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SHORT COMMUNICATION

Matrix metalloproteinases (MMP-2,9) and their tissue inhibitors (TIMP-1,2) as novel markers of stress response and atherogenesis in children with chronic kidney disease (CKD) on conservative treatment

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Abstract The system of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) may play a key role in atherogenesis of chronic kidney disease (CKD) patients by its impact on matrix accumulation. Connections with inflammation, stress, or endothelial dysfunction are also probable. However, the data on correlations between these parameters in CKD patients are scarce in adults and absent in children. The aim of our study was to evaluate serum concentrations of MMP-2, MMP-9, TIMP-1, and TIMP-2, as well as their correlations with markers of stress response (Hsp90-α, anti-Hsp60), endothelial dysfunction (sE-selectin), and inflammation (high-sensitivity C-reactive protein) in CKD children treated conservatively. Thirty-seven patients were divided into two groups according to the CKD stage (gr.CKDI, 19 children with CKD stages 2–3; gr.CKDII, 18 subjects with CKD stages 4–5). Twenty-four age-matched healthy subjects served as controls. Serum concentrations of MMP-2, MMP-9, TIMP-1, TIMP-2, Hsp90-α, anti-Hsp60, and sE-selectin were assessed by ELISA. Median values of MMP-2, MMP-9, TIMP-1, and TIMP-2 were significantly higher in all CKD children vs. controls and were increased in patients with CKD stages 4–5 vs. CKD stages 2–3. Hsp90-α, anti-Hsp60, sE-selectin, and glomerular filtration rate predicted the values of MMPs and TIMPs. Chronic kidney disease in children is characterized by MMP/TIMP system dysfunction, aggravated by the progression of renal failure. Correlations between examined parameters, heat shock proteins, and markers of endothelial damage suggest the possibility of MMP/TIMP application as indicators of stress response and atherogenesis in children with CKD on conservative treatment.

Keywords Autoimmunity · Heat shock proteins · Inflammation · Lipids · Matrix destruction

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Introduction

Endothelial dysfunction, inflammation, dyslipidemia, and autoimmune reactions are key elements in the pathogenesis of atherosclerosis (Blasi 2008; Nilsson and Hansson 2008). Heat shock proteins (HSPs) and their antibodies also influence the process of atherosclerosis (Wick et al. 2004; Rigano et al. 2007). The best described example of such impact is that of Hsp60 and anti-Hsp60 working together. Anti-Hsp60 is generated in response to both bacterial and human Hsp60 and triggers autoimmune reactions against one's own HSPs (Pockley et al. 1999; Perschinka et al. 2003; Wu and Tanguay 2006). The impact of anti-Hsp60 on innate immunity is also projected by the activation of macrophages and by stimulation of nuclear factor (NF)-κB, which is one of the regulators of matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP) secretion (Schett et al. 1995). Similar activity has been described very recently in the case of Hsp90-α (Madrigal-Matute et al. 2010). Moreover, the role of HSPs in predicting risk of acute coronary syndrome and progression of atherosclerosis has also been confirmed (Dulin et al. 2010; Zhang et al. 2010).

Accumulating data have pointed to the disturbed extracellular matrix metabolism in myocardial and vascular remodeling as being another new component of the atherosclerotic puzzle (Johnson et al. 2005, 2006; Kuzuya et al. 2006). Therefore, matrix metalloproteinases, endopeptidases with proteolytic activity, as well as their tissue inhibitors, have been proposed as a group of factors that add to the pathogenesis of atherosclerosis. Animal models and in vitro investigations have shown their multifaceted actions, varying from protective and antiatherogenic in the case of TIMP-2, through neutral of TIMP-1 or ambiguous of MMP-9, to proatherogenic of MMP-2 (Luttun et al. 2004; de Nooijer et al. 2006; Johnson et al. 2005, 2006; Kuzuya et al. 2006).

Moreover, gelatinases A and B (MMP-2 and MMP-9) occupy an established position among risk factors for myocardial infarction (Jefferis et al. 2010) and as predictors of mortality due to acute coronary syndrome (Dhillon et al. 2010), whereas their tissue inhibitors TIMPs have an impact on postmyocardial infarction remodeling (Kandalam et al. 2010) and correlate positively with left ventricular mass and wall thickness (Hansson et al. 2009). The role of MMPs in kidney disease has been studied extensively (Catania et al. 2007), and special attention has been paid to the impact on ischemic acute renal injury and scarring in the course of glomerulopathies (Caron et al. 2005; Cheng et al. 2006; Johnson et al. 2002). However, the data on their role in chronic kidney disease, characterized by accelerated progression of atherosclerosis, are scarce and come mainly from adult patients on hemodialysis (Preston et al. 2002; Pawlak et al. 2007).

There are no data so far on MMPs and TIMPs in pediatric patients with chronic kidney disease on conservative treatment. Therefore, the first aim of our study was to evaluate the levels of MMP-2, MMP-9, TIMP-1, and TIMP-2 in serum samples from children in different stages of chronic kidney disease treated conservatively. The second goal was to analyze whether there is any relationship between those parameters and other factors predisposing to atherosclerosis, such as disturbed stress response (Hsp90-α, anti-Hsp60), endothelial activation (sE-selectin), inflammation (high-sensitivity C-reactive protein (hsCRP)), or dyslipidemia.

Subjects and methods

Sixty-one patients enrolled in the study were divided into three groups.

The first group (chronic kidney disease (CKD) I) consisted of 19 patients (10 girls, nine boys, median age of 8.5 years, interquartile range of 4.5–15 years) with CKD stages 2–3 treated conservatively (median glomerular filtration rate (GFR) calculated according to the Schwartz formula 51 ml/min per 1.73 m^2). The factors causing CKD were: reflux nephropathy (seven cases), chronic glomerulonephritis (five), chronic pyelonephritis (one), polycystic kidney disease (four), hemolytic uremic syndrome (one), and cystinosis (one).

The second group (CKD II) contained 18 patients (10 girls, eight boys; median age of 11 years, interquartile range of 5–17.5 years) with CKD stages 4–5 on conservative treatment (median GFR 23 ml/min per 1.73 m^2). Primary diseases causing CKD were reflux nephropathy (nine cases), chronic glomerulonephritis (six), lupus nephropathy (one), neurogenic bladder (one), hemolytic uremic syndrome (one). In all patients, phosphate binders and vitamin D metabolites were supplemented.

Twenty-four children (13 girls, 11 boys, median age of 10.5 years, range of 5–16.5 years) with primary nocturnal enuresis, with normal kidney function, served as controls.

None of the patients showed clinical evidence of infection, malignancy, or vasculitis, suffered from diabetes, smoked, and took antibiotics, corticosteroids, or immunosuppressive therapy. All the CKD children had blood pressure values below the 90th percentile for smaller children and below 120/80 mmHg for adolescents, according to the criteria of the fourth report on high blood pressure in children and adolescents (National 2004). CKD stage 2–3 children, except for three subjects receiving angiotensin-converting enzyme (ACE) inhibitors in nephroprotective doses, did not require antihypertensive drugs. In the CKD stage 4–5 group blood pressure was well controlled either without medication (13 children) or with the use of ACE inhibitors (three patients) or ARB (one child).

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

Blood samples were drawn from peripheral veins after an overnight fast. Samples were clotted for 30 min and centrifuged at 4°C for 10 min, and then serum was stored at −20°C until assayed. Serum concentrations of MMP-2 (gelatinase A), MMP-9 (gelatinase B), TIMP-1, TIMP-2, sE-selectin, Hsp90-α, and anti-Hsp60 were evaluated by commercially available ELISA kits (Stressgen, R&D Systems, UK). In the case of MMP/TIMP, standards and serum samples were transferred to 96-well microplates precoated with recombinant antibodies to human MMP-2, MMP-9, TIMP-1, and TIMP-2. 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 methods of evaluation of Hsp90-α, anti-Hsp60, and sE-selectin were described in our previous publication (Musiał et al. 2010).

In all patients, the kidney function was assessed, calculated by the Schwartz formula. The lipid profile (total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, triglycerides by BioSystems, Barcelona, Spain) and hsCRP as a marker of inflammation (nephelometry by Dade Behring, Marburg, Germany) were also evaluated.

Statistical analysis

Results are expressed as median values and interquartile ranges. Differences between all groups were evaluated using nonparametric tests (Kruskal–Wallis, Mann–Whitney *U*). The relations between parameters were assessed by linear regression analysis. The linear regression equations were calculated as $y = \beta \times + a$ (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. 8.0. A p value of <0.05 was considered significant.

Results

MMP-2 and MMP-9

MMP-2 and MMP-9 concentrations in all CKD children were significantly higher vs. controls ($p<0.000001$), and the levels in the CKD stage 4–5 subgroup were increased when compared to CKD stages 2–3 (Fig. 1a, b).

TIMP-1 and TIMP-2

In the case of TIMP-1, the concentrations in the CKD group were also elevated vs. controls ($p<0.001$). However, the median TIMP-1 values in the CKD stages 2–3 were comparable, whereas in the CKD stages 4–5 they were higher than in the control group (Fig. 1c). TIMP-2 levels were increased in CKD children vs. controls ($p<0.000001$) and rose with the progression of renal failure (Fig. 1d).

MMP/TIMP ratios

When MMP-9/TIMP-1 and MMP-2/TIMP-2 ratios were assessed, the first was significantly higher ($p<0.000001$) and the second was significantly lower ($p<0.000001$) in all CKD children than in the control group. However, none of them could differentiate between the CKD stage 2–3 and the CKD stage 4–5 groups (Fig. 1e, f).

Hsp90-α, anti-Hsp60, sE-selectin

Hsp90-α, anti-Hsp60, and sE-selectin serum concentrations in the CKD population were significantly higher than in the control group (Table 1).

hsCRP, lipid profile

High-sensitivity CRP and total cholesterol levels did not show significant differences between CKD children and controls (Table 1). HDL concentrations were increased, whereas those of LDL and triglycerides were decreased in CKD patients versus controls (Table 1).

Linear regression analysis

In all CKD children, MMPs and TIMPs correlated with each other. We have also found correlations between MMPs, TIMPs, and other examined parameters. In detail, Hsp90-α, anti-Hsp60, sE-selectin, and GFR predicted the values of MMP-2, MMP-9, and TIMP-1 (Table 2). In the case of TIMP-1, such prediction concerned sE-selectin and GFR (Table 2).

No associations between MMP/TIMP, the lipid profile, and hsCRP were observed.

Discussion

In our study, we have shown for the first time the dysfunctional MMP/TIMP system and its correlation to markers of stress response in children with chronic kidney disease treated conservatively.

Serum MMP-2 concentrations in the examined population were elevated, when compared to controls, similar to the results obtained in adults by others (Peiskerova et al. 2009; Chang et al. 2006). The novelty of our observation was the fact that MMP-2 levels increased gradually with the progression of CKD and correlated with GFR. Although Chang et al. (2006) have previously reported the correlation between gelatinases and serum creatinine, the interpretation of these results should be viewed with caution due to methodological inconsistencies (e.g., plasma collection in ethylenediaminetetraacetic acid (EDTA)-containing tubes, although the use of EDTA is not recommended by the manufacturer due to its chelating properties). We have also revealed the correlations between MMP-2, stress response, and endothelial activation in the pediatric CKD population. These findings add new data to the knowledge of cardiovascular complication pathogenesis in CKD children (Mitsnefes 2005). Since MMP-2 has recently been defined as a predictor of mortality in acute coronary syndrome (Dhillon et al. 2010) and its correlation with arterial

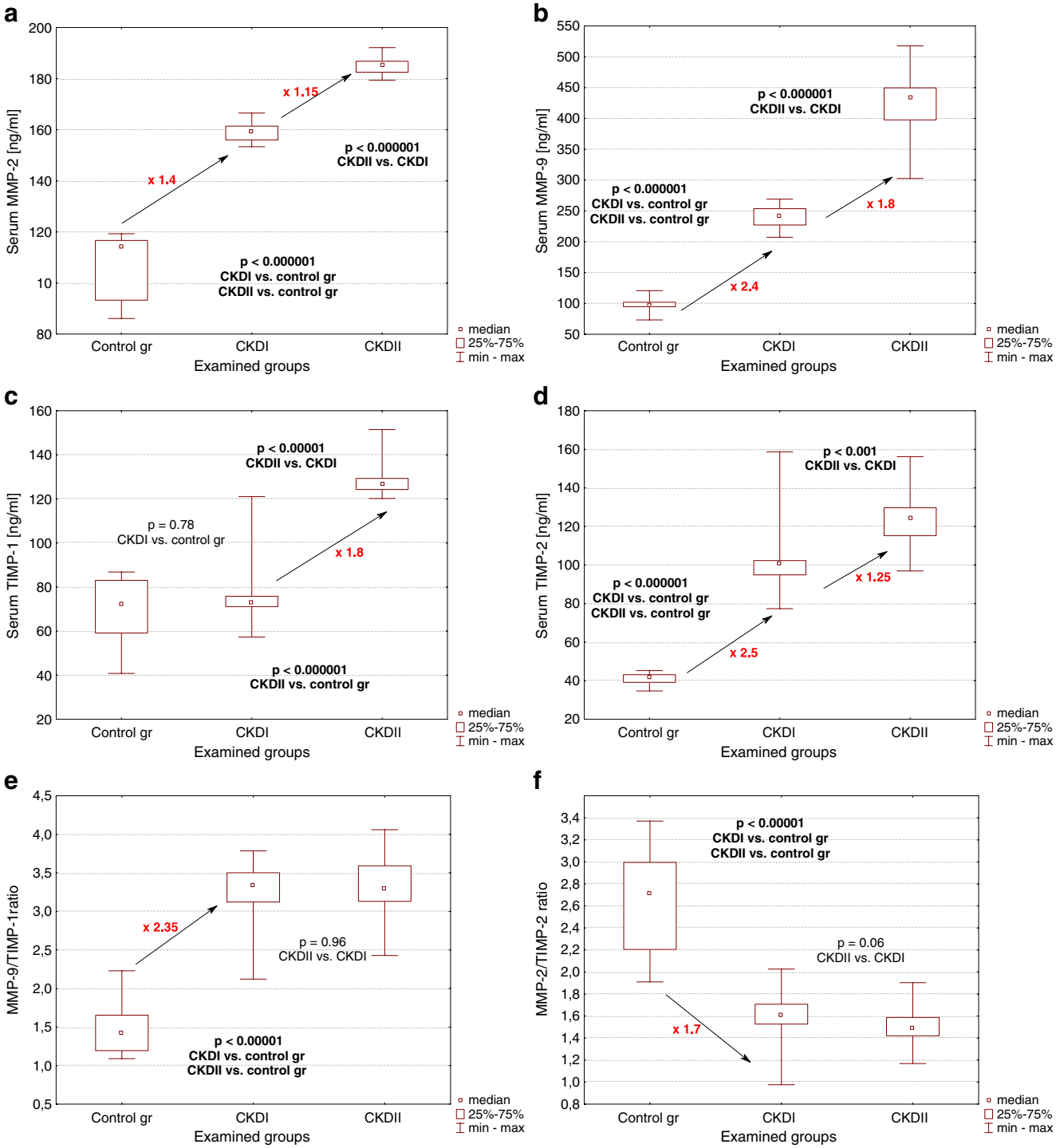

Fig. 1 Serum concentrations of examined parameters (**a** MMP-2; **b** MMP-9; **c** TIMP-1; **d** TIMP-2; **e** MMP-9/TIMP-1; **f** MMP-2/TIMP-2) in the CKD children and in the control group

stiffness in CKD patients has been revealed (Chung et al. 2009), the usefulness of gelatinase A as one of the potential markers of atherogenesis in children with CKD seems justified.

The data on MMP-9 concentrations in CKD patients are scarce and contradictory and concern only adults. Chang et al. (2006) found decreased levels of gelatinase B, whereas Peiskerova et al. (2009) noticed no difference between controls and CKD patients. In our group, a gradual increase was observed, together with an inverse correlation to GFR values. Such discrepancies may result from methodology or differences in the profile of examined

Table 1 The median values and interquartile ranges of examined parameters in the patients with CKD and in the control group

Parameter	Median values (lower–upper quartile) of analyzed parameters	
	Control group n=24	CKD patients n=37
Hsp90-α (ng/ml)	5.0 (4.5–5.5)	20.0 (15.0–25.0)****
Anti-Hsp60 (ng/ml)	11.3 (8.2–26.7)	46.0 (14.9–81.7)*
sE-selectin (ng/ml)	26.1 (22.2–28.0)	68.0 (54.0–80.0)****
hsCRP (mg/l)	0.33 (0.21–0.63)	0.28 (0.16–0.81) NS
CHOL (mg/dl)	188.0 (181.0–196.0)	193.0 (167.8–235.3) NS
HDL (mg/dl)	62.0 (59.0–67.0)	52.0 (49.5–61.0)**
LDL (mg/dl)	99.5 (91.0–109.0)	100.0 (95.0–147.0)**
TGL (mg/dl)	86.0 (66.5–100.0)	115.0 (100.0–159.0)***

NS nonsignificant

*p<0.01; **p<0.001; ***p<0.0001; ****p<0.00001 CKD vs. controls

groups. In detail, the CKD population examined by Chang et al. (2006) contained adults with mean creatinine clearance (CCr) of 11 ml/min and was divided into subgroups with CCr less than 10 ml/min, between 10 and 15 ml/min, and over 15 ml/min. Our patients had relatively higher GFR values (CKD 2–3 51 ml/min; CKD 4–5 23 ml/min), and we had no subjects with CCr less than 15 ml/min. Another reason for difficulties in the interpretation of MMP-9 elevation may be the fact of its ambiguous activity in in vitro investigations. Gelatinase B was described as protecting plaque stability in its early stage and increasing its vulnerability in the case of advanced lesion (Luttun et al. 2004; de Nooijer et al. 2006).

Moreover, matrix metalloproteinases are subject to different factors modifying their activity. The levels of MMPs depend on the presence of diabetic nephropathy (Rysz et al. 2007), hypercholesterolemia (El Messal et al. 2006), inflammation (Addabbo et al. 2007), increased oxidative stress (Valentin et al. 2005; Castro et al. 2009), ACE inhibitors (Lods et al. 2003), and hypertension (Castro et al. 2008). Although we did not observe the correlation of examined enzymes with classical indicators of atherosclerotic changes, such as lipids or hsCRP, we have added sE-selectin, a marker of endothelial dysfunction, and heat shock proteins, to that list. Moreover, serum Hsp90-α, anti-Hsp60, sE-selectin, and GFR predicted the concentrations of MMPs and TIMPs in all CKD children. Therefore, it is probable that in the pediatric CKD population metalloproteinases share common features with stress markers rather than with those typical for atherosclerosis. The enzyme levels, increasing progressively with aggravation of kidney failure, stress conditions, and toxemia, would seem to confirm such a theory. However, correlations of MMPs and TIMPS with Hsp90-α and anti-Hsp60 in CKD patients are new findings, not confirmed by any earlier study. The possible explanation for such links might lie in the common points of signaling pathway regulation. Both Hsp90-α and Hsp60 increase secretion of cytokines (e.g.,

Table 2 The statistically significant correlations between the examined parameters assessed by linear regression analysis in the CKD patients

Dependent variable	Independent variable	Regression coefficient β	Constant term	95% confidence interval (CI)	p
CKD patients					
MMP-2 (ng/ml)	Hsp90-α (ng/ml)	−1.18	196.28	169.09–176.22	0.00
MMP-2 (ng/ml)	Anti-Hsp60 (ng/ml)	−0.13	179.31	168.96–177.96	0.02
MMP-2 (ng/ml)	sE-selectin (pg/ml)	0.70	123.93	168.60–174.59	0.00
MMP-2 (ng/ml)	GFR (ml/min)	−0.49	186.93	166.64–177.58	0.00
MMP-9 (ng/ml)	Hsp90-α (ng/ml)	−8.82	513.79	311.73–362.88	0.00
MMP-9 (ng/ml)	Anti-Hsp60 (ng/ml)	−0.85	381.53	308.97–375.92	0.03
MMP-9 (ng/ml)	sE-selectin (pg/ml)	4.60	17.42	305.03–355.08	0.00
MMP-9 (ng/ml)	GFR (ml/min)	−3.99	460.64	301.59–379.89	0.00
TIMP-1 (ng/ml)	Hsp90-α [ng/ml]	−2.20	147.27	95.09–111.35	0.00
TIMP-1 (ng/ml)	Anti-Hsp60 (ng/ml)	−0.29	118.59	95.79–114.40	0.01
TIMP-1 (ng/ml)	sE-selectin (pg/ml)	1.53	−2.89	95.09–106.95	0.00
TIMP-1 (ng/ml)	GFR (ml/min)	−1.17	137.92	91.98–113.91	0.00
TIMP-2 (ng/ml)	sE-selectin [pg/ml]	0.54	75.71	106.22–118.67	0.01
TIMP-2 (ng/ml)	GFR (ml/min)	−0.58	128.80	91.98–113.91	0.03

TNF-α) and adhesion molecules (e.g., sE-selectin) and trigger NF-κB activity, which then upregulate the synthesis of MMPs and TIMPs (Zhang et al. 1998; Kol et al. 1999; Businaro et al. 2009; Madrigal-Matute et al. 2010). Therefore, there is a functional chain suggesting the reason for the observed correlations. Such connection has not been proven in the case of TIMP-2, thus explaining the lack of relation between Hsp90-α and anti-Hsp60 and TIMP-2 in our population. The question whether any direct regulation between HSP and MMP/TIMP system takes place, although intriguing, requires further detailed investigation.

We have also revealed that all examined MMPs and TIMPs correlate with each other, thus aggravating the complexity of interrelations between them and forming a kind of functional network including processes engaged in reactions to stress conditions.

TIMP-1 concentrations remained unchanged in children with CKD stages 2–3 and increased with disease progression, being higher in CKD stages 4–5 than in controls and in CKD stages 2–3. As far as we are concerned, there are no available data on other studies evaluating TIMP-1 in various CKD stages. One of the possible explanations is that TIMP-1 elevation, seen barely in advanced renal failure, is a compensatory response to MMP-9 increase in early CKD stages. Secondly, according to the hypothesis of Newby (2008), the prevalence of MMP over TIMP activity might result from the fact that in the early stages of atherosclerosis, when monocytes migrate across the endothelial monolayer, mainly MMPs are needed to facilitate the penetration of cells. Subsequently, the TIMP overactivity would be more evident when the in situ differentiation of monocytes into macrophages takes place. The TIMP-2 high concentrations, seen already in CKD stages 2–3, would suggest the early response to the increase of MMP-2, known for its proatherogenic activity. Thus, the progressive elevation in CKD stages 4–5 would be a consequence of further MMP-2 elevation.

However, the best way to evaluate the net effect of balance between MMPs and their inhibitors was to assess the values of MMP/TIMP ratios. Both MMP-9/TIMP-1 and MMP-2/TIMP-2 ratio values in CKD patients differed significantly from those in controls. In the case of the MMP-9/TIMP-1 ratio, MMP-9 increased 2.4-fold in CKD stage 2–3 patients vs. controls, whereas TIMP-1 did not, causing the net 2.4-fold rise of the ratio value. When children in CKD stages 2–3 and CKD stages 4–5 were compared, both MMP-9 and TIMP-1 increased 1.8-fold, so the ratio did not change. Taken together, the MMP-9 response dominated over that of TIMP-1 only in the early CKD, and there was no difference between stages 2–3 and 4–5.

When the MMP-2/TIMP-2 ratio was taken into account, both MMP-2 and TIMP-2 concentrations were higher in CKD stages 2–3 vs. controls. The elevation of protective TIMP-2 was more pronounced (2.5-fold) than that of proatherogenic MMP-2 (1.4-fold), thus causing the net decrease of a ratio value. In CKD stages 4–5, when compared to stages 2–3, the rise of both MMP-2 and TIMP-2 was proportional and the ratio value, as in the case of MMP-9/TIMP-1, did not change. Therefore, it seems that disturbances in MMP/TIMP balance are already noticeable in early CKD, whereas their correction and stabilization take place in later stages of renal failure.

Conclusions

Our study describes for the first time the network of MMP/TIMP systems in children with chronic kidney disease. Relationships between these parameters, heat shock proteins, and markers of endothelial dysfunction suggest the possible role of MMPs and TIMPs in the stress response and atherogenesis of CKD children. The progressive changes in concentrations of MMPs and their inhibitors may illustrate the adaptive response to unfavorable conditions resulting from chronic dysfunction of the kidney. However, the precise role of MMP/TIMP balance and its relation to the stress response in the CKD pediatric population need further investigation.

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