Endocrine Journal 2003, **50** (2), 209–213

NOTE

The Higher Immunoreactivity to ACE (Angiotensin Converting Enzyme) in Patients with Type 2 Diabetes Mellitus than in Non-Diabetic Individuals

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Abstract. In a random sample of 200 patients with type 2 diabetes mellitus, immunoreactivities to ACE (angiotensin converting enzyme) were measured by ELISA. Immunoreactivities were positive for 129 (64.5%) patients, and were positive in 30 (83.3%) out of 36 patients in the early stage of clinical diabetic nephropathy. Serum ACE activity in rabbits immunized with ACE decreased to 50% of the control level after 7 months (78.0 ± 3.8 IU/L/37°C, basal, 42.0 ± 5.0 at 7 months and 33.3 ± 3.5 IU/L/37°C at 8 months, respectively). When rabbit serum containing antiACE antibodies was mixed, after heat-treatment at 56°C for 30 min, with normal human serum, the ACE activity was reduced in a concentration-dependent manner. These results suggested that anti-ACE autoantibody may be present in patients with type 2 diabetes mellitus. However, the absence of data on the epitope for the antibody does not allow any conclusion except that the immunoreactivities to ACE are higher in type 2 diabetic patients than in non-diabetic individuals.

Key words: Immunoreactivity to ACE, ELISA assay, Type 2 diabetes mellitus, Diabetic nephropathy
(*Endocrine Journal* **50**: 209–213, 2003)

IT is suggested that ACE (angiotensin converting enzyme) may have a proximate effect on the etiology and pathogenesis of atherosclerosis [1, 2]. In addition, an immunologic reaction to ACE may occur during the process of atherogenesis, related to a specific antigen, as autoantibodies to lipoprotein have been found in atherosclerosis [3]. In this study, we examined the possibility that the immunoreactivity to ACE, if raised, has some effect on the etiology or pathogenesis of diabetic nephropathy in patients with type 2 diabetes mellitus, since atherosclerotic changes that increase with atherosclerosis and diabetes mellitus, are increased more remarkably in diabetes mellitus.

Received: May 7, 2002
Accepted: November 12, 2002
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Materials and Methods

Informed consent was obtained and the Human Research Committee of the Kawasaki Medical School approved this study on human subjects.

1. In control tests, 30 control patients over 40 years old (15 males and 15 females), who had no diseases such as diabetes mellitus, cardiac disease, renal disease, hepatic disease, hypertension, hyperlipidemia, etc., 60 healthy subjects from 21 to 60 years old (30 males and 30 females), and 30 healthy subjects from 18 to 20 years old (15 males and 15 females) were studied. The AI (atherogenic index) was 2.1 ± 0.8 (2.3 ± 0.9 for male and 1.8 ± 0.6 for female patients, respectively) in the control patients. In the healthy subjects from 21 to 40 years old, the AI was 2.0 ± 0.7 and there was a significant difference between male and female subjects (2.4 ± 0.6 for 30 male and 1.8 ± 0.6 for 30 female subjects, respectively). In the

healthy subjects from 18 to 20 years old, the AI was 1.6 ± 0.4 (1.7 ± 0.4 for male and 1.6 ± 0.4 for female subjects, respectively).

2. Out of random 200 patients with type 2 diabetes mellitus, 118 patients were male, 9 patients were less than 40 years old, and the mean duration time of the disease was 17 years (from 1 year to 39 years). These patients were divided into 5 stages according to first morning urinary albumin index (mg/g·creatinine). The first morning urinary albumin index in normal subjects was less than 11 mg/g · Cr. Therefore, in stage 1 (no nephropathy), the index is below 11 mg/g · Cr; for stage 2 (pre-nephropathy), the index is 11–30 mg/g · Cr; in stage 3 (incipient-nephropathy), the index is 30–100 mg/g · Cr; for stage 4 (overt-nephropathy I), the index is 100–300 mg/g · Cr ; and in stage 5 (overt-nephropathy II), the index is over 300 mg/g · Cr.

Hypertension was accompanied in 116 out of the 200 patients (58.0%) : 35.7% in stage 1, 59.0% for stage 2, 58.3% in stage 3, 76.2% for stage 4 and 91.1% in stage 5 (Table 2). Diabetic retinopathy was exhibited in 48.1% (64/133 patients examined): 25.0%

Table 1. Immunoreactivities to ACE determined by ELISA in 200 patients with type II diabetes mellitus

Stage*	Stage 1 (n = 70)	Stage 2 (n = 39)	Stage 3 (n = 36)	Stage 4 (n = 21)	Stage 5 (n = 34)
Urinary albumin index (mg/g · creatinine)	below 11	11–30	30–100	100–300	over 300
Nephropathy	no-	pre-	incipient-	overt-nephropathy	
Patients with no immunoreactivity 71/200 (35.5%)	29/70 (41.4%)**	11/39 (28.2%)	6/36 (16.7%)**	6/21 (28.6%)	19/34 (55.9%)
	40/109 (36.7%)		31/91 (34.1%)		
Patients with IgM immunoreactivity 56/200 (28.0%)	19/70 (27.1%)	10/39 (25.6%)	11/36 (30.6%)	8/21 (38.1%)	8/34 (23.5%)
	29/109 (26.6%)		27/91 (29.7%)		
Patients with IgG immunoreactivity 35/200 (17.5%)	8/70 (11.4%)***	6/39 (15.4%)	13/36 (36.1%)***	5/21 (23.8%)	3/34 (8.8%)
	14/109 (12.8%)		21/91 (23.1%)		
Patients with IgM & IgG immunoreactivities 38/200 (19.0%)	14/70 (20.0%)	12/39 (30.8%)	6/36 (16.7%)	2/21 (9.5%)	4/34 (11.8%)
	26/109 (23.9%)		12/91 (13.2%)		

* cf. text. .

** or *** There was a significant difference between the two means.

Table 2. Hypertension in 200 patients with type II diabetes mellitus

Stage*	Stage 1 (n = 70)	Stage 2 (n = 39)	Stage 3 (n = 36)	Stage 4 (n = 21)	Stage 5 (n = 34)
Urinary albumin index (mg/g · creatinine)	below 11	11–30	30–100	100–300	over 300
Nephropathy	no-	pre-	incipient-	overt-nephropathy	
Hypertensive patients accompanied by no antiACE immunoreactivity 43/71 (60.6%)	8/29 (27.6%)	6/11 (54.5%)	5/6 (83.3%)	5/6 (83.3%)	19/19 (100%)
	14/40 (35.0%)**		29/31 (93.5%)**		
Hypertensive patients accompanied by antiACE immunoreactivities 73/129 (56.6%)	17/41 (41.5%)	17/28 (60.7%)	16/30 (53.3%)	11/15 (73.3%)	12/15 (80.0%)
	34/69 (49.3%)***		39/60 (65.0%)***		
Total patients with hypertension	25/70 (35.7%)	23/39 (59.0%)	21/36 (58.3%)	16/21 (76.2%)	31/34 (91.1%)

* cf. text.

** There was a significant difference between the two means.

*** There was no significant difference between the two means.

Table 3. Retinopathy in 133 patients with type II diabetes mellitus*

Stage**	Stage 1 (n = 44)	Stage 2 (n = 31)	Stage 3 (n = 23)	Stage 4 (n = 12)	Stage 5 (n = 23)
Patients with retinopathy	11/44 (25.0%)	13/31 (41.9%)	14/23 (60.9%)	10/12 (83.3%)	16/23 (69.6%)
Patients with IgM immunoreactivity	3/11 (27.3%)	2/13 (15.4%)	4/14 (28.6%)	5/10 (50.0%)	5/16 (31.3%)
Patients with IgG immunoreactivity	2/11 (18.2%)	4/13 (30.8%)	1/14 (7.1%)	2/10 (20.0%)	0/16 (0.0%)
Patients with IgM & IgG immunoreactivities	1/11 (9.1%)	5/13 (38.5)	9/14 (64.3%)	1/10 (10.0%)	4/16 (25.0%)
Total Patients with immunoreactivity	6/11 (54.5%)	11/13 (84.6%)	14/14 (100.0%)	8/10 (80.0%)	9/16 (56.3%)
Total Patients with immunoreactivity in patients with no retinopathy	22/33 (66.7%)	14/18 (77.8%)	6/9 (66.7%)	1/2 (50.0%)	5/7 (71.4%)

* Diabetic retinopathy was examined in 133 of the 200 patients.
** cf. text.

(11/44) in stage 1, 41.9% (13/31) for stage 2, 60.9% (14/23) in stage 3, 83.3% (10/12) for stage 4 and 69.6% (16/23) in stage 5 (Table 3). Atherogenic index was 3.8 ± 1.5 in 43 patients examined.

ELISA for anti-rabbit ACE (Sigma) antibodies was done as reported [4] using the anti-human IgM antibody or IgG antibody conjugated with HRP purchased from DAKO A/S, Denmark. Absorbance rate was measured by an ELISA reader at 630 nm ($A^{630\,nm}$) or $A^{630\,nm}/A^{450\,nm}$ ratio and serum immunoreactivities were evaluated by comparing the difference of titer in doubling dilutions and normal pooled serum. Scatchard analysis was done by precipitating with polyethyleneglycol (PEG), anti-human IgG or anti-human IgM [5].

Statistical analysis: The relationship of a binary variable with other variables was examined with multiple logistic regression analysis, and that of a continuous variable with other variables was examined by multiple regression analysis. Analysis of variance and Student's paired or unpaired t tests were also performed, and the correlation between two variables was analyzed by linear regression analysis.

The Animal Research Committee of the Kawasaki Medical School approved the study on rabbits.

Nine New Zealand white female rabbits fed RC4-Oriental Yeast containing 0.18 g Na and 1.87 g K per 100 g were immunized with rabbit lung ACE from Sigma after purification by polyacrylamide gel electrophoresis. Monthly injections were administered with 150 μg of the purified ACE dissolved in saline and emulsified with an equal volume of complete Freund's adjuvant (Gibco) intradermally at multiple sites in the back and soles. After initial immunization, the rabbits were boosted every 2 weeks by injecting 10 μg of the ACE into the ear vein. In 2 or 3 days following the booster injection, the rabbits were bled once a month and then sera were tested for production of the antibody to ACE by ELISA. ELISA for anti-rabbit ACE antibodies was done using the anti-rabbit IgM antibody or IgG antibody conjugated with HRP purchased from DAKO A/S, Denmark. Serum ACE activity was measured by the method previously reported [6].

Results and Discussion

1. In the control patients, 3 patients had IgM or IgG immunoreactivities to ACE, and in 4 healthy subjects from 21 to 60 years old and in 3 healthy subjects from 18 to 20 years old the immunoreactivities were detected.

2. In 129 (64.5%) of the 200 patients with type 2 diabetes immunoreactivities to ACE were recognized on two consecutive assays. Titers of serum immunoreactivities ranged from 2^1 to 2^3: titer 2^1 for 67 (51.9%) patients of the 129 patients, titer 2^2 for 48 (37.2%) patients and titer 2^3 for 14 (10.9%) patients, respectively. The highest titer of 2^3 was seen in 14 patients; most of them belonged to stages 1 and 2 of nephropathy and the mean of age or duration of the disease were 60.1 years and 12.1 years, respectively. Immunoreac-

tivity appeared in 67 (50%) out of the 129 patients within 10 years and 90% within 20 years. As shown in Table 1, the immunoreactivities to ACE were positive in over 70% of the patients in the pre- or incipient-nephropathy stages. Titers of serum immunoreactivities determined by ELISA do not correlate necessarily with the urinary albumin index. In the incipient-nephropathy stage (stage 3), there was a significant correlation between values of the urinary albumin index and the number of patients with total immunoreactivities ($z = 2.209$, $P = 0.0272$) and between values of the index and the number of patients with IgG immunoreactivity ($z = 2.515$, $P = 0.0119$) in an evaluation by multiple logistic regression analysis.

In some patients with the immunoreactivities to ACE, those immunoreactivities were confirmed also by Scatchard analysis, but the immunoreactivities were low (the mean affinity constant was 0.3×10^8 M^{-1} for IgM and 0.7×10^8 M^{-1} for IgG, respectively, and the mean binding capacity was 12.1×10^{-11} M for IgM and 6.4×10^{-11} M for IgG, respectively in 3 diabetic patients), though the immunoreactivities were 83.3% positive in the early stage of clinical diabetic nephropathy of the type 2 diabetic patients in the present study. This may be due to the fact that ACE concentration in serum is very low in humans and the relative specific activities of the enzyme in crude extracts are as follows: serum, 1; lung, 30; kidney, 150 [7].

The correlation between hypertension and antiACE immunoreactivity in the 200 patients is shown in Table 2. Hypertension correlated significantly with age ($z = 3.957$, $P = 0.0001$) and also with the urinary albumin index ($z = 4.382$, $P = 0.0000$). During the stages 3, 4 and 5, hypertension showed a significant positive correlation with the urinary albumin index ($z = 3.341$, $P = 0.0008$), and it also showed a significant inverse correlation with anti-ACE immunoreactivities ($z = 2.651$, $P = 0.0080$). This may be due to the hypotensive effects of ACE inhibition caused by the immunoreactivity. In 3 out of the 9 immunized rabbits, the anti-ACE antibody was detected after 4 months and finally in 8 rabbits the antibody became positive by 8 months. Serum ACE activity in 4 immunized rabbits tested decreased to 50% of the control level after 7 months (Table 4). When rabbit serum containing anti-ACE antibodies was mixed, after heat-treated at 56°C for 30 min, with normal human serum, the ACE activity was reduced in a concentration-dependent manner (Table 5). The rabbit antiserum had no converting enzyme activity after the heat-treatment. This indicates that, if titers are high enough to induce an inhibition of ACE activity, an ACE antibody recognized by a common epitope may result in enzyme inhibition in both humans and rabbits. As shown in Table 3, during the stages 1, 2, 3 and 4, the development of retinopathy correlated with that of ACE immunoreactivities, especially those of IgM and IgG ($z = 2.3675$, $P = 0.0179$), and it was also correlated with urinary albumin index ($z = 2.2017$, $P = 0.0276$).

In our study, immunoreactivities to ACE, which

Table 4. Serum ACE activity, sodium and postassium concentrations and PRA in rabbits after immunization with ACE

	ACE actiity	Serum Na	Serum K	PRA
Control values	78.0 ± 3.8 IU/l/37°C (n = 20)	145 ± 0.5 mEq/l (n = 20)	4.4 ± 0.1 mEq/l (n = 20)	7.3 ± 0.5 ng/ml/hr (n = 10)
After 3 months	68.7 No. 1 No. 2 No. 4 No. 5 (63.7, 70.3, 62.7, 78.1)			
After 4 months	59.9 (57.6, 62.6, 54.0, 65.3)	144 (145, 143, 143, 143)	4.0 (3.8, 4.0, 4.2, 4.0)	
After 6 months	46.0 (48.4, 49.9, 41.6, 44.2)	143 (144, 144, 143, 141)	4.1 (3.9, 4.1, 4.1, 4.2)	27.4 (23.9, 25.0, 26.7, 34.2)
After 7 months	42.0 (46.2, 29.6, 36.2, 55.9)	141 (144, 137, 142, 140)	4.5 (3.6, 5.7, 4.6, 4.2)	
After 8 months	33.3 (39.5, 22.3, 29.1, 32.1)	142 (143, 143, 141, 141)	4.7 (3.9, 5.9, 4.6, 4.5)	14.4 (18.3, 5.6, 9.7, 23.9)
After 9 months	40.1 No. 1 No. 5 (31.7, 48.4)	141 (143, 139)	4.0 (3.6, 4.4)	23.2 (18.6, 27.8)

Table 5. ACE activity in the pooled serum from normal subjects after addition of the rabbit anti-ACE antisera

Rabbits antisera added (v/v)	0% (control)	20% (v/v)	50% (v/v)	70% (v/v)
		ACE activity, % remaining		
After 3 months	100%	98.5% rabbits No. 1 No. 2 No. 4 No. 5 (100, 99.0, 98.6, 96.4)	94.2% (100, 98.0, 91.9, 86.9)	91.3% (100, 96.0, 84.0, 85.3)
After 4 months	100%	96.7% (93.8, 100, 95.5, 97.3)	84.0% (82.1, 83.2, 82.1, 88.4)	76.5% (74.1, 76.5, 74.1, 81.2)
After 6 months	100%	94.6% (92.0, 94.2, 94.9, 97.1)	85.3% (79.8, 86.2, 81.7, 93.6)	76.3% (69.1, 78.4, 69.1, 88.7)
After 8 months	100%	99.2% (100, 98.4, 98.4, 100)	87.1% (87.1, 79.6, 84.9, 96.8)	76.9% (75.3, 66.2, 71.4, 94.8)

were recognized most frequently in the pre-nephropathy and incipient-nephropathy stages, gradually decreased in frequency towards the final stage. This may be due to the loss of immunoreactivity in urine, and this loss in urine was most prominent in the IgG of small molecule. These results suggest that anti-ACE autoantibody may be present in patients with type 2 diabetes mellitus. However, data on the epitope for the antibody is not available in the present study.

In conclusion, immunoreactivities to ACE are higher in patients with diabetes than in non-diabetic individuals.

Acknowledgements

The authors thank following coworkers for their contribution to this research : Shoji Sumitomo and Kazue Takafuchi (Division of Endocrinology, Department of Medicine), Satoru Ogo (Laboratory Animal Center), Kenzo Uehira (Electronmicroscopy Center) and Teruo Shirabe (Division of Neuropathology, Department of Pathology).

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