Acid Sphingomyelinase Gene Deficiency Ameliorates the Hyperhomocysteinemia-Induced Glomerular Injury in Mice

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Hyperhomocysteinemia (hHcys) enhances ceramide production, leading to the activation of NADPH oxidase and consequent glomerular oxidative stress and sclerosis. The present study was performed to determine whether acid sphingomyelinase (Asm), a ceramide-producing enzyme, is implicated in the development of hHcys-induced glomerular oxidative stress and injury. Uninephrectomized Asm-knockout (Asm−/−) and wild-type (Asm+/+) mice, with or without Asm short hairpin RNA (shRNA) transfection, were fed a folate-free (FF) diet for 8 weeks, which significantly elevated the plasma Hcys level compared with mice fed normal chow. By using in vivo molecular imaging, we found that transfected shRNAs were expressed in the renal cortex starting on day 3 and continued for 24 days. The FF diet significantly increased renal ceramide production, Asm mRNA and activity, urinary total protein and albumin excretion, glomerular damage index, and NADPH-dependent superoxide production in the renal cortex from Asm+/+ mice compared with that from Asm−/− or Asm shRNA-transfected wild-type mice. Immunofluorescence analysis showed that the FF diet decreased the expression of podocin but increased desmin and ceramide levels in glomeruli from Asm+/+ mice but not in those from Asm−/− and Asm shRNA-transfected wild-type mice. In conclusion, our observations reveal that Asm plays a pivotal role in mediating podocyte injury and glomerular sclerosis associated with NADPH oxidase-associated local oxidative stress during hHcys. (*Am J Pathol 2011, 179:2210–2219; DOI: 10.1016/j.ajpath.2011.07.019)
activation of NADPH oxidase in different cells and tissues. Enhanced plasma Hcys concentrations increase the ceramide production and NADPH activity in the kidney of hyperhomocysteinemic rats. Inhibition of ceramide production improved glomerular injury in those hyperhomocysteinemic rats. However, it remains unknown whether alterations of Asm gene expression and regulation are implicated in the development of hHcys-induced glomerular oxidative stress and injury, ultimately resulting in glomerulosclerosis.

An inherited deficiency of Asm activity results in the type A and B forms of Niemann-Pick disease, and Asm-knockout mice are resistant to radiation and other forms of stress-induced apoptosis. Asm inhibition has rendered cells and animals resistant to the apoptotic effects of diverse stimuli, including Fas-CD95, ischemia, radiation, chemo-therapy, and tumor necrosis factor-α. Asm knockout or Asm inhibition had protective action during lung inflammation and fibrosis, cystic fibrosis, obesity and associated glomerular injury, liver fibrogenesis, and renal fibrosis.

The present study hypothesized that Asm gene deficiency protects glomeruli from hHcys-induced glomerular oxidative stress and injury and thereby ameliorates glomerulosclerosis under such pathological conditions. To test this hypothesis, we first performed a series of experiments using Asm−/− and their wild-type (WT) littermates fed the normal chow or folate-free (FF) diet to determine whether lack of the Asm gene alters renal ceramide production, glomerular local oxidative stress, and podocyte injury in mice during hHcys. Then, we locally silenced the renal Asm gene using short hairpin RNA (shRNA) and observed the effects of renal Asm deficiency on hHcys-induced glomerular oxidative stress and corresponding injury. Our results demonstrate that an Asm gene defect or deficiency in the kidney improves the hHcys-induced local oxidative stress and glomerular injury, ultimately preventing glomerulosclerosis.

**Materials and Methods**

**Animals**

Eight-week-old male C57BL/6J WT Asm−/− mice and their WT littermates were used in the present study. To speed up the damaging effects of hHcys on glomeruli, all mice were uninephrectomized, as previously described. This model has induced glomerular damage unrelated to the uninephrectomy and arterial blood pressure but specific to hHcys. After a 1-week recovery period from uninephrectomy, mice were fed either a normal chow or an FF diet (Dyets Inc., Bethlehem, PA) for 8 weeks to induce hHcys. In another series of C57BL/6J mice, Asm shRNA or a scrambled shRNA (Sigma, St Louis, MO) plasmid with a luciferase expression vector was cotransfected into the kidneys via intrarenal artery injection using the ultrasonographic microbubble system, as previously described. After delivery of plasmids into the kidney, these uninephrectomized mice were maintained on a normal or an FF diet for 8 weeks. All protocols were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University (Richmond, VA).

**HPLC Analysis**

Plasma and renal tissue Hcys levels were measured by the high-performance liquid chromatography (HPLC) method, as previously described. A 100-μL plasma sample or standard solution mixed with 10 μL of internal standard, thioglycolic acid (2.0 mmol/L) solution, was treated with 10 μL of 10% tri-n-butylphosphine solution in dimethyldiformamide at 4°C for 30 minutes. Then, 80 μL of ice-cold 10% trichloroacetic acid in 1 mmol/L EDTA was added and centrifuged to remove proteins in the sample. The supernatant, 100 μL, was transferred into the mixture of 20 μL of 1.55 mol/L sodium hydroxide, 250 μL of 0.125 mol/L borate buffer (pH 9.5), and 100 μL of 1.0 mg/mL 4-fluoro-7-aminosulfonylbenzofurazan solution. The resulting mixture was incubated at 60°C for 30 minutes to accomplish derivatization of thiols. HPLC was performed with an HP 1100 series instrument (Agilent Technologies, Waldbronn, Germany) equipped with a binary pump, a vacuum degasser, a thermostated column compartment, and an autosampler (Agilent Technologies). Separation was performed at an ambient temperature on an analytical column, Supelco LC-18-DB (Supelco, Bellefonte, PA; 150 × 4.6 mm i.d., 5 μm particle size) with a Supelcosil LC-18 guard column (Supelco; 20 × 4.6 mm i.d., 5 μm particle size). Fluorescence intensities were measured with an excitation wavelength of 385 nm and an emission wavelength of 515 nm by a Hewlett-Packard model 1046A fluorescence detector (Agilent Technologies). The peak area of the chromatographs was quantified with a Hewlett-Packard 3392 integrator (Agilent Technologies). The analytical column was eluted with 0.1 mol/L potassium dihydrogen phosphate buffer (pH 2.1) containing 6% acetonitrile (v/v) as the mobile phase, with a flow rate of 2.0 mL/minute.

**Morphological Examination Findings**

The fixed kidneys were paraffin embedded, and sections were prepared and stained with PAS stain. The glomerular damage index (GDI) was calculated from 0 to 4 on the basis of the degree of glomerulosclerosis and mesangial matrix expansion, as previously described. In general, we counted 50 glomeruli in total in each kidney slice under a microscope, when each glomerulus was graded with level 0 to 4 damage, as follows: 0 represents no lesion; 1+, sclerosis of <25% of the glomerulus; 2+, sclerosis of 25% to 50% of the glomerulus; 3+, sclerosis of >50% to 75% of the glomerulus; and 4+, sclerosis of >75% of the glomerulus. A whole kidney average sclerosis index was obtained by averaging scores from counted glomeruli. This observation was examined by two independent investigators (K.M.B. and M.X.) who were blinded to the treatment of the experimental groups.
Asm Activity in Mice Lacking Asm

The activity of Asm was determined as previously described. Briefly, N-methyl-[14C]-sphingomyelin was incubated with renal cortex tissue homogenates, and the metabolite of sphingomyelin, [14C]-choline phosphate, was quantified. An aliquot of homogenates (20 μg) was mixed with 0.02 μCi of N-methyl-[14C]-sphingomyelin in 100-μL acidic reaction buffer containing 100 mmol/L sodium acetate and 0.1% Triton X-100, pH 5.0, and incubated at 37°C for 15 minutes. The reaction was terminated by adding 1.5 mL of chloroform-methanol (2:1) and 0.2 mL of double-distilled water. The samples were then vortex mixed and centrifuged at 1000 × g for 5 minutes to separate into two phases. A portion of the upper aqueous phase containing [14C]-choline phosphate was transferred to scintillation vials and counted in a Beckman liquid scintillation counter. The choline phosphate formation rate (nmol/min/mg protein) was calculated to represent the enzyme activity.

LC–Electrospray Ionization Tandem MS for Quantitation of Ceramide

The separation, identification, and quantitation of ceramide in plasma were performed by LC–mass spectrometry (MS). The HPLC instrument was equipped with a binary pump, a vacuum degasser, a thermostat column compartment, and an autosampler (Waters, Milford, MA). The HPLC separations were performed at 70°C on an RP C18 Nucleosil AB column (5 μm, 70 × 2-mm i.d.) from Macherey Nagel (Duren, Germany). The mobile phase was a gradient mixture formed as previously described. The plasma lipids were extracted according to previous studies. The plasma lipids were extracted according to previous studies. To avoid any loss of lipids, the whole procedure was performed in siliconized glassware. MS detection was performed using a Quattro II quadrupole MS instrument (Micromass, Altrincham, UK) operating under Mass Lynx 3.5 and configured with a Z-spray electrospray ionization source. Source conditions were previously described.

Cell Culture

The conditionally immortalized mouse podocyte cell line, provided by Dr. P. E. Klotman (Division of Nephrology, Department of Medicine, Mount Sinai School of Medicine, New York, NY), was cultured on type I collagen–coated flasks or plates in RPMI 1640 medium supplemented with recombinant mouse interferon-γ at 33°C. After differentiation at 37°C for 10 to 14 days without interferon-γ, podocytes were used for the proposed experiments.

Confocal Microscopic Detection of MR and Its Colocalization with Asm in Podocytes

Podocytes were seeded on poly-L-lysine–coated chambers and treated with L-Hcys (40 μmol/L) or vehicle for 30 minutes. In an additional group of cells, amitriptyline (20 μM; Sigma) was added to pretreat cells for 30 minutes before the addition of L-Hcys. The detection of membrane raft (MR) clusters and their colocalization were performed as previously described. Briefly, podocytes were washed with cold PBS, fixed for 15 minutes in 4% paraformaldehyde, and then blocked with 1% bovine serum albumin in PBS for 30 minutes. For detection of colocalization of MR and Asm, podocytes were first incubated with Alexa488-labeled cholera toxin B at 1 μg/mL (Molecular Probes, Eugene, OR), as previously described. Then, the podocytes were incubated overnight with goat anti-Asm (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with 5 μg/mL Texas Red-conjugated anti-goat IgG for an additional 1 hour at room temperature. After mounting, the slides were observed using a confocal laser-scanning microscope (Fluoview FV1000; Olympus, Tokyo, Japan).

Urinary Total Protein and Albumin Excretion Measurements

The 24-hour urine samples were collected using metabolic cages and subjected to total protein and albumin excretion measurements. The total protein content in the urine was detected by the Bradford method using a UV spectrophotometer. Urine albumin was detected using a commercially available albumin enzyme-linked immunosorbent assay kit (Bethyl Laboratories, Montgomery, TX). The urinary creatinine concentrations were measured by using a QuantiChrom creatinine assay kit (BioAssay System, Hayward, CA).

Delivery of Asm shRNA into the Kidneys by an Ultrasonographic Microbubble Technique

After a 1-week recovery period from uninephrectomy, Asm shRNA or a scrambled shRNA plasmid with a luciferase expression vector was cotransfected into the kidneys via intrarenal artery injection using the ultrasonographic microbubble system. A full description of the procedures for the ultrasonographic microbubble gene transfer technique can be found in our previous studies. To daily monitor the efficiency of gene expression through somatic plasmid transfection, mice were anesthetized with isoflurane and an aqueous solution of luciferase-expressing plasmid. Photons emitted from luciferase-expressing cells within the animal body and transmitted through tissue layers were quantified over a defined period, ranging up to 5 minutes, using the software program Living Image (Xenogen, Hopkinton, MA). Phosphons emitted from luciferase-expressing cells within the animal body and transmitted through tissue layers were quantified over a defined period, ranging up to 5 minutes, using the software program Living Image (Xenogen). The inhibitory efficiency of gene expression by Asm shRNA was further confirmed by detection of the Asm mRNA level in mouse renal cortex using real-time RT-PCR.

ESR Analysis of O2− Production

For detection of Nox-dependent superoxide (O2⋅−) production, proteins from mouse renal cortex were extracted using sucrose buffer and resuspended with
modified Krebs-HEPES buffer containing deferoxamine (100 mmol/L; Sigma) and diethylthiocarbamate (5 mmol/L; Sigma). The Nox-dependent \( \text{O}_2^- \) production was examined by the addition of 1 mmol/L NADPH as a substrate in 50 mg of protein and incubated for 15 minutes at 37°C in the presence or absence of superoxide dismutase (200 U/mL) and then supplied with 1 mmol/L \( \text{O}_2^- \)-specific spin-trapping substance, 1-hydroxy-3-methoxyxyl-2,2,5,5-tetramethylpyrrolidine (Noxygen, Elzach, Germany). The mixture was loaded in glass capillaries and immediately analyzed for \( \text{O}_2^- \) production kinetically for 10 minutes in a Miniscope MS200 electromagnetic spin resonance (ESR) spectrometer (Magnettech Ltd, Berlin, Germany). The ESR settings were as follows: biofield, 3350; field sweep, 60 gauss; microwave frequency, 9.78 GHz; microwave power, 20 mW; modulation amplitude, 3 gauss; 4096 points of resolution; receiver gain, 20 for tissue and 50 for cells. The results were expressed as the fold changes of control.

**Real-Time RT-PCR**

Total RNA from isolated mouse renal tissue was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA), according to the protocol described by the manufacturer. RNA samples were quantified by measurement of optic absorbance at 260 and 280 nm in a spectrophotometer. The concentrations of RNA were calculated according to A260. Aliquots of total RNA (1 \( \mu \)g) from each sample were reverse transcribed into cDNA, according to the instructions of the first-strand cDNA synthesis kit manufacturer (Bio-Rad, Hercules, CA). Equal amounts of the reverse transcriptional products were subjected to PCR amplification using SYBR Green as the fluorescence indicator on a Bio-Rad iCycler system (Bio-Rad). The primers used in this study were synthesized by Operon (Huntsville, AL). The sequences were for the following: Asm, 5’-CACGTGGATGAGTTGAGT-3’ (sense) and 5’-AGAGCTCCACAGTAGTTAC-3’ (antisense); and \( \beta \)-actin, 5’-TCGTCCGGCTGGTCGTC-3’ (sense) and 5’-GCCCTCGTACCCACATAGGA-3’ (antisense). The mRNA levels of the target gene were normalized to the \( \beta \)-actin mRNA levels detected from the same samples.

**Double-Immunofluorescent Staining**

Double-immunofluorescent staining was performed using frozen slides of mouse kidneys. After fixation with acetone, the slides were incubated with rabbit anti-podocin antibody at 1:100 (Sigma), anti-desmin (1:50; BD Biosciences, San Jose, CA), or anti-ceramide (1:50; Enzo Life Sciences) antibodies overnight at 4°C. Then, these slides were washed and incubated with corresponding Texas Red–labeled secondary antibodies. Finally, the slides were washed, mounted, and subjected to fluorescent microscopy examination. The images were captured with a spot charge-coupled device camera and a pseudocolor was added to fluorescence showing in this slide (Diagnostic Instruments Inc., Sterlin Heights, MI). All exposure settings were kept constant for each group of kidneys.

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**Statistical Analysis**

Data are provided as arithmetic mean ± SEM; \( n \) represents the number of independent experiments. All data were tested for significance using analysis of variance or paired and unpaired Student’s t-test, as applicable. The GDI was analyzed using a nonparametric U-test. Only results with \( P < 0.05 \) were considered statistically significant.

**Results**

**Asm Expression and Activity in the Kidneys of Mice Lacking the Asm Gene during hHcys**

As shown in Figure 1, A and B, Asm mRNA expression and its activity were significantly lower in Asm/−/− than in Asm+/+ mice fed a normal diet. FF diet treatment significantly increased the Asm activity and mRNA expression in Asm+/+ mice when compared with normal diet–fed mice. However, this FF diet–induced Asm activity and Asm mRNA expression were significantly attenuated in Asm−/− mice (Figure 1, A and B). The total renal ceramide levels were similar in Asm+/+ and Asm−/− mice fed a normal diet. The FF diet significantly increased the total renal ceramide levels in Asm+/+ mice but not in Asm−/− mice (Figure 1C). Immunofluorescent analysis revealed that ceramide staining was more pronounced in glomeruli of FF diet–fed Asm+/+ mice than in normal diet–fed mice. However, FF diet–induced ceramide production was less in Asm−/− mice (Figure 1D). In addition, confocal microscopic studies using podocin as a podocyte marker showed that Hcys-induced ceramide expression in glomeruli was mostly located in podocytes, as demonstrated by the colocalization of ceramide with podocin. This colocalization was substantially blocked in Asm−/− mice (see Supplemental Figure S1 at [http://ajp.amjpathol.org](http://ajp.amjpathol.org)). The FF diet did not alter the neutral sphingomyelinase expression in glomeruli of both Asm+/+ and Asm−/− mice (see Supplemental Figure S1 at [http://ajp.amjpathol.org](http://ajp.amjpathol.org)). The plasma Hcys concentration was similar in Asm+/+ and Asm−/− mice. However, the FF diet significantly increased the plasma Hcys con-
Desmin staining was more pronounced in glomeruli of FF diet–fed Asm+/+ mice than normal diet–fed mice, as shown by more remarkable red fluorescence detected with glomeruli, which indicates podocyte injury. The lack of Asm decreased the FF diet–induced increase in desmin expression within glomeruli of mice (Figure 3A). Desmin is an intermediate filament protein and may be an injured podocyte marker. The expression of desmin is often increased in various glomerular diseases in which podocyte damage is involved.6 Conversely, another podocyte marker, podocin, was markedly reduced in glomeruli of Asm−/− mice fed the FF diet compared with those fed the normal diet. However, this reduced podocin expression or production did occur in Asm−/− mice, even receiving the FF diet (Figure 3B).

**Lack of Local Oxidative Stress in the Glomeruli of hHcys Mice Lacking the Asm Gene**

As illustrated in Figure 4A, the ESR spectrometric curve exhibited a significant increase in the amplitude of NOX-dependent O2− signals in the glomeruli of WT mice fed the FF diet compared with that observed in WT mice fed the normal diet. In Asm−/− mice, hHcys induced by the FF diet failed to increase glomerular O2− production. These results were summarized in Figure 4B, showing that glomerular O2− production was similar in Asm−/− and Asm+/+ mice fed the normal diet but increased by 2.5-fold in Asm+/+ mice only when fed the FF diet. FF diet–induced glomerular O2− production was much less in Asm−/− mice compared with Asm+/+ mice.

**Improvement of hHcys-Induced Glomerular Injury in Mice Lacking the Asm Gene**

As presented in Figure 2, A and B, urinary protein and albumin excretion was similar in Asm+/+ and Asm−/− mice fed a normal diet. However, an elevated plasma Hcys level, as the result of the FF diet, significantly increased the urinary total protein and albumin excretion in Asm+/+, but not in Asm−/−, mice. By PAS staining, we observed a typical pathological change in glomerular sclerotic damage in Asm+/+ mice fed the FF diet, such as mesangial expansion and glomerular capillary collapse. This pathological feature was not observed in Asm−/− mice. The average GDI was significantly higher in Asm+/+ than in Asm−/− mice when they were fed the FF diet (Figure 2, C and D).

An immunofluorescent histological analysis showed that desmin staining was more pronounced in glomeruli of FF diet–fed Asm+/+ mice than normal diet–fed mice, as shown by more remarkable red fluorescence detected with glomeruli, which indicates podocyte injury. The lack of Asm decreased the FF diet–induced increase in desmin expression within glomeruli of mice (Figure 3A). Desmin is an intermediate filament protein and may be an injured podocyte marker. The expression of desmin is often increased in various glomerular diseases in which podocyte damage is involved.6 Conversely, another podocyte marker, podocin, was markedly reduced in glomeruli of Asm−/− mice fed the FF diet compared with those fed the normal diet. However, this reduced podocin expression or production did occur in Asm−/− mice, even receiving the FF diet (Figure 3B).

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Decreased Asm Expression and Activity in the Kidney with Local Gene Silencing

To further determine the implication of Asm in glomerular injury during hHcys, an shRNA strategy was used to locally silence this gene in the kidney and then observe the changes in glomerular function and pathological features during hHcys. As illustrated by images obtained by an in vivo molecular imaging system, luciferase gene expression cotransfected with Asm shRNA could be detected even on the third day after the kidney was transfected by ultrasonographic microbubble plasmid introduction. In the hemidissected kidney, all of the cortical regions exhibited efficient gene transfection, as shown in green and red fluorescence (Figure 5, A and B). This is consistent with previous studies showing that ultrasonographic microbubble gene introduction is an efficient technique for delivery of the gene into the glomerular cells, vascular endothelial cells, and fibroblasts.

Figure 3. Immunofluorescence staining of desmin and podocin in glomeruli from Asm+/+ and Asm−/− mice, with or without the FF diet. A: Typical images of desmin staining in glomeruli from Asm+/+ and Asm−/− mice, with or without the FF diet (n = 6 in each group). B: Typical images of podocin staining in glomeruli from Asm+/+ and Asm−/− mice, with or without the FF diet (n = 6 in each group).

Figure 4. Effects of the normal diet (ND) and FF diet on glomerular O2− production in Asm+/+ and Asm−/− mice. A: Representative ESR spectra traces for O2− production in Asm+/+ and Asm−/− mice. B: Values are arithmetic mean ± SEM (n = 5 in each group) of O2− production in Asm+/+ and Asm−/− mice fed with the ND or FF diet. *P < 0.05 versus values from Asm+/+ mice fed the ND; **P < 0.05 versus values from mice fed the FF diet.

Figure 5. Renal Asm gene silencing efficiency in C57BL/6J mice, with or without the FF diet. A: Daily imaging confirmation of gene transfection in the kidney by an in vivo molecular imaging system. D, days. B: Localization of transfected gene expression in the hemidissected kidney on day 12 after gene delivery. Ctrl, control; Luci, luciferase. Values are arithmetic mean ± SEM (n = 4 to 8 in each group) of Asm mRNA expression (C), Asm activity (D), total ceramide concentrations (E), and ceramide staining (F) in ND- or FF diet-fed C57BL/6J mice, with or without Asm shRNA transfection. *P < 0.05 versus values from control mice fed the normal diet (ND); **P < 0.05 versus values from mice fed the FF diet.
The efficiency of local Asm gene silencing was also examined by measurement of its expression and activity when animals were sacrificed after completion of functional studies. Real-time RT-PCR analysis demonstrated that Asm mRNA expression was significantly decreased in C57BL/6J WT mice transfected with Asm shRNA compared with control mice fed a normal diet. Compared with the normal diet, the FF diet significantly increased Asm mRNA expression in renal cortical tissue from mice receiving scrambled shRNA, but it had no effect on Asm mRNA abundance in mice receiving Asm shRNA (Figure 5C). Correspondingly, the FF diet significantly increased the Asm activity in renal cortical tissue from mice receiving scrambled shRNA, but it was without effect in mice receiving Asm shRNA (Figure 5D). Moreover, the FF diet–induced decrease in podocin expression (Figure 7A) was significantly higher in FF diet–fed mice compared with that in normal diet–fed mice. However, the Asm shRNA transfection significantly attenuated FF diet–induced decrease in podocin expression (Figure 8). By immunofluorescent microscopic analysis, desmin had more profound abundance in glomeruli of FF diet–fed mice compared with that in normal diet–fed mice. Asm shRNA transfection abrogated the FF diet–induced increase in desmin expression (Figure 7A). In contrast, podocin was markedly reduced in glomeruli from FF diet–fed mice compared with those from normal diet–fed mice. However, the Asm shRNA transfection in FF diet–fed mice completely attenuated the decrease in podocin staining (Figure 7B).

**Blockade of hHcys-Induced Glomerular Injury by Asm Gene Silencing**

Similar to the results obtained from Asm−/− mice, HPLC analysis demonstrated that local silencing of the Asm gene in the kidney had no significant effect on the plasma Hcys level. In mice receiving either scrambled or Asm shRNA, the FF diet significantly increased the plasma Hcys concentrations (data not shown). This confirms that Asm gene manipulation in the kidney does not alter the plasma Hcys concentration levels in mice.

We further determined whether Asm gene silencing locally in the kidney has any beneficial action on the FF diet–induced glomerular injury. As shown in Figure 6, A and B, urinary protein and albumin excretion was similar in both scrambled and Asm shRNA–transfected mice fed the normal diet. FF diet treatment significantly increased the urinary total protein and albumin excretion when compared with the normal diet–fed mice. However, the Asm shRNA transfection significantly attenuated FF diet–induced urinary total protein and albumin excretion. Morphological analysis showed a typical pathological change in glomerular sclerotic damage, such as an expanded glomerular mesangium with capillary collapse, hypercellularity, and fibrous deposition in mice fed the FF diet. The average GDI was significantly higher in FF diet–fed mice compared with normal diet–fed mice receiving scrambled shRNA. However, when Asm shRNA was transfected into the kidney, the FF diet–induced glomerular injuries were substantially attenuated (Figure 6, C and D).

By immunofluorescent microscopic analysis, desmin had more profound abundance in glomeruli of FF diet–fed mice compared with that in normal diet–fed mice. Asm shRNA transfection abrogated the FF diet–induced increase in desmin expression (Figure 7A). In contrast, podocin was markedly reduced in glomeruli from FF diet–fed mice compared with those from normal diet–fed mice. However, the Asm shRNA transfection in FF diet–fed mice completely attenuated the decrease in podocin staining (Figure 7B).

**Suppression of hHcys-Induced Glomerular O2·− Production by Asm Gene Silencing**

As depicted in Figure 8, the glomerular O2·− production was similar in both scrambled and Asm shRNA transfected mice when they were fed a normal diet. However, the FF diet significantly increased the glomerular O2·− production by 1.9-fold in scrambled shRNA transfected mice, but it had no significant effect on glomerular O2·− production in mice with Asm shRNA transfection (Figure 8).

**Attenuation of L-Hcys–Induced MR Clustering in Podocytes by Asm Inhibition**

In addition, we determined the colocalization of MR and Asm in the plasma membrane on Hcys stimulation. In this regard, the podocytes were treated with Texas Red–anti-Asm and fluorescein isothiocyanate–cholera toxin B, and the distribution of anti-Asm within MR clusters was visualized in the podocyte membrane by confocal microscopy. Under the control condition, Asm was evenly spread throughout the whole cell. On Hcys stimulation, Asm translocated to the membrane, as shown by red fluorescence spots or patches. Yellow patches or dots in the overlaid images were considered a colocalization of Asm in MR clusters. Pretreatment of podocytes with the Asm inhibitor, amitriptyline, led to the disruption of MR and abrogated...
patching and clustering of both fluorescein isothiocyanate–
cholera toxin B and Asm after Hcys stimulation (see Sup-
plemental Figure S2 at http://ajp.amjpathol.org).

Discussion

The goal of the present study is to determine whether
Asm, a ceramide-producing enzyme, is implicated in the
development of hHcys-induced glomerular oxidative
stress and injury. We found that FF diet treatment en-
hanced the Asm activity, Asm mRNA expression, and
ceramide production, which was attributed to Nox-de-
pendent O$_2$•⁻ production and local oxidative stress in
glomeruli and ultimately led to glomerulosclerosis. Our
results demonstrate, for the first time to our knowledge,
FF diet–fed Asm\(^{+/+}\) mice but not in Asm\(^{-/-}\) mice. In addition, our studies demonstrated that Hcys stimulation enhanced the colocalization of MR and Asm in the plasma membrane and confirmed the translocation of Asm into the cell membrane. These results together suggest that FF diet–induced Hcys increases the renal and glomerular ceramide levels, mainly because of activation of Asm and an increase in its expression.

Furthermore, we demonstrated that decreased ceramide production via Asm has a protective role in the glomerular injury associated with hHcys. In accordance with lowered ceramide production in Asm\(^{-/-}\) mice fed the FF diet, urinary albumin, protein excretion, and glomerular podocyte injury and sclerosis were also significantly decreased compared with Asm\(^{+/+}\) mice fed the FF diet, suggesting that ceramide-associated renal injury during hHcys is alleviated in these Asm gene knockout mice. Therefore, this sphingomyelinase could be a target of a therapeutic strategy for hHcys-induced glomerular injury or sclerosis.

To further explore the mechanism of glomerular injury during hHcys, we observed changes in podocyte function in Asm\(^{+/+}\) and Asm\(^{-/-}\) mice. It has been well documented that podocyte loss and dysfunction occur with the onset and magnitude of glomerulosclerosis. Because podocytes serve as the final barrier against urinary protein loss in the normal glomeruli, any change in podocyte structure and function may be intimately associated with proteinuria and consequent glomerular sclerosis. The present study showed that podocin protein was markedly decreased in FF diet–fed Asm\(^{+/+}\) mice but not in mice lacking Asm. In addition, we found that desmin, an intermediate filament protein and a specific and sensitive podocyte injury marker, was increased in the glomeruli when Asm\(^{+/+}\) mice received the FF diet. This increased desmin expression in the glomeruli was attenuated in FF diet–fed Asm\(^{-/-}\) mice. These results further support the view that hHcys-induced glomerular injury is associated with increased ceramide production via Asm.

Reportedly, oxidative stress has been implicated in the development of glomerular injury and end-stage renal disease. In our previous studies, Nox-derived \(O_2^-\) production was an important mechanism mediating hHcys-induced glomerular injury or damage. NADPH-dependent \(O_2^-\) production is an early event for homocysteine-induced glomerular cell damage and glomerular sclerosis.\(^{4,35}\) It is possible that hHcys-induced Nox activation is mediated by enhanced Asm activity. The present study hypothesized that Asm may be associated with local oxidative stress in the glomeruli of mice with hHcys. Indeed, electron-spin resonance analysis showed that FF diet treatment significantly increased Nox-dependent \(O_2^-\) production in Asm\(^{+/+}\), but not in Asm\(^{-/-}\), mice. These results confirm the imperative role of Asm in mediating \(O_2^-\) production through the activation of Nox in the glomeruli during hHcys.

To further address the role of the Asm gene in mediating hHcys-induced podocyte and glomerular injury, a local gene-silencing strategy was used in the present study, in which ultrasonographic microbubble–mediated plasmid delivery was used to introduce Asm shRNA into the kidney. This method was highly efficient in delivering plasmids into renal cells in vivo, which led to gene transfection and expression in most renal cells (90%), as confirmed by earlier reports.\(^{2,45,48}\) By using an in vivo molecular imaging system to daily monitor the efficiency of Asm gene transfection in the kidney in living animals, we showed that the transgene or shRNA expression vector (with the luciferase gene as an indicator) could be detected even 3 days after gene transfection and lasted for 4 weeks by observation. This in vivo transgene monitoring importantly guided our functional studies to define the role of the Asm gene in mediating glomerular damage associated with hHcys. After completion of functional protocols, Asm mRNA expression, Asm activity, and renal and glomerular ceramide production were analyzed to confirm the efficient silencing of the Asm gene in shRNA-transfected kidneys. In such local Asm gene–silenced kidney, we found that the glomerular ceramide level was significantly decreased and that \(O_2^-\) production was markedly reduced. Silencing the Asm gene in the kidney ameliorates proteinuria/albuminuria and podocyte injury, whereby glomerular sclerotic changes were substantially suppressed. These results from mice with local renal Asm gene silencing further support the previous conclusion, drawn from studies using Asm-knockout mice that Asm is importantly implicated in the development of podocytes and glomerular injury. The reduction of ceramide production or the inhibition of Nox is preventive to hHcys-induced glomerular injury. Further studies with a careful experimental design for therapeutic action via this signaling system are needed to confirm its therapeutic potential. In conclusion, the present study demonstrated that Asm gene deficiency attenuates the hHcys-induced glomerular oxidative stress and injury. The amelioration of glomerular injury by Asm deficiency during hHcys implicates the pivotal role of Asm in hHcys-induced glomerulosclerosis.

References


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