Circulating antibodies to nephrin in patients with type 1 diabetes

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Abstract

Background. Patients with type 1 diabetes typically develop autoantibodies to antigens of the pancreatic islet cells including insulin, glutamic acid decarboxylase and the protein tyrosine phosphatase-related islet antigen 2 protein. Nephrin is a protein shared by the kidney glomeruli, pancreatic β-cells and islet microendothelia. Since circulating antibodies to nephrin have been shown to cause proteinuria, we wanted to test whether such autoantibodies can be detected in diabetic patients.

Methods. We developed a radioimmunoprecipitation assay and analysed samples in a follow-up series of 66 patients with type 1 diabetes.

Results. A total of 24% of the patients tested positive for nephrin autoantibodies at diagnosis, whereas 23, 14 and 18% had these antibodies at 2, 5 and 10 years, respectively. During the follow-up at 16–19 years after diagnosis, 14 patients had signs of renal injury and 29% of them tested positive for nephrin autoantibodies in at least one sample.

Conclusions. We conclude that a subset of patients with type 1 diabetes present with circulating autoantibodies to nephrin. However, the present data do not allow conclusions of a causative role for these antibodies in the pathogenesis of proteinuria in diabetic patients.

Keywords: autoimmunity; diabetic nephropathy; nephrin; radioimmunoprecipitation; type 1 diabetes

Introduction

Diabetic nephropathy is a severe complication of all types of diabetes. The precise mechanisms leading to perturbation of the kidney glomerular filter and subsequent escalation of functional damage presenting with proteinuria remain partly unknown. The pathological process involves at least accumulation of reactive oxygen species and non-enzymatic glycosylation of circulating and structural proteins and carbohydrate residues, as well as dysregulation of extracellular matrix production within the glomerulus [1].

The interpodocyte slit diaphragm plays a key role in maintaining the integrity of the glomerular filtration barrier. A protein encoded by the gene NPHS1, nephrin, has been established as a key structural and functional component of the slit diaphragm [2]. Evidence for the essential role of nephrin for the filtration function derives from a series of observations. First, a disease presenting with massive, treatment-resistant proteinuria, the congenital nephrotic syndrome of Finnish type (CNF), is caused by specific mutations in the NPHS1 gene resulting in severe structural changes of the podocytes and absence of the slit diaphragms. These changes can be reproduced in nephrin knockout mice [3]. Second, nephrin mRNA and protein expression levels in the kidney are characteristically altered during the course of diabetic [4,5] and other proteinuric nephropathies [6]. Third, circulating antinephrin antibodies are associate with the recurrence of proteinuria in CNF patients having received a normal kidney transplant [7]. In line with this, in vivo injection of antinephrin antibodies in the rat produces massive proteinuria [8]. Together, these data show the crucial functional role of nephrin and its potential to act as an autoantigen.

In extension to the initial description of specific nephrin expression in the kidney, additional sites of expression have been characterized. Particularly interesting is the nephrin expression in the pancreas and more specifically in the insulin-producing β-cells in the islets of Langerhans [9]. While still little is known of the specific functions of nephrin in the β-cells, a variety of β-cell epitopes are known to act as autoantigens in diabetes. The functional significance of these in diabetic complications is unclear but they offer practical diagnostic tools for early...
Detection of insulitis. The best characterized autoantigens include the 65 kDa isoform of glutamic acid decarboxylase (GAD) [10], the protein tyrosine phosphatase-related islet antigen-2 protein (IA-2) [11] and insulin [12].

Our study tested whether nephrin may act as an autoantigen and whether patients with type 1 diabetes show nephrin autoantibodies. We also wanted to know whether these antibodies predict development of proteinuria in diabetes.

Subjects and methods

Subjects and study design

The patient cohort comprised 66 children and adolescents (43 male, 23 female) diagnosed with type 1 diabetes at the Department of Pediatrics, University of Oulu, Finland in a consecutive series between 1983 and 1986. The mean age at diagnosis was 8.2 years (range 0.9–15.6 years). The control group comprised 96 non-diabetic subjects (48 male, 48 female) with a mean age of 11.3 years (range 0.5–18.2 years). These control samples, collected between 1989 and 1994, were measured in parallel to the patient samples and the results were then used to set the cut-off limit for our assay. Serum samples from the patients were taken at diagnosis and at 2, 5 and 10 years later. All samples were stored at −20°C until analysed. Detailed clinical data were available including clinical history, total glycated haemoglobin, HLA-DR alleles and titres of four diabetes-associated autoantibodies, islet cell antibodies (ICA), insulin autoantibodies (IAA) or antibodies (IA; antibodies to exogenous insulin), glutamic acid decarboxylase antibodies (GADA) and protein tyrosine phosphatase-related islet antigen 2 protein antibodies (IA-2A), in the same samples from which nephrin autoantibodies were quantified. Microalbuminuria was defined as an albumin excretion ranging from 30 to 300 mg/24h and proteinuria as a rate exceeding 300 mg/24h. At the time of this study, 10 patients had been lost to follow-up. Of these, nine patients had moved to another region and one had died with a diagnosis of multiple sclerosis. The mean duration of diabetes when lost to follow-up was 8.7 years (range 4–13 years). This study was approved by the ethics committee of the University of Oulu. The participants and/or their parents gave appropriate written informed consent.

Labelled recombinant nephrin

Normal human kidney cDNA was obtained from our previous experiments. PCR primers 5’-tcaagactggcc cacatgtcactggagaacctg-3’ and 5’-tagtcatagcttassocac gatgtcctgctggga-3’ were used to produce the nephrin-specific insert, which was cloned under a T7 promoter sequence in the circular plasmid vector pGEM-4z (Promega, Madison, WI, USA). The recombinant nephrin had 470 amino acid residues spanning from the extracellular side (772 methionine; GenBank accession number AF035835) to the intracellular C-terminal end (1241 valine). 35S-methionine labelled recombinant protein was produced with in vitro transcription/translation using the TnT T7 Coupled Reticulocyte lysate System (Promega) and Redivue L-[35S] methionine (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturers’ instructions. Unincorporated 35S-methionine was removed by gel chromatography through a NAP-5 column (Amersham Biosciences).

Radioimmunoprecipitation assay

The radioimmunoprecipitation assay was performed essentially as described earlier [13]. Patient serum of 2 µl and 15 000 counts per minute (cpm) of labelled nephrin were incubated for 7–15h in a buffer containing 50 mM Tris, 150 mM NaCl and 0.1% Tween-20 (TBST; pH 7.4). Then, Protein A-Sepharose 4B conjugate (Zymed Laboratories, San Francisco, CA, USA) was added, and incubation was continued for 1–2h. After this, the samples were washed eight times with 140 µl of TBST, and scintillation cocktail (OptiPhase ‘HiSafe’ 3; Wallac, Turku, Finland) was added and the activity measured. Each serum sample was measured at least twice. A dilution series (1:25, 1:50, 1:100, 1:200, 1:400) of a polyclonal nephrin antibody (#1222) [14] was used for standardization. These dilutions were given a relative value of 1, 0.5, 0.25, 0.125 and 0.0625, respectively. The actual test samples were given a value in relative units (RU) using the standard curve of the dilution series to diminish the effect of inter-assay variation on the overall levels of measured activity. The intra- and inter-assay coefficients of variation were 4.7 and 17.2%, respectively. In our assays, the mean level of bound nephrin label in the 96 healthy controls was 0.066 RU (SD = 0.015). The respective mean level in the 234 separate patient serum samples was 0.077 RU (SD = 0.026), which was significantly higher when compared to the 96 control samples (P < 0.01). The cut-off limit for antibody positivity used was 0.10 RU and was defined as the 99th percentile of the values in the control group (mean + 2.09 SD). We selected this cut-off limit because of the background cpm in the normal control sera. Such background was also found in a similar radioligand binding assay for the detection of GADA [15]. To verify the specificity of our assay, we tested different sets of nephrin-specific and control antibodies with our assay. The nephrin-specific antibodies were against epitopes of the extracellular (#1052 and #1125; both produced as described previously [16]) or the intracellular (#1222) part of human nephrin. The control antibodies were against the irrelevant proteins FAT [17] or zonula occludens 1 (ZO-1; Zymed Laboratories). These antibodies were diluted in 1:25, 1:50, 1:100 and 1:200 in a pool of five healthy control sera. The radioimmunoprecipitation assay was performed as described above and the assay curves are presented in Figure 1A. Furthermore, we produced non-radiolabelled recombinant nephrin and performed blocking reactions. Briefly, to the normal assay reaction containing 15 000 cpm labelled nephrin, we added an amount of non-labelled recombinant nephrin equivalent to 20 000, 40 000 or 60 000 cpm of labelled recombinant nephrin. Samples of nephrin autoantibody positive and negative diabetic patients were tested as well as a standard series sample of polyclonal nephrin antibody at 1:50 dilution. Except for the negative patient samples, the blocking reactions resulted in a uniform and dose-dependent decrease of the measured mean cpm levels to approximately 30, 14 and 0%, compared to the respective unblocked reaction and standardized to the background cpm levels of...
negative patient samples or TBST controls (Figure 1B). Addition of irrelevant luciferase control protein to the reactions did not affect the results.

Immunofluorescence
Normal rat kidney tissue was obtained from our previous experiments. Animals were treated according to the rules and regulations of the Ethical Committee of the University of Helsinki. Rat kidney tissue, instead of human kidney tissue, was chosen to avoid unspecific staining possibly given by human serum samples. The 6 μm thick rat cortex cryostat sections were air-dried, fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and then blocked with CAS-Block (Zymed Laboratories). The sections were incubated with positive control antibody, patient serum or control serum diluted 1:20, 1:50 or 1:100 in ChemMate Antibody Diluent (Dako, Glostrup, Denmark) over night at +6°C. We used a polyclonal protein A-purified rabbit antibody for intracellular rat nephrin as the positive control [14]. After three washes in PBS, the sections were incubated with fluorescein isothiocyanate (FITC)-conjugated rabbit antihuman IgG (1:100; Dako) or tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat antirabbit IgG (1:100; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h at room temperature. After washes, the slides were covered with Vectashield Hard Set mounting medium (Vector Laboratories, Burlingame, CA, USA). Fluorescence microscopy was performed with an Olympus BX50 microscope (Olympus Optical, Tokyo, Japan) equipped with a cooled digital camera (Hamamatsu Photonics, Hamamatsu City, Japan). Corel Photo-Paint 12.0 and CorelDraw 12.0 (Corel Corporation, Ottawa, Canada) software were used for image documentation.

Statistical analysis
Statistical analysis software (SPSS 12.0.1 for Windows; SPSS, Chicago, IL, USA) was used for all statistical analyses. Normally distributed scale variables were compared with the use of the two-tailed independent samples t-test. The non-parametric Mann–Whitney U-test was applied to compare skewed variables. Categorical variables were tested with chi-square statistics. A Kaplan–Meier survival curve was constructed for the time of diagnosis of diabetes to the time of diagnosis of microalbuminuria or proteinuria. By applying multivariate tests in a general linear model, we analysed the correlations of consecutive nephrin autoantibody titres within-subjects across the follow-up time and also compared the titre profiles between groups categorized by the appearance of microalbuminuria during the follow-up period. All given P-values are two-sided and P-values <0.05 were considered significant.

Results
Clinical and immunological profile of patients
The baseline clinical data categorized by the presence of nephrin autoantibodies at diagnosis can be seen in Table 1. A similar comparison between the respective patient characteristics categorized by the renal outcome did not reveal any statistically significant differences either. The occurrence of type 1 diabetes in a first-degree family member was not associated with nephrin autoantibody titres in the patients (data not shown). Of the 66 patients in the study, 11 (16.7%) had developed microalbuminuria during
the total observation period of 16–19 years, and three (4.5%) other patients had progressed to overt proteinuria. The mean duration of diabetes at the manifestation of microalbuminuria and proteinuria was 9.5 and 12.0 years, respectively. The time from diagnosis of diabetes to manifestation of renal injury varied considerably; this is further demonstrated by a Kaplan–Meier survival curve observing the development of microalbuminuria among our patient cohort (Figure 2). The prevalences of IA-2A and GADA followed closely the prevalence of ICA (Table 2). We also searched for a correlation between the antibody titres and the ICA titre of each serum sample at diagnosis, and at 2, 5 and 10 years. Particularly, we noted that nephrin autoantibody titres did not correlate with ICA titres, unlike the IA-2A or GADA titres. Interestingly, patients presenting with nephrin autoantibodies always had an HLA-DR4 and/or HLA-DR3 allele present as well (Table 3). Out of 66 patients, 18 (27.2%) were diagnosed with diabetic retinopathy during the follow-up period. There was no association with the presence of nephrin autoantibodies and the development of diabetic retinopathy.

**Autoantibodies to nephrin**

At the time of diagnosis 16/66 (24.2%) patients tested positive for nephrin antibodies. The respective proportions at 2, 5 and 10 years were 14/61 (23.0%), 8/58 (13.8%) and 8/44 (18.2%). These and the prevalences of other diabetes-associated autoantibodies are shown in Table 2. Altogether, 29 out of the 66 patients (43.9%) gave at least one sample positive for nephrin autoantibodies. Substantial fluctuation was seen in the levels of nephrin antibodies over the 10-year measurement period. The analysis of consecutive nephrin autoantibody titres within-subjects excluded any significant correlation ($P = 0.14$), as did the comparison between subject groups defined by the appearance of diabetic nephropathy ($P = 0.39$).

Only four patients (6.1%) tested positive in all measured samples.

**Nephrin autoantibodies and renal injury**

Among the 14 patients with signs of renal injury, four (28.6%) were positive for nephrin autoantibodies on at least one occasion. Particularly, we note that in patients with nephrin autoantibodies at any time point, the mean duration of diabetes at manifestation of microalbuminuria was 7.7 years (95% CI 0.0–20.4 years), whereas the patients free of nephrin

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**Table 1. Clinical and biochemical characteristics at diagnosis categorized by the presence of nephrin autoantibodies**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Positive</th>
<th>Negative</th>
<th>Test value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>8.4 ± 3.4</td>
<td>8.2 ± 4.0</td>
<td>$t = -0.134$ df = 64</td>
<td>0.893</td>
</tr>
<tr>
<td>Male proportion</td>
<td>63%</td>
<td>66%</td>
<td>$\chi^2 = 0.065$ df = 1</td>
<td>1.000</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>110 ± 12</td>
<td>110 ± 14</td>
<td>$T = 0.111$ df = 49</td>
<td>0.912</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>72 ± 11</td>
<td>68 ± 9</td>
<td>$t = -1.034$ df = 47</td>
<td>0.306</td>
</tr>
<tr>
<td>Serum creatinine (µmol/l)</td>
<td>67 ± 13</td>
<td>67 ± 20</td>
<td>$t = -0.024$ df = 53</td>
<td>0.981</td>
</tr>
<tr>
<td>Glycated haemoglobin (%)</td>
<td>15.9 ± 3.4</td>
<td>14.5 ± 3.3</td>
<td>$t = 0.118$ df = 62</td>
<td>0.906</td>
</tr>
<tr>
<td>Blood pH</td>
<td>7.34 ± 0.08</td>
<td>7.35 ± 0.10</td>
<td>$T = 0.416$ df = 61</td>
<td>0.679</td>
</tr>
<tr>
<td>Ketosis present at diagnosis</td>
<td>88%</td>
<td>65%</td>
<td>$\chi^2 = 2.861$ df = 1</td>
<td>0.117</td>
</tr>
<tr>
<td>C-peptide (nmol/l)</td>
<td>0.15 ± 0.06</td>
<td>0.18 ± 0.16</td>
<td>$t = 0.839$ df = 61</td>
<td>0.405</td>
</tr>
<tr>
<td>Initial insulin dose (U/kg/d)</td>
<td>0.46 ± 0.19</td>
<td>0.39 ± 0.20</td>
<td>$t = -1.210$ df = 62</td>
<td>0.231</td>
</tr>
</tbody>
</table>

*aExcept for the proportions, all values shown are the mean ± SD.*
Autoantibodies had a later manifestation of microalbuminuria at a mean of 10.1 years (95% CI 7.0–13.2 years). However, the mean difference of 2.4 years remained statistically insignificant ($P = 0.42$).

**Immunofluorescence**

We tested in immunofluorescence, using rat kidney cortex sections, the three diabetic serum samples with the highest titres of nephrin autoantibodies and three control samples negative for nephrin autoantibodies. Immunofluorescence microscopy revealed an epithelial-type glomerular staining typical for nephrin with two of the highest serum samples as one being negative. Stainings with negative control sera and secondary antibodies alone showed negligible reactivity. A polyclonal antibody against rat nephrin was used as a positive control (Figure 3).

**Discussion**

In this study, we show that a considerable subset of patients with type 1 diabetes present with circulating autoantibodies to nephrin, mostly during the early years of the disease. Furthermore, the patient sera with the highest titres are shown to stain glomeruli in an epithelial-like pattern. Based on the key position of nephrin at the slit diaphragm bridging the interpodocyte space and the direct effect of *in vivo* injected [8] or naturally occurring antinephrin antibodies [7] causing direct glomerular damage and proteinuria, we hypothesized that such circulating antibodies may be involved in the development of diabetic damage to the glomerular filtration barrier.

Over the last three decades, an exhaustive series of evidence has been generated to show that type 1 diabetes is an autoimmune disease. Autoimmunity is a frequent, transient phenomenon in many inflammatory-infectious diseases. The initial proof of autoimmunity in diabetes was the discovery of circulating antibodies against the islets of Langerhans, now known to comprise antibodies to a heterogeneous set of islet cell antigens. These antibodies are proposed to emerge when sequestered proteins are exposed to autoreactive T cells after an exogenous, e.g. viral, insult of the pancreatic β-cells. Humoral immune responses to β-cell autoantigens such as insulin, GAD and IA-2 have been shown to associate with the early pre-clinical insulitis stage of diabetes [10–12]. Indeed, radiobinding assays for these autoantibodies have been introduced for clinical use as early markers or predictors of type 1 diabetes. However, the contribution of these antibodies to disease progression is not completely clear. Compared with GADA or IA-2A, at least either one of which is present in >90% of the cases with recent onset type 1 diabetes [13], nephrin antibodies have a low prevalence at the time of diagnosis of clinical diabetes and thus may not function as clinical markers of the disease process.
of diabetes itself. Our results show dissimilar levels of ICA and nephrin antibodies (Table 1). Nevertheless, the profile over time of the nephrin autoantibody prevalence compared with that of the ICA, IA-2A or GADA is similarly declining, suggesting that the nephrin autoantibodies are also generated during the early autoimmune process leading to type 1 diabetes. It is noteworthy that the patients developing kidney complications and those remaining unaffected present with a roughly similar prevalence of nephrin autoantibodies. During the follow-up period, only four of the 29 (13.8%) patients positive for nephrin autoantibodies at any time point developed microalbuminuria or overt proteinuria. It is, therefore, unlikely that the nephrin autoantibodies significantly affect the development of diabetic nephropathy. Whether these patients represent distinct subgroups among diabetic patients remains to be studied in detail. Our test screened the samples mainly for autoantibodies against epitopes of the intracellular part of nephrin. In addition to covering one-third of the extracellular part, we chose to cover the whole intracellular portion with our labelled nephrin. The autoimmune response in type 1 diabetes leads to β-cell damage, which may then lead to the exposure of hidden cryptic cytoplasmic epitopes rendering them available to an autoimmune attack. Cryptic epitopes are considered to be important to the pathogenesis of autoimmunity [18]. This is why we considered the cytoplasmic region of nephrin to be the most likely immunogenic area of nephrin in the pancreatic β-cells. Studies on T-cell epitope spreading suggest that once the CD4+ T-cell tolerance is broken for one epitope, the autoimmune response can spread to other epitopes within the same autoantigen and even to nearby autoantigens [19]. Such epitope spreading is likely to occur during the generation of intracellular nephrin autoantibodies as well and lead to generation of extracellular nephrin autoantibodies. This phenomenon occurs also in the autoimmune response to GAD during the pre-clinical stages of type 1 diabetes [20]. Due to the above mentioned factors, it seemed conceivable that the presence of autoantibodies against the intracellular epitopes of nephrin could be associated with the development of diabetic nephropathy in type 1 diabetes. Furthermore, intracellular autoantibodies have pathogenic importance in other conditions, and the CNF patients with recurrent nephrosis after kidney transplantation had equal quantities of antibodies against intra- and extracellular nephrin.

Fig. 3. Immunofluorescence microscopy. Patient serum positive for nephrin autoantibodies stained glomeruli in an epithelial-like pattern (A), similarly to a polyclonal nephrin antibody used as a positive control (B). Arrows in A and B point out such areas of linear epithelial staining. Serum from a control subject (C) or conjugate control (D) did not stain glomeruli. Scale bar shown in D is valid for the entire figure.
in a previous study [7]. The limited serum sample volume and the inefficiency of in vitro production of a full-length nephrin probe further supported the screening of the intracellular region of nephrin in type 1 diabetes.

One explanation for the absence of significant damage to the kidneys could thus be that the intracellular nephrin epitopes are normally protected in the podocyte cytoplasm. Formation of linear deposits of IgG in the diabetic glomeruli has been reported earlier [21]. Although fluctuation in the individual nephrin autoantibody titres was seen in the present study, the subset of patients with constantly prevailing nephrin antibodies may increasingly accumulate these antibodies in the kidney. We would like to point out that the follow-up study was not originally designed to find out whether circulating autoantibodies could end up in the kidney glomerulus and attach to their respective epitopes. The immunohistochemical stainings with patient sera did not exclude this possibility, but the issue requires further study.

Only 25–30% of the patients with type 1 diabetes develop diabetic nephropathy during the course of the disease [22]. Some patients experience a rapidly progressive glomerular injury, while others develop this complication only after decades. Accordingly, diabetic nephropathy appears as a multifactorial disease. An early intervention with angiotensin converting enzyme (ACE) inhibitors postpones diabetic nephropathy. ACE inhibition is a routine yet a relatively costly treatment if administered in every patient and it may also be associated with considerable side effects. This emphasizes the importance of accurate early diagnostics to identify patients benefiting from intensive therapy. In an attempt to meet the needs of early diagnosis, microalbuminuria has been used as a marker for diabetic nephropathy [23]. However, ~7% of non-diabetic, non-hypertensive subjects present with microalbuminuria as well. Moreover, not all diabetic patients with microalbuminuria develop overt nephropathy and, furthermore, clear histopathological changes can develop without long-standing microalbuminuria. Together, these facts call for novel early molecular markers of diabetic nephropathy [24].

In conclusion, our current results show the presence of nephrin autoantibodies in patients with type 1 diabetes. We are aware of the limitations caused by the relatively small number of patients in our study. Nevertheless, the collection of this well-characterized material was started more than two decades ago and it provides the possibility of follow-up. The precise role of these antibodies in the development of diabetic nephropathy remains to be studied in detail.

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Conflict of interest statement. None declared.

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