Plasma kininogen and kininogen fragments are biomarkers of progressive renal decline in type 1 diabetes

Michael L. Merchant[1,2], Monika A. Niewczas[3,4], Linda H. Ficociello[3,6], Janice A. Lukenbill[1,2], Daniel W. Wilkey[1,2], Ming Li[1,2], Syed J. Khundmiri[1,2], James H. Warram[3], Andrzej S. Krolewski[3,4] and Jon B. Klein[1,2,5]

[1]Kidney Disease Program, University of Louisville, Louisville, Kentucky, USA; [2]Clinical Proteomics Center, University of Louisville, Louisville, Kentucky, USA; [3]Division of Research, Joslin Diabetes Center Boston, Boston, Massachusetts, USA; [4]Department of Medicine, Brigham & Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA and [5]Veterans Administration Medical Center, Louisville, Kentucky, USA

The ability of microalbuminuria to predict early progressive renal function decline in type 1 diabetic patients has been questioned. To resolve this, we determined the plasma proteome differences between microalbuminuric patients with type 1 diabetes and stable renal function (controls) and patients at risk for early progressive renal function decline (cases) and asked whether these differences have value as surrogate biomarkers. Mass spectrometry was used to analyze small (<3 kDa) plasma peptides isolated from well-matched case and control plasma obtained at the beginning of an 8–12 year follow-up period. A Spearman analysis of plasma peptide abundance and the rate of renal function decline during follow-up identified seven masses with a significant negative correlation with early progressive renal function decline. Tandem mass spectrometry identified three fragments of high-molecular-weight kininogen. Increased plasma high-molecular-weight kininogen in the cases was confirmed by immunoblot. One peptide, des-Arg9-BK(1–8), induced Erk1/2 phosphorylation when added apically to two proximal tubular cell lines grown on permeable inserts. Thus, we have identified plasma protein fragments, some of which have biological activity with moderate to strong correlation, with early progressive renal function decline in microalbuminuric patients with type 1 diabetes. Other peptides are candidates for validation as candidate biomarkers of diabetes-associated renal dysfunction.

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Correspondence: *Andrzej S. Krolewski, Division of Research, Joslin Diabetes Center, Room 368, One Joslin Place, Boston, Massachusetts 02215, USA. E-mail: andrzej.krolewski@joslin.harvard.edu or Jon B. Klein, Donald Baxter Research Building, Room 110E, 570 S. Preston Street, Louisville, Kentucky 40202, USA. E-mail: jon.klein@louisville.edu*

[6]Currently at Fresenius Medical Care–North America.

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Microalbuminuria (MA) has been considered the primary diagnostic tool to identify type 1 diabetes mellitus (T1D) patients at risk for progressive renal dysfunction.[1,2] However, the correlation of MA with future renal dysfunction in diabetics has now been called into question. Several findings indicate that MA may not reliably herald the beginning of renal dysfunction. First, only ~20% of patients with MA will progress to proteinuria;[3] second, many patients with MA can revert to normoalbuminuria;[4–6] and third, many individuals with T1D have already experienced early progressive renal function decline (ERFD) before or coincidental with MA onset.[7,8] These findings have called into question the model of diabetic nephropathy in which MA conveyed a high risk of progressive renal dysfunction and support a new model in which only a subset of those with MA develop progressive ERFD. This change in our understanding of diabetic renal disease also is indicative of our incomplete understanding of the mechanisms of ERFD, a process that takes place while measured renal function is still in the normal or even elevated range. These findings emphasize on the need for further studies to understand the pathophysiology of ERFD in patients with MA and to identify those T1D patients at risk for early renal damage.

We addressed the hypothesis that qualitative differences in plasma proteins might provide insight into ERFD pathophysiology and serve as candidate biomarkers of the risk for progressive ERFD and progressive renal function loss. To address this hypothesis, we have analyzed plasma samples obtained during the first Joslin Study of the Natural History of Microalbuminuria in Type 1 Diabetes using LC-MALDI-TOF mass spectrometry (MS) to compare the low-molecular-weight protein (<3000 Da) or peptidomic plasma fraction. We analyzed the plasma peptidome of patients matched for

cystatin C estimated glomerular filtration rate (eGFR), MA, and medications (among other clinical parameters) comparing those who retained stable renal function with those who developed ERFD during subsequent 8–12 years of follow-up. We hypothesized that qualitative differences in the low-molecular-weight plasma proteome (the peptidome) might provide insight into the etiology of early progressive RFD and serve as putative biomarkers of future progression. We observed a striking correlation between the rate of future renal function decline and components of the kallikrein–kininogen system. These protein fragments should now be considered as candidates for confirmation in larger studies as candidate biomarkers of ERFD and predictors of renal dysfunction in T1D.

RESULTS

Characteristics of the study population

The study population comprised the patients whose onset of MA was documented in the first Joslin Study of the Natural History of Microalbuminuria in Type 1 Diabetes. Additional eligibility criteria included follow-up examinations spanning at least 8–12 years after MA onset for estimating the rate of GFR decline and availability of a 6 ml aliquot of stored urine for peptide analysis.[9] Thirty-three patients (16 cases and 17 controls) were selected from a previous urinary biomarker study who met all eligibility criteria (cases with renal function decline defined as a decline of 3.3% or more per year (range: –3.3 to –16.1% per year), and controls with lesser rates of renal function decline (range: +1.9 to −3.2% per year) had contemporaneous plasma samples available for the current study.

Correlation of discriminating peptides with the rate of future renal function decline

To detect peptides whose abundance strictly correlated with the linear estimate of renal function and not simply a discrete clinical group, a Spearman rank order correlation analysis was performed comparing peptide abundance with the rate of renal function decline. A total of seven peptides were identified with the Spearman correlation value ranked between an absolute value of −0.45 and −0.51 ($P<0.001$) (Table 1). As such if validated, they may have a value to identify patients with an increased risk for the development of ERFD.

Identification of peptide amino-acid sequence by mass spectrometry

To better understand the possible role of the selected peptide masses in the etiology of ERFD, we sought to identify partial amino-acid sequence tagging information using tandem mass spectrometry. Two of the seven masses were not fragmented because of low intensity and proximity of the monoisotopic peak to larger more intense ions. By using the parent peptide mass, the peptide fragmentation data (see Supplementary Data online for peak list information and fragmentation spectra) gained from the tandem MS experiments, and the data analysis software, Matrix Science Mascot, amino-acid sequences were assigned to a total of four of the five peptides submitted for MS/MS analysis (Table 1). The results of these analyses identified fragments of two plasma proteins including kininogen-1 (three fragments) and a fragment of plasma kallikrein-sensitive glycoprotein (inter-α-trypsin inhibitor heavy chain H4, ITIH4). The comparison of the fragmentation spectra for synthetic peptides with experimental data supported these peptide assignments (see Supplementary Data online). The kinin peptides were modified by C-terminal proteolysis and/or prolyl-specific hydroxylation. Although the strength of the assignments for these peptides as candidate biomarkers for ERFD in T1D is based on the Spearman correlation analysis, we provide a figure (Figure 1) to illustrate the comparison of the means for these plasma peptide abundances. These data suggest that in these patients the abundances of specific plasma peptides can begin to predict significant declines in GFR. In general, a comparison between the cases and controls for these peptides identified a 30–50% increased mean abundance in patients who were at risk for ERFD (Figure 1, Table 1).

Table 1 | Characterization of plasma peptides whose abundance strongly correlates with the rate of future renal function decline

Peptide mass (m/z)	Spearman's rank correlation coefficient (r_s)	*P*-value[a] for comparison of cases and controls	Mascot MOWSE score[b]	Peptide or protein fragment
Identified				
757.402	−0.51	0.006	36	des-Phe8-des-Arg9-Bradykinin (1-7) (K)-RPPGFSP-(F)
904.472	−0.46	0.03	44	des-Arg9-Bradykinin (1–8) (K)-RPPGFSPF-(R)
920.467	−0.45	0.06	44	Hyp3-des-Arg9-Bradykinin (1–8) (K)-RP-Hyp-GFSPF-(R)
1675.794	−0.50	0.03	96	Kallikrein-sensitive glycoprotein (ITIH4) (P)-GPPDVPDHAAYHPFR-(R)
Unidentified				
789.394	−0.48	0.01	NA	
945.453	−0.46	0.01	NA	
1050.099	−0.46	0.02	NA	

Abbreviation: NA, not applicable.
The integrated area under the curve for plasma peptide data was extracted from aligned LC-MALDI-TOF MS data sets and analyzed using the Spearman correlation analysis to the estimated rate of renal function decline (estimated using serum concentration of cystatin C, and changes in renal function were estimated by slopes). Peptides with strong correlation (between −0.45 and −1.0) to progressive renal function decline are listed. The difference in the observed abundance of plasma peptides selected by the Spearman correlation was estimated using the unpaired *t*-test with Welch's correction for unequal variation in case and control peptide abundance data sets.
[a]Analysis by *t*-test (with Welch's correction for unequal variances) of differences in abundance of peptides in case ($n=16$) and control ($n=17$) samples.
[b]Mascot MOWSE scores >20 are considered significant (*P*-value <0.05) and provide for identity.

Differential abundance of high-molecular-weight kininogen in plasma

We then examined whether the increased abundance of bradykinin (BK) forms in case plasma might result from