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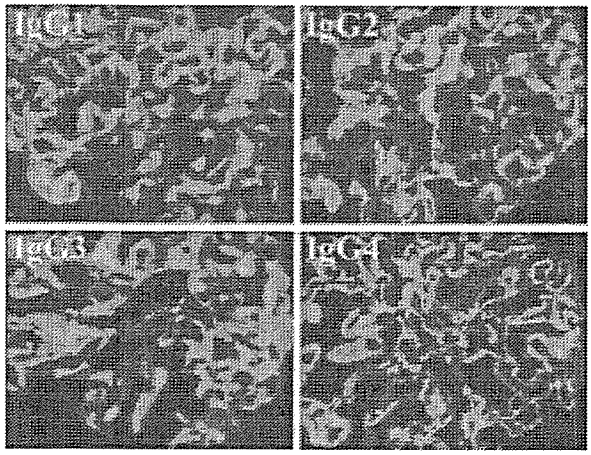

Figure 2. Immunofluorescence showed the depositions of IgG1 to 4 along the glomerular capillary walls.

showed a strong deposition of complement 3 (C3) in the mesangial area (Fig. 1G). IgG, IgA and IgM were all negative. Electron microscopy showed mesangial proliferation, matrix increase and mesangial interposition. No virus-like particles were found in endothelial cells. Only a few electron-dense positive deposits were observed in mesangial areas (Fig. 1H). These findings were compatible with MPGN. After three cycles of steroid pulse (methylprednisolone 1 g/day×3 days/cycle) and oral corticosteroid treatment (prednisolone 30 mg/day, 2 weeks later to 28 mg/day), the patient showed clinical remission. During the subsequent seven-year follow-up period, her urinalysis, serum chemistry and renal function tests showed normal findings. ANA, anti-DNA antibodies and CRP were negative. Serum IgA and IgM level were in normal range. Serum IgG level was increased slowly and reached the upper limit of the normal range in the last three years of follow-up. The steroid dosage, the complement level, and the ASO and C1qIC titers are shown in Fig. 3. Although C3 complement and CH50 were normal, the C4 complement level fluctuated around lower limit of normal range. Serum C1qIC showed a high level during the whole follow-up duration. ASO titer was rather increased even with steroid treatment (normal range< 160 folds) and without infection. The patient dropped out of the follow-up observations when she was 17 years old. When she was 25, urine abnormality was again found in a health examination (1+ of proteinuria and 3+ of hematuria). ANA showed a positive result of 160 fold, serum IgG 2400 mg/dl (normal range 1110-1820 mg/dl), C3 53 mg/dl, C4 12 mg/dl and CH50 38.1 U/ml, gamma globulin 23.8% (normal range 11.0-19.5%), but she was not diagnosed as SLE at that time and she again dropped out after 2-3 times of follow-up until she came to our hospital this time at the age of 30. During the whole pregnancy period, she only had mild proteinuria once at the 38th week of pregnancy.

Table 1. Clinical Parameters of One Months and Ten Months after Treatment

	One months after treatment	Ten months after treatment
ANA	20	160
Anti-ss-DNA (AU/ml)	42.4	Unchecked
Anti-ds-DNA (IU/ml)	7.5	80.1
C3 (mg/dl)	53.9	46.8
C4 (mg/dl)	4.4	1.1
CH50 (U/ml)	21.6	<30
Serum Alb (mg/dl)	2.7	3.2
Proteinuria (g/day)	2.39	0.21

Discussion

This is a rare case report that showed the development of two distinct types of glomerulonephritis in one female patient. One was MPGN at age 10 and the other was membranous lupus nephritis at age 30. When we diagnosed this patient with membranous lupus nephritis, we wondered if she might have suffered from SLE about 20 years previously she had had mild lupus nephritis class IV in the past and as time passed her renal involvement was transformed into lupus nephritis class V.

Based on this suspicion, we re-evaluated her clinical record of 20 years previously. At that time, she showed no photosensitivity or facial butterfly erythema. Anti-nuclear antibody, the most sensitive test for SLE (9), was negative. The well-known immunofluorescence characteristics of lupus nephritis, i.e. C1q deposition on the glomeruli and the frequently observed IgG on glomeruli were not found in this patient. Glomerular IgG deposits reportedly observed in almost all proliferative lupus nephritis patients and C1q deposits are found in 3/4 of these patients (10). Further, virus-like particles, which are considered to be associated with lupus nephritis, were not found in endothelial cells (11). Instead, only marked C3 deposition was observed in mesangial areas, which supported the diagnosis of MPGN together with the light microscopic features and clinical data. In addition, a second renal biopsy at age 30 showed equal densities of mesangial, subepithelial and intramembranous deposits. It was suggested that the deposits observed in the re-biopsy specimen were formed simultaneously and not gradually. Otherwise, the deposition of C1q in glomeruli (12) and electron microscopic discovery of numerous mesangial dense deposits disaffirmed the diagnosis of idiopathic membranous nephropathy. The depositions of IgG1, 2, 3 and 4 along the glomerular capillary walls also suggested membranous lupus nephritis, but not idiopathic membranous nephropathy, which is considered as an IgG4-mediated disease (13, 14). Therefore, membranous lupus nephritis was a newly presented disease in this patient. We were convinced that this patient suffered from two types of glomerulonephritis,

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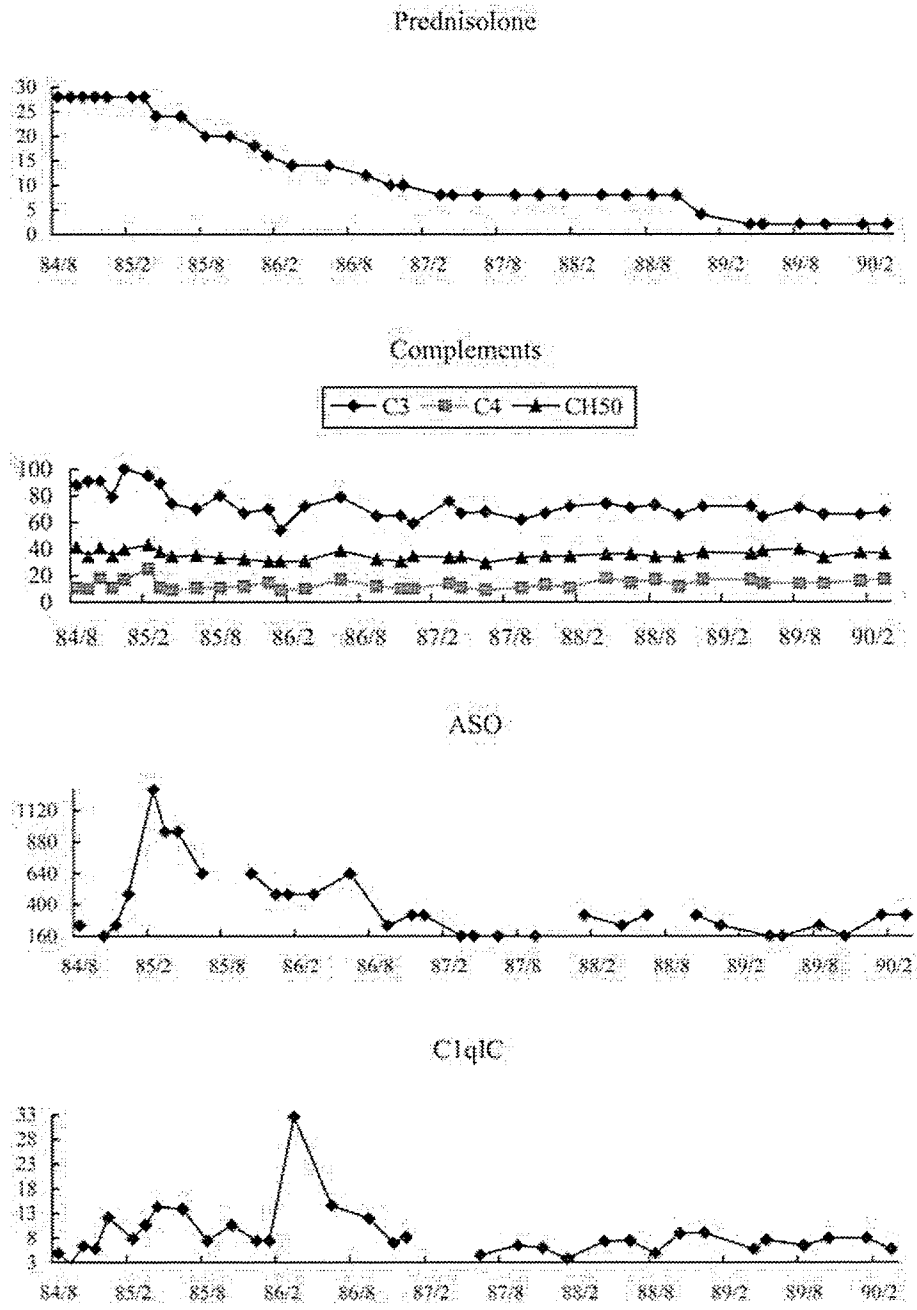

Figure 3. Clinical parameters in the 7 year follow-up. The normal range of C4 complement was considered to be 12-39 mg/dl before 1985 and 15-44 mg/dl after that.

MPGN and membranous lupus nephritis.

The clinical characteristics of membranous lupus nephritis are summarized as: less common hypertension, nephrotic range proteinuria, slow progressing renal disfunction, commonly absent anti-ds-DNA antibody and variable hypocomplementemia (15). The present patient had normal blood pressure, 2.59 g/day proteinuria, normal renal function and severe hypocomplementemia. Anti-ds-DNA is always absent in membranous lupus nephritis while our patient showed an increased level even with a markedly increased anti-ss-DNA antibody. Anti-ds-DNA antibody has been used as a marker of disease activity in SLE for over 35 years (16). In our patient, anti-ds-DNA titer returned to normal range after the steroid pulse treatment and became elevated again when SLE was activated. The value of anti-ss-DNA antibody was not considered before, but it has been regarded recently for its predictive effect by ELISA method (16). Our patient had a high level of anti-ss-DNA at the onset of SLE and it was decreased by the steroid pulse treatment. It is regrettable that its titer was not detected when the disease activated

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again. A similar case was reported by Yumura et al (5). In their case, anti-ss-DNA showed a good predictive effect in disease activity. Immunosuppressive therapy is recommended in lupus nephritis patients with protracted nephrotic syndrome (15). Our patient benefited from this therapy as it reduced her proteinuria to a low level, but it was not sufficient to suppress the disease activity. The combination with cyclosporine or cyclophosphamide may be necessary for this patient.

The second question was then why two types of glomerulonephritis developed in the same patient. Did she have special constitution that made her more likely to contract different immune complex-associated diseases? We collected the laboratory data available for this patient and tried to answer this question. The clinical feature of this patient was that she had a high level of ASO in the follow-up duration and persistent high titers of C1qIC, which showed no responses to steroid therapy. The relationship of ASO and nephritis was unclear in this case because ASO was in the normal range at the onset of MPGN while it increased after the steroid treatment. One case with elevated ASO and nephropathy was reported by Ito et al (17). In their case, the patient had proteinuria, hematuria, lymphopenia, hypocomplementemia, positive ANA and "full-house" nephropathy accompanied with mesangial, subendothelial and subepithelial deposits. In that case, SLE was not diagnosed although the pathologic features supported lupus nephritis. This is quite a different case from the present case. So, we speculate that the elevated ASO may merely indicate an active situation of immune system. It is considered that ineffective immune complex clearance could cause tissue injury and may in turn stimulate an autoantibody response (18). The present patient was positive for C1qIC in every clinical examination from her onset of MPGN to her development of SLE. The persistent existence of immune complex may damage her immune system to produce autoantibodies with time and showed an abnormality in her clearance system. Immune complex processing is proved abnormal in patients with hypocomplementemia. Our patient showed normal findings of serum C3 and CH50, while her serum C4 was at a low level although barely within the normal range. Considering the accompanying high level of C1qIC, an activity of classical complement pathway is suggested and a low level of serum C1q is conceived. Experimental investigations have validated that some C1q-deficient mice generate high titers of antinuclear antibodies and some of them later develop glomerulonephritis with immune deposits (18). This process was also thought to occur in our patient. The increasing serum IgG level could not be considered a good sign retrospectively. The final warning may be the abnormalities of clinical parameters when she was 25 years old: positive ANA, high serum IgG and low serum C3 and C4. All these findings suggest that the immune system of this patient was active before the development of SLE. So, we speculate that she might have entered into an abnormal immunologically active phase from her teens, which would account for the production of autoantibodies. Pregnancy may have been the trigger which finally induced SLE because her body had reached the utmost compensative state before pregnancy.

There have been no similar cases reported: no patient has developed idiopathic glomerulonephritis before the onset of SLE. Therefore, we can only hypothesize according to the evidence of our case that the patient might have a special constitution that facilitates production of various types of immune complexes. She first suffered from MPGN, an immune complex-mediated glomerulonephritis. Steroid pulse treatment might have eliminated the deposits from the glomeruli and controlled the production of autoantibodies. Dose reduction and discontinuation of the steroid treatment reactivated her autoimmune system, as evidenced by her persistently positive serum C1qIC. The systemic damage caused by immune complex and the production of autoantibodies rendered a vicious circle and finally caused the patient to suffer from another immune complex-mediated glomerulonephritis, membranous lupus nephritis.

This appeared to be a very rare and valuable case. It provides excellent data for studying the mechanism of immune complex mediated glomerulonephritis.

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INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

Relationship between the Expression of Advanced Glycation End-Products (AGE) and the Receptor for AGE (RAGE) mRNA in Diabetic Nephropathy

Daisuke Suzuki, Masao Toyoda, Naoyuki Yamamoto, Masaaki Miyauchi, Mayuko Katoh, Moritsugu Kimura, Mayumi Maruyama, Masashi Honma, Tomoya Umezono and Mitsunori Yagame

Abstract

Objective The receptor for advanced glycation end-products (RAGE) is one of several advanced glycation end-product (AGE)-specific cellular receptors. To evaluate the relationship between AGE and RAGE in renal tissues of diabetic nephropathy (DN), we examined the levels of expression of AGE protein and of RAGE mRNA. We also investigated the relationships among the degree of mesangial expansion and the expression of AGE and RAGE mRNA.

Patients and Methods Renal biopsy tissues were obtained from 20 patients with DN. We performed immunohistochemical staining using monoclonal anti-AGE antibody and *in situ* hybridization using non-radioactive oligonucleotide RAGE probe on these tissues. We also examined five control renal samples. We evaluated the intensity of positive anti-AGE antibody staining and the percentage of cells positive for RAGE mRNA. We also measured the total glomerular area and mesangial area in glomeruli using an automatic image analyzer. We then calculated the percentage of mesangial area as a proportion of the total glomerular area (%Mes).

Results Anti-AGE antibody was detected in the expanded mesangial matrix in DN but not in control samples. RAGE mRNA expression was detected mainly in glomerular intrinsic cells, including glomerular mesangial and epithelial cells, in both DN and control. %Mes correlated significantly with both the intensity of anti-AGE antibody positive staining and the percentage of cells positive for RAGE mRNA.

Conclusions Our findings suggest that both AGE and RAGE are associated with the development and progression of DN.

Key words: Advanced glycation end-products (AGE), receptor for advanced glycation end-products (RAGE), diabetic nephropathy, *in situ* hybridization

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Introduction

Diabetic nephropathy (DN) is one of the major complications of both types 1 and 2 diabetes mellitus and the most common cause of end-stage renal failure in many countries. The pathologic changes of DN include progressive expansion of the mesangial matrix and thickening of the glomerular basement membrane (GBM) (1-3). Mesangial expansion is thought to be the most important lesion, and was reported to be the critical lesion that ultimately leads to renal insufficiency in DN (1-3).

Various pathogenic mechanisms have been postulated for DN, although the precise mechanism remains to be clarified. Long-term metabolic aberrations are considered to play a key role, because a number of changes in the kidney are prevented or improved after normalization of glucose metabolism in animal and human models (4, 5). Possible me-

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Table 1. Clinical Parameters of Diabetic Patients and Normal Subjects

	DN	Controls
Gender (M/F)	16 / 4	4 / 1
Age (yr)	42.5±11.8	60.0±8.1*
Known duration (yr)	9.8±5.7	
SBP (mmHg)	141.0±17.1	128.8±14.1
DBP (mmHg)	83.4±10.8	71.2±14.7
S-Cr (mg/dl)	0.81±0.16	0.80±0.16
TP (g/dl)	6.32±1.00	6.54±0.27
HbA1c (%)	9.65±2.58	not done
U-protein (g/d)	0.73±1.02*	0.02±0.01
Ccr (ml/min)	87.6±30.8	not done

DN; diabetic nephropathy, SBP; systolic blood pressure, DBP; diastolic blood pressure, TP; total protein, Ccr; creatinine clearance, *; p<0.01

diators of untoward effects of hyperglycemia include advanced glycation end-products (AGE) generated by the Maillard reaction through non-enzymatic glycation of protein amino groups (6). Recently, we reported that AGE accumulate in the tissues of DN (7, 8) and that their accumulation correlates with the severity of diabetic complications (9, 10). Several lines of evidence suggest that AGE may be involved in the development of diabetic glomerular lesions (7, 8, 11, 12). AGE modification indeed alters the structure and function of matrix tissue proteins (6) and, more interestingly, AGE-modified proteins stimulate a variety of cellular responses via a specific cell-surface receptor (13, 14) on several cell types, including glomerular cells (15-17). The receptor for AGE (RAGE) is one of several AGE-specific cellular receptors. RAGE was first identified as a receptor for AGE using bovine lung extract. It is a signal transduction receptor for AGE of the immunoglobulin super-family that mediates diverse cellular responses (13, 14). Recently, RAGE has been proposed to play an important role in the development of DN (18, 19). However, the AGE-RAGE interaction is not yet fully understood.

The aim of the present study was to evaluate the relationship between AGE and RAGE in tissues from DN. We examined AGE expression using immunohistochemical staining and RAGE mRNA expression using an *in situ* hybridization technique. We also investigated the relationships among the degree of mesangial expansion and the expression of AGE and RAGE mRNA.

Methods

Patients

Renal biopsy specimens were obtained from 20 patients with DN (3, 20). All diabetic patients had macro proteinuria. The presence of DN was confirmed by pathological evaluation of renal biopsy specimens, using techniques such as light microscopy, electron microscopy and immunofluorescence staining. The following clinical parameters were recorded at the time of renal biopsy: gender, age, known duration, blood pressure, serum creatinine, total protein, hemoglobin A1c, urinary protein and creatinine clearance. Clinical features are shown in Table 1. The study was approved by the Human Research Committee of Tokai University School of Medicine, and informed consent was obtained from each patient for renal biopsy and immunohistochemical staining and *in situ* hybridization studies. We also examined five control samples obtained from five subjects using unaffected portions of surgically removed kidneys afflicted with malignancies. After resection, the samples were embedded in OCT compound (Tissue Tek, Miles, Elkhart, IN) and stored until use. The control patients had no urinary abnormalities, and histopathological examination of control tissues excluded any glomerular disease.

Measurement of the percentage of mesangial area with respect to total glomerular area

We calculated the percentage of mesangial area with respect to total glomerular area to determine the degree of mesangial expansion in renal tissues. To minimize bias, 10 glomeruli were chosen from more than 50 glomeruli in each patient and in control samples. The criterion for selection was the cross-section through their vascular poles. We measured the total glomerular area and periodic acid-Schiff (PAS)-positive area using an automatic image analyzer determined by three independent investigators. We then calculated the percentage of mesangial area with respect to total glomerular area (%Mes).

Immunohistochemical staining

Renal biopsy specimens were sectioned at 4 μm and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) on ice for 15 minutes. After washing with PBS, the sections were blocked with 4% skim milk for 60 minutes at room temperature and incubated with peroxidase (HRP)-conjugated anti-AGE antibody (Transgenic, Kumamoto, Japan) at 4℃ overnight. The major epitope-structure of this antibody was recently identified as N^{ε}-carboxymethyllysine (CML) (21). After washing with Tris-saline buffer containing 100 mM NaCl and 150 mM Tris-HCl, pH 7.5, the sections were dehydrated through a graded ethanol series, incubated in methanol with 0.3% H_2O_2 at room temperature for 20 minutes to block endogenous peroxidase, and washed three times with Tris-saline buffer with 0.02% Tween 20 (Wako Pure Chemical Industries, Osaka, Japan). After a final wash with Tris-saline buffer containing Tween 20, they were developed by reaction with diaminobenzidine tetrahydrochloride in 0.05 M Tris-HCl, pH 7.6, and 0.03% H_2O_2. Sections were counterstained briefly with hematoxylin, and then rinsed, dehydrated, cleared in xylene and mounted. The intensity of staining for anti-AGE antibody was evaluated using a score of 0-4 (negative staining: 0, trace: 1, weak: 2,

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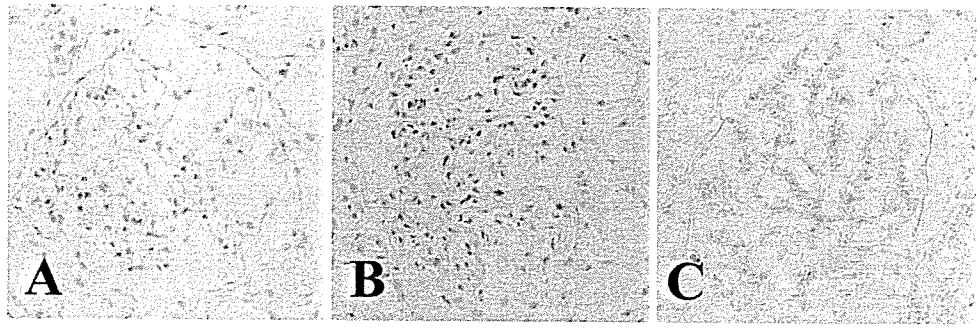

Figure 1. Immunohistochemical detection of anti-AGE antibody in controls (A) and diabetic nephropathy (B, with mild mesangial expansion and C, with moderate mesangial expansion). The AGE antibodies did not stain the glomeruli in control subjects (A). In contrast, in the glomeruli of diabetic nephropathy, AGE stained positively in the expanded mesangial area (B and C). (Original magnification ×100)

Table 2. Results of % Mes, Int AGE ab and % RAGE mRNA in DN and Controls

	DN	Controls
% Mes	23.5±4.8*	14.3±1.6
Int AGE ab	3.1±0.7*	0.0±0.0
% RAGE mRNA	29.6±5.2*	12.0±2.1

DN; diabetic nephropathy
% Mes: the percentage of mesangial area with respect to the total glomerular area
Int AGE ab: the intensity of anti-AGE antibody positive staining
% RAGE mRNA: the percentage of cells positive for RAGE mRNA
*; p<0.01 vs DN

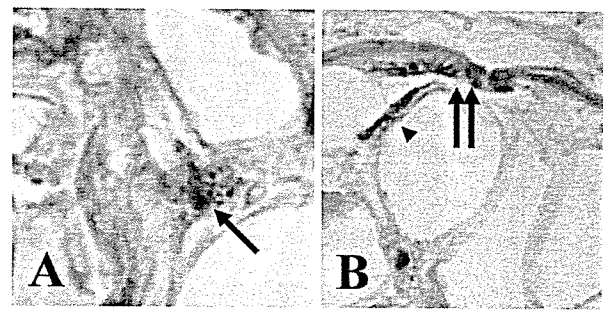

Figure 2. *In situ* hybridization of RAGE in glomeruli of diabetic nephropathy. *In situ* hybridization followed by PAS staining revealed that RAGE mRNAs were localized in glomerular resident cells, mainly glomerular mesangial cells (A: arrow), epithelial cells (B: arrowhead) and epithelial cells of Bowman's capsule (B: double arrow). (Original magnification ×200)

strong: 3, very strong: 4).

in situ hybridization

The probe for RAGE corresponded to sequences No. 784-812 of human RAGE cDNA. One hundred picomoles of the oligonucleotide probe were labeled using a digoxigenin (DIG) oligonucleotide tailing kit according to the standard protocol (Roche, Penzberg, Germany). Free DIG was removed by ethanol precipitation and dissolved in diethylpyrocarbonate-treated water. *In situ* hybridization was performed according to the modified technique developed in our laboratory (22, 23). Briefly, fresh kidney biopsy tissues were embedded in OCT compound and stored at -70℃ until use. Specimens were cut into 4 μm thick sections, fixed in 4% paraformaldehyde, and then deproteinized by HCl and digested with proteinase K (Sigma, St. Louis, MO). Specimens were prehybridized in a prehybridization buffer, drained and hybridized overnight with DIG-labeled oligonucleotide probe in the prehybridization buffer. After hybridization, the DIG-labeled probe was visualized by immunohistochemical staining using a mouse monoclonal anti-DIG antibody (Roche), HRP-conjugated rabbit anti-mouse antibody (Dako, Glostrup, Denmark), and HRP-conjugated swine anti-rabbit antibody (Dako). Color was developed with diaminobenzidine tetrahydrochloride in 0.05 M Tris-HCl, pH 7.6, and 0.03% H_2O_2. Sections were counterstained briefly with hematoxylin, and then rinsed, dehydrated, cleared in xylene and mounted.

To evaluate the specificity of the signals, two types of control experiments were carried out as described previously (22, 23). First, pre-treatment with RNase was performed after proteinase K digestion, and thereafter sections were prehybridized and hybridized. Second, a competitive study was performed by adding 100-fold excess amount of homologous or unrelated, unlabeled oligonucleotides to the hybridization buffer together with the antisense probe.

To quantify the expression of RAGE mRNA, all nuclei as well as nuclei surrounded by RAGE-positive cytoplasm in at least 10 randomly selected cross-sections of nonsclerotic glomeruli were counted in each specimen. Enumeration of these nuclei was performed by three investigators who were

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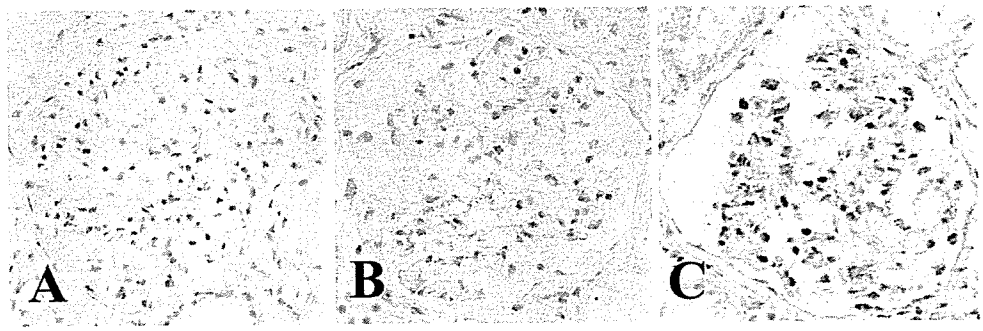

Figure 3. Detection of RAGE mRNA in tissues of normal controls (A) and diabetic nephropathy (B, with mild mesangial expansion and C, with moderate mesangial expansion). (Original magnification ×100)

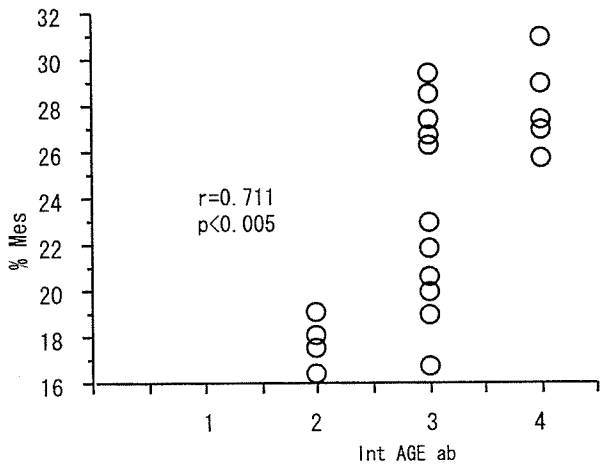

Figure 4. Correlation between percentage of mesangial area with respect to total glomerular area (%Mes) and intensity of staining for anti-AGE antibody (Int AGE ab) in diabetic nephropathy. %Mes correlated significantly with Int AGE ab.

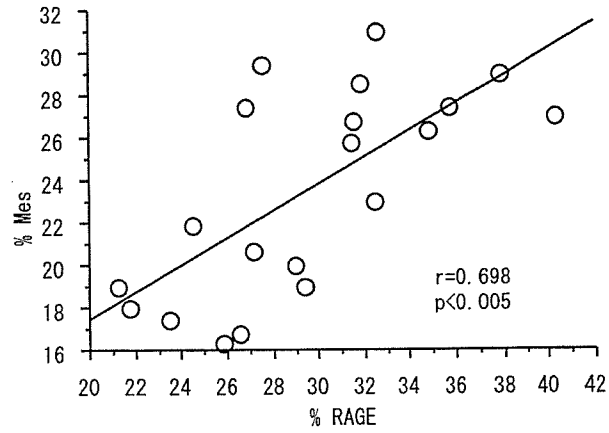

Figure 5. Correlation between percentage of mesangial area with respect to total glomerular area (%Mes) and percentage of RAGE mRNA-positive cells among total glomerular cells (%RAGE) in diabetic nephropathy. %Mes correlated significantly with %RAGE.

blinded to the severity of DN. Results were expressed as the percentage of RAGE mRNA-positive cells among total glomerular cells.

Statistical analysis

The Mann-Whitney U test, regression analysis and Spearman rank correlation test were used for statistical comparison. A P value <0.05 denoted a statistically significant difference between groups.

Results

Immunohistochemical staining demonstrated the protein expression level of AGE in renal tissue of DN and control. The anti-AGE antibody was detected in the expanded mesangial matrix in DN tissues. The intensity of immunostaining appeared to increase with the degree of mesangial expansion in DN (Fig. 1 and Table 2). However, it was not detected in control samples (Fig. 1). The intensity of staining for anti-AGE antibody was stronger in DN than in control samples (Table 2). Immunohistochemical staining using normal mouse IgG in lieu of this antibody was barely detectable in renal tissues from patients with DN (not shown).

Our *in situ* hybridization method also demonstrated cells positive for RAGE mRNA in renal tissue of DN and control. As shown in Fig. 2, *in situ* hybridization followed by PAS staining clearly identified individual cells positive for RAGE mRNA in renal tissue of DN and control. These cells included glomerular mesangial and epithelial cells and cells of Bowman's capsule (Fig. 2). Glomerular expression was noted to increase with the progression of mesangial expansion in DN. The percentage of cells positive for RAGE mRNA was significantly higher in DN than in control samples (Fig. 3, Table 2).

To verify the specificity of the signal obtained in *in situ* hybridization, two control studies were performed. Pre-

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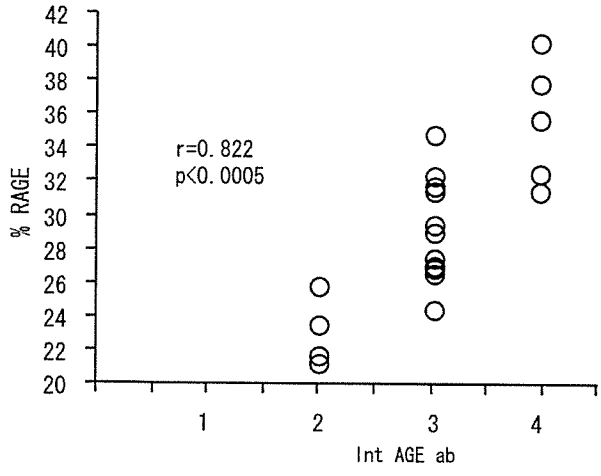

Figure 6. Correlation between percentage of RAGE mRNA-positive cells among total glomerular cells (%RAGE) and intensity of staining for anti-AGE antibody (Int AGE ab) in diabetic nephropathy. %RAGE correlated significantly with Int AGE ab.

treatment of tissue with RNase prior to hybridization eliminated most of the signal obtained with the RAGE probes (data not shown). The signal of each mRNA disappeared when a large amount of unlabeled homologous oligonucleotide was added. In contrast, the addition of non-homologous oligonucleotide did not block the signal (data not shown).

We also examined the relationships among %Mes, the intensity of staining for anti-AGE antibody and the percentage of cells positive for RAGE mRNA. %Mes correlated significantly with both the intensity of anti-AGE antibody-positive staining, and the percentage of cells positive for RAGE mRNA (Figs. 4, 5). The intensity of anti-AGE antibody-positive staining correlated significantly with the percentage of cells positive for RAGE mRNA (Fig. 6).

Discussion

AGE comprise a variety of molecular structures, such as CML (24), pentosidine (25), and pyrraline (26), characterized by different formation mechanisms. We recently demonstrated that anti CML and anti pentosidine antibodies were stained in the expanded mesangial matrix and nodular lesions of DN (8). The staining pattern for these antibodies was similar to the present study. Moreover, the intensity of positive anti-CML and pentosidine antibody staining appeared to increase with the degree of mesangial expansion in DN. The results of our present study also demonstrated positive staining for monoclonal anti-AGE antibody in the mesangium of DN, but this was not detected in control samples. Furthermore, the intensity of positive anti-AGE antibody staining correlated significantly with %Mes. These data suggest that deposition and/or formation of AGE in the mesangium is associated with the progression of DN. In this study, we used just a semi-quantitative method employing the score of 0-4 to evaluate the intensity of staining for anti-AGE antibody. A real quantitative method such as the direct evaluation of intensity of staining for antibodies is necessary to precisely quantify.

AGE mediate their effects through two different pathways via a receptor-independent AGE cross-link formation pathway and through a receptor-dependent pathway where AGE bind to specific cell surface-associated receptors, such as the macrophage scavenger receptor (MSR) type II, OST-48, 80K-H, galectin-3, CD 36 and RAGE (13, 14, 27-30). Among these receptors, RAGE is the best characterized and most implicated with the role of AGE. For that reason, we used the RAGE probe in this study. To evaluate the relationship between AGE and RAGE in tissues from DN, we also examined the expression of RAGE mRNA. Renal cells positive for RAGE mRNA were identified at the cellular level in DN and control. In order to distinguish individual cells positive for RAGE mRNA in the glomeruli, we performed PAS staining after *in situ* hybridization and then defined the topographical relationship of positively stained cells relative to GBM and mesangial matrix. The signal for RAGE mRNA was identified among glomerular resident cells, mainly glomerular mesangial and epithelial cells and cells of Bowman's capsule. Tanji et al (31) reported that RAGE protein expression was restricted to podocytes in normal control human glomeruli and in glomeruli of patients with DN. In another study, Tsuji et al (32) also demonstrated that both RAGE mRNA and RAGE protein were produced by mouse mesangial cells. Taken together, these results suggest that RAGE may be synthesized primarily by glomerular epithelial and mesangial cells. It is very interesting to investigate the expression of anti RAGE antibody. We had tried to do immunohistochemical staining using several anti RAGE antibodies. However, these antibodies did not react with RAGE on the tissues from control and DN. Further studies are warranted.

Our quantitative analysis of *in situ* hybridization studies demonstrated that the percentage of cells positive for RAGE mRNA was significantly greater in DN than in control samples. Tanji et al (31) reported that diffuse low-level expression of anti-RAGE antibody was restricted to the podocytes of normal control samples. On the other hand, there was marked upregulation of RAGE expression in tissues from DN. They also demonstrated RAGE mRNA expression in glomeruli from DN using RT-PCR. However, no expression was observed in glomeruli from normal control samples. Together, these data suggest that RAGE is upregulated in glomeruli in DN.

Recent studies reported that AGE-RAGE interaction is associated with the pathogenesis of DN. Yamamoto et al (33) established transgenic mice that overexpressed human RAGE in vascular cells and crossbred them with another transgenic line that develops insulin-dependent diabetes shortly after birth. The resultant double transgenic mice exhibited increased hemoglobin A1c and serum AGE levels, as did the diabetic controls. However, the double transgenic

mice exhibited enlargement of the kidney, glomerular hypertrophy, increased albuminuria, mesangial expansion, advanced glomerulosclerosis, and increased serum creatinine compared with diabetic littermates lacking the RAGE transgene (33). Furthermore, treatment with neutralizing RAGE antibody or soluble RAGE improved renal function and renal pathological changes in db/db mice (34, 35). To date, no studies have quantified RAGE mRNA and correlated the percentage of RAGE-expressing cells to the severity of diabetic glomerulopathy. The present study is the first to show that the percentage of cells positive for RAGE mRNA correlated significantly with not only %Mes, but also with the intensity of anti-AGE antibody staining in glomeruli from DN. Moreover, it has been reported that AGE can activate the RAGE gene through NF-κB (36). Taken together, these data suggest that AGE accumulation may involve upregulation and activation of RAGE. The AGE-RAGE interaction apparently plays an important role in the pathogenesis of DN. However, it is not clear whether the RAGE-independent AGE cross-link formation pathway or the RAGE-dependent pathway is more closely associated with development of DN. Further studies are required to address this issue.

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