Nephrol Dial Transplant (1999) 14: 1904–1911

Nephrology Dialysis Transplantation

Original Article

Plasma renin and prorenin and renin gene variation in patients with insulin-dependent diabetes mellitus and nephropathy

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Abstract

Background. The most striking abnormality in the renin–angiotensin system in diabetic nephropathy (DN) is increased plasma prorenin. Renin is thought to be low or normal in DN. In spite of altered (pro)renin regulation the renin gene has not been studied for contribution to the development of DN.

Methods. We studied plasma renin, prorenin, and four polymorphic markers of the renin gene in 199 patients with IDDM and DN, and in 192 normoalbuminuric IDDM controls matched for age, sex, and duration of diabetes. Plasma renin and total renin were measured by immunoradiometric assays. Genotyping was PCR-based.

Results. Plasma renin was increased in patients with nephropathy (median (range), 26.3 (5.2–243.3) *vs* 18.3 (4.2–373.5) μU/ml in the normoalbuminuric group, $P<0.0001$). Prorenin levels were elevated out of proportion to renin levels in nephropathic patients (789 (88–5481) *vs* 302 (36–2226) μU/ml, $P<0.0001$). Proliferative retinopathy had an additive effect on plasma prorenin, but not on renin. DN was associated with a *Bgl*I RFLP in the first intron of the renin gene (bb-genotype: $n=106$ *vs* 82 in DN and normoalbuminuric patients respectively, $P=0.037$), but not with three other polymorphisms in the renin gene. A trend for association of higher prorenin levels with the DN-associated allele of this renin polymorphism was observed in a subgroup of patients with DN (bb *vs* Bb + BB, $P=0.07$).

Conclusions. The results indicate that in DN there is an increase in both renin and prorenin levels. A renin gene polymorphism may contribute weakly to DN. Although speculative, one of the renin gene alleles could lead to increased renin gene expression, leading to higher renin and prorenin levels. These may play a role in the pathogenesis of DN.

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Key words: diabetes mellitus; nephropathy; polymorphism; prorenin; renin; retinopathy

Introduction

A major cause of morbidity and death in insulin-dependent diabetes mellitus (IDDM) is diabetic nephropathy (DN), characterized by proteinuria, steady decline of kidney function, hypertension and cardiovascular disease [1]. Although poor metabolic control plays a role in the development of DN, it appears that 60–70% of the diabetic population is protected from nephropathy despite inadequate metabolic control [2]. It has therefore been suggested that susceptibility to DN is innate and determined by genetic factors. This hypothesis is supported by reports on familial clustering of DN [3] and by studies demonstrating association with familial predisposition to hypertension [4].

The favourable effect of ACE inhibition on the development [5] and progression [6] of DN is suggestive evidence that the renin–angiotensin system (RAS) is involved in the pathogenesis of DN and that variation at RAS gene loci might determine susceptibility to DN. In many studies, however, plasma renin and angiotensin II are normal or suppressed in diabetics (for review see [7,8]) and reports on association between DN and polymorphic markers have been equivocal for the angiotensinogen gene ([9–11] positive and [12] negative) and negative for the AT1-receptor [13,14] gene. Three meta-analyses of the numerous studies on association of the ACE I/D polymorphism with DN show that the D-allele may confer an increased risk of DN [15–17]. Interaction between the ACE I/D polymorphism and an angiotensinogen allele may also present elevated risk of DN [18]. Similarly, poor metabolic control may interact with the AT1-receptor polymorphism to augment risk of DN [14]. The renin gene that codes for prorenin and renin has not yet been investigated in DN in large study groups. Prorenin is the enzymatically inactive biosyn-

thetic precursor of renin, the rate-determining enzyme in the proteolytic cascade leading to formation of angiotensin II, the effector of the RAS. Several reports have described increased plasma prorenin levels when diabetes is complicated by microvascular disease like DN and retinopathy [19–22]. In the present case-control study we measured plasma levels of renin and prorenin and determined genotypes of the renin gene in patients with IDDM complicated by DN and in matched normoalbuminuric controls.

Subjects and methods

Subjects

In 1993, 242 IDDM patients with DN had their glomerular filtration rate measured at the Steno Diabetes Centre (Copenhagen, Denmark). Plasma and DNA samples were obtained from 199 patients of this cohort. Nephropathy in this study was defined as the presence of a urinary albumin excretion rate >300 mg/24 h in at least two of three consecutive urine samples, combined with the presence of retinopathy, while clinical or laboratory evidence of urinary tract disease other than diabetic glomerular sclerosis was absent. A group of 192 IDDM patients with persistent normal urinary albumin excretion rate (<30 mg/24 h) and matched for sex, age, and duration of diabetes, served as controls. A detailed description of the subjects with and without diabetic nephropathy can be found in references [23,24].

Methods

Blood pressure was measured with a Hawksley random zero standard sphygmomanometer after a 10 min rest in the sitting position. The disappearance of Korotkoff sounds (phase V) was used to determine diastolic blood pressure. Retinopathy was assessed by fundus photography after pupillary dilatation. Retinal lesions were categorized as either no, background, or proliferative retinopathy. EDTA-anticoagulated blood for measurement of plasma prorenin and renin was collected in the morning after an overnight fast from an indwelling catheter in a forearm vein after the patient had been in the supine position for at least 15 min. The blood was centrifuged at 3000 *g* at room temperature and plasma was stored at -80°C. Sixty-nine patients with nephropathy and nine with normoalbuminuria did not want to stop their antihypertensive drugs at least 8 days before the day the investigations were performed.

Laboratory procedures

All measurements were done by technicians who were unaware of the status of the patients. Urinary albumin excretion rate (AER) was measured from 24 h collections by an enzyme-linked immunoassay [25]. Haemoglobin A_{1c} (HbA_{1c}) was measured by high-performance liquid chromatography (HPLC, Biorad, Diamat, Richmond, CA, USA). The normal range is 4.1–6.4%. Serum creatinine was measured by autoanalyser.

Plasma prorenin and renin determination

Renin and total renin (i.e. renin + prorenin) were measured by an immunoradiometric assay (IRMA), purchased as a kit from Nichols Institute, Wijchen, The Netherlands [26]. In this assay renin is sandwiched between a biotinylated monoclonal antibody (mAb) R3–36–16 against human renin, immobilized on an avidin-coated polystyrene bead, and a ^{125}I-labelled mAb R1–20–5 against a renin-specific epitope not expressed on prorenin. Therefore the radiolabelled mAb does not recognize native prorenin. Yet prorenin can be measured with this assay, after preincubation with the active-site directed renin inhibitor remikiren, which causes exposure of the renin-specific epitope recognized by mAb R 1–20–5 [26]. The difference between the results with and without remikiren, i.e. the difference between total renin and renin, is a measure of the prorenin concentration. The assay was modified by shortening incubation to 6 h and performing the assay at 37°C. These modifications prevent inadvertent activation of prorenin and therefore exposure of the renin-specific epitope during the assay. Intra- and interassay variation for prorenin (320 μU/ml) is 4 and 13% respectively and for renin (22.2 μU/ml) 8 and 15%. Renin and prorenin of each patient were always measured in the same assay. Results of the renin and prorenin measurements are expressed as μU/ml. Standards in the assay were dilutions of the international reference preparation of human kidney renin 68/356 of the National Institute for Biological Standards and Control (Potters Bar, Hertfordshire, UK). The geometric mean and 95% reference limits for 100 normal subjects (50 women) with an age range of 19–62 years was 22.0 (7.7–54.8) μU/ml for renin and 199 (88.1–390) μU/ml for prorenin.

DNA extraction

Genomic DNA was extracted from peripheral leukocytes by a standard technique. It was aliquoted and stored at -80°C until use.

Genotyping

Four polymorphisms were studied at the REN locus (Figure 1). They consist of two restriction fragment length polymorphisms (RFLPs) [27,28] and two microsatellites [29,30]. The *Taq*I RFLP is located 4063 bases upstream of the start codon and consists of a C-T mutation. The *Bgl*I RFLP is a C-T mutation located at base 1161 of intron A. The $(ACAG)_n$ microsatellite is located in intron G. The CA-repeat is located downstream of the REN locus. Genotyping was PCR-based. Primers were obtained from Eurogentec (Seraing, Belgium), as were Goldstar Taq polymerase and the restriction enzymes. Except for haplotyping the *Bgl*I and *Taq*I loci (see below), PCR volume was 25 μl, consisting of 200 ng of DNA, 75 mM Tris–HCl, 20 mM $(NH_4)_2\,SO_4$, 0.01% Tween 20, 0.5 μM of each primer, dNTP

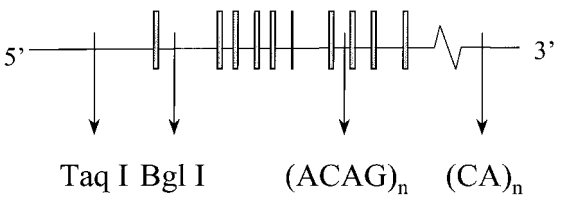

Fig. 1. Schematic representation of the human renin gene. Shown are the exons (boxes) and the sites of the four polymorphic markers that were studied (see text). Length from *Taq*I marker to final exon is about 16 Kb.

concentrations of 10 μM each and a polymerase activity of 1 U/25 μl PCR volume. Primer sequences and cycling conditions are summarized in Table 1.

Genotyping of the RFLPs was performed by PCR of the chromosomal region around the marker locus, followed by digestion by the restriction enzyme. Alleles were called B and T or b and t, indicating respectively digestion by *Bgl*I and *Taq*I or not. Controls with different genotypes, verified by direct sequencing, were run in each assay. Haplotyping of the *Bgl*I and *Taq*I loci in double heterozygotes was performed by a PCR on genomic DNA, using the Expand Long Template PCR System according to instructions by the manufacturer (Boehringer Mannheim, Germany). PCR volume was 50 μl and primer and DNA concentrations were the same as above. Primer sequences and cycling conditions are given in Table 1. PCR products were resolved on a 0.5% agarose gel. Sequencing of the RFLP loci was performed with PCR products as templates and a dye Terminator technique (Perkin–Elmer, Applied Biosystems Division, Foster City, CA) using a nested primer. Electrophoresis was on the ABI-Prism 377 automatic sequencer. Results were analysed with ABI Sequencing software.

Microsatellite loci were amplified in a duplex PCR, with one of each primer pair labelled with the fluorochrome FAM. Labelled PCR products were separated by electrophoresis on a denaturing 5% polyacrylamide gel, with fluorescence detection by an ABI Prism 377 automatic sequencer (Perkin–Elmer). In each lane a TAMRA-labelled calibration ladder was run. Allele lengths were calculated by Genescan software and checked by eye by two independent observers.

Statistical analysis

Continuous normally distributed data were expressed as mean (standard deviation). AER, creatinine, plasma renin and prorenin were log transformed before statistical analysis in order to obtain normal distribution. They are presented as median (range). Comparisons between groups were performed by Student's *t*-test or ANOVA. χ^2 analysis was used to compare the distribution of genotypes and alleles and for comparison between groups for non-continuous variables. Two-sided P values $\leqslant 0.05$ were considered significant. All aforementioned analyses were performed using the statistics package Statgraphics (Manugistics, Princeton NJ). Multiallelic polymorphisms were tested using the CLUMP program. CLUMP employs Monte Carlo simulation and has been developed for use in genetic case-control studies. A description of the program can be found in reference [31].

Results

Patient characteristics

Table 2 describes the features of the patients that were enrolled in the study. Patients with nephropathy and those with normoalbuminuria were well matched with regard to sex, age, and duration of diabetes. In the nephropathic group systolic and diastolic blood pressure were higher and serum creatinine was increased compared to the normoalbuminuric group. Furthermore, HbA_{1c} levels were higher in nephropathic patients.

Renin and prorenin are increased in diabetic nephropathy

When plasma levels were evaluated according to the use of antihypertensive medication (aHT) at the time of blood sampling, both renin and prorenin levels in the 69 patients with nephropathy on aHT were higher than those in the patients not on aHT at the time of blood sampling. Normoalbuminuric patients showed no difference in renin or prorenin levels between patients with or without aHT at the time of blood sampling (Table 3). In the 130 patients with DN and the 183 normoalbuminuric patients that either used no drugs or had stopped aHT for at least 8 days, renin and prorenin levels were elevated in nephropathic patients (26.3 (5.2–243.3) and 789 (88–5481) μU/ml respectively *vs* 18.3 (4.2–373.5) and 302 (36–2226) μU/ml in normoalbuminuric patients, $P < 0.0001$ both for renin and prorenin). If nephropathic

Table 1. Primer sequences and PCR conditions for genotyping of renin gene polymorphisms

Polymorphism	Primers	Product size (bp)	PCR
*Bgl*I-RFLP	5′-GGGGAAGCAGCTTGATATCGTGG 5′-CTAGGCTGGAGCTCAAGCGATC	772 (515/257)[a]	3′95°C, 30 × (30″92°C, 30″60°C, 1″72°C), 4′72°C
*Taq*I-RFLP	5′-GCTGTCTTCTGGTGGTACTGCC 5′-TGCTGGCCATGAACTGGTTCTAGC	964 (394/570)[a]	5′95°C, 30 × (45″95°C, 30″60°C, 1″30′72°C), 6′72°C
*Taq*I/*Bgl*I Haplotype	5′-GCTGTCTTCTGGTGGTACTGCC 5′-CTAGGCTGGAGCTCAAGCGATC	6079 (5324/5687/5717)[b]	2′94°C, 10 × (10″94°C, 30″65°C, 5′68°C), 15 × (10″94°C, 30″65°C, 5′ plus 20″/cycle 68°C), 7′°C
tetranucleotide repeat	5′-AGAGTACCTTCCCTCCTCTACTCA[c] 5′-CTCTATGGAGCTGGTAGAACCTGA	255, 263, 267, 271	5′95°C, 15 × (1′92°C, 1′59°C, 2′72°C[d]), 15 × (1′92°C, 1′59°C, 1′72°C), 5′72°C
dinucleotide repeat	5′-GCGGGATATTTGAGTTGTGT 5′-GAACTGTTCAACTGGAGCCT[c]	126–144 (10 alleles)	

[a]Size of bands after digestion if restriction site is present.
[b]Size of major bands if both *Bgl*I and *Taq*I, only *Taq*I or only *Bgl*I restriction sites are present respectively.
[c]Primer 5′-labelled with FAM.
[d]The polymerization time is shortened in each cycle (4 s per cycle).

Table 2. Clinical characteristics of the case-control study group, consisting of 199 patients with diabetic nephropathy and 192 diabetic patients with normoalbuminuria

	Nephropathy	Normoalbuminuria
n	199	192
Sex (m/f)	122/77	118/74
Age (years)	40.9 ± 9.6	42.7 ± 10.2
Duration of diabetes (years)	27.7 ± 7.9	26.8 ± 8.5
BMI (kg/m^2)	24.0 ± 3.3	23.6 ± 2.5
HbA_{1c} (%)	9.6 ± 1.5	8.5 ± 1.1[a]
UAER (mg/24 h)	796 (16–14 545)	8 (1–30)
Serum creatinine (μM)	103 (54–684)	76[a] (40–116)
Systolic BP (mm Hg)	151 ± 23	132 ± 18[a]
Diastolic BP (mm Hg)	86 ± 13	76 ± 10[a]
Antihypertensive treatment (*n*)	150	23[a]
ACE-inhibitor	106	9
Beta-blocker	28	3
Calcium-entry blocker	44	6
Diuretic	129	15
Retinopathy		
Nil	0	68 (35)
Simplex	62 (31)	105 (55)
Proliferative	137 (69)	19 (10)

Data are means ± SD, median (range), or *n*(%). [a]$P < 0.001$.

Table 3. Plasma renin and prorenin levels in patients with diabetes mellitus with or without diabetic nephropathy

	Nephropathy	Normoalbuminuria
Renin (μU/ml)		
no anti-HT	26.3[a] (5.2–243.3) $n = 130$	18.3 (4.2–373.5) $n = 183$
+ anti-HT	55.2[b] (10.9–506.3) $n = 69$	26.8 (6.6–78.5) $n = 9$
Prorenin (μU/ml)		
no anti-HT	789[a] (88–5481) $n = 130$	302 (36–2226) $n = 183$
+ anti-HT	1085[b] (178–10 740) $n = 69$	288 (173–686) $n = 9$

Data are median (range). [a]$P < 0.0001$ compared to levels in normoalbuminuric patients [b]$P < 0.01$ compared to levels in patients with same renal status but without antihypertensive medication. Anti-HT = antihypertensive therapy.

patients that had never used antihypertensive drugs ($n = 48$) were compared to 48 normoalbuminuric patients, matched for age, sex, and diabetes duration, prorenin levels were 527 (110–1653) *vs* 296 (80–1812) μU/ml and renin levels were 23.3 (5.8–114.8) *vs* 19.4 (4.2–81.6) μU/ml ($P < 0.001$ and $P = 0.059$ respectively). The increase in renin in nephropathic patients was not due to lower sodium intake as assessed by 24 h urinary excretion of sodium chloride. Sodium excretion was the same in both groups (150 ± 84 *vs* 151 ± 66 mmol/24 h, NS). Sodium excretion was also identical in nephropathic patients using aHT at the time of blood sampling, nephropathic patients that had stopped aHT and patients not on aHT medication. Log[renin] and log[creatinine] as well as log[prorenin] and log[creatinine] were correlated in the nephropathic group (R resp. 0.31 and 0.41, $P = 0.0003$ and $P < 0.0001$ respectively).

Influence of retinopathy on renin and prorenin levels

Proliferative retinopathy appears to have an effect on prorenin that is independent of the presence of nephropathy (Table 4). The effect on renin levels is at most modest and equivocal, depending on the test used. The results of the statistical analysis by two-way ANOVA should be considered with caution, because groups are unbalanced.

Relationship between diabetic nephropathy and renin gene polymorphisms

Table 5 gives the frequencies of genotypes or alleles of the renin gene polymorphisms in the two groups. Frequencies of *Taq*I and *Bgl*I polymorphisms and of the microsatellites are in accordance with frequencies found in other Caucasian populations [27–30]. Genotypes of the RFLPs were in Hardy–Weinberg equilibrium. The power of detecting a 20% increase in frequency of allele b of the *Bgl*I polymorphism is 98%, and 60% for a 10% increase. Similarly, the power to detect a 20% increase in frequency of the t-allele of the *Taq*I polymorphism is 99% and 72% for a 10% increase. There were no significant differences in allele frequencies of the *Bgl*I and *Taq*I polymorphism in the two groups. However, genotype frequency distribution appeared to differ. χ^2 analysis of BB *vs* Bb *vs* bb in the two groups yielded a P value of 0.056. Grouping of *Bgl*I genotypes based on the presence of either at least one B-allele (BB + Bb *vs* bb) or at least one b-allele (bb + Bb *vs* BB) gave P values of 0.037 and 0.87. This suggests an overrepresentation of bb-genotypes in the nephropathic group. The unadjusted odds ratio for nephropathy for subjects with a bb-genotype is 1.56 (95% C.I. 1.05–2.33). The odds ratio for nephropathy, as determined by multiple logistic regression analysis, and adjusted for sex, age, duration of diabetes, glycated haemoglobin level, and systolic blood pressure, was 1.70 for the bb-genotype ($P = 0.03$). Haplotyping of the *Bgl*I and *Taq*I loci revealed the absence of bt alleles. Therefore only six different genotypes were observed. We studied with the Clump-package the distribution of two microsatellites at the renin gene locus and the genotypes of the *Bgl*I/*Taq*I haplotypes, arranged in $2 \times N$ tables as shown in Table 5. Estimated '*P*-values' for the least conservative of the four available tests was 0.188 for the *Bgl*I/*Taq*I haplotypes, 0.81 for the tetranucleotide repeat and 0.088 for the dinucleotide repeat (each value calculated after 1000 simulations), arguing against association between nephropathy and these polymorphic markers.

Although a trend towards higher prorenin levels exists in bb homozygotes (bb *vs* BB + Bb, $P = 0.07$)

Table 4. Renin and prorenin plasma levels in patients with or without diabetic nephropathy, according to the presence or absence of retinopathy

	Nephropathy		Normoalbuminuria	
Retinopathy	Renin (μU/ml)	Prorenin[a] (μU/ml)	Renin (μU/ml)	Prorenin[b] (μU/ml)
No	–	–	17.5 (4.2–66.8)	267 (36–711) $n=65$
Background	26.5 (5.2–82.4)	553 (88–2301) $n=43$	18.1 (5.3–373.5)	321 (45–1124) $n=100$
Proliferative	26.1 (7.7–243.3)	869 (167–5481) $n=87$	19.4 (10.5–81.6)	414 (224–2226) $n=18$

Shown are median (range). Patients taking antihypertensive medication are not included.
[a]$P<0.001$ proliferative *vs* background. [b]ANOVA, $P=0.0004$; proliferative *vs* background, $P=0.001$; proliferative + background *vs* nul, $P=0.04$.
Renin levels are not significantly different in different stages of retinopathy by this analysis. A two-way ANOVA of log-transformed renin and prorenin levels in a collapsed table (proliferative retinopathy YES/NO and nephropathy YES/NO) demonstrates a significant effect of the presence of nephropathy and proliferative retinopathy on prorenin levels ($P<0.0001$). In contrast with the results of ANOVA on the Table above a two-way ANOVA on a collapsed table demonstrates an effect of retinopathy on renin levels ($P=0.02$). The effects of retinopathy and nephropathy on renin and prorenin levels are independent ($P=0.30$ and 0.91 resp, two-way ANOVA).

Table 5. Renin gene polymorphisms in patients with insulin-dependent diabetes mellitus

Genotype/alleles	Nephropathy (*n*)	Normoalbuminuria (*n*)
TT	158	140
Tt	36	48
tt	5	4
BB	21	19
Bb	71	91
bb	107	82
Haplotype genotypes		
BT/BT	7	7
Bt/Bt	5	4
bT/bT	107	82
BT/Bt	9	8
BT/bT	44	51
Bt/bT	27	40
Tetranucleotide repeat (length in basepairs)		
255	314	309
259	0	0
263	38	32
267	43	41
271	3	2
Dinucleotide repeat (length in basepairs)		
126	0	4
128	37	32
130	165	149
132	19	20
134	52	53
136	68	80
138	30	34
140	22	12
142	4	0
144	1	0

(Table 6), we did not find significant differences in plasma levels between the other renin genotypes, either in the nephropathic or in the normoalbuminuric group (results not shown).

Table 6. Prorenin levels in diabetic patients with different *Bgl*I genotypes

	Prorenin (μU/mL)	
*Bgl*I genotype	Nephropathy	Normoalbuminuria
BB	726 (269–1400) $n=14$	343 (118–1124) $n=18$
Bb	621 (88–5236) $n=47$	297 (80–1812) $n=88$
bb	877 (167–5481) $n=69$	301 (36–2226) $n=77$

Shown are median (range), bb *vs* (BB + Bb): $P=0.07$ in nephropathic patients. Patients were not taking antihypertensive drugs.

Finally, we analysed the data for association of renin gene polymorphisms with coronary heart disease, hypertension and retinal status. No statistically significant differences were revealed (data not shown).
In multiple logistic regression analysis, with presence or absence of nephropathy as dependent variable and the renin *Bgl*I and *Taq*I polymorphisms, ACE I/D polymorphism, angiotensinogen M235T and T174M polymorphisms and the angiotensin receptor type 1 A/C polymorphism at position 1166 as independent variables, only the renin bb-genotype, not surprisingly, remained in the final model ($P=0.037$), but more importantly, no interactions were observed between the bb-genotype and the polymorphisms in the angiotensinogen, ACE and AT_1-receptor genes.

Discussion

In this report we confirm previous findings that in IDDM patients plasma prorenin levels are increased

by 150% when DN is present. We also found plasma renin to be 50% higher in nephropathic than in normoalbuminuric patients with IDDM. Furthermore, the presence of diabetic nephropathy is weakly associated with one of the genotypes of a *Bgl*I polymorphism in the first intron of the renin gene.

Although there are other reports of increased renin levels in DN [8,20.32], the general impression has always been that renin levels are low in DN (for a list of references see Ref. [7]). The apparent discrepancy with our finding of increased renin levels could be due to various factors. The first may be the poorly developed measurement methodology for renin [33]. Most studies date from the seventies and early eighties and employed variants of an enzyme–kinetic, plasma renin activity (PRA) assay, which measures angiotensin I production by renin from endogenous angiotensinogen. The PRA assay depends on both renin and angiotensinogen concentration. Kinetics of the enzymatic reaction of renin with angiotensinogen, often performed at non-physiological pH in PRA assays, may differ in diabetic plasmas, including altered renin reactivity and non-linear angiotensin I generation, but no reports exist that address this problem. The immunological direct assay we employed, measures molecules and is unlikely to be affected by the diabetic state. Another recent study that measured renin by IRMA, also found a trend to higher renin levels in diabetic subjects with microalbuminuria—the early phase of DN—compared to normoalbuminuric diabetics [21]. This suggests again that the cause of the discrepancy may be assay-related. Renin IRMAs may have a problem in co-measurement of prorenin as renin. Our IRMA employs a monoclonal antibody specific for renin. Prorenin may also assume a renin-like conformation by so-called cryoactivation, even at room temperature, which is the temperature of the assay incubation [34]. Although this activation amounts to not more than 1–2%, renin levels may be overestimated, especially if prorenin levels are high. In our assay co-measurement of prorenin as renin is eliminated by performing the assay for 6 h at 37°C. We are therefore confident that our results on plasma renin in DN are valid. Another explanation for the discrepancy of previous reports with our observation of increased renin may be that study groups were often not homogeneous. Data for NIDDM and IDDM patients were often pooled in earlier studies and it is not known whether this is justified. Moreover, the various stages of DN were not always separated, partly because at the time these stages were not yet discerned. A final possible explanation for our high renin levels is that these are due to previous antihypertensive use. High renin levels may be observed with ACE inhibitors and diuretic use (AT1-receptor antagonists were not yet being prescribed to these patients). Patients had stopped antihypertensive medication at least 8 days before blood-sampling. This is usually sufficient for any effect of ACE inhibition to wear off [35]. After cessation of diuretic use secondary hyperaldosteronism may persist for longer periods than 8 days. However, this, together with a higher blood pressure, is expected to cause decreased renin levels, rather than the increased levels we found. Unfortunately, data on aldosterone that may clarify the issue somewhat, are not available. Finally, nephropathic patients virgin to antihypertensives have somewhat less increase in plasma renin and prorenin levels. This might argue in favour of a carry-over effect of antihypertensives on prorenin and renin levels in the hypertensive subgroup. On the other hand, this normotensive subgroup too does not show suppressed renin levels as is expected from older literature [7], but rather increased levels.

Increased plasma renin and prorenin levels in DN support the hypothesis that the RAS is overactive and possibly even pathogenetic in the development or progression of DN, especially since increase in total renin occurs early [22]. A possible mechanism could be higher intra-glomerular pressure, through angiotensin II-mediated constriction of the efferent arteriole, with resulting proteinuria, but this remains speculative. The origin of elevated renin is most probably the kidney, since no other source of renin has been described [36]. Whether an excess prorenin also originates from the kidney is not known. Franken *et al.* [20] did not observe an increase in renal vein-to-artery ratio for prorenin in patients with near end-stage diabetic nephropathy and high prorenin, suggesting that there is no markedly increased production of prorenin in the kidney. Prorenin may also originate from extrarenal sites like ovaries, testes, and adrenals [36]. Whether prorenin itself acquires enzymatic activity in DN is an unsolved question. Several mechanisms of prorenin activation have been described [36,37].

Some controversy exists concerning the relationship between retinopathy and plasma prorenin. We observed increased prorenin levels with proliferative retinopathy. This is in agreement with Franken *et al.*, who found an increase of plasma prorenin with increasing severity of retinopathy [20]. However, contrary to their findings, we did not find a difference between patients without and those with background retinopathy in the normo-albuminuric group. This is similar to the result obtained by Allen *et al.* [22], who did not observe—in a longitudinal survey—an increase in plasma prorenin when patients progressed from no to background retinopathy. Thus, apparently retinopathy and nephropathy have an additive effect on plasma prorenin levels, suggesting that the increase in plasma prorenin is an aspecific marker for microvascular disease as proposed previously [19]. Yet probably only the advanced, proliferative stage of retinopathy influences plasma prorenin. This is in contrast with DN, where plasma prorenin increases very early in the course of the disease [22].

The derangement in renin and prorenin in DN could be connected to a polymorphism in the renin gene, if this polymorphism is associated with DN. DN is weakly associated with the bb-genotype of a *Bgl*I RFLP in the first intron of the renin gene. This suggests a deleterious recessive effect of the b-allele. The first intron is involved in renin gene transcription regulation

[38] and the *Bgl*I polymorphism may be linked to this regulatory sequence. This may provide a mechanism that explains the association of DN with this marker. It could also explain the findings of Daneman *et al.* [21], who described a genetic influence on plasma prorenin levels in diabetic patients with microalbuminuria, although no genotyping of the renin gene was performed in this study. From this observation we anticipated a relation between *Bgl*I renin genotype and plasma renin or prorenin level. However, we could not demonstrate such an association, although a trend towards higher prorenin levels was observed in bb homozygotes. This trend may have failed to reach significance because only prorenin levels in the subgroup of patients not on antihypertensives were included in our analysis.

There was no interaction of the *Bgl*I marker with variants of other genes of the RAS, although the power of the study was not sufficient to detect any interactions but very strong ones. Three other markers in the renin gene were not associated with nephropathy. There is only one report about polymorphic markers in the renin gene in diabetic patients with nephropathy and their controls [39]. This report did not show an association of renin gene polymorphisms with diabetic nephropathy. However, the report was based on a small group (40 patients) and diabetes duration was shorter (11 years), so that future nephropathic patients may have been present among control subjects.

The polymorphic marker that was first studied in the present study group, the ACE gene I/D polymorphism, was not in Hardy–Weinberg equilibrium in the control group [23], which might cast doubt on any conclusion on the genetics of DN in this study group. However, polymorphic markers in the renin, angiotensinogen [10], and AT1 receptor [13] genes were in Hardy–Weinberg equilibrium in the control group, which makes selection bias of controls unlikely. The absence of Hardy–Weinberg equilibrium in the ACE gene may be caused by misclassification of alleles [40], or may be incidental, the ACE-gene not being linked to the other RAS genes.

Our control group turned out to have better metabolic control. Individuals with the bb-genotype of the renin gene may have been protected from DN if this better metabolic control has been long-term. Whether this may have weakened the association between DN and a renin gene polymorphic marker, we do not know. In subgroup analyses according to glycated hemoglobin levels either matching was lost or groups became very small.

Our study group is unique in that now the renin, angiotensinogen, ACE, and AT1R genes have been studied for association with DN. We can conclude that in this group of all RAS-components [10,13,23] plasma renin and prorenin and possibly the renin gene *Bgl*I polymorphism are associated with DN. Plasma ACE has also been found to be increased in DN in this study group [23], but the ACE gene I/D polymorphism was not associated with DN in these patients. Recent meta-analyses, however, showed that the ACE gene D-allele may confer an increased risk for DN (excess risk up to 56%, depending on ethnic background and reliability of genotyping method) [15–17].

Since the effect of the ACE I/D polymorphism and, in our group, the renin *Bgl*I polymorphism is small and since there is no major interaction between the RAS-gene variants we must assume that the main part of the genetically determined susceptibility to DN lies outside the genes that code for RAS components. Still, in view of our results on plasma renin and prorenin measurements and in view of the favourable reaction to ACE inhibitors, a pathogenetic mechanism of DN in which the RAS, in particular renin and prorenin, is involved, remains likely.

In conclusion, a direct, validated assay showed that both prorenin and renin are increased in plasma of IDDM patients with diabetic nephropathy. In the present patient group a renin gene variant is the sole genetic factor within the RAS that may contribute, albeit weakly, to the genetic susceptibility to diabetic nephropathy. Renin and prorenin could well play a direct pathogenetic role in DN.

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Received for publication: 17.8.98
Accepted in revised form: 9.4.99