NM-3, an inhibitor of angiogenesis, ameliorates renal alterations in obese type 2 diabetic mice

Kunihiro Ichinose, Yohei Maeshima, Yoshihiko Yamamoto, **Masaru Kinomura**, Kumiko Hirokoshi, Hiroyuki Kitayama, Yuki Takazawa, Hitoshi Sugiyama, Yasushi Yamasaki, Naoki Agata# and Hirofumi Makino

Department of Medicine and Clinical Science, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan; #Genzyme, Boston, MA, USA.

Online-Only Appendix

Research design and methods

Experimental protocols. Blood glucose was monitored every week. No mice died and no signs of apparent exhaustion were observed during the experimental period. At 0 and 8 weeks after initiating the intraperitoneal injection of NM-3, the body weight and the individual 24-hr food consumption were measured. At 8 weeks after initiating treatment, individual 24-hr urine sample collection was performed using metabolic cages. Non-fasting blood samples were drawn from the retro-orbital venous plexus using heparinized capillary tubes under anesthesia at the time of sacrifice. Kidney, liver and heart weight was measured just after sacrifice. NM-3 (3-(2-methylcarboxymethyl)-6-methoxy-8-hydroxyisocoumarin, Genzyme, Boston, MA) was dissolved in PBS and filtered to be used for the animal experiments. The dosage of NM-3 used in the present study was determined according to previous reports using NM-3 (1; 2). NM-3 has a serum half-life of 3-10 hours in preclinical models, a low toxicity profile, and thus a potentially wide therapeutic window (2). According to the manufacturer's information, oral dosage of NM-3 at 1g/kg was well tolerated in animals without acute toxicity.

Blood and urine examination. Serum levels of mouse insulin were determined using ultrasensitive rat insulin enzyme-linked immunosorbent assay kit and mouse insulin standard (Morinaga, Inc., Yokohama, Japan) following the manufacturer's instructions. According to the manufacturer's technical information, mouse insulin can be measured in combination with mouse insulin standard due to a high homology among mammalian animals. All samples were examined in duplicate, and mean values of individual sera were utilized for statistical analysis. The intra- and inter assay coefficients of variation for the insulin assays were less than 5% and 10%, respectively.

Histological Analysis. Mean glomerular tuft volume (G_V) was determined from the mean glomerular cross-sectional tuft area (G_A) as described previously (3-5). Thirty glomeruli from each cortical area were observed, images were taken and analyzed by using NIH image to determine the mean G_A. G_V was calculated as $G_V = \beta/k \times (G_A)^{3/2}$, with $\beta = 1.38$, the shape coefficient for spheres and $k = 1.1$, a size distribution coefficient (3). More than 30 glomerular cross-sections were observed by two investigators and averaged to determine glomerular volume.

Mesangial matrix index was defined as the proportion of the glomerular tuft occupied by the mesangial matrix excluding nuclei. The mesangial matrix areas of 20 glomeruli in each kidney were analyzed and averaged. The mesangial matrix areas were selected using Photoshop software (Adobe Systems Inc., San Jose, CA), followed by analysis using NIH image.

Immunohistochemistry. For immunohistochemistry of CD31 and type IV collagen, frozen sections (4-μm) were fixed in acetone. Then, sections were blocked with 10% normal goat serum (Sigma) followed by incubation with rat anti-mouse CD31 monoclonal antibody (Pharmingen, San Diego, CA) or polyclonal rabbit anti-mouse type IV collagen antibody (Chemicon International, Inc., Temecula, CA) overnight. Sections were then washed, and incubated with FITC-conjugated anti-rat IgG

secondary antibodies (CD31) or FITC-conjugated anti-rabbit IgG secondary antibodies (type IV collagen) for 30 min at room temperature. After washing in PBS, sections were observed by a confocal laser fluorescence microscope (LSM-510; Carl Zeiss, Jena, Germany). The immunoreactivity of glomerular CD31 or type IV collagen was quantified as follows; color images were obtained as TIF files by LSM-510. The brightness of each image file was uniformly enhanced by Photoshop software followed by analysis using NIH image. Image files (TIFF) were inverted and opened in gray scale mode. Type IV Collagen or CD31 indices were calculated using the following formula, {[X (density) x positive area (μm^2)]/ glomerular total area (μm^2)}, where the staining density is indicated by a number from 0 to 256 in gray scale.

Glomerular accumulation of monocyte/macrophage was determined by immunohistochemistry using rat anti-mouse F4/80 antibody (Serotec, Oxford, UK). Frozen sections were fixed in acetone and subjected to immunoperoxidase staining using the Vectastain ABC Elite reagent kit as previously described (4; 6; 7). Diamino-benzidine was used as a chromogen. The number of F4/80-positive cells was determined by observing more than 20 glomeruli from each section.

For immunohistochemistry of VEGF, formalin (10%)-fixed, paraffin-embedded sections (4-μm) were used. After deparaffinization, sections were incubated with rabbit polyclonal anti-VEGF antibody (Santa Cruz Biotechnology, Inc.) followed by incubation with biotinylated-secondary antibody, and immunoperoxidase staining was carried out utilizing the Vectastain ABC Elite reagent kit (Vector Labs, Burlingame, CA) as previously described (4; 5). Diamino-benzidine was used as a chromogen. All slides were counterstained with hematoxylin. Normal rabbit IgG was used as a negative control.

RNA Extraction and quantitative real-time polymerase chain reaction (real-time PCR). Kidneys from each mouse were homogenized and total RNA was extracted using RNeasy Midi Kit (Qiagen, Chatsworth, CA) and stored at -

80°C until use. Total RNA was subjected to RT with poly-d (T) primers or random primers and reverse transcriptase (GeneAmp RNA PCR Kit; Applied Biosystems, Foster City, CA). Quantitative real-time PCR was used to quantify the mRNA levels of nephrin, IL-6, MCP-1 and TGF-β1, and the amount of 18s rRNA. cDNA was diluted 1:50 with autoclaved deionized water. For the detection of nephrin mRNA level, 10 μl of the diluted cDNA was added to the Lightcycler-Mastermix, 0.5 μM of specific primer, 3 mM of $MgCl_2$ and 2 μl of Master SYBR Green (Roche Diagnostics, Mannheim, Germany). For the detection of IL-6, MCP-1 and TGF-β1 mRNA levels, 5 μl of the diluted cDNA was added to the Lightcycler-Mastermix, 1 μM of specific primer, 3 mM of $MgCl_2$ and 2 μl of Master SYBR Green. For detecting the level of 18s rRNA, 2 μl of the diluted cDNA was added to the Lightcycler-Mastermix, 0.5 μM of specific primer, 3 mM of $MgCl_2$ and 2 μl of SYBR Premix Ex Taq (Takara Bio, Japan). These reaction mixtures were filled up to a final volume of 20 μl with water. PCR reactions were carried out in a real-time PCR cycler (Lightcycler; Roche Diagnostics). The program was optimized and performed finally as denaturation at 95°C for 10 min followed by 40 cycles of amplification (nephrin; 95°C for 10 s; 60°C for 15 s; 72°C for 9 s, IL-6 and 18s rRNA; 95°C for 10 s; 60°C for 20 s, MCP-1; 95°C for 10 s; 62°C for 10 s; 72°C for 6 s, TGF-β1; 95°C for 10 s; 61°C for 10 s; 72°C for 11 s, respectively). The temperature ramp rate was 20°C/s. At the end of each extension step, the fluorescence was measured to quantitate the PCR products. After completion of the PCR, the melting curve of the product was measured by temperature gradient from 65 to 95°C at 0.1 or 0.2°C/s with continuous fluorescence monitoring to produce a melting profile of the primers. The amount of PCR products was normalized with 18s rRNA to determine the relative expression ratio for nephrin, IL-6, MCP-1 or TGF-β1 mRNA in relation to 18s rRNA. The following oligonucleotide primers specific for mouse nephrin, IL-6, MCP-1, TGF-β1 and 18s rRNA were used: nephrin, 5'-ATCTCCAAGACCCCAGGTACACA-3' (forward) and 5'-AGGGTCAGGACGGCTGA T-3' (reverse); IL-6 ,5'-CCACTTCACAAGTCGGAGGCTTA-3' (forward) and 5'-GCAA GTGCATCATCGTTGTTCATAC-3' (reverse); MCP-1, 5'-AAG CTGTAGTTTTTGTC

ACC-3' (forward) and 5'-GGGCAGATGCAGTTTTAA-3' (reverse); TGF-β1, 5'-AACAA CGCCATCTATCAG-3' (forward) and 5'-TATTCCGTCTCCTTGGTT-3' (reverse); 18s rRNA, 5'-ACTCAACACGGGAAACCTCA-3' (forward) and 5'-AACCA GACAAATC GCTCCAC-3' (reverse). Four independent experiments were performed.

Cell culture. **Conditionally immortalized mouse podocytes, generous gifts from Dr. Peter Mundel (Albert Einstein College of Medicine, Bronx, NY), were cultured on type I collagen-coated flasks (BD Falcon, SanJose, CA) with RPMI 1640 (Invitrogen, Carlsbad, CA) containing 10% fetal calf serum (FCS; Cansera International Inc., Canada), 100 U/mL penicillin, 100 μg/mL streptomycin. To propagate podocytes, cells were cultivated at 33°C and treated with 50 U/mL of recombinant mouse IFN-γ (BD Bioscience, Palo Alto, CA) to enhance expression of the large T antigen. After 90% confluence, the cells were induced to differentiate into podocyte lineage by shifting them to 37°C and culturing in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO) that contained 10%FCS without IFN-γ, *i.e.*, nonpermissive conditions. Under this condition, the majority of cells had an arborized shape and expressed podocyte specific synaptopodin. After 6 days of culture under nonpermissive conditions, the cells were further cultured in DMEM that contained 0.5% FCS for 24 hr, and quiescent mature podocytes were incubated with 5.5 mM normal glucose with PBS-DTT buffer (NG), NG with 19.5 mM mannitol (NG/Manni), 25 mM high glucose with PBS-DTT buffer (HG/N0), HG with 1 μg/ml of NM-3 (HG/N1), 10 μg/ml of NM-3 (HG/N10) or 100 μg/ml NM-3 (HG/N100) for 48 hr.**

Primary murine mesangial cells (MES13) were purchased from the ATCC (Rockville, MD). Mesangial cells in these studies were used between the 10th and 20th passage. Characteristics of mesangial cells were confirmed by immunoreactivity for actin and desmin and lack of staining for factor VIII as previously described (6). Cells were cultured in DMEM containing 10% FCS at 37°C. After subconfluence, cells were starved for 24 hr by incubating them in

DMEM containing 0.4% FCS. Quiescent cells were incubated with 5.5 mM NG, NG with 19.5 mM mannitol (NG/Manni), 25 mM high glucose with PBS-DTT buffer (HG/N0), HG with 1 μg/ml NM-3 (HG/N1), 10 μg/ml NM-3 (HG/N10) or 100 μg/ml NM-3 (HG/N100) for 48 hr. They then were subjected to western blot analysis.

Immunoblot. Briefly, kidneys were homogenized in radioimmunoprecipitation assay (RIPA) buffer (30 μl of 2.2 mg/ml aprotinin, 10 μl of 10 μg/ml phenylmethylsulfonyl fluoride, and 10 μl of 100 mM sodium orthovanadate per 1 ml of RIPA buffer) at 4°C. Similarly, cultured mesangial cells or podocytes were lysed using RIPA buffer as previously described (8). After centrifugation at 14,000 rpm for 30 min at 4°C, supernatant was collected and stored at -80°C until use. Total protein concentration was determined by using DC-protein determination system (Bio-Rad) using bovine serum albumin (BSA) as a standard. Samples were processed for SDS-PAGE and proteins were electrotransferred onto nitrocellulose membrane (Hybond-ECL; Amersham). The membranes were blocked with 5% nonfat dry milk in 1X TBS (0.1% Tween-20) for 1 hr, incubated overnight with polyclonal rabbit anti-mouse angiopoietin-1, angiopoietin-2 (Alpha diagnostics, San Antonio, TX), anti-VEGF-A (Santa Cruz), nephrin (Research Diagnostics, Inc., Flanders, NJ), TGFβ (Santa Cruz), MCP-1 (BioLegend, San Diego, CA) and TNF-α (BD Pharmingen) antibodies at 4°C. After incubation with HRP-labeled secondary antibodies for 1h, signals were detected with ECL system (Amersham). Membranes were re-probed with rabbit polyclonal anti-actin antibodies (Bio-Rad) to serve as controls for equal loading. The density of each band was determined by using NIH image software, and expressed as a value relative to the density of corresponding band obtained from actin immunoblot.

References

1. Salloum RM, Jaskowiak NT, Mauceri HJ, Seetharam S, Beckett MA, Koons AM, Hari DM, Gupta VK, Reimer C, Kalluri R, Posner MC, Hellman S, Kufe DW, Weichselbaum RR: NM-3, an isocoumarin, increases the antitumor effects of radiotherapy without toxicity. *Cancer Res* 60:6958-6963, 2000
2. Reimer CL, Agata N, Tammam JG, Bamberg M, Dickerson WM, Kamphaus GD, Rook SL, Milhollen M, Fram R, Kalluri R, Kufe D, Kharbanda S: Antineoplastic effects of chemotherapeutic agents are potentiated by NM-3, an inhibitor of angiogenesis. *Cancer Res* 62:789-795, 2002
3. Weibel ER: Stereological Methods. In *Practical Methods for Biological Morphometry* London, Academic, 1979, p. 51-57
4. Yamamoto Y, Maeshima Y, Kitayama H, Kitamura S, Takazawa Y, Sugiyama H, Yamasaki Y, Makino H: Tumstatin Peptide, an inhibitor of angiogenesis, prevents glomerular hypertrophy in the early stage of diabetic nephropathy. *Diabetes* 53:1831-1840, 2004
5. Ichinose K, Maeshima Y, Yamamoto Y, Kitayama H, Takazawa Y, Hirokoshi K, Sugiyama H, Yamasaki Y, Eguchi K, Makino H: Anti-angiogenic endostatin peptide ameliorates renal alterations in the early stage of type 1 diabetic nephropathy model. *Diabetes* 54:in press, 2005
6. Maeshima Y, Kashihara N, Yasuda T, Sugiyama H, Sekikawa T, Okamoto K, Kanao K, Watanabe Y, Kanwar YS, Makino H: Inhibition of mesangial cell proliferation by E2F decoy oligodeoxynucleotide in vitro and in vivo. *J Clin Invest* 101:2589-2597., 1998
7. Hashimoto N, Maeshima Y, Satoh M, Odawara M, Sugiyama H, Kashihara N, Matsubara H, Yamasaki Y, Makino H: Overexpression of Angiotensin Type 2 Receptor Ameliorates Glomerular Injury in mouse remnant kidney model. *Am J Physiol Renal Physiol*, 2003

8. Maeshima Y, Sudhakar A, Lively JC, Ueki K, Kharbanda S, Kahn CR, Sonenberg N, Hynes RO, Kalluri R: Tumstatin, an endothelial cell-specific inhibitor of protein synthesis. *Science* 295:140-143., 2002