

Anti-angiogenic Endostatin peptide ameliorates renal alterations in the early stage of type 1 diabetic nephropathy model

Kunihiro Ichinose, Yohei Maeshima, Yoshihiko Yamamoto, Hiroyuki Kitayama, Yuki Takazawa, Kumiko Hirokoshi, Hitoshi Sugiyama, Yasushi Yamasaki, *Katsumi Eguchi and Hirofumi Makino

*Department of Medicine and Clinical Science, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan; *First Department of Internal Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan.*

Online-Only Appendix

Research design and methods

Measurement of blood pressure. Arterial blood pressure was measured before sacrifice using a programmable sphygmomanometer (BP-98A; Softron, Tokyo, Japan) by the tail-cuff method as described previously [Hashimoto, 2003 #102].

Histological Analysis. Mean glomerular tuft volume (G_v) was determined from the mean glomerular cross-sectional tuft area (G_A) as described previously [Weibel, 1979 #127; Yamamoto, 2004 #162]. 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 [Weibel, 1979 #127]. More than 30 glomerular cross-sections were observed by two investigators and averaged to determine glomerular cell number and capillary number.

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, $\{[X (\text{density}) \times \text{positive area } (\mu\text{m}^2)] / \text{glomerular total area } (\mu\text{m}^2)\}$, where the staining density is indicated by a number from 0 to 256 in gray scale.

Double immunofluorescent staining was performed as previously described [Hamano, 2003 #141]. Briefly, frozen sections (4- μm) were fixed in cold (-20°C) acetone for 3 min and then air dried. Sections were incubated with primary antibodies, rat anti-mouse $\alpha 5$ integrin (BD Pharmingen), hamster anti-mouse CD31 (Chemicon) at 4°C overnight. Subsequently, sections were washed three times in PBS and incubated with Alexa Fluor 546-labeled goat anti-rat IgG or Alexa Fluor 488-labeled goat anti-hamster IgG (Molecular Probes) at room temperature for 1hr. After three washes with PBS, Vectashield anti-fade mounting medium (Vector Laboratories) was applied and sections were observed by a confocal laser fluorescence microscope (LSM-510; Carl Zeiss, Jena, Germany) and images were obtained. Normal rat and hamster IgG were used as negative controls.

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 [Maeshima, 1998 #63; Hashimoto, 2003 #102; Yamamoto, 2004 #162]. 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 (3- μ m) were used. After deparaffinization, sections were incubated with rabbit polyclonal anti-human 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 [Yamamoto, 2004 #162]. 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- β 1, and the amount of 18s rRNA. cDNA was diluted 1:50 with autoclaved deionized water. For the detection of nephrin mRNA level, 10 μ l of the diluted cDNA was added to the Lightcycler-Masternix, 0.5 μ M of specific primer, 3 mM of MgCl_2 and 2 μ l of Master SYBR Green (Roche Diagnostics, Mannheim, Germany). For the detection of IL-6, MCP-1 and TGF- β 1 mRNA levels, 5 μ l of the diluted cDNA was added to the Lightcycler-Masternix, 1 μ M of specific primer, 3 mM of MgCl_2 and 2 μ l of Master SYBR Green. For detecting the level of 18s rRNA, 2 μ l of the diluted cDNA was added to the Lightcycler-Masternix, 0.5 μ M of specific primer, 3 mM of MgCl_2 and 2 μ l of SYBR Premix Ex Taq (Takara Bio, Japan). These reaction mixtures were filled up to a final

volume of 20 μ l 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- β 1; 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- β 1 mRNA in relation to 18s rRNA. The following oligonucleotide primers specific for mouse nephrin, IL-6, MCP-1, TGF- β 1 and 18s rRNA were used: nephrin, 5'-ATCTCCAAGACCCCAGGTACACA-3' (forward) and 5'-AGGGTCAGGACGGCTGAT-3' (reverse); IL-6, 5'-CCACTTCACAAGTCGGAGGCTTA-3' (forward) and 5'-GCAAGTGCATCATCGTTGTTTCATAC-3' (reverse); MCP-1, 5'-AAG CTGTAGTTTTTGTCACC-3' (forward) and 5'-GGGCAGATGCAGTTTTAA-3' (reverse); TGF- β 1, 5'-AACAA CGCCATCTATCAG-3' (forward) and 5'-TATTCCGTCTCCTTGGTT-3' (reverse); 18s rRNA, 5'-ACTCAACACGGGAAACCTCA-3' (forward) and 5'-AACCA GACAAATCGCTCCAC-3' (reverse). Four independent experiments were performed.

Immunoblot. Briefly, kidneys were homogenized in radioimmunoprecipitation assay (RIPA) buffer (30 μ l of 2.2 mg/ml aprotinin, 10 μ l of 10 μ g/ml phenylmethylsulfonyl fluoride, and 10 μ l of 100 mM sodium orthovanadate per 1 ml of RIPA buffer) at 4°C. 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](#), flk-1, Tie2 (SantaCruz), [nephrin \(Research Diagnostics, Inc., Flanders, NJ\)](#) and [endostatin \(R&D systems, Inc.\)](#) 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.

[***ELISA.***](#) Serum levels of mouse VEGF-A were determined by using [Quantikine Mouse VEGF Immunoassay kit \(R&D Systems, Inc., Minneapolis, MN\)](#) following the manufacturer's instructions. Serum levels of mouse endostatin were determined by using the [ACCUCYTE Murine Endostatin EIA kit \(Cytoimmune Sciences, Inc., Rockville, MD\)](#) following the manufacturer's instructions. The latter kit is designed to measure the total (bound and free) amount of endostatin in serum and plasma. According to the manufacturer's technical information, measurement of each protein was not interfered in the presence of VEGF-B, VEGF-D, VEGF-R2 or VEGF-R3 (VEGF-A-ELISA) and cytokines, growth factors or heat-inactivated mouse endostatin (endostatin-ELISA). All samples were examined in duplicate, and mean values of individual sera were utilized for statistical analysis. The mean minimum detectable dose of VEGF-A was 7.8 pg/mL. The intra- and inter assay coefficients of variation were less than 8.2% and 8.4%, respectively (VEGF-A). The mean minimum detectable dose of endostatin was 1.95 ng/mL. Both the intra- and inter assay coefficients of variation were less than 10.0% (endostatin). In every assay, we observed proper standard curve by using serial dilutions of recombinant mouse VEGF-A or endostatin protein as described in the manufacturer's instructions.