"A severe diabetic nephropathy model with early development of nodule-like lesions induced by megsin overexpression in RAGE/iNOS transgenic mice"

Inagi, R ; Yamamoto, Y ; Nangaku, M ; Usuda, N ; Okamato, H ; Kurokawa, K ; Van Ypersele de Strihou, Charles ; Yamamoto, H ; Miyata, T.

ABSTRACT

Many factors are involved in the pathogenesis of diabetic nephropathy. A single gene abnormality may be prerequisite but insufficient to the disease to manifest. It is therefore only when a second or sometimes a third damage is associated that the consequences of pathogenic phenotypes become evident. We generated the triple transgenic mice overexpressing megsin (a novel glomerular-specific serpin), a receptor for advanced glycation end products (RAGE), and inducible nitric oxide synthase (iNOS). Compared with the single- or two-gene transgenic mice, the triple transgenic mice developed, at an early age (16 weeks), severe albuminuria and renal damage with all of the characteristics of human diabetic nephropathy (i.e. glomerular hypertrophy, diffuse mesangial expansion, inflammatory cell infiltration, and interstitial fibrosis). Interestingly, 30-40% of glomeruli exhibit nodule-like lesions. Oxidative and carbonyl stress markers (pentosidine, N-carboxymethyllysine, and 8-hydroxy-deoxyguanosine) were significantly higher in the triple transgenic mice. The iNOS transgenic mice have a diabetes phenotype, the renal consequences of which are moot, and the superimposition of RAGE leads to more conspicuous manifestations. By additional overexpression of megsin, a gene known to be involved in mesangial proliferation and expansion, these local consequences become dramatically manifest and approximate those observed in human pathology. This multiple hit approach is of interest in consideration of the sequential events during development of diabetic nephropathy.

CITE THIS VERSION

A Severe Diabetic Nephropathy Model With Early Development of Nodule-Like Lesions Induced by Megsin Overexpression in RAGE/iNOS Transgenic Mice

Reiko Inagi,1 Yasuhiko Yamamoto,2 Masaomi Nangaku,3 Nobuteru Usuda,4 Hiroshi Okamoto,5 Kiyoshi Kurokawa,1 Charles van Ypersele de Strihou,6 Hiroshi Yamamoto,2 and Toshio Miyata1

Many factors are involved in the pathogenesis of diabetic nephropathy. A single gene abnormality may be prerequisite but insufficient to the disease to manifest. It is therefore only when a second or sometimes a third damage is associated that the consequences of pathogenic phenotypes become evident. We generated the triple transgenic mice overexpressing megsin (a novel glomerular-specific serpin), a receptor for advanced glycation end products (RAGE), and inducible nitric oxide synthase (iNOS). Compared with the single- or two-gene transgenic mice, the triple transgenic mice developed, at an early age (16 weeks), severe albuminuria and renal damage with all of the characteristics of human diabetic nephropathy (i.e., glomerular hypertrophy, diffuse mesangial expansion, inflammatory cell infiltration, and interstitial fibrosis). Interestingly, 30–40% of glomeruli exhibit nodule-like lesions. Oxidative and carbonyl stress makers (pentosidine, N\textsubscript{-}carboxymethyllysine, and 8-hydroxy-deoxyguanosine) were significantly higher in the triple transgenic mice. The iNOS transgenic mice have a diabetes phenotype, the renal consequences of which are moot, and the superimposition of RAGE leads to more conspicuous manifestations. By additional overexpression of megsin, a gene known to be involved in mesangial proliferation and expansion, the local consequences become dramatically manifest and approximate those observed in human pathology. This multiple hit approach is of interest in consideration of the sequential events during development of diabetic nephropathy. Diabetes 55:356–366, 2006

D iabetic nephropathy is increasing dramatically worldwide and is now the most common cause of end-stage renal failure requiring renal replacement therapy (1,2). Its molecular pathogenesis is thus becoming a target of active research. Many factors and pathways are involved in the pathogenesis of diabetic nephropathy. A single gene abnormality may be prerequisite but not sufficient to the disease to manifest as the resilience of the various parts of our biological network: the absence or excess of one element is readily compensated for by other elements. It is therefore only when a second-hit or sometimes a third-hit damage is associated that the consequences of the various pathogenic phenotypes are evident.

The transgenic mice overexpressing a receptor for advanced glycation end products (RAGE) in vascular endothelial cells and inducible nitric oxide synthase (iNOS) in islet β-cells develop insulin-dependent diabetes and eventual nephropathy characterized by albuminuria with mild glomerular hypertrophy and mesangial expansion at the age of 16 weeks, followed by development of glomerulosclerosis at 34 weeks (3). On the other hand, overexpression of megsin, a novel serine protease inhibitor (serpin), predominantly expressed in mesangial cells (4,5) and upregulated in renal diseases, including diabetic nephropathy (6,7), in mice induces progressive mesangial cell proliferation and expansion at the age of 40 weeks (8).

In the present study, we generated the triple transgenic mice overexpressing megsin, RAGE, and iNOS. Compared with the age-matched single- or two-gene transgenic mice, the triple transgenic mice developed severe nephropathy at an early age, characterized by prominent albuminuria, and glomerular and tubulointerstitial damage at the early age of 16 weeks after birth. Of note, 30–40% of glomeruli exhibit segmental sclerosis reminiscent of diabetic nodular lesions.

Here, we demonstrate an experimental model of severe diabetic nephropathy that exhibits all of the characteristics of human diabetic nephropathy, including mesangial expansion and nodular-like lesions. The renal consequences are moot in a single genetic abnormality, whereas the superimposition of a second-hit damage leads to more conspicuous manifestations. When, in addition, a third hit is associated, the local consequences become markedly manifest and approximate those observed in human pathology. Although it remains to be determined to what extent the three gene abnormalities are relevant to the
development of human diabetic nephropathy, the present multiple hit approach is of interest in consideration of the sequential events during development of diabetic nephropathy in which various pathogenic factors are superimposed.

**RESEARCH DESIGN AND METHODS**

**Triple transgenic mice overexpressing megsin, RAGE, and iNOS.** Megsin transgenic mice (line A, genetic background C57BL/6N) (8) were cross-bred with an insulin-dependent diabetic mouse line overexpressing human RAGE in vascular endothelial cells and iNOS in Langerhans islet β-cells, respectively (RAGE/iNOS transgenic mice, line 102, genetic background CD-1) (3). After verification of the transgenes by PCR analysis according to the previous procedures (3,8), the obtained triple transgenic mice overexpressing megsin, RAGE, and iNOS were backcrossed with CD-1 mice more than four times. They were fed a high-calorie diet food (Labo H Standard; Nousan, Kanagawa, Japan). Twenty consecutive glomeruli in the midcortex were measured. The serum samples were analyzed using by a Biochemical autoanalyzer DRI-CHEM 3500V (Fujifilm, Tokyo, Japan). Ten areas in each slide from wild-type, RAGE/iNOS transgenic, or the triple transgenic mice (n = 5) were examined.

**Biochemical analysis of serum and urine sample.** The serum samples were analyzed using by a Biochemical autoanalyzer DRI-CHEM 3500V (Fujifilm, Tokyo, Japan) and a Creatinine-Test Wako (Wako Pure Chemical Industries, Osaka, Japan). For morphometric analysis in glomeruli, the glomerular tuft was measured in a blinded manner using the software NIH ImageJ (National Institutes of Health, Bethesda, MD). Ten areas in each slide from wild-type, RAGE/iNOS transgenic, or the triple transgenic mice (n = 5) were examined.

**Immunohistochemistry.** To examine mesangial matrix deposition, 4-μm sections of methyl Carnoy’s fixed tissues were stained with antibodies as follows, according to the method described previously (8). In brief, type IV and type I collagens were localized using an indirect immunoperoxidase method with polyclonal goat anti–type IV or anti–type I collagen antibodies (1:100 dilution), respectively (Southern Biotechnology Associates, Birmingham, AL). Fibronectin or laminin was localized using the rabbit antifibronectin antibody (1:100 dilution). The serum samples were analyzed using by a Biochemical autoanalyzer DRI-CHEM 3500V (Fujifilm, Tokyo, Japan).

**Kidney weight/body weight**

<table>
<thead>
<tr>
<th></th>
<th>Wild-type</th>
<th>RAGE/iNOS Tg</th>
<th>Triple Tg</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>6</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>2.5 ± 0.1</td>
<td>7.8 ± 1.1*</td>
<td>7.4 ± 2.5*</td>
</tr>
<tr>
<td>Blood urea nitrogen (mg/dl)</td>
<td>30.6 ± 4.6</td>
<td>39.6 ± 7.0*</td>
<td>38.3 ± 18.7*</td>
</tr>
<tr>
<td>Creatinine/body weight (mg · dl⁻¹ · kg⁻¹)</td>
<td>0.013 ± 0.002</td>
<td>0.017 ± 0.003*</td>
<td>0.018 ± 0.009*</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>196 ± 52</td>
<td>794 ± 152*</td>
<td>808 ± 288*</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>6.78 ± 5.3</td>
<td>0.27 ± 0.2*</td>
<td>0.32 ± 0.21*</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>153 ± 50</td>
<td>149 ± 19</td>
<td>167 ± 15</td>
</tr>
</tbody>
</table>

Data are means ± SE. *P < 0.05 vs. wild-type mice. Tg, transgenic.

**Histology.** Kidney tissues were fixed in 10% neutral buffered formaldehyde, embedded in paraffin, and sectioned at 4-μm thickness, followed by the periodic acid Schiff (PAS), periodic acid–methenamine silver (PAM), or Masson trichrome staining for light microscopic analysis and morphometry. Morphometry. Kidney sections were stained by PAS or Masson trichrome or immunostained with antibodies to α-smooth muscle actin (α-SMA) or F4/80. Each staining picture was scanned using a 3CCD camera (Olympus Optical, Tokyo, Japan). For morphometric analysis in glomeruli, the glomerular tuft areas, mesangial areas (the percentage of glomerular tuft area), and the number of glomerular cell nuclei were analyzed in a blinded manner using the software Image Grabber PCI (Fujifilm) and Mac Aspect (Mitani, Tokyo, Japan). Twenty consecutive glomeruli in the midcortex were measured. Glomerular cross-sections containing only a minor portion of the glomerular tuft (<=20 discrete capillary segments per cross-section) were not used. To avoid examiner’s bias, both the largest and the smallest glomeruli in the 20 glomeruli were excluded. Eighteen glomeruli from each section were examined, and averages were expressed as means ± SD. For morphometric analysis in the tubulointerstitium, positive area for α-SMA expression or the number of positive cells for F4/80 expression in the interstitium per high-power field was measured in a blinded manner using the software NIH ImageJ (National Institutes of Health, Bethesda, MD). Ten areas in each slide from wild-type, RAGE/iNOS transgenic, or the triple transgenic mice (n = 5) were examined.

**Electron microscopy.** Renal tissues were fixed with 0.1 mol/l sodium phosphate (pH 7.4) containing 2.5% glutaraldehyde and 1% osmium tetroxide solution. They were embedded in an epoxy resin (TAAB Laboratories Equipments, Berkshire, U.K.). Ultrathin sections were doubly stained with uranyl acetate and lead citrate solutions.

**Immunohistochemistry.** To examine mesangial matrix deposition, 4-μm sections of methyl Carnoy’s fixed tissues were stained with antibodies as follows, according to the method described previously (8). In brief, type IV and type I collagens were localized using an indirect immunoperoxidase method with polyclonal goat anti–type IV or anti–type I collagen antibodies (1:100 dilution), respectively (Southern Biotechnology Associates, Birmingham, AL). Fibronectin or laminin was localized using the rabbit anti-fibronectin antibody (1:100 dilution). The serum samples were analyzed using by a Biochemical autoanalyzer DRI-CHEM 3500V (Fujifilm, Tokyo, Japan).

**Histology.** Kidney tissues were fixed in 10% neutral buffered formaldehyde, embedded in paraffin, and sectioned at 4-μm thickness, followed by the periodic acid Schiff (PAS), periodic acid–methenamine silver (PAM), or Masson trichrome staining for light microscopic analysis and morphometry. Morphometry. Kidney sections were stained by PAS or Masson trichrome or immunostained with antibodies to α-smooth muscle actin (α-SMA) or F4/80. Each staining picture was scanned using a 3CCD camera (Olympus Optical, Tokyo, Japan). For morphometric analysis in glomeruli, the glomerular tuft areas, mesangial areas (the percentage of glomerular tuft area), and the number of glomerular cell nuclei were analyzed in a blinded manner using the software Image Grabber PCI (Fujifilm) and Mac Aspect (Mitani, Tokyo, Japan). Twenty consecutive glomeruli in the midcortex were measured. Glomerular cross-sections containing only a minor portion of the glomerular tuft (<=20 discrete capillary segments per cross-section) were not used. To avoid examiner’s bias, both the largest and the smallest glomeruli in the 20 glomeruli were excluded. Eighteen glomeruli from each section were examined, and averages were expressed as means ± SD. For morphometric analysis in the tubulointerstitium, positive area for α-SMA expression or the number of positive cells for F4/80 expression in the interstitium per high-power field was measured in a blinded manner using the software NIH ImageJ (National Institutes of Health, Bethesda, MD). Ten areas in each slide from wild-type, RAGE/iNOS transgenic, or the triple transgenic mice (n = 5) were examined.

**Electron microscopy.** Renal tissues were fixed with 0.1 mol/l sodium phosphate (pH 7.4) containing 2.5% glutaraldehyde and 1% osmium tetroxide solution. They were embedded in an epoxy resin (TAAB Laboratories Equipments, Berkshire, U.K.). Ultrathin sections were doubly stained with uranyl acetate and lead citrate solutions.

**Immunohistochemistry.** To examine mesangial matrix deposition, 4-μm sections of methyl Carnoy’s fixed tissues were stained with antibodies as follows, according to the method described previously (8). In brief, type IV and type I collagens were localized using an indirect immunoperoxidase method with polyclonal goat anti–type IV or anti–type I collagen antibodies (1:100 dilution), respectively (Southern Biotechnology Associates, Birmingham, AL). Fibronectin or laminin was localized using the rabbit anti-fibronectin antibody (1:100 dilution). The serum samples were analyzed using by a Biochemical autoanalyzer DRI-CHEM 3500V (Fujifilm, Tokyo, Japan).

**Histology.** Kidney tissues were fixed in 10% neutral buffered formaldehyde, embedded in paraffin, and sectioned at 4-μm thickness, followed by the periodic acid Schiff (PAS), periodic acid–methenamine silver (PAM), or Masson trichrome staining for light microscopic analysis and morphometry. Morphometry. Kidney sections were stained by PAS or Masson trichrome or immunostained with antibodies to α-smooth muscle actin (α-SMA) or F4/80. Each staining picture was scanned using a 3CCD camera (Olympus Optical, Tokyo, Japan). For morphometric analysis in glomeruli, the glomerular tuft areas, mesangial areas (the percentage of glomerular tuft area), and the number of glomerular cell nuclei were analyzed in a blinded manner using the software Image Grabber PCI (Fujifilm) and Mac Aspect (Mitani, Tokyo, Japan). Twenty consecutive glomeruli in the midcortex were measured. Glomerular cross-sections containing only a minor portion of the glomerular tuft (<=20 discrete capillary segments per cross-section) were not used. To avoid examiner’s bias, both the largest and the smallest glomeruli in the 20 glomeruli were excluded. Eighteen glomeruli from each section were examined, and averages were expressed as means ± SD. For morphometric analysis in the tubulointerstitium, positive area for α-SMA expression or the number of positive cells for F4/80 expression in the interstitium per high-power field was measured in a blinded manner using the software NIH ImageJ (National Institutes of Health, Bethesda, MD). Ten areas in each slide from wild-type, RAGE/iNOS transgenic, or the triple transgenic mice (n = 5) were examined.
tin or anti-laminin antibodies (1:100 dilution), respectively (Chemicon International, Temecula, CA).

For detection of glomerular damage and macrophage infiltration, 4-μm sections of methyl Carnoy’s fixed tissues were stained with mouse monoclonal antibody to α-SMA (1:400 dilution; Boehringer Mannheim, Mannheim, Germany), a marker for mesangial activation (9), and rat monoclonal antibody to F4/80 (1:400 dilution; Caltag Laboratories, Burlingame, CA), a cell surface marker for murine macrophages (10), respectively. The localization of the first antibody was visualized by an indirect immunoperoxidase method (11,12).

Immunofluorescence studies. Frozen sections cut at 4 μm thickness were reacted with fluorescein isothiocyanate–conjugated goat anti-mouse IgG or C3 antibodies (MP Biomedicals, Irvine, CA).

Measurements of pentosidine and carboxymethyllysine. For detection of pentosidine or carboxymethyllysine (CML), the cortex of murine kidney tissue (∼100 mg) was minced, followed by reduction for 4 h at room temperature by addition of excess of NaBH₄ in 0.2 mol/l borate buffer (pH 9.1). Proteins were then precipitated by addition of an equal volume of 20% trichloroacetic acid and were centrifuged at 2,000 g for 10 min. The supernatant was discarded, and the pellet was washed with 1,000 μl 10% trichloroacetic acid. After being dried under vacuum, the pellet was acid hydrolyzed in 500 μl 6 N HCl for 16 h at 110°C in screw-cap tubes that were purged with nitrogen. Hydrolysates were dried in vacuo, rehydrated in water, and used for measuring pentosidine or CML.

Pentosidine was analyzed on a reverse-phase high-performance liquid chromatography as previously described (13). In brief, 20 μl of the test sample diluted by PBS was injected into a high-performance liquid chromatography system and separated on a C18 reverse-phase column (Waters, Tokyo, Japan). The effluent was monitored with a fluorescence detector (RF-10A Shimadzu, Kyoto, Japan) at an excitation-emission wavelength of 335/385 nm. Synthetic pentosidine was used as a standard. Detection limit was 0.1 pmol pentosidine/mg protein.

CML content of the test samples was measured as its pentafluoroacetyl treatment.
methyl esters by selected-ion monitoring gas chromatography/mass spectrometry (14) after the addition of heavy labeled internal standards (d4-CML). The CML and d4-CML standards were gifts from Dr. John W. Baynes (Department of Chemistry and Biochemistry, University of South Carolina, Columbia, SC). Limit of detection was 1.0 pmol CML/mg protein.

Detection of urine 8-hydroxy-deoxyguanosine. Urine 8-hydroxy-deoxyguanosine (8-OHdG) was measured by an 8-OHdG detection ELISA kit (8-OHdG Check [High sensitive]; Japan Aging Control Laboratories, Shizuoka, Japan). The tested urine was ultrafiltrated with Vivaspin (Sartorius, Goettingen, Germany) for >10,000 molecular weight cutoffs before the assay. The data were expressed per milligram urine creatinine.

Statistical analysis. Data were expressed as means ± SD. ANOVA was used to evaluate the statistical significance of various differences. If the analysis detected a significant difference, the Scheffe’s t test was used to compare results obtained from the experimental animals. The difference in the degree of albuminuria in between the triple and RAGE/iNOS transgenic mice was analyzed by the χ² test for independence. Values are considered significant at P < 0.05.

RESULTS

Generation of the triple transgenic mice of megsin, RAGE, and iNOS. Megsin transgenic mice were crossed with RAGE/iNOS transgenic mice to establish a megsin/RAGE/iNOS triple transgenic mouse strain. The transgene expression was confirmed by genome PCR described previously (data not shown). The triple transgenic mice were backcrossed with CD-1 seven times to unify the genetic background. Transmission of the three transgenes to the progeny and litter sizes were comparable with controls, suggesting normal reproductive behavior of the triple transgenic mice. Renal phenotypes of the triple transgenic mice were analyzed and compared at 16 weeks of age with those of RAGE/iNOS or other transgenic mouse lines, including megsin/iNOS transgenic mice.

Development of severe diabetic nephropathy in the triple transgenic mice. Both RAGE/iNOS and the triple transgenic mice developed diabetes at 16 weeks after birth (Table 1). The levels of serum glucose, HbA1c, concentrations, or serum insulin concentrations were similar in the two strains; the degrees of hyperglycemia and hypoinsulinemia were equivalent.

Both strains developed a nephropathy characterized by kidney hypertrophy and an increase in serum blood urea nitrogen and creatinine levels (Table 1). At 16 weeks, the proportion of mice with severe albuminuria (defined as an urine albumin-to-creatinine ratio above 1) was only 15.6% (5 of 32) in the RAGE/iNOS transgenic mice versus 38.5% (10 of 26) in the triple transgenic mice (P < 0.05) (Fig. 1). PAS-stained kidney tissues were analyzed at 16 weeks. The RAGE/iNOS transgenic mice exhibited the previously reported features of diabetic nephropathy with enlargement of the glomerular tufts and mesangial matrix expansion (Fig. 2C) (3). Compared with RAGE/iNOS transgenic mice, the age-matched triple transgenic mice developed more severe, diffuse glomerular lesions such as glomerular hypertrophy, global mesangial expansion, and adhesion of
ules were observed in sclerotic lesions reminiscent of Kimmelstiel-Wilson nodules. The majority of glomeruli of the triple transgenic mice showed global mesangial sclerosis. Noteworthy, some segmental sclerotic lesions were observed in ~30–40% of glomeruli of 57% (12 of 21) of the triple transgenic mice. Tubulointerstitial damage was also prominent, as witnessed by expanded interstitium and inflammatory cell infiltration (Fig. 2F). Hyaline deposits in the arterioles of 1–2 glomeruli per 50 glomeruli, suggesting the presence of arteriolaropathy (Fig. 2F). Transgenic mice with megsin gene alone did not show any pathological changes at such an early age (Fig. 2B).

The nodule-like lesions were further assessed by PAM staining. Dark silver staining of the basement membrane was observed in wild-type mice (Fig. 3A) together with an increased mesangial matrix in RAGE/iNOS transgenic mice (Fig. 3B). In contrast, PAM staining of the triple transgenic mice confirmed nodule-like sclerosis with loss of capillaries and glomerular cells as well as glomerular hypertrophy, severe mesangial expansion, and thickening of basement membrane (Fig. 3C). Electron microscopic analysis revealed basement membrane thickening and foot process effacement in the triple transgenic mice (Fig. 4).

In another strain of megsin/iNOS transgenic mice, hyperglycemia and hypoinsulinemia were similar to those of the RAGE/iNOS or the triple transgenic mice. Nevertheless, they developed only negligible mesangial expansion (data not shown).

**Acceleration of mesangial matrix expansion by megsin overexpression.** Glomerular abnormalities were quantitated in 16-week-old mice by computer-assisted morphometry. Glomerular size was significantly larger in both RAGE/iNOS and triple transgenic mice than in the wild-type and megsin transgenic mice (Fig. 5A). Obviously, megsin overexpression in triple transgenic mice failed to aggravate the glomerular hypertrophy observed in RAGE/iNOS transgenic mice.

The mesangial matrix ratio was similar in the wild-type, megsin transgenic, and RAGE/iNOS transgenic mice. By contrast, it was significantly augmented in the triple transgenic mice overexpressing megsin (Fig. 5B). Taken together, these observations account for the severe mesangial expansion and nodule-like lesions observed in the triple transgenic mice by standard light microscopy.

Observations of PAS staining at 8 weeks (Fig. 6A–C) disclose an early, significant (*P < 0.05) increase in glomerular tuft area only in the double or triple transgenic mice (Fig. 6D). The mesangial matrix ratio also tended to increase in the transgenic mice, but the difference with the wild-type, RAGE/iNOS transgenic mice failed to reach significance (Fig. 6E).

**Early proliferation of glomerular cells by megsin overexpression.** The number of glomerular cells expressed as nuclei per glomerulus rose mildly (*P < 0.05) in megsin transgenic mice and markedly (*P < 0.01) in both RAGE/iNOS and the triple transgenic mice, compared with that of wild-type control mice at 16 weeks (Fig. 5C). Interestingly, the number of glomerular cells was significantly lower in the triple transgenic mice than in RAGE/iNOS transgenic mice (*P < 0.05) (Fig. 5C). By contrast, at an early age of 8 weeks, elevated glomerular cell number was observed only in the triple transgenic mice (Fig. 5F).

**Mesangial matrix expansion, immune complexes deposits, and glomerular or tubulointerstitial damages in the triple transgenic mice.** Immunohistochemical studies were performed at 16 weeks. Type IV collagen, fibronectin, and laminin accumulated markedly in the mesangial area of the triple transgenic mice (Fig. 7). No type I collagen accumulation was observed in the various transgenic mice strains (data not shown). These changes are in part consistent with those observed in human diabetic nephropathy.

At the age of 16 weeks, deposition of IgG and C3 in glomeruli of the triple transgenic mice increased significantly compared with that of RAGE/iNOS transgenic mice (Fig. 8). They deposited mainly in the mesangium and weakly in the basement membrane and capillary walls.

At 16 weeks, glomerular damage was associated with mesangial activation only in the triple transgenic mice, as shown by immunostaining of α-SMA (Fig. 9A). In the same strain, α-SMA expression was also observed in some tubular and interstitial cells, demonstrating tubulointerstitial injury. Inflammatory cell infiltration in the tubulointerstitial compartment was also revealed by staining of F4/80 (Fig. 9A). Morphometrical analysis of the sections immunostained with antibodies to α-SMA or F4/80 showed a significant increase in tubulointerstitial damage (*P < 0.005) and inflammatory cell infiltration (*P < 0.05) in the...
triple transgenic mice compared with those in RAGE/iNOS transgenic mice (Fig. 9B). The degree of tubulointerstitial matrix expansion, assessed in Masson trichrome–stained sections, was significantly higher ($P < 0.01$) in the triple transgenic mice that in RAGE/iNOS transgenic mice (Fig. 10).

**Augmented oxidative/carbonyl stress in the triple transgenic mice.** The status of oxidative and carbonyl stress in RAGE/iNOS and in the triple transgenic mice was assessed by the measurement, in the renal cortex, of two advanced glycation end products (AGEs), pentosidine and CML. A statistically significant accumulation of pentosidine was observed in the triple transgenic mice, compared with age-matched wild-type mice (Fig. 11A). CML accumulation also tended to be higher in the triple transgenic mice that in the wild-type mice, but the difference did not reach significance (Fig. 11B).

Another marker of oxidative stress, 8-OHdG, was measured in the urine of experimental animals. Its concentration in the urine of the triple transgenic mice was above that of RAGE/iNOS transgenic mice ($P < 0.05$) and more than twice higher than that of wild-type mice ($P < 0.001$) (Fig. 11C).

**DISCUSSION**

The triple transgenic model used in this study leads to general considerations. Knockout mice or transgenic animals burdened by the overexpression of a single gene often exhibit biological markers of their condition in the absence of a defined pathological phenotype. However, the addition of a second hit or sometimes, as in this study, a third hit disclose marked abnormalities and a pathological phenotype. This sequence of events is best accounted for by the resilience of the various parts of our biological network: the absence or excess of an element is readily compensated for by other elements. It is only when a second or sometimes a third damage is associated that
the consequences of the various genetic changes are evident. In this contest, the pure iNOS transgenic mice have a diabetes phenotype (15) whose renal consequences are moot at an early age (16 weeks). However, the superimposition of RAGE in the vasculature leads to more conspicuous clinical manifestations probably as a consequence of increased AGE accumulation (3). When, in addition, the mesangial expansion is augmented because of inhibition of glomerular-specific serine protease inhibitor, megsin, the local, glomerular consequences of the previous two abnormalities (iNOS and RAGE) become markedly manifest and now approximate those observed in human pathology. In this multiple-hit approach, it becomes difficult to untangle the respective consequences.
of the three transgenic changes: the triple transgenic mice exhibit the integrated consequences of all three genes abnormalities.

The present experimental model of diabetic nephropathy is unique because it exhibits all of the characteristics of human diabetic nephropathy, including mesangial expansion and nodule-like lesions in the glomeruli associated with the local collapse of the glomerular tuft. This model is also accompanied with the tubulointerstitial damage associated with inflammatory cell infiltration. These lesions occur as early as at 16 weeks of age. Lack of phenotypic changes in control mice with sole overexpression of megsin at this early stage was consistent with our previous studies (8).

Megsin was identified as a novel serine protease inhibitor (serpin) predominantly expressed in mesangial cells, using “the gene profile” of cultured human mesangial cells (4,5,16). Its gene and protein expressions are exclusively localized within the glomerulus and upregulated in human and experimental renal diseases, in which mesangial expansion and proliferation are associated (e.g., IgA nephropathy, diabetic nephropathy, and rat Thy-1 nephritis) (6,17,18). Of note, overexpression of megsin in mice induced progressive mesangial cell proliferation and expansion at the age of 40 weeks (8). The RAGE/iNOS transgenic mice were reported to develop insulin-dependent diabetes and eventual nephropathy characterized by albuminuria with mild glomerular hypertrophy and mesangial expansion at the age of 16 weeks, followed by development of glomerulosclerosis at 34 weeks of age (3).

The contribution of megsin, a kidney-specific novel serpin, to the development of severe diabetic nephropathy in our model is noteworthy. Biological significance of megsin has been supported by several lines of evidence. Megsin has the consensus sequence among functional serpins (4). Binding and functional assays in vitro using recombinant megsin revealed its inhibitory action on plasmin (8). Overexpression of megsin in normal mice is associated with mesangial proliferation and expansion, as shown in our previous report (8), and aggravated diabetic nephropathy, as shown in this study. However, molecular mechanisms of megsin in the pathogenesis of mesangial proliferation and expansion remain elusive. Mesangial extracellular matrix mass is constantly regulated by its synthesis and degradation, the latter being determined by the balance existing between proteases and their inhibitors. If protease inhibitors predominate, increased matrix accumulation and glomerulosclerosis will ensue (19,20). Our previous experiments demonstrated that anti–glomerular basement membrane nephritis in megsin transgenic

---

**FIG. 9.** A: Detection of mesangial activation and tubulointerstitial injury and macrophage infiltration in wild-type (a), RAGE/iNOS transgenic (b), and the triple transgenic (c and d) mice at the age of 16 weeks. Mesangial activation and tubular and interstitial cell injury, estimated by immunohistochemical analysis using anti–α-SMA antibody, was accelerated more in the triple transgenic mice than in RAGE/iNOS transgenic mice (upper panels). The number of macrophages infiltrating into the interstitium, detected by immunostaining using anti-F4/80 antibody, was also increased in the triple transgenic mice compared with that in RAGE/iNOS transgenic mice (lower panels). Original magnification ×200 in a–c and ×400 in d. B: Morphometrical analyses for tubulointerstitial damage (a) and macrophage infiltration into interstitium (b) in the experimental mice at the age of 16 weeks. Kidney sections from wild-type, RAGE/iNOS, or triple transgenic mice were stained for α-SMA or F4/80, followed by the morphometric analysis. Tubulointerstitial damage (**P < 0.005) and macrophage infiltration (##P < 0.0001) significantly increased in the triple transgenic mice compared with those in RAGE/iNOS transgenic mice. *P < 0.0001 versus wild-type mice. Tg, transgenic.
mice developed persistent mesangial matrix expansion, supporting a possible role of megsin as a functional serpin in regulating matrix deposition.

Overexpression of megsin in the RAGE/iNOS transgenic mice induced mesangial activation and dysfunction, as demonstrated by expression of a marker of mesangial activation, α-SMA (9), as well as upregulation of various mesangial matrix components (type IV collagen, fibronectin, and laminin). The RAGE/iNOS transgenic mice harbor the RAGE transgene under the endothelium-specific promoter, flk-1. The fact that, at the early age of 8 weeks (Fig. 5), pathological changes were significant only in the triple transgenic mice suggests that the combination of endothelial damage (due to the RAGE transgene) and mesangial dysfunction (due to megsin overexpression) is critical to the development of severe diabetic nephropathy.

One can argue that the pathological features represent only the addition of two independent phenomena, i.e., the spontaneous glomerulopathy in megsin transgenic mice and the diabetic nephropathy of iNOS/RAGE transgenic mice. However, this hypothesis appears unlikely, because at an early age (16 weeks), pathological changes are absent in megsin transgenic mice, whereas they are full blown in the triple transgenic model and only moderate in the RAGE/iNOS strain.

Most experimental models of diabetic nephropathy do not exhibit characteristic histological lesions. Streptozocin-induced diabetic mice, ob/ob mice, and db/db mice develop extremely weak mesangial hypertrophy or expansion in an early age, and these phenotypic changes are obvious but still mild in transgenic mice overexpressing RAGE and iNOS, whereas the changes in triple transgenic mice are marked at such an early age.

The number of glomerular cells in the triple transgenic mice was higher at 8 weeks than in both wild-type and RAGE/iNOS transgenic animals. We reported a similar finding in megsin transgenic mice with or without anti-glomerular basement membrane nephritis (8) and attributed it to alterations of the microenvironment of mesangial matrix, reputedly responsible for mesangial cell proliferation and differentiation. The number of glomerular cells further rose at 16 weeks in the triple transgenic mice but rose unexpectedly less than in RAGE/iNOS transgenic in which it became significantly higher. This effect in the triple transgenic mice at the age of 16 weeks may result from the progression of severe sclerosis, leading to acellular nodule-like lesion. Imbalance of mesangial extracellular matrix synthesis and degradation due to predomination of a protease inhibitor, i.e., megsin (21–24), is also likely to contribute to development of nodule-like lesions.

This model is also associated with existence of oxidative stress and carbonyl stress, which have been implicated in the pathogenesis of diabetes complications. The biomarkers (i.e., renal contents of two well-known AGEs, pentosidine and \( N^\epsilon \)-carboxymethyl-lysine, as well as urinary 8-OHdG concentration) were markedly higher in the triple transgenic mice, whereas they were not elevated or were only slightly elevated in the iNOS/RAGE transgenic mice. It remains unknown whether this model is eventually associated with continuous renal dysfunction leading to end-stage renal failure. Further studies are necessary to unravel the progression of the disease at a later stage in this model.

Because diabetic nephropathy is the major cause of end-stage renal injury in many countries, development of drugs effective to retard the progression of diabetic nephropathy has become a pharmaceutical goal. However, it has been hampered at least in part by the lack of adequate experimental models (7). Altogether, the present model...
offers an attractive target for the study of measures preventing the onset of diabetic nephropathy. Still, the characteristics of the model should be kept in mind. The overexpression of iNOS exposes tissues to extremely high levels of peroxynitrate with an attendant destruction of islet β-cells, whereas overexpression of RAGEs increases markedly the susceptibility of endothelial cells to AGEs. Similarly, overexpression of megsin inhibits markedly proteases, favoring matrix accumulation (8). Thus, it remains to be determined to what extent these various abnormalities are also implicated in the development of human diabetic nephropathy. The prevention or lack of prevention of diabetic nephropathy by various drugs should therefore be interpreted cautiously.

In conclusion, we established a new diabetic nephropathy model in triple transgenic mice overexpressing RAGE, iNOS, and megsin. In this model, the triple-hit damage develops severe diabetic nephropathy, at an early age of 16 weeks after birth, which is characterized by development of mesangial expansion, nodule-like lesion, and tubulointerstitial damage with an increase in local oxidative stress. These phenotypes closely resembled those in advanced diabetic nephropathy in humans. This multiple-hit approach is of interest to consider the sequential events during the development of diabetic nephropathy.

ACKNOWLEDGMENTS

This study was supported in part by grants from the Japan Science and Technology Agency.

We thank Professors Satoshi Sugiyama, Nobuaki Yamanaka, and Charles Alpers for their helpful discussions on renal pathology of triple transgenic mice.

REFERENCES


