n = at least 7 mice/group. (b-f) ADR generates oxidative stress: effect of sRAGE. At the indicated times after ADR, kidneys were retrieved, and cortices were prepared for examination of NADPH oxidase activity (b), malondialdehyde epitopes (c), iNOS (laser capture-microdissected glomeruli), and total nitrate and nitrite (d-e), and nitrotyrosine ELISA (f). n = at least 7 mice/group. *P < 0.05, **P < 0.01.

Figure 5e). Pretreatment of the podocytes with anti-RAGE IgG significantly attenuated CTGF transcripts by S-100b (P < 0.0002; Figure 5f), as did introduction of Ad DN RAGE (P < 0.01; Figure 5g).

Effects of RAGE Ligands on Murine Podocytes: Activation of NADPH Oxidase via p44/42 MAP Kinase Signaling

Our in vivo findings implicated RAGE and generation of ROS in ADR nephropathy; thus, we tested the impact of RAGE ligands on regulation of NADPH oxidase in cultured podocytes. Incubation with S-100b rapidly activated NADPH oxidase; at 5 and 15 min, approximately 2.1-fold and approximately 1.7-fold increases in activity were noted versus sham, respectively (P < 0.05; Figure 6a). The effects of S-100b were dose- and RAGE-dependent (Figure 6b), as reduction was observed with anti-RAGE IgG (Figure 6c) and Ad DN RAGE (P < 0.01; Figure 6d).

Activation of p44/p42 and p38 MAP kinases is suggested to play key roles in ADR-mediated nephropathy. Incubation of
cultured podocytes with S-100b enhanced phosphorylation of p44/p42 MAP kinase at 5 and 15 min (~11.0-fold and ~15.0-fold versus sham; \( P < 0.01 \); Figure 6e), in a manner blocked by anti-RAGE IgG \( (P < 0.01); \) Figure 6f). Incubation with S-100b resulted in significant, time-dependent increases in p38 MAP kinase phosphorylation with peak at 15 min (~5.8-fold; \( P < 0.05 \); Figure 6g).

To address by what mechanism RAGE ligands activated
NADPH oxidase, podocytes were treated with inhibitors of either p44/42 MAP kinase or p38 MAP kinase, and then stimulated with S-100b. These experiments identified roles for p44/p42 MAP kinase, as PD98059 resulted in nearly complete suppression of activation of NADPH oxidase by S-100b ($P < 0.01$), whereas SB203580 had no effect (Figure 6h).

**DISCUSSION**

Ligand-RAGE interaction promotes generation of ROS, at least in part via NADPH oxidase, here shown for the first time in the podocyte. As ROS may trigger AGE generation, we propose that ADR initiates events, which, via
RAGE, contribute to podocyte stress. Our studies do not exclude that ADR-RAGE directly initiates the burst of ROS. Immediate roles for RAGE are less likely, however, as ADR is not a RAGE ligand. Furthermore, although oxidative stress is suppressed in RAGE null and sRAGE-treated mice, it is not abolished. ADR also stimulates increases in S-100/calgranulins, ligands linked to cell stress, in at least in part in podocytes.

Signal transduction via RAGE is essential for modulation of cellular properties by ADR. In murine podocytes, S-100b increases phosphorylation of both p44/p42 and p38 MAP kinases, implicated in mitogenic, inflammatory, and death- or survival-provoking pathways. We examined these two MAP kinase family members, as recent studies elucidated specific roles for p44/p42 and p38 MAP kinase in podocyte stress. In vivo, administration of inhibitors of these pathways to rodents receiving ADR attenuated proteinuria. Our survival-provoking pathways. We examined these two nases, implicated in mitogenic, inflammatory, and death- or survival-provoking pathways,21–24 We examined these two MAP kinase family members, as recent studies elucidated specific roles for p44/p42 and p38 MAP kinase in podocyte stress.25–27 In vivo, administration of inhibitors of these pathways to rodents receiving ADR attenuated proteinuria.25 Our data suggest that RAGE ligands signal through p44/p42 MAP kinase in the podocyte, and not p38 MAP kinase, to stimulate activation of NADPH oxidase.

Our studies confirmed that an early effect of ADR was extensive podocyte foot process effacement. Key podocyte actin-binding proteins, such as α-actinin 4 and synaptopodin, maintain podocyte shape and integrity. Although podocytes express RAGE, RAGE does not appear essential for maintenance of normal glomerular structure and function, as RAGE null mice at baseline do not display foot process effacement, glomerular sclerosis, or detectable albuminuria.

Importantly, RAGE null mice were vulnerable to ADR. First, these mice were homozygous for BALB/cJ susceptibility alleles at the DOXNP/H locus (chromosome 17). The gene encoding RAGE is in the MHC class III region on chromosome 17. Second, our studies revealed that p44/42 MAP kinase was a central signaling pathway by which RAGE ligand evoked activation of NADPH oxidase in podocytes. In distinct studies, p44/42 MAP kinase was found not to be impacted by deletion of the RAGE gene in smooth muscle cells. Lastly, RAGE null mice demonstrated only partial protection from the effects of ADR.

Podocyte depletion may follow lethal oxidative stress and the failure of this terminally differentiated cell to proliferate. The significant protection afforded by RAGE deletion or antagonism in preserving podocytes is likely linked mechanistically to protection from glomerulosclerosis, as previous studies established the etiologic link between progressive podocyte loss and glomerulosclerosis by diphertheria toxin-induced podocyte depletion using transgenic rats. Importantly, the mice used in this study were globally devoid of RAGE. As others have suggested contributory roles for macrophages and certain T lymphocyte subsets in the pathogenesis of ADR nephropathy,7,36–41 it is possible that RAGE-dependent non-podocyte roles contributed to ADR injury. Future studies, using tissue-targeted RAGE null mice, will explore this concept.

Taken together, we propose that consequent to ADR, ligand-RAGE interaction amplifies generation of ROS, at least in part via NADPH oxidase. Diverse changes in cellular properties ensue, which evoke albuminuria and glomerulosclerosis. The identification of AGEs and RAGE in glomeruli of human focal segmental glomerulosclerosis and other progressive glomerular diseases suggests that the ligand-RAGE mechanism may contribute to diverse sclerosing glomerulopathies.

**CONCISE METHODS**

**Animal Studies**

Animal experiments were performed with the approval of the Institutional Animal Care and Use Committee of Columbia University. Homozygous RAGE null mice were bred into BALB/cJ background to the 12th generation. Wild-type BALB/cJ mice (Jackson Laboratories, Bar Harbor, ME) were controls. Male mice (age 8 wk), body weight 20 to 25 g, were treated with a single dose of ADR (doxorubicin HCI) (Ben Venue Laboratories, Bedford, OH), 10.5 mg/kg, by tail vein injection. Urine sRAGE, was prepared as described.1–3,5

**Albuminuria**

Urine at 24 h was collected in metabolic cages, and urine albumin and creatinine were determined using kits from Exocell (Philadelphia, PA).

**Morphometry**

Kidney sections, 3 μm, were stained with periodic acid-Schiff (PAS) (Sigma-Aldrich, St Louis, MO). Images were scanned into a computer and quantification of mesangial matrix in nuclei-free regions, and glomerular area was performed using a Zeiss microscope (Thornwood, NY) and an image analysis system. Forty glomeruli per animal were selected randomly. Podocyte foot process effacement was determined by electron microscopy in at least five glomeruli per mouse.

**Confocal Microscopy**

Confocal microscopy was performed using a Lasersharp 2000 BioRad scanning confocal microscope (Bio-Rad, Hercules, CA). Polyclonal rabbit anti-human RAGE IgG4 and mouse anti-human synaptopodin IgG (Maine Biotechnology, Portland, ME) were used. Frozen sections (5 μm) were incubated with anti-RAGE IgG followed by biotin-conjugated anti-rabbit IgG (Sigma-Aldrich) and with streptavidin-conjugated Alexa Fluor 555 (Invitrogen, Carlsbad, CA). Other antibodies included antisynaptopodin IgG, rabbit antinitrotyrosine IgG (Upstate Group, Lake Placid, NY), and anti-S-100 IgG, Sigma-Aldrich).

**ELISA for Detection of AGE and Nitrotyrosine Epitopes**

Kidney cortex was homogenized and protein concentrations were measured (Bio-Rad). AGE ELISA was performed using T-gel (Pierce Chemical, Rockford, IL)-affinity-purified chicken anti-AGE followed by anti-chicken IgY (Sigma-Aldrich). Ribose glycated albumin was the standard. ELISA for the detection of nitrotyrosine epitopes was performed using a kit from Oxis Research (Oxis Research, Portland, OR).
Assay for Detection of Malondialdehyde (MDA) and NOS Activity

Assay for detection of MDA was performed using the MDA-586 Assay kit, and levels of total nitrite and nitrate were measured using a kit from Oxis Research Products.

NADPH Oxidase Activity

Kidney protein (25 μg) and lucigenin (250 μM) (Alexis, San Diego, CA) were added to HEPES buffer (Sigma-Aldrich), followed by NADPH (10 mM; Sigma-Aldrich). The samples were immediately examined in a Lumat LB9501 luminometer (PE Wallac, Gaithersburg, MD) for 10 s.

Laser Capture and Real-time Quantitative PCR

Laser capture of 150 glomeruli from three sections per mouse was performed with PixCell II (Arcturus, CA) and pooled for RNA extraction. cDNA was synthesized with TaqMan Reverse Transcription Reagents Kit (PE Applied Biosystems, Foster City, CA). The primers and probes for RAGE, TGF-β1, CTGF, and β-actin were purchased from PE Applied Biosystems. Real-time PCR was performed in an ABI Prism 7900 Sequence Detection System (PE Applied Biosystems) with TaqMan PCR Master Mix. The relative target mRNA level was calculated by the comparative Ct method as instructed by the manufacturer.

Cell Culture

Murine podocytes (provided by Dr. Paul Klotman, Mount Sinai School of Medicine, NY) were propagated and differentiated as described. Cells were stimulated with ADR, 3 μg/ml (5 μM) for 3 d. Supernatants were retrieved for AGE ELISA. In other studies, podocytes were stimulated with S-100B. Certain cells were preincubated with 0.2 mg/ml ADR in normal saline. The beads were washed and eluted with low pH buffer (pH 2.5). The eluate was applied on IgG-coupled AffiGel 15 beads. sRAGE would bind ADR was performed.

Statistical Analysis

Mean ± SE is reported. A one-way ANOVA was performed; if the F test resulted in a P value less than 0.05, multiple comparisons were made by Tukey’s pair-wise testing, which conserves the overall type I error of 0.05. All data were analyzed by the SAS system software (SAS Institute, Cary, NC).

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