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Development of a standardized ELISA for the determination of autoantibodies against human M-type phospholipase A2 receptor in primary membranous nephropathy

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ABSTRACT

Background: Autoantibodies against the M-type phospholipase A2 receptor (PLA_2R1) are specific markers for primary membranous nephropathy (pMN) and anti-PLA_2R1 serum levels may be useful to monitor disease activity. So far, a recombinant cell-based indirect immunofluorescence assay (RC-IFA) using recombinant PLA_2R1 as a substrate has been widely available but lacks a finely graduated assessment of antibody concentrations.

Methods: In order to setup a standardized ELISA, the extracellular domain of human PLA_2R1 was expressed in HEK293. The purified protein was used to form the solid-phase in an ELISA which was then employed to analyze sera from 200 patients with primary MN, 27 patients with secondary MN, 230 patients with other glomerular diseases, 316 patients with systemic autoimmune diseases, and from 291 healthy blood donors.

Results: At a set specificity of 99.9% the sensitivity of the anti-PLA_2R1 IgG ELISA was found to be 96.5%. A similar sensitivity (98.5%) was obtained when binding of only subclass IgG_4 was analyzed. The calibrated assay showed a good class correlation with the results of the RC-IFA, was robust and could be stored for several months without any loss of quality.

Conclusion: The results demonstrate that the new test system is qualified for routine use and that it has an almost perfect agreement with both, the clinical characterization of the patients and the results generated with RC-IFA.

1. Introduction

Membranous nephropathy (MN) is a leading cause of nephrotic syndrome in adults with varying natural history and course [1]. Whereas primary MN (pMN) is considered to be an organ-specific autoimmune disease, secondary forms are associated with malignant tumors, bacterial or viral infections, systemic autoimmune diseases and toxic drugs. Whereas secondary MN mostly responds to underlying diseases pMN may range from end stage renal disease to spontaneous remission [2,3]. Even though there have been risk assessments based on clinical and laboratory data for the eventual outcome [4,5], it remains uncertain which patient should solely receive supportive care or eventually needs to undergo an immunosuppressive therapy. The discovery of the M-type phospholipase A2 receptor (PLA_2R1) as the major target antigen of autoantibodies in about 70% of patients with MN [6,7] has led to the hypothesis that circulating autoantibodies bind to PLA_2R1 on podocytes leading to destruction of the glomerular filtration barrier and the onset of proteinuria [8]. Several independent retrospective follow-up studies have confirmed this specific association between anti-PLA_2R1 and pMN [9–12]. The autoantibodies can be considered as pathognomonic markers [8,12,13].

Increases of anti-PLA_2R1 levels precede the exacerbation of symptoms whereas decreases are associated with amelioration or remission

Abbreviations: MALDI-TOF, Matrix-Assisted Laser Desorption/Ionization-Time Of Flight mass spectrometry; PAGE, polyacrylamide gel electrophoresis; FITC, fluorescein isothiocyanate; IFA, indirect immunofluorescence assay; RC-IFA, recombinant cell-based indirect immunofluorescence assay; HRP, horse radish peroxidase; AP, alkaline phosphatase; PLA_2R1, M-type phospholipase A2 receptor; MN, membranous nephropathy; pMN, primary membranous nephropathy.

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[12,13]. This relationship between these autoantibodies and the disease activity paves the road for the integration of quantitative antibody determination in the monitoring of treatment in patients with chronic MN or after kidney transplantation [13]. Moreover, the magnitude of anti-PLA_2R1 titer itself has been proposed as a predictive marker for disease outcome and, in turn, as a valuable tool for the decision on treatment strategies [8].

In order to use PLA_2R1 autoantibodies as clinical markers for disease activity validated test systems with high sensitivity and specificity are necessary. In the initial publication, Beck et al. used a Western blot after non-reducing SDS-PAGE [6]. Despite unconfirmed descriptions about an improved variant of the method with a sensitivity of >90% [14], it is difficult to use Western blotting outside of an expert laboratory and the method is not suited for the evaluation of large sample numbers. These limitations led to the development of a recombinant cell-based indirect immunofluorescence assay (RC-IFA) that uses the human cell line HEK293 over-expressing the full-length human PLA_2R1 as substrate [9]. The assay can be used both as a diagnostic tool and for the approximation of antibody concentrations during monitoring of patients. More recently, an ELISA protocol was published that uses a recombinantly produced extracellular domain of PLA_2R1 to form the solid-phase [12]. The report indicated a superior grading of antibody titers following this approach but the study did neither evaluate the robustness of the method nor its specificity on a large number of disease controls.

The aim of the current study was to develop a standardized ELISA for the determination of autoantibodies against PLA_2R1 that can be made widely available and used in a routine setting for clinical applications. To this end, the extracellular domain of human PLA_2R1 was purified to homogeneity and used to build a robust solid-phase in an ELISA that was then evaluated in a large cohort of clinically well-characterized patients with primary and secondary MN from three different centers. The findings were compared to a cohort of disease controls and healthy blood donors. Our data show a very high sensitivity and specificity of the ELISA as well as a very close correlation with the RC-IFA making the ELISA a very good tool for accurate PLA_2R1 antibody assessment.

2. Materials & methods

2.1. Human sera

Sera of patients with biopsy-proven primary and secondary membranous nephropathy were collected from three centers. One cohort consisted of 122 patients with primary MN and 15 secondary MN from the University Hospital Hamburg-Eppendorf, Germany. A second cohort consisted of 66 primary MN and 7 secondary MN patients from the Radboud University Nijmegen, The Netherlands. The third cohort consisted of 12 primary MN and 5 secondary MN patients from the CNRS, Nice, France. This resulted in a total of 200 sera from patients with primary MN and 27 with secondary MN, respectively. All patients with primary and secondary MN were prospectively or retrospectively identified and were only included if the time between diagnosis by renal biopsy and blood withdrawal was less than 8 weeks. The definition of primary MN also included a serological pre-characterization and positivity in the anti-PLA_2R1 RC-IFA (see below). The definition of secondary MN was fulfilled if another underlying disease was identified and anti-PLA_2R1 RC-IFA was negative.

Disease controls included samples from patients with Rheumatoid Arthritis, psoriatic arthritis, systemic lupus erythematosus without renal involvement, mixed connective tissue disease, systemic sclerosis, Sjoegren's syndrome, polymyositis, undifferentiated collagenosis, autoimmune thyroiditis, and other glomerular diseases (see further details in Table 1). Finally, 291 serum samples from healthy blood donors were included. These samples were obtained from the University Hospital of Lübeck, Germany.

Table 1
Characteristics of the patients enrolled in this study. The diagnosis of MN was based on an unequivocal biopsy within 8 weeks prior to the blood sampling. Primary MN was defined as positive determination of anti-PLA_2R1 by RC-IFA. Secondary MN was defined as biopsy proven MN with a clinical underlying disease known to be associated with MN and a negative test for PLA_2R1 antibodies by RC-IFA. FSGS, Focal Segmental Glomerular Sclerosis; IgAN, IgA nephropathy; MCD, Minimal Change Disease; Membranoproliferative Glomerulonephritis, MPGN; GN-nos, other glomerular diseases.

Group	n	Female/male	Median age (range)
Primary MN	200	67/133	54 (16–86)
Secondary MN	27	12/15	57 (25–79)
Other renal diseases	230		
FSGS	20	8/12	52 (16–68)
IgAN	9	5/4	50 (23–58)
MCD	16	9/7	39 (20–62)
MPGN	2	1/1	45, 54
Alport's syndrome	1	0/1	19
GN-nos	182	57/125	55 (18–83)
Other autoimmune diseases	316		
Rheumatoid arthritis	39	36/3	50 (29–86)
Psioriatic arthritis	30	14/16	51 (19–73)
Systemic lupus erythematosus	55	45/10	52 (34–86)
Mixed connective tissue disease	15	15/0	52 (23–77)
Systemic sclerosis	86	74/12	62 (1–93)
Sjoegren's syndrome	27	24/3	65 (13–83)
Polymyositis	8	6/2	63 (50–85)
Undifferentiated collagenosis	6	6/0	52 (26–73)
Autoimmune thyroiditis	50	42/8	49 (22–90)
Healthy individuals	291	112/179	38 (18–68)

All serum samples were collected within the standard diagnostic patient work-up in the respective centers. In adherence to the Helsinki principles, informed consent from all patients, whose material was used in this study, was obtained.

2.2. Detection of anti-PLA_2R1 antibodies by indirect immunofluorescence

Anti-PLA_2R1 antibodies were determined using a recombinant cell-based indirect immunofluorescence test (RC-IFA, Euroimmun, Germany) containing a BIOCHIP mosaic of formalin-fixed HEK293 cells over-expressing PLA_2R1 and mock-transfected HEK293 cells as negative control. To achieve a semi-quantitative measurement of PLA_2R1 autoantibody levels, different dilutions of serum samples were prepared in phosphate-buffered saline, 0.2% Tween-20 and incubated for 30 min. A fluorescein isothiocyanate-conjugated goat anti-human IgG polyclonal antibody (Euroimmun) was used to detect bound IgG antibodies. All slides were evaluated by two-independent observers using a microscope with 460–490 nm LED excitation (EUROStar; Euroimmun). In all cases, the incubated slides were evaluated independently by two experts. A specific fluorescence of the transfected cells at a dilution of 1:10 or higher was considered to be as positive (Supplementary Fig. 1).

2.3. Purification of recombinant human PLA_2R1 from HEK293 cells

The extracellular domain of the human PLA_2R1 isoform 1 (Acc No. NP_031392.3, amino acid residues 1–1397 [15]) was expressed in HEK293 cells essentially as described for the full-length protein [16]. The cell culture supernatant was harvested after expression for 5 days. The recombinant protein was purified by immobilized metal affinity chromatography as described for the extracellular domains of desmogleins 1 and 3 [17]. All steps were carried out at 4 °C. The fractionation of PLA_2R1 was monitored by non-reducing Western blot described by Beck et al. [6] using a human sample containing high-titer autoantibodies against PLA_2R1 as determined by RC-IFA. The purified antigen was characterized by SDS-PAGE, Western blot and PNGase F digestion (Supplementary Fig. 2).