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Effects of Short-Term Glycemic Control, Low Protein Diet and Administration of Enalapril on Renal Hemodynamics and Protein Permselectivity in Type 2 Diabetic Patients with Microalbuminuria

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Narita, T., Koshimura, J., Suzuki, K., Murata, M., Meguro, H., Fujita, H., Kitazato, H. and Ito, S. *Effects of Short-Term Glycemic Control, Low Protein Diet and Administration of Enalapril on Renal Hemodynamics and Protein Permselectivity in Type 2 Diabetic Patients with Microalbuminuria.* Tohoku J. Exp. Med., 1999, **189** (2), 117-133 —— To determine whether each of glycemic control (GC), low protein diet (LPD) or administration of angiotensin converting enzyme inhibitor (ACEI) has beneficial effects on diabetic nephropathy through the different mechanisms, changes in charge and size selectivity of glomerulus and renal hemodynamics were analyzed in microalbuminuric type 2 diabetic patients after additive combination therapy (first period: GC only, second period: GC+LPD, third period: GC+LPD+ACEI). To detect improvement of the impairments of glomerular charge selectivity and size selectivity, changes in the ratio of the renal clearance of two plasma proteins with similar molecular radii and different isoelectric points (pIs) (ceruloplasmin and IgG: CRL/IgG) and changes in the ratio of the renal clearance of two plasma proteins with similar pIs and different molecular radii (α2-macroglobulin and albumin: α2/Alb) were examined before and after each therapy. Creatinine clearance decreased significantly in the first and third periods although slight but not significant decrease was detected in the second period. Filtration fraction was significantly decreased only in the third period. Although renal clearances of Alb, IgG and CRL were decreased in periods of all three therapies, that of α2-macroglobulin with a large molecular radius was decreased significantly only after the third therapy. Neither CRL/IgG nor α2/Alb changed during these three therapies. These findings suggest that each of three short-term therapies consisting of GC, GC+LPD and GC+LPD+ACEI, reduced proteinuria in microalbuminuric type 2 diabetic patients not through the improvement of renal size and charge selectivities, but through improvement of renal hemodynamics. —— early diabetic nephropathy; renal hemodynamics;

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decreased proteinuria; charge selectivity; size selectivity

Diabetic nephropathy has become the leading cause (25–35%) of end stage renal disease in the United States and Europe (Parving et al. 1996), and more than 30% of patients who were newly accepted for regular dialysis treatment had diabetic end stage renal failure in Japan (Japanese Society for Dialysis Therapy 1996). To prevent renal failure, early detection and intervention of diabetic nephropathy is necessary (Mogensen et al. 1995; Molitch 1997). The importance of microalbuminuria in the prediction of overt nephropathy has been known in diabetic nephropathy for more than a decade (Mogensen et al. 1995; Molitch 1997). The strategies for management of microalbuminuria have emphasized early intervention, focusing on aggressive glycemic control (Ohkubo et al. 1995; The DCCT Research Group 1995), blood pressure control with angiotensin converting enzyme inhibitor (ACEI) (Ravid et al. 1994; Sano et al. 1996; The Microalbuminuria Captopril Study Group 1996) and low protein diet therapy (LPD) (Cohen et al. 1987).

Aggressive glycemic control in both type 1 and type 2 diabetic patients has been shown to reduce the risk of the development of overt nephropathy (Ohkubo et al. 1995; The DCCT Research Group 1995). ACEI has been shown to attenuate urinary albumin excretion and to reduce the development of overt nephropathy even when blood pressure of patients was normotensive (Ravid et al. 1994; Sano et al. 1996; The Microalbuminuria Captopril Study Group 1996). Use of a low protein diet in diabetic patients with microalbuminuria may modify the underlying glomerular injury and delay progression of nephropathy (Cohen et al. 1987). Furthermore, a combination therapy consisting of the 3 therapies mentioned above may be more effective than one alone, since not only reduced proteinuria but also a rise in glomerular filtration rate (GFR) was demonstrated after 3 years of intensive therapy (Manto et al. 1995). These findings led us to consider that each of these 3 therapies may have effects on diabetic nephropathy through different mechanisms.

To answer this question, changes in constituents of proteinuria and renal hemodynamics after additive combination therapy of glycemic control, LPD and ACEI were analyzed in type 2 diabetic patients with microalbuminuria in the present study.

Subjects and Methods

Subjects

Ten type 2 diabetic patients with microalbuminuria (7 males and 3 females: Group Mic), and for comparison, ten type 2 diabetic patients with macroalbuminuria (6 males and 4 females: Group Mac) and seven type 2 diabetic patients with normoalbuminuria (3 males and 4 females: Group Nor) were recruited from

patients who were admitted to our hospital in the present study. Microalbuminuria, macroalbuminuria and normoalbuminuria were defined as a urinary albumin excretion rate (AER) of 15-200 μg/minutes, more than 200 μg/minutes and less than 15 μg/minutes in 24-hour urine samples, respectively. Absence of clinical renal disease other than diabetic nephropathy was established by clinical history, normal urinary sediment and qualitative urinanalysis and the lack of detectable lesions in the kidneys by ultrasound examination, such as unilateral or bilateral atrophy, urinary stones, hydronephrosis or tumor. None of the patients had received any medication other than insulin, oral hypoglycemic agent (sulfonylureas) or antihypertensive agent. As one patient in group Nor, 4 patients in group Mic and 5 patients in group Mac had hypertension, they had been treated with calcium antagonists or α blockers under the conditions of systolic blood pressure of less than 140 mmHg and diastolic blood pressure of less than 80 mmHg 1 month before hospitalization for the protocol of the present study. Additionally, 10 healthy male volunteers aged 23-35, were recruited as a normal control population from the members of the medical research staff of our hospital (Group C). They had no history of diabetes mellitus, heart disease, hypertension or known renal disease. They were normoglycemic, normotensive and normoal-

TABLE 1. *Clinical characteristics of control subjects and type 2 diabetic patients with normoalbumrinuria, microalbuminuria and macroalbuminuria*

	Group C	Group Nor	Group Mic	Group Mac
Numbers (Females)	10 (0)	7 (4)	10 (3)	10 (4)
Body mass index (kg/m^2)	21.5±1.2	22.9±4.2	24.6±5.9	24.6±2.9
Age (Years)	26.5±4.5	65.0±10.5	63.1±5.31	56.6±10.0
Duration (Years)		10.7±11.4	14.5±7.5	21.3±13.4
SBP (mmHg)	111.6±7.0	124.6±15.6	127.0±21.2	154.8±11.3[b]
DBP (mmHg)	74.8±6.3	69.4±7.0	70.2±8.8	82.6±5.6[b]
Hypertension (numbers)	0	1	4	5
Total cholesterol (mg/100 ml)		219.1±69.8	204.5±55.3	260.3±77.6
Triglyceride (mg/100 ml)		139.1±103.0	109.4±43.8	208.9±142.8
HDLc (mg/100 ml)		64.9±25.4	52.1±12.3	40.7±11.2[a]
Hemoglobin Alc (%)		9.5±2.1	9.3±1.3	9.5±2.2
Retinopathy (numbers) (nil/S/P)		(6/0/1)	(2/4/4)	(0/3/7)

Data are expressed as mean±S.D.

Group C, control subjects; Group Nor, type 2 diabetic patients with normoalbuminuria; Group Mic, type 2 diabetic patients with microalbuminuria; Group Mac, type 2 diabetic patients with macroalbuminuria; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDLc, high density lipoprotein cholesterol; S, simple retinopathy; P, proliferative retinopathy (including patients who had been treated with photocoagulation therapy).

[a] $p<0.05$ when compared with group Nor and Mic.

[b] $p<0.01$ when compared with group C, Nor and Mic.

buminuric and had normal urinary sediment and qualitative urinanalysis.

All the subjects were fully informed before they gave their consent to this study.

Clinical characteristics of control subjects and the 3 groups of type 2 diabetic patients are shown in Table 1.

Protocol

We have studied the effects of a short-term period of glycemic control, low protein diet and administration of ACEI on renal hemodynamics and protein permselectivity in type 2 diabetic patients with microalbuminuria.

After hospitalization, patients in group Mic received insulin therapy or sulfonylurea and placed on the diabetic standard diet, which contained approximately 1.2 g•day^{-1}•kg ideal body weight (IBW)$^{-1}$ of protein and 30 kcal•day^{-1}•kg IBW^{-1} of total energy intake (normal protein diet). IBW was calculated as (height [m])$^2\times$22 (kg). Treatment of insulin or sulfonylurea was individualized. All patients had been instructed to monitor their blood glucose levels before each meal or at bed time by reagen-strips and to adjust their insulin or sulfonylurea doses to maintain pre-meal blood glucose concentrations in the range of 120–150 mg/100 ml. It took 1 to 3 weeks to attain these blood glucose levels (glycemic control [GC] period). After blood glucose levels had been controlled, the patients were placed on a 1-week low protein diet. The patients received a diet containing 0.7 g • kg IBW^{-1} protein (GC+LPD period). A 1-week low protein diet regimen was chosen for the present study because a significant effect of protein restriction on the renal hemodynamics was reported to be demonstrated at only 1 week after initiation of the low protein diet (Kupin et al. 1987). Thereafter, a 1-week administration of enalapril was added to the preceding glycemic control and low protein diet therapy (GC+LPD+ACEI period). A dose of 2.5 mg/day of enalapril was administered to the patients with less than 120 mmHg and 80 mmHg of systolic and diastolic blood pressure levels, and 5 mg/day of enalapril was administered to the other patients.

Twenty-four-hour urine samples and blood samples taken in a fasting state were collected from all the subjects on the first day of the GC period and the last day of each therapy for measurements of albumin (Alb), IgG, ceruloplasmin (CRL), α2-macroglobulin (α2), creatinine and urea nitrogen, calculating the 24-hour endogenous creatinine clearance (Ccr), the 24-hour renal clearance of each plasma proteins (C-Alb, C-IgG, C-CRL and C-α2) and the protein intake.

To evaluate effects of LPD and ACEI on the renal hemodynamics, filtration fraction, which may be considered to be parallel variations of intraglomerular pressure (Böhlen et al. 1997), was estimated as the ratio of the renal clearance of thiosulphate (Thios; Banyu Pharmaceutical Co., Ltd., Tokyo) to the renal clearance of *p*-aminohippurate (PAH; Daiichi Pharmaceutical Co., Ltd., Tokyo). The studies of renal hemodynamics were performed in the morning after an

overnight fast on the first day of each GC+LPD and GC+LPD+ACEI periods and on the day after a 1-week therapy of GC+LPD+ACEI period had been completed. A steady state of water diuresis was induced by oral water loading. A teflon cannula was inserted into an antecubital vein of each arm for infusion and blood sampling. After injection of priming doses of Thios and PAH, a sustaining dose of continuous injection was started to maintain constant plasma concentration of 15-25 mg/100 ml and 2-4 mg/100 ml, respectively. After a 1-hour equilibration period, the patients were required to void completely and to collect 1-hour exact timed urine. Blood samples were drawn at the midpoint of the 1-hour urine collection period. Then plasma and urinary concentrations of Thios and PAH were measured to calculate the clearance of Thios (C-Thios) and PAH (C-PAH), respectively.

For comparison, exact 24-hour urine samples and blood samples taken in a fasting state were collected from the patients of group Nor and Mac, who were admitted to our hospital, and the control subjects (Group C) to calculate Ccr, C-Alb, C-IgG, C-CRL and C-α2.

Measurements

Urinary and serum IgG, CRL (Yamazaki et al. 1995) and α2 (Ito et al. 1995) were measured by immuno-radiometric assay in our laboratory. In brief, 0.3 ml of coating antiserum was added to the assay tubes (Nunc tubes, Roskilde, Denmark) and the reaction was allowed to proceed at room temperature for 24 hours. The tubes were washed 3 times with 0.4 ml of 0.1 M phosphate buffer (pH 7.4) containing 0.05% Tween 20, 0.3% BSA (bovine serum albumin; SIGMA Chemical Co., St. Lous, MO, USA) and 0.01% NaN_3 (washing buffer). Then, 0.1 ml of 0.1 M phosphate buffer (pH 7.4) containing 0.15% Tween 20, 0.9% BSA and 0.03% NaN_3, and either 0.2 ml of standard (N Protein Standard SY, Behring Diagnostics Inc., Marburg, Germany) or samples were added to the tubes. After 16 hours of incubation at room temperature, the tubes were washed 3 times with 0.4 ml of washing buffer. Then 0.3 ml of radioiodinated antibody was added to the tubes and allowed to react at room temperature for 16 hours. The tubes were washed with 0.4 ml of washing buffer, and the radioactivity was counted. As a coating antiserum, 1 : 2000 diluted rabbit antiserum to human IgG (Organo Teknika Corporation, Durham, NC, USA), 1 : 1000 diluted goat anti-human CRL antiserum (Dia Sorin, Stilwater, MN, USA) and 1 : 2000 diluted rabbit anti-human α2 antiserum (raised in rabbit No. 1 in our laboratory) were used in IgG, CRL and α2 assays, respectively. As a radioiodinated antibody, affinity purified rabbit anti-human IgG antiserum (MBL Co., Ltd., Tokyo), affinity purified rabbit anti-human CRL antiserum (DAKO Co., Ltd., Glostrup, Denmark) and affinity purified rabbit anti-human α2 antiserum (raised in rabbit No.2 in our laboratory) were used in IgG, CRL and α2 assays, respectively. Radioiodinated antibody was prepared by the chloramin T method. Intra- and inter-assay coefficient

variances for the immunoradiometric assays of these plasma proteins were almost 3% and 10%, respectively.

Albumin levels in urine and serum were measured by radioimmunoassay (RIA) using double antibody technique (Brodows et al. 1986). Radioiodinated human albumin (Organo Teknika Corporation, Durham, NC, USA) was prepared by the chloramin T method. As a first antibody, rabbit anti-human albumin antiserum, and as a second antibody, goat anti-rabbit IgG antiserum were purchased from Organo Teknika Corporation. Intra- and interassay coefficients of variation for this method were 5 and 12%, respectively. Creatinine in urine and serum were measured by Follin's method. Urinary urea nitrogen was determined by urease-UV method. Serum concentration of total cholesterol, triglyceride and HDL-cholesterol were measured by enzymatic methods using an automated multi-analyzer (7600, Hitachi, Tokyo). HbA1c was measured by high performance liquid chromatography method using an automated analyzer (HLC-723GHb V A1c 2.2, Tosoh, Tokyo). Thiosulphate and *p*-aminohippurate concentration in plasma and urine were measured according to the standard methods (Brun 1950, 1951).

All the samples of urine and serum of the subjects were stored at −80°C until measurement.

Calculations

Ccr, C-Thios, C-PAH and each of renal clearance of Alb, IgG, CRL and $\alpha 2$ were corrected for body surface area and were expressed as /1.48 m^2. Body surface area was calculated from height and body weight. Filtration fraction (FF) was calculated as the ratio of the renal C-Thios to the renal C-PAH.

Protein intake was calculated from urinary urea nitrogen ($g \cdot day^{-1}$; UUN) and estimated non-urea nitrogen (NUN) excretion of 0.031 g N·kg body $weight^{-1} \cdot day^{-1}$ (Maroni et al. 1985) as following formula (Paul and Southgate 1978; Isaksson 1980): Protein intake = (UUN + NUN) × 6.25 (g/day).

To detect improvement of the impairments of glomerular charge selectivity and size selectivity before and after each therapy, the ratio of the renal clearance of two plasma proteins with similar molecular radii and different isoelectric points (pIs) (ceruloplasmin and IgG: CRL/IgG) and the ratio of the renal clearance of two plasma proteins with similar pIs and different molecular radii ($\alpha 2$-macroglobulin and albumin: $\alpha 2$/Alb) were calculated as a glomerular charge selectivity index and as a glomerular size selectivity index, respectively.

Statistics

Values are expressed as mean values ± S.D. or median with range. To test for differences among 3 groups or repeated values in each individual, the Kruskal-Wallis test or the repeated Friedman test were used, respectively. After multiple comparisons revealed a significant difference, statistically significant differences

between 2 groups or 2 repeated values in each individual were calculated using the Mann-Whitney test or the Wilcoxon signed-ranks test, respectively. All calculations were made using Stat View software package (Abacus Concept, Inc., Berkeley, CA, USA).

Results

Table 1 shows the clinical characteristics of groups C, Nor, Mic and Mac. No differences were found between group Nor and Mic except the rates of hypertension and retinopathy. The rate of patients with any retinopathy (including patients who had been treated with photocoagulation therapy) was high in group Mac, Mic and Nor in that order. Both systolic and diastolic blood pressure levels in group Mac were significantly higher than the other three groups ($p<0.01$). High-density-lipoprotein-cholesterol level was lower in group Mac than group Nor and Mic ($p<0.05$).

Table 2 shows creatinine clearance, urinary albumin excretion rate and renal clearance of Alb, CRL, IgG and α2 in each group. In group Mic, values before GC periods are presented. Since 3 of 10 patients in group Mic had already targeted glycemic control levels on admission as premeal blood glucose concentra-

Table 2. *Creatinine clearance, urinary albumin excretion rate and renal clearance of 4 plasma proteins in control subjects and type 2 diabetic patients with normoalbuminuria, microalbuminuria and macroalbuminuria before glycemic control therapy*

	Group C ($n=10$)	Group Nor ($n=7$)	Group Mic ($n=7$)	Group Mac ($n=10$)
Creatinine clearance ($ml \cdot min^{-1} \cdot [1.48\ m^2]^{-1}$)	103.8±13.0	114.8±37.5	108.8±12.8	62.9±26.6[b]
Urinary albumin excretion rate ($\mu g/min$)	4.7 (2.1–9.8)	6.0 (2.1–13)	83[a] (44–170)	1100[c] (270–3900)
C-Albumin ($\mu l \cdot min^{-1} \cdot [1.48\ m^2]^{-1}$)	0.077 (0.035–0.17)	0.12 (0.045–0.22)	2.2[a] (0.81–4.4)	27[c] (5.8–110)
C-IgG ($\mu l \cdot min^{-1} \cdot [1.48\ m^2]^{-1}$)	0.12 (0.052–0.16)	0.18 (0.057–0.22)	0.74[a] (0.67–3.1)	8.9[c] (1.3–40)
C-ceruloplasmin ($\mu l \cdot min^{-1} \cdot [1.48\ m^2]^{-1}$)	0.17 (0.060–0.26)	0.19 (0.047–0.29)	2.9[a] (1.6–7.2)	40[c] (4.3–71.1)
C-α2-macroglobulin ($nl \cdot min^{-1} \cdot [1.48\ m^2]^{-1}$)	4.1 (1.2–9.1)	6.9 (1.2–22)	43[a] (19–110)	380[c] (53–930)

Data are expressed as mean±S.D. (creatinine clearance) or median (range) (urinary excretion rate of 4 plasma proteins). Group C, control subjects; Group Nor, type 2 diabetic patients with normoalbuminuria; Group Mic, type 2 diabetic patients with microalbuminuria; Group Mac, type 2 diabetic patients with macroalbuminuria; C-, renal clearance of each plasma protein.

[a] $p<0.01$ when compared with group C and Nor.

[b] $p<0.01$ when compared with group C, Nor and Mic.

[c] $p<0.001$ when compared with group C, Nor and Mic.

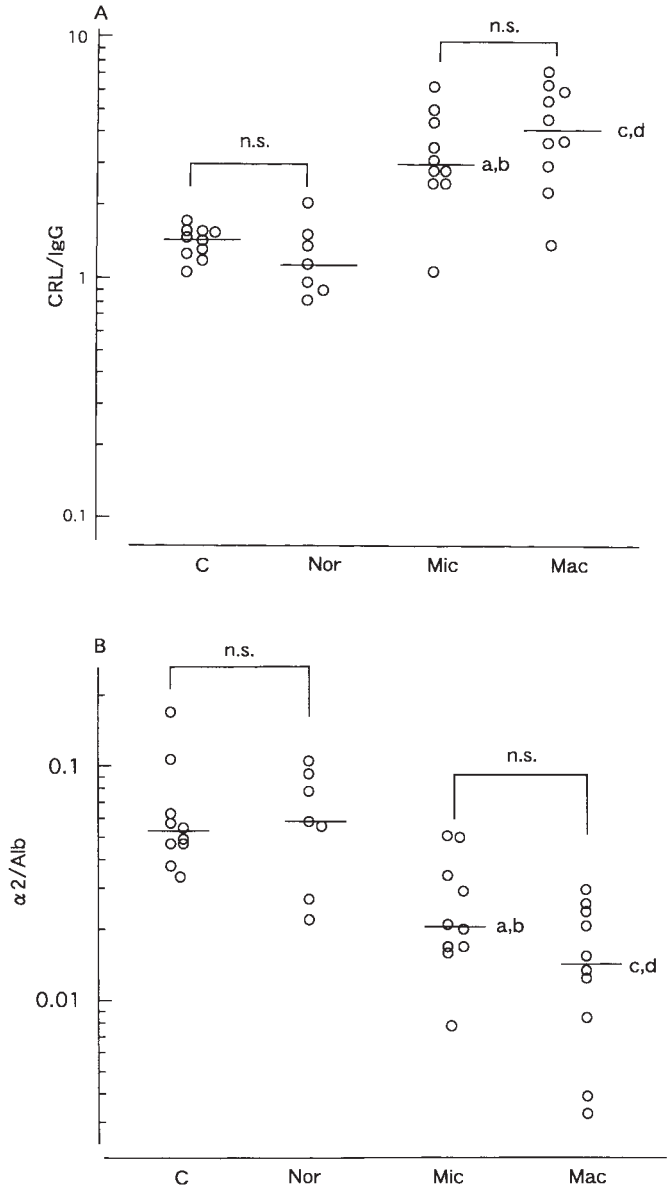

Fig. 1. Glomerular charge and size selectivity in control subjects and type 2 diabetic patients with normoalbuminuria, microalbuminuria and macroalbuminuria. A: glomerular charge selectivity index estimated as the ratio of the renal clearance of ceruloplasmin to the renal clearance of IgG. B: glomerular size selectivity index estimated as the ratio of the renal clearance of α2-macroglobulin to the renal clearance of albumin. C, control subjects; Nor, type 2 diabetic patients with normoalbuminuria; Mic, type 2 diabetic patients with microalbuminuria; Mac, type 2 diabetic patients with macroalbuminuria; CRL/IgG, the ratio of the renal clearance of IgG to the renal clearance of ceruloplasmin; α2/Alb, the ratio of the renal clearance of α2-macroglobulin to the renal clearance of albumin. Horizontal bars indicate the median of each group. [a]$p<0.01$ when compared with group C. [b]$p<0.05$ when compared with group Nor. [c]$p<0.001$ when compared with group C. [d]$p<0.01$ when compared with group Nor. n.s., indicates not significant.

tion <150 mg/100 ml, data on these 3 patients were excluded from the values for group Mic in Table 2. Renal clearances of Alb, CRL, IgG and $\alpha 2$ in group Mic were significantly higher than both group C and group Nor ($p<0.01$). In group Mac, all of renal clearances of Alb, CRL, IgG and $\alpha 2$ were higher compared with the other three groups ($p<0.001$). Creatinine clearance in group Mac was significantly lower, compared with the other three groups ($p<0.01$). Thus, all the renal clearances of Alb, IgG, CRL and $\alpha 2$ increased in parallel with the progression of diabetic nephropathy.

Analysis of urine samples after glycemic control showed that CRL/IgG as a glomerular charge selectivity index was significantly higher in groups Mic and Mac compared with groups C and Nor as seen in Fig. 1A. The ratio of $\alpha 2$/Alb as a glomerular size selectivity index was significantly lower in groups Mic and Mac compared with groups C and Nor (Fig. 1B).

The individual average of the 3 daily premeal blood glucose levels before treatment (baseline values), after GC period (the first period), after GC+LPD period (the second period) and after GC+LPD+ACEI period (the third period) were 232.0 ± 57.4, $166.0^{*}\pm24.2$, $141.1^{*}\pm23.1$ and $150.4^{*}\pm24.6$ mg/100 ml, (Mean$\pm$S.D., $^{*}p<0.05$ vs. baseline values), respectively (Table 3). Thus, glycemic control levels were improved significantly during the first period and maintained at stable levels during the second and third periods. Protein intake was decreased significantly after the second period and maintained until the endpoint of the protocol (Table 3), indicating accurate compliance of patients during LPD. Blood pressure levels did not change through the present study.

Although C-Alb, C-IgG and C-CRL significantly decreased in steps after each therapy, C-$\alpha 2$ decreased slightly but not significantly during the first and second

TABLE 3. *Changes in blood glucose levels, protein intake and blood pressure levels of type 2 diabetic patients with microalbuminuria*

	Average of 3 premeal blood glucose levels (mg/100 ml)	Protein intake (g/day)	Blood pressure (mmHg)
Baseline	232.0 ± 57.4	67.9 ± 10.7	126.3 ± 18.0 /70.3 ± 6.6
Aater GC therapy	166.0 ± 24.2^{a}	64.9 ± 11.3	127.1 ± 22.4 /69.1 ± 8.6
After GC+LPD therapy	141.4 ± 23.1^{a}	44.5 ± 10.1^{b}	124.0 ± 22.3 /70.9 ± 12.6
After GC+LPD+ACEI therapy	150.4 ± 24.6^{a}	40.5 ± 7.6^{b}	122.6 ± 16.7 /76.3 ± 15.4

Data are expressed as mean$\pm$S.D.

GC, glycemic control; LPD, low protein diet; ACEI, angiotensin converting enzyme inhibitor.

[a] $p<0.05$ when compared with baseline.

[b] $p<0.01$ when compared with baseline.

periods whereas C-α2 significantly decreased only after the third period (Table 4). Since 3 of 10 patients in group Mic had left the hospital before the third period has been completed because of their individual circumstances, data of the 7 remainders were showed in Tables 3 and 4, and Fig. 2.

After the first period, creatinine clearance significantly decreased from 108.8 ± 12.8 ml $\cdot$ min^{-1} $\cdot$ (1.48 m^2)$^{-1}$ to 90.3 ± 13.3 ml $\cdot$ min^{-1} $\cdot$ (1.48 m^2)$^{-1}$ (Mean $\pm$ S.D., $p<0.05$). During the second period, Ccr slightly decreased from 90.3 ± 13.3 ml $\cdot$ min^{-1} $\cdot$ (1.48m^2)$^{-1}$ to 86.1 ± 9.8 ml $\cdot$ min^{-1} $\cdot$ (1.48 m^2)$^{-1}$ (Mean $\pm$ S.D., $p=0.068$) and during the third period, Ccr significantly decreased from 86.1 ± 9.8 ml $\cdot$ min^{-1} $\cdot$ (1.48 m^2)$^{-1}$ to 73.5 ± 20.1 ml $\cdot$ min^{-1} $\cdot$ (1.48 m^2)$^{-1}$ (Mean $\pm$ S.D., $p<0.05$) (Table 4). Thus, Ccr decreased in steps after each therapy. Changes in FF in the second and the third periods are given in Fig. 2. During the second period, FF showed a tendency of decrease from 0.28 ± 0.070 to 0.23 ± 0.023 (Mean $\pm$ S.D., not significantly), while it was significantly decreased from 0.23 ± 0.023 to 0.18 ± 0.029 (Mean $\pm$ S.D., $p<0.05$) during the third period.

Glomerular charge selectivity index calculated as C-CRL/C-IgG (CRL/IgG) and glomerular size selectivity index calculated as C-α2/C-Alb (α2/Alb) did not change during any of the 3 therapies (Figs. 3 and 4).

TABLE 4. *Changes in creatinine clearance and renal clearances of albumin, IgG, ceruloplasmin and α2-macroglobulin in response to serial ⟨glycemic control⟩ therapy, ⟨glycemic control + low protein diet⟩ therapy and ⟨glycemic control + low protein diet + angiotensin converting enzyme inhibitor⟩ therapy in type 2 diabetic patients with microalbuminuria*

	Baseline ($n=7$)	After GC ($n=10$)	After GC+LPD ($n=10$)	After GC+LPD +ACEI ($n=7$)
Creatinine clearance (ml$\cdot$min$^{-1}\cdot$[1.48 m^2]$^{-1}$)	108.8 ± 12.8	90.3 ± 13.3^a	86.1 ± 9.8	73.5 ± 20.1^c
C-Albumin (μl$\cdot$min$^{-1}\cdot$[1.48 m^2]$^{-1}$)	2.2 (0.81–4.4)	0.89^a (0.49–2.5)	0.76^b (0.29–1.6)	0.57^c (0.26–1.1)
C-IgG (μl$\cdot$min$^{-1}\cdot$[1.48 m^2]$^{-1}$)	0.74 (0.67–3.1)	0.56^a (0.35–0.20)	0.36^b (0.19–1.2)	0.24^c (0.11–1.1)
C-ceruloplasmin (μl$\cdot$min$^{-1}\cdot$[1.48 m^2]$^{-1}$)	2.9 (1.6–7.2)	1.8^a (0.80–4.0)	1.5^b (0.47–2.6)	0.92^c (0.15–1.9)
C-α2-macroglobulin (nl$\cdot$min$^{-1}\cdot$[1.48 m^2]$^{-1}$)	43 (19–110)	23 (11–120)	19 (6.0–52)	11^c (7.4–44)

Data are expressed as mean $\pm$ S.D. (creatinine clearance) or median (range) (urinary excretion rate of 4 plasma proteins) C-, renal clearance of; GC, glycemic control; LPD, low protein diet; ACEI, angiotensin converting enzyme inhibitor.

[a] $p<0.05$ when compared with baseline values.

[b] $p<0.05$ when compared with values after glycemic control therapy.

[c] $p<0.05$ when compared with values after the combination therapy of glycemic control and low protein diet.

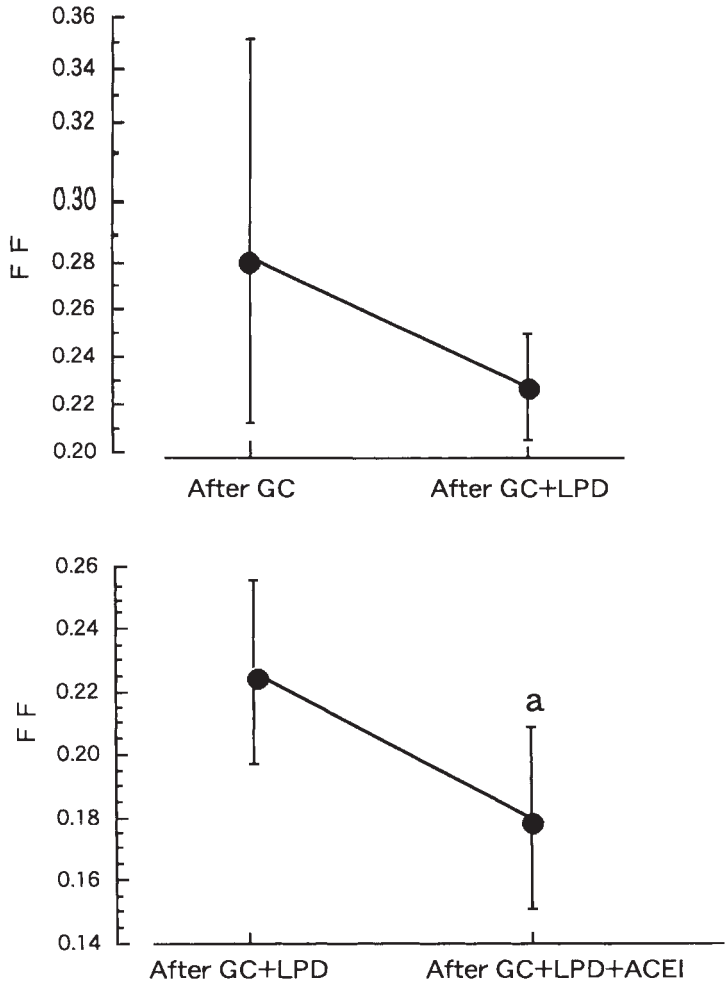

Fig. 2. Changes in filtration fraction in type 2 diabetic patients with microalbuminuria.

Filtration fraction (FF) were measured before and after addition of low protein diet therapy to glycemic control therapy, and further administration of angiotensin converting enzyme inhibitor to the combination therapy of glycemic control and low protein diet.

Values are expressed mean±S.D. Vertical bars indicate S.D. GC, glycemic control therapy; LPD, low protein diet therapy; ACEI, administration of angiotensin converting enzyme. $^{a}p<0.05$ when compared with the values after the combination therapy of glycemic control and low protein diet.

Discussion

To examine the degree of impairment of charge selectivity of the glomerulus, the ratio of the renal IgG4 clearance to the renal IgG clearance (IgG4/IgG: charge selectivity index) has been used, since the two plasma proteins have identical molecular radii and different pIs (Pietravalle et al. 1991; Deckert et al. 1993; Morano et al. 1993). As serum IgG4 levels in control subjects and diabetic patients showed a remarkably wide range (0.012–2.0 mg/ml), however, the degree of change in charge selectivity of glomerulus estimated from IgG4/IgG may tend to be underestimated. Therefore, instead of IgG4, the charge selectivity index was calculated as the ratio of the renal CRL clearance to the renal IgG clearance in this study. The reasons of the use of CRL are as follows. First, the glomer-

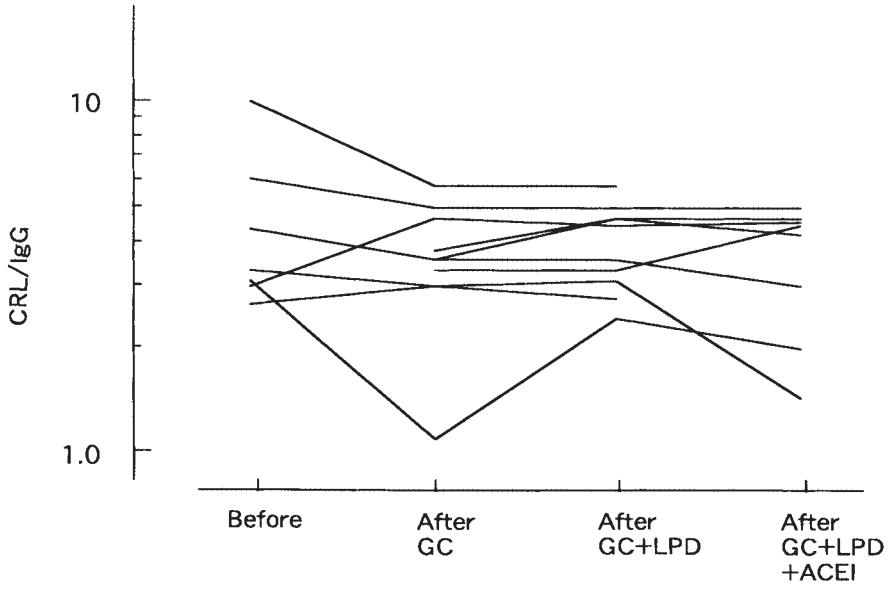

Fig. 3. Changes in glomerular charge selectivity after additive 3 kinds of therapies in type 2 diabetic patients with microalbuminuria.

Glomerular charge selectivity index, estimated as the ratio of the renal clearance of ceruloplasmin to the renal clearance of IgG (CRL/IgG) did not change after each of glycemic control therapy (GC), additive low protein diet therapy (LPD) to glycemic control therapy or further administration of angiotensin converting enzyme inhibitor (ACEI) to the combination therapy of glycemic control and low protein diet.

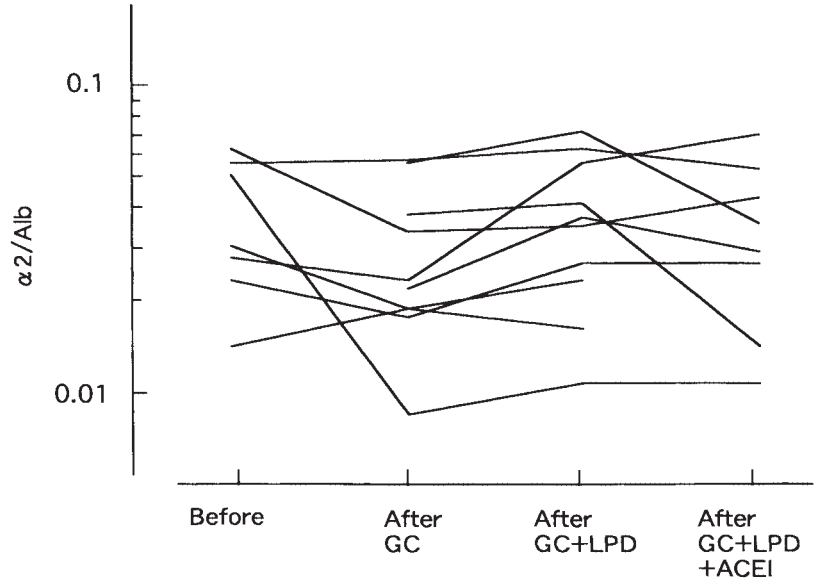

Fig. 4. Changes in glomerular size selectivity after additive 3 kinds of therapies in type 2 diabetic patients with microalbuminuria.

Glomerular size selectivity index estimated as the ratio of the renal clearance of α2-macroglobulin to the renal clearance of albumin (α2/Alb) did not change after each of glycemic control therapy (GC), additive low protein diet therapy (LPD) to glycemic control therapy or further administration of angiotensin converting enzyme inhibitor (ACEI) to the combination therapy of glycemic control and low protein diet.

ular handling of CRL and anionic IgG by anionic components of basement membrane was reported to be similar in experimental proteinuric rats (Bertolatus et al. 1987). Second, serum CRL levels were measured within a narrow range (0.11–0.20 mg/ml). Third, the molecular radii of CRL and IgG are almost similar, and their pIs are 4.4 and 7.4, respectively. Based on these aspects of biochemical features of CRL, CRL/IgG can be considered as a reliable marker of charge selectivity of glomerulus.

The charge selectivity index measured as the ratio of CRL/IgG was higher in type 2 diabetic patients with microalbuminuria and with macroalbuminuria than in patients with normoalbuminuria in the present study. This finding suggests that at least an impairment of charge selectivity in the glomerulus may be initiated in diabetic patients with microalbuminuria. Loss of charge selectivity in the stage of early diabetic nephropathy was demonstrated in previous studies (Pietravalle et al. 1991; Deckert et al. 1993; Morano et al. 1993). The present result seems to support their findings.

In addition to loss of charge selectivity, an impairment of pore size selectivity was suspected in patients with microalbuminuria, since urinary excretion of $\alpha 2$ was significant higher in patients with microalbuminuria than in control subjects and diabetic patients with normoalbuminuria. The molecular radius of $\alpha 2$ is 88 Å, and its pI is 5.4. Our recent study (Narita et al. 1999) showed that $\alpha 2$ with its large molecular radius did not increase its flux through physiological pore of the glomerulus in healthy subjects even when GFR was increased by oral protein loading. Therefore, it seems reasonable to think that urinary excretion of $\alpha 2$ increased its flux through the pores of the glomerular basement membrane with impaired size selectivity in microalbuminuric diabetic patients. Although several investigators reported that impairment of glomerular pore size selectivity was present in type 1 diabetic patients with macroalbuminuria (Friedman et al. 1983; Tomlanovich et al. 1987; Deckert et al. 1993), recent analysis using neutral charged dextran clearance revealed that the rise in proportion of large, so called non-discriminatory pores (shunt pathway) of glomerular basement membrane is present in microalbuminuric type 1 diabetic patients (Scandling and Myers 1992). The radius of this large pore was thought to be approximately 110 to 115 Å (Tencer et al. 1998a). Our present result regarding C-$\alpha 2$, taken together with these findings mentioned above, suggests that an impairment of pore size selectivity due to the rise in proportion of large and non-discriminatory pores of glomerular basement membrane may be present in microalbuminuric type 2 diabetic patients.

To examine the degree of impairment of pore size selectivity of the glomerulus during each therapies, the ratio of the renal $\alpha 2$ clearance to the renal Alb clearance ($\alpha 2$/Alb) was calculated in this study. The molecular radii of Alb and $\alpha 2$ are 36 Å and 88 Å , respectively, and the pIs are 4.8–5.4 and 5.4, respectively. Thus, the molecular radii of these plasma proteins are different, but these pIs are

similar. Furthermore, the ratio of α2/Alb was recently reported to be superior to that of IgG/Alb in characterization of glomerular diseases (Tencer et al. 1998b). Based on these findings, we used the ratio of α2/Alb as a indicator of the degree of impairment of pore size selectivity of the glomerulus during each therapy.

α2/Alb values in groups Mic and Mac in this study was comparable to that in the recent analysis using selectivity index of α2/Alb (Tencer et al. 1998b). This levels of size selectivity index were judged to be non-selective in various renal diseases (Tencer et al. 1998b). According to their idea, so called non-selective proteinuria was present in both macroalbuminuric and microalbuminuric stages in diabetic nephropathy. Additionally, lower α2/Alb in groups Mic and Mac than that in group C and Nor is in agreement with the results that decrease in size selectivity index was found in parallel with progression of diabetic nephropathy (Scandling and Myers 1992; Gall et al. 1994), although size selectivity index in their studies was estimated as IgG/Alb. The phenomenon in which renal clearance of albumin is higher than that of IgG or α2 may be explained by the idea proposed by Scandling and Myers (1992). They suggest that albumin (molecular radius=36 Å) can be filtered through the major portion of the restrictive pores of glomerular basement membrane (pore radius=56 Å), whereas IgG (molecular radius=55 Å) and α2 can not be filtered through this restrictive pore, even when impaired charge selectivity is present. In contrast, these three serum proteins could be filtered through large non-discriminatory pore (the so called, shunt pathway). Then, α2/Alb or IgG/Alb was decreased in parallel with the progression of diabetic nephropathy.

Glycemic control caused significantly decreased Ccr, C-Alb, C-IgG and C-CRL, and slightly but not significantly C-α2. In view of the fact that molecular radii of IgG and CRL are similar and their pIs are different, it seems unlikely that a parallel decrease in C-IgG and C-CRL was due to modification of glomerular charge selectivity by glycemic control. Furthermore, considering that the pIs of Alb, CRL, α2 are anionic and molecular radii of these plasma proteins are different and that α2/Alb was not affected, it seems probable that significantly decreased urinary excretions of the former two proteins and slight decrease in C-α2 did not result from improvement of pore size selectivity. Significantly decreased creatinine clearance suggests that glycemic control may cause the decreased proteinuria through improvement of intraglomerular hydraulic pressure.

When a low protein diet therapy was added to the glycemic control therapy, it caused significantly decreased C-Alb, C-IgG and C-CRL, and slightly reduced Ccr and C-α2. Furthermore, FF decreased slightly but not significantly. Charge selectivity index (CRL/IgG) and size selectivity index (α2/Alb) did not change. Therefore, the effects of a low protein diet for 1 week on decreased proteinuria and changes in renal hemodynamics seemed to be similar to that of glycemic control. This finding, therefore, suggests that short-term therapy with a low protein diet may improve pathophysiological condition of the diabetic

nephropathy through the mechanism of reduced intraglomerular hydraulic pressure.

When administration of ACEI was added to the therapies of combined glycemic control and low protein diet, C-Alb, C-IgG, C-CRL, C-α2, Ccr and FF decreased significantly. In contrast, charge and size selectivity index did not change. Thus, it seems likely that the anti-proteinuric effect of ACEI for 1 week is similar to the effects of precedent two therapies through reduction of intraglomerular hydraulic pressure. It has been established that ACEI may play an effective role in slowing the progression of experimental diabetic nephropathy through the mechanism of reducing the intraglomerular hydraulic pressure that was induced by dilatation of the efferent arteriole in the glomerulus (Zatz et al. 1986; Anderson et al. 1989). In human diabetic nephropathy, recent meta-analysis indicated the renoprotective effect of ACEI independently of reduced systemic blood pressure (Weidmann et al. 1995). The present results confirmed that ACEI has a beneficial effect on diabetic nephropathy through improvement of renal hemodynamics without reduced systemic blood pressure levels. In contrast to glycemic control therapy alone and combined therapy of glycemic control and low protein diet, additive administration of ACEI caused a statistically significant decrease in C-α2. The decrease in FF found only after administration of ACEI may cause significant decrease in C-α2. The finding, therefore, suggests that administration of ACEI is the most effective therapy for reducing intraglomerular pressure among the 3 therapies.

The present study showed that significant decrease in urinary excretion of proteins with small molecular radii was transformed to ones with large molecular radii in steps when a gradual decrease in the intraglomerular hydraulic pressure induced decreased urinary excretion of plasma proteins, and urinary excretion of proteins with quite large molecular radii, such as α2, decreased only after remarkable decrease in intraglomerular hydraulic pressure was obtained. This idea is supported by the report that filtration fraction may be considered to be a parallel variation of intraglomerular pressure (Böhlen et al. 1997).

It must be emphasized in the present study that the addition of each therapy caused additive improvement of proteinuria in type 2 diabetic patients with microalbuminuria. The result agreed with the previous report, in which intensive therapy consisting of glycemic control, low protein diet and blood pressure control with ACEI in type 1 diabetic patients with early stage diabetic nephropathy may be effective in slowing the progression to renal failure (Manto et al. 1995). Thus, combination therapies should be carried out in diabetic patients with nephropathy.

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