Soluble Receptor for Advanced Glycation End Products Alleviates Nephritis in (NZB/NZW)F1 Mice

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Objective. To investigate the efficacy of different doses of the soluble form of the receptor for advanced glycation end products (sRAGE) (conjugated to the Fc portion of immunoglobulin) in the treatment of nephritis in lupus-prone mice, in comparison with the efficacy of combination therapy with mycophenolate mofetil plus prednisolone.

Methods. Twenty-eight female (NZB/NZW)F1 mice were divided into 5 groups (untreated, sRAGE [dose groups of 0.5, 1, or 2 μg], or mycophenolate mofetil plus prednisolone). Proteinuria and histologic damage were evaluated. Immune complex deposition and the nuclear translocation of NF-κB in the kidney tissue were assessed by immunofluorescence staining. Serum concentrations of anti–double-stranded DNA (anti-dsDNA) and IgG subclasses were also measured. The population of T cells was evaluated using a fluorescence-activated cell sorter, and expression of intracellular adhesion molecule 1 and vascular cell adhesion molecule 1 in the kidney tissue was assessed by immunohistochemical staining.

Results. In comparison with untreated mice, mice treated with 1 or 2 μg sRAGE showed significantly reduced proteinuria and attenuated histologic renal damage, with efficacy comparable to that of combination therapy. Treatment with 2 μg sRAGE significantly reduced immune complex deposition and decreased the serum concentrations of anti-dsDNA, IgG2a, IgG2b, and IgG3. In addition, sRAGE interrupted the nuclear translocation of NF-κB in the kidney, resulting in reduction in the expression of downstream genes of NF-κB in vivo and in vitro. Furthermore, sRAGE effectively modified T cell populations.

Conclusion. Treatment with sRAGE significantly improved nephritis in lupus-prone mice, with efficacy comparable to that of standard induction treatment for lupus nephritis. These data suggest that sRAGE has antiinflammatory effects on the pathophysiology of lupus nephritis and could serve as a potent new therapy for this disease.

Advanced glycation end products (AGEs) are glycoproteins that are produced through nonenzymatic reactions between sugars and free amino groups of proteins. AGEs are formed in pathologic and diverse physiologic settings, such as diabetes mellitus, hypoxia, ischemia-reperfusion injury, chronic renal disease, and inflammation (1,2). The receptor for AGEs (RAGE) is a member of the immunoglobulin superfamily (3), and it is a pattern-recognition receptor that is involved in various innate immune responses (4). Ligands for RAGE include AGEs, members of the S100/calgranulin protein family, high mobility group box chromosomal protein 1 (HMGB-1), and amyloid β-sheet fibrils, which are involved in tissue injury and necrosis (5). In contrast to Toll-like receptors (TLRs), RAGE usually interacts with endogenous ligands, and thereby participates in the pathogenesis of diseases triggered by chronic inflammation, which produces multiple endogenous ligands (5).
The membrane-bound form of RAGE (mRAGE) consists of 3 types of domains: extracellular immunoglobulin-like domains, a single hydrophobic membrane-spanning domain, and a short cytoplasmic domain, which is essential for RAGE-mediated signaling (6). Ligands bound to RAGE activate various intracellular signaling cascades (4,7). Importantly, RAGE signaling enhances the nuclear translocation of NF-κB, and in turn may increase the expression of tumor necrosis factor α and interleukin-6 (IL-6). These cytokines induce tissue inflammation and are involved in the pathogenesis of autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus (SLE) (4,8–10).

The soluble form of RAGE (sRAGE) possesses the same extracellular region, but lacks membranous and cytoplasmic domains. Soluble RAGE is produced from mRAGE by alternative splicing and cleavage and is also produced directly by gene expression of endogenous secretory RAGE (4). Soluble RAGE can bind to RAGE ligands in the extracellular space, and thus competitively inhibits the binding of ligands to mRAGE, resulting in a reduction of the inflammation induced by NF-κB activation (4,11).

SLE is an autoimmune disease that affects multiple organs, and in lupus nephritis, one of the manifestations of SLE, the deposition of immune complex, including anti–double-stranded DNA (anti-dsDNA) and complement (mostly, C3), plays a crucial role in the pathophysiologic processes of SLE (12). The amount of proteinuria reflects the extent of peripheral glomerular capillary loop involvement, which tends to increase along with mesangial proliferation and membranous nephropathy (12).

In this study, we used (NZB/NZW)F1 lupus-prone mice, a model in which anti-dsDNA–mediated immune complex deposition leads to glomerulonephritis, which is a profile that is similar to that of human SLE. In (NZB/NZW)F1 mice, glomerulonephritis can occur at 5–6 months of age, resulting in death from severe renal failure at an average of 12 months of age (13). Proliferative lupus nephritis (class III or class IV) is the most severe form of lupus nephritis (14). Intravenous or oral prednisolone plus either intravenous cyclophosphamide or oral mycophenolate mofetil (MMF) are currently recommended as standard induction therapies for proliferative lupus nephritis (12,15). Unfortunately, despite the fact that improved outcomes have been achieved, end-stage renal disease still occurs at a relatively high rate in the long-term disease course (12). Therefore, new therapies are needed.

To date, several reports have demonstrated that serum HMGB-1 levels are elevated in SLE patients, particularly in those with active renal disease, in comparison with that in healthy controls (16). HMGB-1 was also reported to act as a proinflammatory mediator in anti-dsDNA–induced kidney damage in SLE (17). In addition to antibody-induced nephritis, the link between RAGE and glomerular stress was recently elucidated; RAGE is expressed by podocytes and glomerular endothelial cells. Ligands for RAGE can induce up-regulation of the chemokine C-C motif ligand CCL2 and generation of reactive oxygen species by podocytes, and can induce apoptosis in podocytes. RAGE ligands also suppress glomerular endothelial cell growth (18). Moreover, RAGE has been found on the surface of B cells, and HMGB-1 was thought to be part of immunostimulatory complexes that bind to and activate autoreactive B cells (19). Therefore, it can be reasonably speculated that sRAGE can reduce the production of nephritis-related autoantibodies specific to SLE, leading to a reduction in NF-κB activity, and can thereby improve lupus nephritis by inhibiting the binding of RAGE ligands to mRAGE (20,21).

To address these issues, we treated mice with different doses of sRAGE. The sRAGE was conjugated to the Fc portion of immunoglobulin, as this extends the half-life of sRAGE in murine serum. We investigated the efficacy of sRAGE in terms of alleviating the severity of lupus nephritis, in comparison with no treatment or combination treatment, in lupus-prone mice.

**MATERIALS AND METHODS**

**Expression and purification of fusion protein.** To construct the sRAGE–Fc portion, a leader sequence (gene K02149, protein AAS31633), mouse IgG H-chain (primer set 5’-ATAGGCTAGCGCCACCATGGGATGG-3’ and 5’-TGTTGAGTTTTTGTCCGAGTGAGACATCTGT-3’), amino acids 23–341 of mouse sRAGE (primer set 5’-GGTCAGAA-CATCACAGCCCGGATTG-3’ and 5’-GTGAGTTTTGTCCGAGTGGACATCTGT-3’), and mouse IgG H-chain (primer set 5’-CCAGCTCGAGCTATTTACCCGGAGACAG-3’ and 5’-CATCACAGCCCGGATTG-3’) were amplified, and overlap-extension polymerase chain reaction (PCR) was performed. The PCR product was digested with sfi I and inserted into the pYK602 vector. Mouse sRAGE-Fc was expressed in HEK 293E cells and purified on a protein A–Sepharose column (Amersham Biosciences) according to the manufacturer’s instructions. The purified recombinant sRAGE was dialyzed with phosphate buffered saline (PBS) and analyzed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis. In addition, a Limulus amebocyte lysate test kit (Associates of Cape Cod) was used to determine the endotoxin level.
Animals and treatment protocol. All animals were treated in accordance with the Guidelines and Regulations for the Use and Care of Laboratory Animals of Yonsei University (Seoul, Republic of Korea). Twenty-eight 18-week-old female (NZB/NZW)F1 mice were purchased and housed individually in a specific pathogen–free barrier facility under standard sterile conditions. In our previous study, 25–30% of untreated mice died during the experimental period (20). Therefore, we assigned 8 mice to the untreated group and 5 mice to each treatment group, as follows: group 1 was untreated, group 2 received 0.5 μg sRAGE, group 3 received 1 μg sRAGE, group 4 received 2 μg sRAGE, and group 5 received MMF (33.3 mg/kg) plus prednisolone (1.5 mg/kg). All treatments began at 24 weeks of age. The different doses of sRAGE (groups 2–4) were injected intraperitoneally twice a week, and MMF and prednisolone (group 5) was orally administered daily. Treatment in all groups was continued until 31 weeks of age. PBS was injected intraperitoneally into the untreated or MMF plus prednisolone–treated mice in accordance with the same schedule.

Measurement of proteinuria. Twice daily during the experimental period, urinary proteinuria was measured with albumin reagent strips for spot urine (Yongdong Pharmaceutical) using urine samples collected from each mouse. Proteinuria was assessed semiquantitatively using the following scale: 0 = none or trace; 1+ = ≤100 mg/dl; 2+ = ≤300 mg/dl; 3+ = ≥1,000 mg/dl; and 4+ = >1,000 mg/dl.

Histopathologic assessment. Kidney tissue samples were obtained from all mice, and the tissue was fixed in buffered formalin and stored in liquid nitrogen. Formalin-fixed kidney specimens were embedded in paraffin, cut into 4-μm-thick sections, and stained with periodic acid–Schiff according to conventional procedures. Renal histologic abnormalities, including glomerular, tubular, and vascular damage, were scored semiquantitatively on a 4-point scale by 2 individual pathologists working independently and in a blinded manner. The average score from the 2 pathologists was calculated, as described in our previous report (20). At least 50 glomeruli per mouse were examined.

Immunofluorescence staining. All kidney tissues were embedded in OCT compound and frozen at −20°C. Samples were cut into 4-μm-thick sections, fixed in 4% paraformaldehyde for 15 minutes, and washed 3 times in cold PBS. Nonspecific binding was blocked with 1% normal goat serum in PBS with Tween for 30 minutes. The sections were incubated with goat anti-mouse IgG (1:100; Sigma–Aldrich) and rabbit anti-mouse C3 (1:100; Abcam) at room temperature for 1 hour, and washed 3 times in cold PBS, incubated with Alexa Fluor 488–labeled donkey anti-goat IgG and Alexa Fluor 568–labeled donkey anti-rabbit IgG (each 1:100; Invitrogen) at room temperature for 1 hour, and washed 3 times with PBS. Some sections were treated with goat anti-mouse NF-κB (1:100; Abcam) followed by Alexa Fluor 488–labeled donkey anti-goat IgG (1:200; Invitrogen) according to the same method. Nuclei were stained with DAPI (1:1,000; Invitrogen). Sections were mounted in a mounting medium (Vector Laboratories) and examined under a laser scanning confocal microscope (LSM 710; Carl Zeiss). Two pathologists independently assigned semiquantitative scores for the intensity and distribution of immunofluorescence staining for IgG and C3, on a scale ranging 0–3 for staining intensity, in which 0 = no staining, 1 = weak staining, 2 = moderate staining, and 3 = strong staining. The average score from the 2 pathologists was calculated.

To analyze the extent of nuclear translocation of NF-κB, the relative area of colocalization (expressed as a percentage) and colocalization coefficient (Ch1–T1) for nuclei occupied by NF-κB were calculated from merged images using the LSM ZEN 2009 image analysis program (Carl Zeiss). Colocalization coefficients (for nuclei–NF-κB colocalization) were calculated using Zeiss colocalization coefficient function software, in which all background pixels were considered, and the number of colocalizing pixels (for NF-κB) in channel 1 (Ch1) was calculated relative to the total number of pixels (for the nuclei).

Serum concentrations of anti-dsDNA and IgG subclasses. We obtained blood samples at the end of the experiment, and then separated the sera and stored the serum samples at −80°C. The anti-dsDNA IgG concentration was measured by sandwich enzyme-linked immunosorbent assay (Alpha Diagnostic International). IgG subclasses were analyzed using a Milliplex MAP Mouse Immunoglobulin Isotyping kit (Millipore).

Immunohistochemistry. Immunohistochemical analysis was performed using a Vectastain ABC kit (Vector). Tissues were stained with goat antibodies against mouse intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) overnight at 4°C, and then stained with biotinylated secondary linking antibody and, finally, with streptavidin–peroxidase complex for 1 hour. The final color product was developed using aminoethylcarbazole (Dako). Sections were counterstained with hematoxylin, and then photographed with an Olympus photomicroscope. A semiquantitative score for the intensity and distribution of ICAM-1 or VCAM-1 staining on glomeruli and tubules was assigned as follows: 0 = normal or general slight staining; 1 = focal, mildly increased staining; 2 = focal, moderately increased staining; and 3 = diffuse, markedly increased staining. The scoring was performed independently by 2 pathologists in a blinded manner, and the average score was calculated.

Fluorescence-activated cell sorting (FACS) analysis. Immune cells from murine spleens were obtained by mechanical shredding and collected in complete RPMI medium (Invitrogen). T cells were isolated using a CD4 T Cell Isolation Kit II (Miltenyi Biotec), in which non–T cells were depleted. Expression of intracellular cytokines by the cells was determined following cell stimulation with 75 ng/ml phorbol 12-myristate 13-acetate and 750 ng/ml ionomycin (both from Sigma–Aldrich) for 5 hours, together with GolgiPlug/brefeldin A (BD Biosciences). CD4+ T cells were stained with anti-CD4–fluorescein isothiocyanate (FITC) (BD Biosciences). Intracellular cytokines were detected using antibodies obtained from eBioscience (interferon-γ [IFNγ]–PerCP, IL-17A–allophycocyanin, and IL-4–phycoerythrin [PE]). Intracellular flow cytometry staining was carried out using a BD Cytofix/Cytoperm Fixation/Permeabilization Solution kit with BD GolgiPlug (BD Biosciences), according to the manufacturer’s protocols. Treg cells were stained with anti-CD4–FITC and anti-CD25–PerCP. The cells were fixed and permeabilized with FoxP3 staining buffer, followed by staining for FoxP3–PE (eBioscience). Flow cytometric analysis of surface staining was
conducted on an LSR II FACS (BD Biosciences). Analysis of the results was conducted using FlowJo software (BD Biosciences).

**Western immunoblotting.** Murine podocytes were treated with 1 µg/ml of HMGB-1 for various lengths of time, and then immunoblotted with p65 and VCAM-1 antibodies. The cells were preincubated with 10 µg/ml of sRAGE for 1 hour before being treated with 1 µg/ml of HMGB-1, followed by immunoblotting with p65 and VCAM-1 antibodies. Extracts of the cytoplasm and nuclei were prepared from the treated cells for detection of VCAM-1 and p65, respectively. The extracts were analyzed via electrophoresis on a 4–20% Tris-glycine–polyacrylamide gel (Koma Biotech) before being transferred to nitrocellulose membranes using iBlot (Invitrogen). The membranes were incubated with washing buffer containing 5% bovine serum albumin (BSA) for 2 hours. The primary antibodies NF-κB p65 (1:1,000; Cell Signaling Technology), VCAM-1 (1:2,000; Abcam), TATA box binding protein (1:5,000; Abcam), and β-actin (1:5,000; Sigma-Aldrich) were diluted in washing buffer with 5% BSA, and then applied to the membrane for 3 hours at room temperature. After washing, the membranes were incubated with the horseradish peroxidase–conjugated secondary antibodies rabbit IgG (1:5,000; KPL) and mouse IgG (1:2,000; KPL) in washing buffer with 5% BSA for 45 minutes at room temperature. Immuno-reactive bands were detected using an enhanced chemiluminescence system (GE Healthcare). Western immunoblotting was performed 4 times with each antibody.

**Evaluation of sRAGE toxicity.** Blood samples obtained from untreated, MMF plus prednisolone–treated, and 1 or 2 µg sRAGE–treated mice were examined using blood chemistry and standard liver and kidney function tests to determine the toxic effects of sRAGE on the murine bone marrow, liver, and kidney.

**Statistical analysis.** All statistical analyses were conducted using SPSS for Windows, version 18. Representative results are the mean values from each mouse in each group. All values in the treatment groups were compared with values in untreated mice. The results and measurements are expressed as the mean ± SD. The Kaplan-Meier plot for survival rate was analyzed using the log rank test. Statistical comparisons between 2 groups were evaluated with the Mann-Whitney U test. Multiple-group comparisons were performed using analysis of variance followed by Tukey’s method for multiple comparisons. In all analyses, P values less than 0.05 were considered statistically significant.

**Figure 1.** Proteinuria and survival rate in lupus-prone mice. Beginning at age 24 weeks, mice were injected intraperitoneally with phosphate buffered saline alone (untreated), treated orally with mycophenolate mofetil (MMF) (33.3 mg/kg) plus prednisolone (PL) (1.5 mg/kg) daily, or injected intraperitoneally with 0.5, 1, or 2 µg soluble receptor for advanced glycation end products (sRAGE) twice a week up to age 31 weeks. A, Proteinuria levels over time, as measured twice weekly by urine dipstick. Levels were determined on a scale of 0 (none or trace) to 4+ (>1,000 mg/dl). Asterisks indicate significant differences (P < 0.05) compared with the untreated group. Results are the mean ± SD scores from 2 independent pathologists for each group (n = 8 untreated, n = 5 per treatment group). B, Kaplan-Meier plots for survival rate over time.

**Figure 2.** Histologic changes in the murine kidney tissue. A, Samples of kidney tissue were collected from untreated mice (panel 1), mice treated with 0.5, 1, or 2 µg soluble receptor for advanced glycation end products (sRAGE) (panels 2–4), and mice treated with mycophenolate mofetil (MMF) plus prednisolone (PL) (panel 5). Immunohistochemical staining of the kidney tissue sections was used to assess renal histologic abnormalities, including glomerular, tubular, and vascular damage. Representative results are shown. B, Glomerular, tubular, and vascular damage in the kidney tissue was scored semiquantitatively on a 4-point scale. Asterisks indicate significant differences (P < 0.05) compared with the untreated group. Results are the mean ± SD scores from 2 independent pathologists for each group (n = 8 untreated, n = 5 per treatment group).
RESULTS

Proteinuria and survival rate in lupus-prone mice. At the beginning of the experiment (with mice at age 24 weeks), the mean proteinuria level was more than 2+ in most mice. Thereafter, in mice at age 31 weeks, treatment with 1 μg sRAGE resulted in a slight reduction in proteinuria (decrease of 24% compared with baseline). Administration of 2 μg sRAGE or MMF plus prednisolone each significantly decreased the level of proteinuria, by 75% or 62%, respectively, by the end of treatment, compared with that in untreated mice. Furthermore, the efficacy of 2 μg sRAGE became apparent at an earlier time point (in mice at age 27 weeks) than that with MMF plus prednisolone (Figure 1A). Two untreated mice died as a result of disease progression during the experiment, whereas all mice in the treatment groups survived (Figure 1B). Taken together, these observations indicate that 2 μg sRAGE alleviated nephritis in (NZB/NZW)F1 mice, to an extent comparable to that following combination treatment with MMF plus prednisolone.

Histologic changes in the murine kidney tissue. In untreated mice, severe glomerulonephritis and tubular damage were frequently observed, but vascular changes such as wall thickening or capillary thrombosis were not apparent. In contrast, mild glomerular and tubular changes or normal structures were observed in the kidney tissue of mice treated with 1 or 2 μg sRAGE or MMF plus prednisolone (Figure 2A). Histologic evaluation showed that treatment with 1 μg sRAGE, 2 μg sRAGE, or MMF plus prednisolone each signifi-
significantly decreased the glomerular damage scores, by 65%, 81%, or 81%, respectively, and tubular damage scores were decreased by 61%, 78%, or 72%, respectively, when compared with no treatment (Figure 2B). However, treatment with 1 or 2 μg sRAGE or MMF plus prednisolone had little influence on vascular damage scores.

**Immune complex deposition in the murine kidney tissue.** In untreated mice, heavy accumulation of IgG (red) and C3 (green) was observed within the glomeruli, and immune complex deposition was observed in the mesangium as well as in the capillary loops. The yellow-orange color resulting from coincident fluorescence was indicative of the colocalization of C3 and IgG as an immune complex. The fluorescence intensity observed in the kidney tissue of mice treated with 0.5 μg sRAGE was similar to that in untreated mice. The fluorescence intensity was slightly decreased in mice treated with 1 μg sRAGE, and treatment with 2 μg sRAGE or MMF plus prednisolone significantly reduced immune complex deposition when compared with that in untreated mice (Figures 3A and B). These data show that sRAGE attenuates nephritis in lupus-prone mice by reducing the deposition of glomerular immune complex, IgG, and C3.

**Serum concentration of anti-dsDNA and IgG subclasses.** Treatment with 2 μg sRAGE and treatment with MMF plus prednisolone each significantly decreased the serum concentrations of anti-dsDNA, by 33% and 24%, respectively, in comparison with that in untreated mice. Although the serum concentration was reduced to a greater extent by 2 μg sRAGE compared with MMF plus prednisolone, the difference was not significant (Figure 4A). Neither sRAGE nor MMF plus
prednisolone affected the concentration of IgG1, whereas treatment with 2 µg sRAGE or MMF plus prednisolone significantly reduced the serum concentrations of IgG2a, IgG2b, and IgG3. In addition, serum concentrations of IgG2a and IgG3 were reduced by sRAGE at a dose of 1 µg, and the IgG3 concentration was reduced with a dose of 0.5 µg sRAGE (Figure 4B). These findings suggest that sRAGE can decrease the production of anti-dsDNA as well as the levels of IgG2a, IgG2b, and IgG3 and may contribute to a reduction in immune complex deposition in the kidneys, leading to alleviation of nephritis in (NZB/NZW)F1 mice.

**Effects of sRAGE on T cell populations.** Treatment of mice with 1 or 2 µg sRAGE significantly reduced the numbers of CD4+IFNγ+ T cells (proportion of Th1 cells, 8.2% or 7.4%, respectively, versus 11.8% with no treatment) and also significantly reduced the numbers of CD4+IL-17A+ T cells (proportion of Th17 cells, 4.8% or 3.5%, respectively, versus 8.6% with no treatment) (Figures 5A and B). In addition, there was a tendency toward a reduction in the number of CD4+IL-4+ T cells in mice treated with either 1 or 2 µg sRAGE in comparison with untreated mice, and the difference was significant (proportion of CD4+IL-4+ T...
cells, 2.8% for each sRAGE dose group versus 3.1% with no treatment), whereas this trend was not seen following treatment with MMF plus prednisolone (Figure 5C).

Furthermore, treatment with 1 or 2 μg sRAGE significantly increased the number of CD4+FoxP3+ T cells as compared with no treatment or MMF plus prednisolone (proportion of CD4+FoxP3+ T cells, 6.1% or 6.7% with 1 or 2 μg sRAGE, respectively, versus 3.5% or 5.4% with no treatment or MMF plus prednisolone, respectively) (Figure 5D). When we analyzed the effect of sRAGE on the total number of CD4+ T cells, treatment with 1 or 2 μg sRAGE and treatment with MMF plus prednisolone each significantly decreased the total number of CD4+ T cells as compared with no treatment (proportion of total CD4+ T cells, 37.6%, 33.8%, 37.2%, and 44.6%, respectively) (Figure 5E). (More details on the mean levels of each T cell population are available from the corresponding author upon request.)

Transcriptional activity of NF-κB. Treatment with 1 μg of sRAGE, 2 μg of sRAGE, or MMF plus prednisolone each significantly decreased the colocalization area of nuclei occupied by NF-κB, by 71%, 96%, or 99%, respectively, and the Ch1-T1 colocalization coefficient was reduced by 78%, 96%, or 99%, respectively, as compared with that in untreated mice (results available from the corresponding author upon request). Furthermore, in untreated mice and mice treated with 0.5 μg sRAGE, the kidney tissue showed strong staining for ICAM-1 and VCAM-1, both of which are well-known downstream genes regulated by NF-κB and mediators that amplify inflammation (22). In contrast, treatment with 1 or 2 μg sRAGE and treatment with MMF plus prednisolone each significantly reduced the staining intensity of ICAM-1, by 69%, 94%, and 94%, respectively, and also significantly reduced the staining intensity of VCAM-1, by 69%, 90%, and 84%, respectively, when compared with no treatment (Figures 6A and B).

In addition, Western immunoblotting showed that treatment of murine podocytes with HMGB-1 significantly increased the expression of p65 after 15 minutes. HMGB-1 also showed a tendency to enhance the expression of VCAM-1, but this increase was not statistically significant. Meanwhile, preincubation of the cells with sRAGE prior to HMGB-1 treatment inhibited the HMGB-1–mediated induction of p65 and VCAM-1 expression (Figures 6C and D). Thus, sRAGE appears to interrupt the nuclear translocation of NF-κB and to inhibit its transcriptional activity.

Toxicity of sRAGE. There were no notable differences in the white blood cell or platelet counts, hemoglobin levels, or liver or kidney function test results between the groups, although the platelet count and blood urea nitrogen levels did differ between the treated and untreated mice. Untreated mice had a lower platelet count and a higher blood urea nitrogen concentration compared with treated mice (results available from the corresponding author upon request). Relative thrombocytopenia and an increased blood urea nitrogen concentration are features that are thought to be related to lupus nephritis.

DISCUSSION

HMGB-1, the major RAGE-related ligand, activates NF-κB and contributes to the pathophysiology of various diseases such as cardiovascular diseases, vascular complications of diabetes mellitus, and chronic renal diseases (2,4,18,23). There have been many attempts to prevent HMGB-1 from interacting with RAGE in order to modify disease activity. In animal models, HMGB-1 neutralization attenuated experimental autoimmune myocarditis (24), slowed the development of arthritis and ameliorated its severity (25), and reduced vascular inflammation and the development of atherosclerosis (26). HMGB-1 is also thought to play a key role in the pathophysiology of SLE. Serum levels of HMGB-1 and anti–HMGB-1 autoantibodies were elevated in SLE patients and correlated with disease activity (16,27). In addition, HMGB-1 interacts with RAGE on B cells and plasmacytoid dendritic cells, resulting in TLR9–dependent IFNα secretion and autoreactive B cell activation, and it acts as an inflammatory mediator in anti–dsDNA–induced kidney damage in SLE (28,29). Thus, as in other inflammatory diseases, the neutralization of HMGB-1 can be a novel therapeutic strategy in lupus-like disease.

Nevertheless, there have been no reports regarding the efficacy of sRAGE in SLE. In the present study, we first demonstrated that treatment with sRAGE reduced the severity of nephritis in lupus-prone mice. Specifically, treatment with 2 μg sRAGE significantly improved proteinuria and histologic renal damage, reduced the extent of glomerular immune complex deposition and the serum concentrations of lupus nephritis–related autoantibodies, and inhibited the nuclear translocation of NF-κB and the expression of its downstream genes in (NZB/NZW)F1 mice, with an efficacy comparable to that of standard induction treatment.
Furthermore, 1 or 2 μg sRAGE exhibited a more rapid therapeutic effect than that of MMF plus prednisolone.

Anti-dsDNA is the most important autoantibody in the pathogenesis of lupus nephritis and it has been found to modulate the expression of genes involved in renal damage (12,17). The present study produced similar results, indicating that glomerular anti-dsDNA deposition and its serum concentration were closely correlated with the severity of kidney tissue damage. However, in addition to anti-dsDNA, various other autoantibodies produced by autoreactive B cells can accumulate in the kidney tissue and lead to immune-mediated nephritis (10). Moreover, previous studies indicated that IgG2a and IgG2b were pathogenic and that class-switching to these subclasses required TLR-9 and its signaling molecules (30). The administration of nonspecific IgG2a aggravated lupus-like disease in lupus-prone mice (31). IgG3 has also been reported to be associated with lupus-like disease. Both IgG2a and IgG3 were pathogenic in IFNα-induced lupus-like disease, and their serum concentrations were reduced by immunosuppressive treatment (32). Anti-estrogen treatment was found to have beneficial effects in lupus nephritis by reducing the levels of IgG3, but not IgG2, against DNA or nuclear extract (33). Therefore, because of the recognized roles of autoantibodies beyond anti-dsDNA and IgG subclasses in the pathogenesis of lupus nephritis, we measured nonspecific IgG subclasses, as well as anti-dsDNA, to determine autoantibody production by autoreactive plasma cells.

In this study, sRAGE significantly altered T cell populations, by enhancing Treg cell numbers while reducing Th1 cell, Th17 cell, and Th2 cell numbers. In previous studies, HMGB-1 administration reduced the expression of CTLA-4 and FoxP3 and the secretion of IL-10 in murine splenic Treg cells, when compared with that in normal mice (34,35). In contrast, HMGB-1 strengthened the endogenous signaling for dendritic cell maturation as well as the Th1 cell polarization, and it may contribute to activation of Th17 cells (24,36). In lupus nephritis patients, Treg cell numbers are usually reduced, while Th1 cell numbers are increased significantly (37), and the persistent activation of Th17 cells with defective Treg cell function are thought to further contribute to the progression of lupus nephritis (38,39). In the present study, in comparison with untreated mice, mice treated with sRAGE had significantly decreased numbers of Th1, Th17, and Th2 cells, and increased Treg cell numbers, resulting in the alleviation of lupus nephritis in lupus-prone mice. In addition, sRAGE significantly reduced the number of total CD4+ T cells, suggesting that sRAGE may act by both reducing the total CD4+ T cell numbers and contributing to the alteration of T cell subset polarization in a favorable direction for the improvement of lupus nephritis.

NF-κB enhances proinflammatory cytokines that up-regulate Fas ligand in human mesangial cells. Up-regulation of Fas ligand may contribute to glomerular inflammation in lupus nephritis (40). Unlike that in other classes of lupus nephritis and in normal controls, NF-κB activation in class IV lupus nephritis is enhanced within glomerular, endothelial, and mesangial cells or infiltrating cells (41). Moreover, proteasome inhibitor has been reported to improve lupus nephritis, mainly by depleting autoreactive plasma cells and by inhibiting the nuclear translocation of NF-κB (20,21,42). In this study, sRAGE reduced the expression of ICAM-1 and VCAM-1 in the kidney tissue of (NZB/NZW)F1 mice. Furthermore, in vitro results using murine podocytes indicated that sRAGE significantly inhibited the HMGB-1–induced expression of p65 and also showed a tendency to reduce the expression of its downstream gene, VCAM-1. In addition, we found that sRAGE decreased the colocalization between NF-κB and nuclei. Thus, we conclude that sRAGE slows the progression of lupus nephritis by interrupting the binding of RAGE to its ligands and inhibiting the transcriptional activity of NF-κB.

We used doses of MMF plus prednisolone that are clinically equivalent to those used in patients with lupus nephritis (33.3 mg/kg of MMF and 1.5 mg/kg of prednisolone, equating to 2,000 mg of MMF and 90 mg of prednisolone, respectively, for a 60-kg patient). In general, the metabolic rate of mice is higher than that of humans. Thus, to compensate for the differences in the metabolic rates between the two species, a higher dose of MMF or prednisolone would have been reasonable (43). Nonetheless, the observation that human-level doses of MMF plus prednisolone significantly improved nephritis in (NZB/NZW)F1 mice should lessen concerns over insufficient doses (14,15). Previous studies used 100 μg sRAGE in murine models of atherosclerosis in diabetes mellitus (26,44).

In this study, we administered 0.5, 1, and 2 μg sRAGE and found that the latter two doses alleviated nephritis in lupus-prone mice in a dose-dependent manner. Previous studies used daily injections of sRAGE, while we injected sRAGE twice per week. Our experiment had two major strong points. First, we demonstrated the efficacy of sRAGE for nephritis in lupus-prone mice. Second, we utilized lower doses and less frequent dosing of sRAGE than has been utilized in
previous studies, and still demonstrated effectiveness (26,44).

In summary, sRAGE significantly improved nephritis in lupus-prone mice, to an extent comparable to that of MMF plus prednisolone. In (NZB/NZW)F1 mice, treatment with sRAGE significantly improved proteinuria and histologic renal damage, reduced glomerular immune complex deposition and the serum concentrations of lupus nephritis–related autoantibodies, and interrupted the nuclear translocation of NF-κB in the kidney tissue, leading to reduced expression of ICAM-1 and VCAM-1. These data suggest that sRAGE may have antiinflammatory effects in the pathophysiology development of lupus nephritis and could serve as a potent additional therapy for lupus nephritis.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Drs. Choi and Y.-B. Park had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.


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