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Original Article

Urinary endothelin-1 excretion according to morpho-functional damage lateralization in reflux nephropathy

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Abstract

Background. Reflux nephropathy (RN) is a patho-physiological human model of reduced nephron reserve, due to loss of renal mass, but little information exists about the role of urinary endothelin-1 (uET-1) in this disease. The aim of this study was to assess the presence of uET-1-like-immunoreactivity (uET-1L) in RN patients, particularly if lateralized renal damage existed.

Methods. Thirty patients with vesico-ureteral reflux (VUR) and consequent RN, were studied. The presence of VUR was established by voiding cysto-urethrography. RN was assessed and graded by 99mTc-dimercapto-succinic acid scan (DMSA). Renal plasma flow (ERPF) was evaluated by 123I-Hippuran renal sequential scintigraphy, and glomerular filtration rate (GFR) by creatinine clearance. Forty-five healthy subjects were selected as a control group. uET-1L excretion, in both affected and control groups, was assayed.

Results. Mann–Whitney U test showed a significant difference between control and patient groups in both GFR and uET-1L. A good correlation between DMSA grading, single kidney clearance and VUR grade was shown. A significant relationship was also shown between uET-1L and both ERPF and GFR. Patients with RN were divided into two subgroups according to functional damage lateralization. Between the two groups, a significant difference was found only for uET-1L when GFR was applied as a covariate in ANCOVA analysis.

Conclusion. Our preliminary results confirmed the increase of urinary ET-1 excretion in RN, especially when renal functional injury was lateralized.

Keywords: endothelin-1; ERPF; GFR; 123-I-OIH; reflux nephropathy; vesicoureteral reflux

Introduction

The endothelins are potent vasoconstrictor peptides produced by vascular endothelium in many different tissues. Endothelin-1 (ET-1) is the major renal isoform, produced by mesangial and tubular epithelial cells. ET-1 acts in a paracrine and autocrine manner on ETA and ETB receptors on endothelial and smooth-muscle cells. ET-1 modulates vascular tone and extracellular volume, induces vascular smooth muscle and mesangial cell growth, as well as extracellular matrix protein accumulation. These processes are associated with vascular remodelling, renal fibrosis and glomerular sclerosis [1]. In the kidney the ETA receptor-type predominantly controls the haemodynamic effects of ET-1, while the ETB receptor is involved in the tubular regulation of salt and water re-absorption [2]. Increased urinary ET-1 excretion (uET-1) has been reported in progressive and end-stage renal disease and correlated with deterioration of renal function in animal models and human studies [3–5]. Reflux nephropathy (RN) is a pathophysiological human model of nephron reserve reduction due to loss of renal mass, but little evidence exists about the role of uET-1 in this disease [6,7].

The aim of this study was two-fold: (i) to assess changes in uET-1-like-immunoreactivity (uET-1L) in a selected cohort of paediatric patients with RN and evaluate the relationship between uET-1L and renal function; (ii) to test if differences in uET-1L excretion exist between patients with disease affecting predominantly one kidney and those with bilateral RN, with an equal degree of renal impairment in each kidney.
Subjects and methods

Patient population

Thirty consecutive patients (20 males and 10 females; median age, 9.5 years; age range, 4–20 years) with vesico-ureteral reflux (VUR) and RN were selected for this study. The presence of VUR and renal parenchymal scarring were established previously by voiding cysouretrography (VCUG) and 99mTcdimercaptosuccinic acid scan (DMSA), respectively. Effective renal plasma flow (ERPF) was evaluated by $^{123}$I-Hippuran ($^{123}$I-OIH) RSS; glomerular filtration rate (GFR) was evaluated by creatinine clearance. ERPF and GFR were normalized to body surface area (BSA) of 1.73 m$^2$ and expressed in ml/min. Lateralized damage was defined as when the most injured kidney contributed less than 1/6 of total renal function. According to this definition, the patient population was divided into two subgroups: group A (GR A) (lateralized damage) and group B (GR B) (no-lateralized damage). Among patients admitted to our paediatric unit and submitted to nephrologic evaluation for history of abdominal pain or fever, 45 subjects (23 males and 22 females; median age, 10 years; age range, 4–21 years) with normal creatinine clearance, normal urinalysis, without urinary tract infection and ultrasonographic renal parenchymal abnormalities, were selected as a control group. uET-1L excretion, in both affected and control subjects, was assessed by commercial radioimmunoassay.

Voiding cysouretrography

VCUG was performed without sedation and reported by an experienced paediatric radiologist (I.R.); all children were prepared previously with prophylactic broad-spectrum antibiotic treatment. The bladder was catheterized by an appropriate Foley catheter and gently filled with radiographic contrast material. Voiding was then allowed during fluoroscopy. According to the recommendations of the International Reflux Study in Children [8], VUR was graded from I–V.

DMSA scan and RSS: technical features

DMSA scintigraphy was performed according to our usual clinical protocol. Three hours following DMSA injection (0.74 MBq/kg body weight, 10 MBq at least), one posterior projection and two posterior-oblique ones were recorded with the patient supine and the gamma camera head directly set beneath the examination bed. A Siemens ZLC large field of view gamma camera interfaced with an Icon workstation, and equipped with a low energy (140 KeV), ultra-high resolution collimator and a 128×128-matrix acquisition. Each image total count was equal to 500 000. Images were stored on a computer to allow digital image enhancement for improved evaluation. An experienced observer (L.C.G.), blinded to the other patient data, evaluated the images. The scintiscans were graded according to the severity of abnormality using the Goldraich system, modified by Craig et al. [9]: grade I, no more than two cortical defects; grade II, more than two cortical defects but remnant areas of normal renal parenchyma; grade III, diffuse reduction in uptake of DMSA throughout the whole kidney with or without focal defects; grade IV, shrunken kidney contributing <10% of the overall renal functional mass. To obtain the overall parenchymal renal damage, the DMSA score was calculated from the sum of the Goldraich DMSA grade of both kidneys. $^{123}$I-OIH RSS was performed with a Siemens ZLC large field of view gamma camera interfaced with an Icon workstation, and equipped with a low energy (140 KeV), ultra-high resolution collimator. Before starting the study, a syringe with 20–37 MBq of $^{123}$I-OIH was positioned on a specially prepared 5-cm thick plexiglas support and placed on the examination table, in close contact with the collimator surface. Activity was measured by the gamma camera. A 50-s static image, $64 \times 64$ matrix with a 20% window centred over the $^{123}$I 159 KeV energy peak, was acquired.

Commencing 20 s before the radiotracer injection (bolus), data was acquired by a dynamic acquisition ($80 \times 5$ frames, followed by $65 \times 20$ frames for $^{123}$I-OIH).

One lateral view after the examination, using a $^{57}$Co marker, was used to determine the kidney depth and the distance to the camera was measured in centimetres. Single kidney $^{123}$I-OIH clearance rate ($Cl$) was then determined using a method validated previously in our institution [10]. Briefly, this was based on time–activity curves generated from the heart and kidney areas using the region of interest (ROI) technique. The kidney background was subtracted, taking into account the intravascular radioactivity. The influence of kidney surface dimensions and depth on the system counting efficiency was determined previously using phantoms, and the appropriate correction factors were calculated and introduced in the computer program. $^{123}$I-OIH clearance was closely related ($r=0.97$, $P < 0.00001$) to the fraction of tracer accumulated by the kidney during the first minute after administration (D1). The radioactivity measured in each renal ROI was divided by the injected dose. Hence, $Cl$ was calculated by multiplying D1 by a proportionality factor established previously, which transforms the D1 in the related $Cl$.

ERPF was expressed as the sum of right and left $^{123}$I-OIH $Cl$. Reference $Cl$ values for an individual kidney have been established previously in an age-matched population, as $333 \pm 49$ ml/min/1.73 m$^2$ BSA [11].

Endothelin assay

Uriney uET-1L was measured by radioimmunoassay using a commercially available kit (Endothelin 1/2 RIA Kits; Biomedica-Vienna-Austria), after extraction with C18 Sep-Pak cartridges (Waters-Millipore) according to the method described by Ando et al. [12]. Because of co-extraction of all three isoforms of endothelin and because of the cross-reactivity of the antibody, the radioimmunoassay did not discriminate between ET-1 and ET-2, nor between ET-1 and ET-3. No cross-reaction occurred with Big-ET-1–38 or Bigendothelin fragments 22–38. Nonetheless, because ET-1 has been demonstrated to be the predominant isoform in the human kidney [13] the substance that we measured was assumed to predominantly represent ET-1. The recovery was $97.9 \pm 2.36\%$ (mean $\pm$ SD); the detection limit was 1.99 ng/ml (95% confidence limits), and the precision profile showed a coefficient of variation from 25.5 to 6.3% with a working range (uET-1L values detected with an error <10%) from 11.2 to 327.9 ng/ml. uET-1L values expressed as pg/ml were multiplied by the respective 24-h urine volumes, and expressed as ng/24 h.
Creatinine clearance

Creatinine clearance was computed from the creatinine excretion in a 24-h urine collection and a single measurement of serum creatinine. Urine and serum creatinine were determined by Dimension Flex reagent cartridge CREA (Dade Behring, Marburg, Germany). This spectrophotometric method for direct determination of serum and urine creatinine is derived from the kinetic alkaline picrate assay, based on a variant of Jaffe’s reaction, published by Larsen [14].

Statistical analysis

Results are expressed as median and range and as mean±SD in ANOVA and ANCOVA. The relationships between the considered variables were assessed using the Spearman non-parametric correlation coefficient. The Mann–Whitney U test was used for group comparisons. The Kolmogorov–Smirnov test was used as a normal significance test. Analysis of variance and covariance by the LSD post hoc test was employed for GRA and GRB comparisons. The STATISTICA software package (www.Statsoft.com) was used for statistical data analysis. A P value < 0.05 was considered statistically significant.

Results

In patients with RN, VCUG showed VUR in all, unilateral in 12 (right-sided in five, left sided in seven) and bilateral in 18. VUR was grade IV in 17 kidneys, III in 14, II in eight and I in nine. DMSA scans showed renal damage in 42 kidneys (bilateral in 12 cases; unilateral in 18 cases) and was negative in the remaining 18. DMSA scan was grade 0 in 18 kidneys, grade I in eight, grade II in seven, grade III in 14 and grade IV in 13. ERPF median value was 387 ml/min/1.73 m² BSA (range, 114–640). GFR median value was 85 ml/min/1.73 m² BSA (range, 18–120). uET-1L median value was 6.55 ng/24 h (range, 2.4–18) (Table 1). Spearman non-parametric correlation analysis demonstrated a strong relationship between DMSA grading and both Cl (R = 0.88, P < 0.00001) and VUR grade (R = 0.86, P < 0.00001). A significant relationship was also shown between uET-1L and ERPF (R = 0.64, P < 0.00002), GFR (R = 0.70, P < 0.00002) and DMSA score (R = 0.46, P < 0.01). In the control group, absence of any parenchymal abnormalities was confirmed by Ultrasonography. Creatinine plasma level was < 1 mg/dl, median GFR value was 119 ml/min/1.73 m² BSA (range, 87–130) and median uET-1L value was 3.5 ng/24 h (range, 1.9–6.9) (Table 1).

The Mann–Whitney U test showed a significant difference for both GFR (Z = −4.7, P < 0.00001) and uET-1L (Z = −4.4, P < 0.00002) between control and patient groups. Following analysis of the renal damage to each kidney, 11 of 30 patients (36.7%) were included in GRA, with the remaining 19 patients forming GRB (Table 2). No significant difference was found between GRA and GRB with respect to GFR [68 (28–120) vs 104 (18–120) ml/min/1.73 m² BSA], ERPF [285 (136–608) ml/min/1.73 m² BSA] and uET-1L [7.2 (3.1–18) vs 5.1 (2.4–9.6) ng/24 h] median values (Table 3).

Table 1. Patients with RN and control group characteristics (results are expressed as median and range)

<table>
<thead>
<tr>
<th></th>
<th>Patients with RN</th>
<th>P</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>30</td>
<td></td>
<td>45</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>20 M, 10 F</td>
<td>ns</td>
<td>23 M, 22 F</td>
</tr>
<tr>
<td>Age (year)</td>
<td>9.5 (4–20)</td>
<td>&lt;0.00001</td>
<td>10 (4–21)</td>
</tr>
<tr>
<td>GFR (ml/min/1.73 m² BSA)</td>
<td>85 (18–120)</td>
<td></td>
<td>119 (87–130)</td>
</tr>
<tr>
<td>ERPF (ml/min/1.73 m² BSA)</td>
<td>387 (114–640)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UET-1L (ng/24h)</td>
<td>6.55 (2.4–18)</td>
<td>&lt;0.00002</td>
<td>3.5 (1.9–6.9)</td>
</tr>
</tbody>
</table>

Table 2. 123I-OIH Cl, ERPF and ERPF % of more affected kidney of patients with lateralized kidney damage

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Right 123I-OIH Cl</th>
<th>Left 123I-OIH Cl</th>
<th>ERPF</th>
<th>ERPF % of more affected kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16</td>
<td>592</td>
<td>608</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>550</td>
<td>0</td>
<td>550</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>33</td>
<td>475</td>
<td>508</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>453</td>
<td>0</td>
<td>453</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>319</td>
<td>0</td>
<td>319</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>275</td>
<td>10</td>
<td>285</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>12</td>
<td>210</td>
<td>222</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>182</td>
<td>0</td>
<td>182</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>180</td>
<td>180</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>173</td>
<td>173</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>136</td>
<td>0</td>
<td>136</td>
<td>0</td>
</tr>
</tbody>
</table>

Cl, single kidney clearance rate; ERPF = right Cl + left Cl; Cl are expressed in ml/min/1.73 m² BSA.
Conversely, the DMSA score was significantly different (Z = −2.8, P < 0.005): 5 (4–7) vs 3 (1–7) between GR A and GR B, respectively, indicating greater parenchymal involvement in GR A.

Because a significant relationship exists between GFR and uET-1L in the whole patient population (R = −0.70, P < 0.00002) and in both GR A (R = −0.78, P < 0.005) and GR B (R = −0.58, P < 0.00501) subgroups, the effect of inequality of renal damage may be masked by variance related to total renal function. The results of the Mann–Whitney U test, in elucidating this lateralization effect, are biased by function related variance, as the non-parametric tests are unable to evaluate GFR as a covariate. ANCOVA analysis may be employed to explain the main effect (lateralization) free from the function related variance between and within these groups. To investigate this hypothesis, the Kolmogorov–Smirnov test was used to test the hypothesis of normality for GFR and uET-1L in the patient group and subgroups. No significant difference from the normal distribution was found in the considered variables of each patient group. The LSD test showed uET-1L values to be significantly different (P < 0.05) between GR A (mean ± SD, 8.6 ± 4.9 ng/24 h) and GR B (mean ± SD, 5.7 ± 2.2 ng/24 h). When GFR was considered as a covariate in the analysis, the ANOVA significance increased (P < 0.005) (Table 3). The inclusion of this additional factor (GFR) reduced the error of sums of deviation square and increased the statistical power (sensitivity) of our study design.

Discussion

ET-1 was identified initially by Yanagisawa et al., in 1988 [15]. The effects of ET-1 in biological systems are well documented in both animal and human studies [15]. Komeyama et al. [6] and Takeda et al. [7] analysed uET-1L excretion in patients with primary VUR. They described significantly higher uET-1L values in patients compared with normal subjects, increasing in proportion to the grade of reflux. Nevertheless, they found no relationship between uET1-L and both renal parenchymal damage and function, expressed as DMSA uptake.

Our study has shown higher uET-1L levels in RN patients compared with controls. Furthermore, the uET-1L level was related to DMSA score, ERPF and GFR, indices of parenchymal and functional renal damage.

Moreover, in the whole population (controls and patients) a close relationship was found between uET-1L levels and creatinine clearance. Indeed, as GFR and ERPF fell, urinary ET-1L levels increased.

There is evidence of a complex role of this autacoid in several pathologic and physiologic conditions [16], as assessed by Mattyus et al. [17]. They found that renal excretion of ET is influenced by several factors, which modulate intrarenal ET production. Castellani et al. [18] have demonstrated that the renal haemodynamic response induced by mental stress is a complex reaction in which ET-1 plays a part. They found that uET-1L increased during mental stress, even in healthy subjects. In the light of our findings, we propose that ET-1 is not merely a marker of renal damage, but that the increase in urinary ET-1L excretion, related to its renal production, indicates abnormal renal haemodynamic modulation. This is supported by the finding of higher ET-1L levels in patients with a lateralized renal injury in comparison with patients with bilateral damage and similar renal function.

However, our data may be affected by a bias because of the reduced power of the normality tests, ANOVA and ANCOVA in the analysis of small sample sizes. Indeed, it is recognized that the results of ANCOVA may be influenced by the violation of the hypothesis of normality of the linked distributions and of linearity and parallelism of the relationships between groups. However, an aim of the study was to test the significance of the lateralization effect on uET-1L excretion, irrespective of the total function as measured by GFR. Conversely, there is no simple non-parametric test able to take into account the effect of GFR as covariate as well as ANCOVA.

The reason to stress the effect of renal function lateralization on uET-1L excretion is supported by the work of Takeda et al. [19]. They measured urinary ET-1L before and after unilateral nephrectomy in 15 patients and found that values after surgery were significantly greater than preoperative ones, suggesting the role of uET-1L as an indicator of kidney overload.

It is also found that in a physiologic condition of renal mass loss, as happens in old age, urinary excretion of ET-1L is increased in comparison with younger healthy subjects [20]. Furthermore, kidneys in old age react to adrenergic stimulation with a more pronounced and prolonged vasoconstriction, which is probably caused by a defect in prostaglandin modulation of endothelin activity.
Our preliminary results confirm the role of ET-1L as a marker of both kidney damage and overload in a human model of lateralized functional injury. If this data is confirmed in a larger cohort of patients, it offers a new approach to the management of RN, with the possibility of a therapeutic role for ET-1 receptor antagonists in the treatment of patients with extensive renal mass loss.

Conflict of interest statement. None declared.

References