



Novel indicators of fibrosis-related complications in children with chronic kidney disease



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ABSTRACT

Objectives: Tumor growth factor (TGF)β1 initiates renal fibrosis, whereas matrix metalloproteinases (MMPs), their tissue inhibitors (TIMPs), adhesion molecules and heat shock proteins (hsps) may act in further stages of this process. The aim of this study was to assess the concentrations of Hsp90α, sE-selectin, MMP-2, TIMP-1, TIMP-2 and TGFβ1 in children with advanced chronic kidney disease (CKD) and their role as markers of fibrosis. **Methods:** 80 children with CKD stages 1–5 and 30 controls were enrolled in the study. Serum concentrations of examined parameters were assessed by ELISA.

Results: Median values of all markers were significantly elevated in CKD patients vs. controls. sE-selectin and MMP-2 concentrations kept growing from the beginning of renal failure progression. TIMP-1, TIMP-2 and TGFβ1 levels remained unchanged in the late CKD stages, whereas Hsp90α concentrations decreased significantly in CKD stage 5. All parameters, except for MMP-2, correlated with TGFβ1, but the strongest predictive value was seen in the case of TIMP-1 and TIMP-2.

Conclusions: The increased concentrations of examined parameters indicate enhanced cell damage, inflammation and aggravation of proteolytic processes in CKD children. Variability in behavior of selected markers and existing correlations point at the complexity of relations between different elements responsible for the fibrosis puzzle.

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1. Introduction

Renal interstitial fibrosis is the final common pathway in chronic kidney disease, independent of its origin. This multistep process is stimulated by pro-fibrotic agents like TGFβ1 [1,2]. The latter governs response to tissue injury, including cell migration, inflammation, extracellular matrix accumulation and irreversible damage of the tissue [2–4]. TGFβ1 is also the main regulator of epithelial–mesenchymal transition and subsequent differentiation of fibroblasts into myofibroblasts, resulting in the production of proteolytic enzymes [1,5]. Their role in fibrosis has been well documented in a series of animal models and in vitro experiments [6–8].

The activity of matrix metalloproteinases firstly stimulates the elimination of damaged cells, thus preventing the expansion of extracellular matrix components [9]. However, when proteolytic overactivity endures, the balance between protective and noxious agents is disturbed, the activity of tissue inhibitors of metalloproteinases becomes inefficient and fibrosis starts heading towards its irreversible phase. The essential point is tubular atrophy and further nephron destruction, resulting in end stage kidney disease.

Although heat shock proteins are mainly acting as chaperones, the key element in the fibrosis-related processes might be their behavior in stress conditions. Their intracellular content increases, then they are released and may serve as markers of cell damage. Recent investigation has suggested that inhibition of Hsp90 degrades the TGFβ type II receptor and by that way it may attenuate renal fibrosis [10]. However, nothing is known about the role of the extracellular form of Hsp90 as a marker of fibrosis in chronic kidney disease or its probable connection with TGFβ1.

Selectins form another group of important players in the fibrosis-related processes. They mainly take part in leukocyte capture and rolling along the vascular wall, enabling further arrest and migration towards inflamed and damaged tissues. Special engagement of soluble E-selectin in this process raises the question about its potential role as an indicator of kidney damage in the course of fibrosis [11].

The unfavorable prognosis connected with fibrosis stimulates the search for factors that could potentially slow down or reverse its progression [12]. Although the abovementioned preliminary results of the in vitro investigations are promising, there are no available data on the role of those markers in renal interstitial fibrosis assessment in CKD patients – either adults or children.

Our aim was to evaluate the serum concentrations of Hsp90α, sE-selectin, MMP-2, TIMP-1, TIMP-2 and TGFβ1 in children with chronic kidney disease in the pre-dialysis period and in those with normal kidney function, forming a control group. We also searched for correlations

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between these parameters and assessed their potential role as markers of fibrosis-related complications.

2. Methods

2.1. Patient characteristics

110 patients enrolled in this retrospective cross-sectional study were divided into 4 groups.

The first group (CKD I) contained 15 patients (6 girls, 9 boys, median age 8.5 years, interquartile range 4.5–11 years) with CKD stages 1–2 (median GFR calculated according to the Schwartz formula 78 ml/min/1.73 m²) [13]. The diseases leading to CKD were: reflux nephropathy (8 cases), chronic pyelonephritis (6), and polycystic kidney disease (1).

The second group (CKD II) consisted of 39 patients with CKD stages 3–4 (18 girls, 21 boys, median age 9 years, interquartile range 3.5–14 years) treated conservatively (median GFR calculated according to the Schwartz formula 36 ml/min/1.73 m²). The diseases leading to CKD were: reflux nephropathy (16 cases), chronic glomerulonephritis in the course of nephrotic syndrome (12), chronic pyelonephritis (5), polycystic kidney disease (4), hemolytic uremic syndrome (1) and cystinosis (1).

The third group (CKD III) contained 26 patients with CKD stage 5 (10 girls, 16 boys; median age 10.5 years, interquartile range 2–16.5 years), yet on conservative treatment (median GFR calculated according to the Schwartz formula 13 ml/min/1.73 m²). The factors causing CKD were: reflux nephropathy (13), chronic glomerulonephritis in the course of nephrotic syndrome (9), neurogenic bladder (3), and hemolytic uremic syndrome (1). In all patients with CKD stages 3–5 and 5 children with CKD stage 2 phosphate binders and vitamin D metabolites were supplemented.

30 children (16 girls, 14 boys, median age 10.0 years, range 5.5–15.5 years) with primary nocturnal enuresis and normal kidney function, served as controls.

None of the patients has shown clinical evidence of infection, had diabetes, malignancies or vasculitides, smoked, or took antibiotics, statins, corticosteroids or immunosuppressive therapy. They were also free of such co-morbidities as cardiovascular disease, peripheral vascular disease or obesity. In the CKD group 56 children were normotensive according to the criteria of the fourth report on high blood pressure in children and adolescents [14], in 39 patients blood pressure was well controlled with the use of ACE inhibitors (22), calcium channel blockers (10 patients) and β -blockers (3 children), and 4 patients needed combined therapy.

Informed consent was obtained from the subjects and their parents, if necessary. The research project has been approved by the University ethics committee, in accordance with the Helsinki declaration.

Blood samples were drawn after an overnight fast from peripheral veins in CKD patients and in controls. Samples were clotted for 30 min, centrifuged at room temperature (with the exception of Hsp90 α , centrifuged at 4 °C), 1000 g for 15 min, and then serum was stored at –20 °C until assayed.

2.2. Analyte and assay characteristics

Serum concentrations of Hsp90 α (molecular mass – 90 kDa), sE-selectin (115 kDa), MMP-2 (72 kDa), TIMP-1 (~20 kDa), TIMP-2 (~20 kDa) and TGF β 1 (25 kDa) were evaluated by commercially available ELISA kits (Hsp90 α – Enzo Life Sciences, reagent kit ADI-EKS-895; sE-selectin – R&D Systems, reagent kit DSLE00; MMP-2 – R&D Systems, reagent kit DMP2F0; TIMP-1 – R&D Systems, reagent kit DTM100; TIMP-2 – R&D Systems, reagent kit DTM200; TGF β 1 – R&D Systems, reagent kit DB100B). Standards and serum samples were transferred to 96 well microplates pre-coated with recombinant antibodies to human Hsp90 α , sE-selectin, MMP-2, TIMP-1, TIMP-2 and TGF β 1. Captured

proteins were then detected using monoclonal antibodies against Hsp90 α , sE-selectin, MMP-2, TIMP-1, TIMP-2 and TGF β 1 conjugated to horseradish peroxidase. Next, the assay was developed with tetramethylbenzidine substrate and blue color was developed proportionately to the amount of captured protein. The addition of acid stop solution ended the color development and converted it to the endpoint yellow. The intensity of the latter was measured in a microplate reader at 450 nm, with the correction wavelength at 550/650 nm. Each sample was tested in duplicate and the arithmetical mean was considered a final result. Measurements were performed according to the manufacturer's instructions, results were calculated by reference to standard curves.

The intra-assay and inter-assay coefficients of variation (%CV) for examined parameters did not exceed 6.0% and 8.5%, respectively, and were as follows: Hsp90 α – \leq 5.2% and \leq 7.5%, sE-selectin – \leq 5.3% and \leq 6.5%, MMP-2 – \leq 4.7 and \leq 7.2%, TIMP-1 – \leq 4.2% and \leq 5.1%, TIMP-2 – \leq 4.5% and \leq 6.6%, TGF β 1 – \leq 6.0% and \leq 8.5%. Limits of detection: Hsp90 α – 50 pg/ml, sE-selectin – 0.027 ng/ml, MMP-2 – 0.16 ng/ml, TIMP-1 – 0.08 ng/ml, TIMP-2 – 0.01 ng/ml, and TGF β 1 – 4.6 pg/ml.

Serum creatinine was assessed with the Creatinine (Enzymatic) OSR61204 reagent on the Beckman Coulter AU2700 analyzer. High sensitivity (hs)CRP was assessed by immunonephelometry with Siemens CardioPhase hsCRP reagent on the BN II System analyzer.

2.3. Statistical analysis

Results are expressed as median values and interquartile ranges. Since the null hypothesis of normality of distribution was rejected by a Shapiro–Wilk test, multiple comparisons and comparisons in pairs were evaluated by using nonparametric tests (Kruskal–Wallis, Mann–Whitney U). The relations between parameters were assessed in the whole group of 80 CKD patients with the use of Pearson's correlation coefficient.

The statistically significant correlations were then analyzed by linear regression analysis in CKD stages 1–2, stages 3–4 and stage 5 separately, in order to identify any differences in strength of relations, depending on the renal failure aggravation. The linear regression equations were calculated as $y = \beta x + a$ (y – dependent variable, β – regression coefficient, x – independent variable, a – constant term). We presented only those equations where both regression coefficient and constant term were statistically significant. Statistical analysis was performed using the package Statistica ver. 10.0. A p value < 0.05 was considered significant.

3. Results

Median values of Hsp90 α , sE-selectin, MMP-2, TIMP-2 and TGF β 1 in CKD patients were significantly elevated since the earliest stages of CKD when compared to those in the controls, whereas TIMP-1 levels started growing within stages 3–4 (Table 1). sE-selectin and MMP-2 concentrations rose gradually and became even higher in children with end stage kidney disease, whereas those of TIMP-1, TIMP-2 and TGF β 1 have remained stable since stages 3–4 despite the renal failure progression. The Hsp90 α values peaked down with kidney function deterioration in stage 5, although remained increased in comparison to the control group. hsCRP values have not changed with renal failure progression (Table 1).

3.1. Correlations and regression analysis

Hsp90 α and sE-selectin correlated significantly with MMP-2, its tissue inhibitors (TIMP-1, TIMP-2) and eGFR in the whole group of CKD patients (Table 2). All parameters, excluding MMP-2, correlated with TGF β 1 (Table 2).

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