Urinary endothelin-1 as a marker of renal damage in sickle cell disease

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Abstract

Background. Sickle cell disease (SCD) affects the kidney by acute mechanisms as well as by insidious renal medullary/papillary necrosis, resulting in tubular defects, which increase the risk of dehydration and subsequent sickle crisis. Hypoxia has been reported to stimulate endothelin-1 (ET-1) synthesis by endothelial cells and also in the renal tubule.

Methods. This case-control study measured ET-1 in urine as a marker of its renal synthesis in asymptomatic SCD patients. Baseline plasma and urinary ET-1 levels were measured and followed during a water deprivation study and a subsequent administration of desmopressin.

Results. Urine and plasma levels of ET-1 were elevated in patients with SCD, compared with carefully matched African-French and African controls, and urine ET-1 excretion was associated with a marked urine-concentrating defect. Moreover, urinary ET-1 output was correlated with microalbuminuria in SCD patients.

Conclusions. ET-1 is known to antagonize the tubular effects of vasopressin and to promote renal scarring; increased renal production of ET-1 could produce nephrogenic diabetes insipidus and dehydration in SCD patients through a combination of fibrosis and functional resistance to vasopressin. This study provides a rationale for trials with endothelin receptor antagonists in sickle cell disease nephropathy.

Keywords: endothelin-1; hypoxia; nephrogenic diabetes insipidus; sickle cell disease

Introduction

Chronic renal disease is one of the main determinants of quality of life and prognosis in patients with sickle cell disease (SCD). Due to its important epithelial metabolic activity [1], the renal medulla is very susceptible to hypoxia, and in patients with SCD, it is constantly damaged [2,3]. Endothelin-1 (ET-1), a potent vasoconstrictor and proinflammatory factor is an endothelial and tubular cell-derived peptide, the synthesis of which is extremely sensitive to cell injury and renal ischaemia [4–6], and its concentration is elevated in the plasma of patients with sickle cell disease [7–9]. Because most of the plasma ET-1 filtered through glomeruli is subject to degradation by neutral endopeptidase (EC 3.4.24.11) in the proximal tubule and by an acidic, highly specific ET-1-inactivating metalloendopeptidase, ET-1 in urine is of renal origin [10]. Thus, urinary ET-1 reflects the production of this peptide by the kidneys and is a hallmark of specific pathophysiological processes. Exaggerated excretion of ET-1 in urine has been documented in experimental chronic ischaemic nephropathies; and the blockade of endothelin receptors could prevent the development of severe renal damage [11]. Moreover, because urinary ET-1 has its own biological effects on tubular cells, it could promote renal functional disturbances, such as an increased clearance of free water with the associated elevated risk of dehydration [12,13]. However, no data on the actions of ET-1 are currently available for patients with SCD.

The purpose of this prospective study was to determine the relevance of such pathophysiological mechanisms that implicate renal ET-1 in patients with sickle cell nephropathy in the steady state. Accordingly, we wanted to determine if urinary excretion of ET-1 was increased in young adults with sickle cell anaemia compared with carefully matched African-French and African subjects without SCD [subjects were all...
descended from Black African parents and were residents of France; some of them were born in France (mostly in the French West Indies—Antilles), the others were born in West and Central Africa. In addition, we tested for any correlation between the urine concentrating defect observed in SCD and renal ET-1 production; and since we observed microalbuminuria in most of the patients in this study, we tested for any correlation between albuminuria and renal ET-1 production.

### Patients and methods

**Patients**

Patients with HbS were identified in the records of the Department of Hematology at Tenon Hospital. Phenotyping and genotyping were performed on all patients and documented in the records before the selection of patients as previously described [14]. Healthy volunteers were identified based on the records of the Clinical Investigation Center at Saint-Antoine Hospital, Paris. We compared 17 individuals with HbS with 17 healthy individuals matched for sex, age, ethnicity and body mass index (BMI), because these factors may affect plasma ET-1 levels [15–17]. Healthy volunteers were identified based on the records before the selection of patients as previously described [14]. Healthy volunteers were identified based on the records of the Clinical Investigation Center at Saint-Antoine Hospital, Paris. We compared 17 individuals with HbS with 17 healthy individuals matched for sex, age, ethnicity and body mass index (BMI), because these factors may affect plasma ET-1 levels [15–17]. The protocol was approved by the local Ethics Committee, and abides by the tenets of the Helsinki protocol. The main characteristics of the subjects are listed in Table 1.

The key criteria for exclusion from the study were: (1) current treatment with hydroxyurea, (2) blood transfusion during the 100 days preceding the study, (3) non-steroidal anti-inflammatory drugs used during the preceding two weeks, (4) BMI > 30, (5) positive serology for HIV or hepatitis B or C, (5) pregnancy and (6) hypertension. The patients and the controls were taking no medications.

**Protocol**

The ability of the kidney to concentrate urine was examined after overnight water deprivation and a subsequent intranasal administration of 1-desamino-8-D-arginine vasopressin (dDAVP) (Ferring Pharmaceuticals AB, Malmo, Sweden), in all subjects, to specifically test the tubular response to vasopressin.

In the morning following a first normal ambulatory visit, each subject arrived at the Clinical Investigation Center after an overnight fast, having been instructed to discard the first morning-voided urine. After the collection of the next spontaneously voided urine, each received 20 µg of dDAVP intranasally. Urine voided between 0 and 180 min after the administration of dDAVP was collected, and creatinine clearance measured. Urine osmolality was determined by measuring freezing point depression using a micro-osmometer. Plasma and urine analyses were performed on three sets of samples: one collected the day before the overnight water deprivation (day 0), and on the following day (day 1), and two samples taken before and 3 h after dDAVP administration, respectively.

To minimize the risk and the effect of major dehydration due to environmental reasons, the pursuit of the protocol was postponed if the ambient temperature was more than 25°C.

The criteria to stop the study were: a clinical painful crisis, fever (body temperature >37.5°C), the increase of serum creatinine by more than 20%, or the decision of the subject to stop. Follow-up of the subjects was performed by phone 6 to 8 h after discharge from the Clinical Investigation Center and the end of the study, which was 24 h later in case delayed acute clinical events occurred.

### Endothelin assay

The urinary and plasma levels of immunoreactive active mature ET-1 were measured using a radio-immunoassay method (Peninsula Laboratories Inc., Meyerside, UK). Inter- and intra-assay variations were <10%. The mean recovery of the complete procedure, i.e. from extraction to radioimmunoassay, was >75%. According to the manufacturer, the cross-reactivity of the antibody to ET-1 with big-ET-1, ET-2 and ET-3 was <16, 7 and 7% respectively. After its collection, blood with EDTA was immediately centrifuged at 4°C at 1600 G for 15 min. Plasma and urine were immediately transferred into chilled polypropylene tubes containing 1500 KIU aprotinin (500 KIU/ml of blood or urine) and were stored at −80°C. Urine (3 ml) and the plasma from 1 ml of blood were acidified each with 0.25 ml 1 N HCl and were loaded onto Sep-Pak.

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**Table 1. Characteristics of the study population**

<table>
<thead>
<tr>
<th>Subjects</th>
<th>SCD (n = 17)</th>
<th>Healthy controls (n = 17)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>27 ± 2</td>
<td>27 ± 2</td>
<td>ns</td>
</tr>
<tr>
<td>Sex ratio M/F</td>
<td>5/12</td>
<td>5/12</td>
<td>ns</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>21 ± 1</td>
<td>22 ± 1</td>
<td>ns</td>
</tr>
<tr>
<td>Height, cm</td>
<td>171 ± 2</td>
<td>170 ± 2</td>
<td>ns</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>63 ± 2</td>
<td>65 ± 2</td>
<td>ns</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>121 ± 3</td>
<td>124 ± 3</td>
<td>ns</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>64 ± 2</td>
<td>63 ± 2</td>
<td>ns</td>
</tr>
<tr>
<td>Plasma osmolality, mosm/kg</td>
<td>293.7 ± 1.5</td>
<td>290 ± 1.1</td>
<td>ns</td>
</tr>
<tr>
<td>Plasma Na, mmol/l</td>
<td>138.0 ± 0.5</td>
<td>137.1 ± 0.3</td>
<td>ns</td>
</tr>
<tr>
<td>Plasma K, mmol/l</td>
<td>4.1 ± 0.1</td>
<td>4.0 ± 0.1</td>
<td>ns</td>
</tr>
<tr>
<td>Fasting glycaemia, mmol/l</td>
<td>4.6 ± 0.1</td>
<td>4.7 ± 0.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Blood urea nitrogen, mmol/l</td>
<td>2.5 ± 0.2</td>
<td>3.9 ± 0.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Plasma creatinine, µmol/l</td>
<td>51.0 ± 3.1</td>
<td>73.7 ± 3.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Creatinine clearance (ml/min/1.73/m²)</td>
<td>166.9 ± 10.2</td>
<td>130.3 ± 4.5</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SEM.
C18 columns (Peninsula Laboratories Inc.). ET-1 was eluted from the columns with 2 ml 80% methanol and 0.1% trifluoroacetic acid, and the eluate was lyophilized and the pellet reconstituted in 0.25 ml assay buffer. Urinary and plasma ET-1 levels were assessed prior to the overnight fast and, after it, before and after dDAVP administration. The mean intra-individual variabilities of plasma ET-1 concentration was 2.36±4.15 pg/ml and 1.88±4.2 pg/ml in the SCD patients and controls, respectively (ns). The mean intra-individual variabilities of the ratio of urinary ET-1 to urinary creatinine was 2.13±2.8 pg/mmol and 0.73±0.44 pg/mmol in SCD patients and controls, respectively (ns). Plasma and urine osmolality, urine creatinine and plasma antidiuretic hormone (ADH) concentrations were concomitantly also assessed without any knowledge of the subjects’ status.

**Statistical methods**

Results are expressed as mean±SEM. Statistical analysis was performed with ANOVA for repeated measures and Wilcoxon’s test for matched pairs, when appropriate. Correlation analysis was performed with Spearman’s non-parametric correlation test.

**Results**

**Plasma ET-1**

The mean level of plasma ET-1 for SCD patients in steady state was 66.7±27.5 pg/ml, not significantly higher than that of control subjects, 31.1±5.3 pg/ml ($P = 0.06$). This lack of significance, however, was due to high variability resulting from the fact that one subject had unusually high plasma ET-1 concentrations (472.3±21 pg/ml) (verified in duplicate in two different plasma samples). The mean level of plasma ET-1 was 41.4±5.5 pg/ml for the 16 other SCD patients in steady state ($P < 0.05$ vs control subjects). In addition, the difference in mean plasma ET-1 levels was significant after overnight water deprivation in SCD patients (38.1±5.2 pg/ml), compared with controls (29.07±4.2 pg/ml) ($P < 0.05$) (Figure 1). Analysis of variance indicated that SCD status was associated with significantly higher plasma ET-1 levels over both periods compared with matched controls ($P < 0.01$).

**Nephrogenic diabetes insipidus**

The ability to concentrate urine was impaired in SCD subjects after overnight water deprivation (410.8±8.3 vs 845.1±54.9 mosm/kg H$_2$O; $P < 0.001$ vs controls). Compliance with water deprivation in both groups was excellent, for individual ADH levels were significantly increased after the overnight fast (9.8±2.0 and 5.9±1.4 pg/ml in SCD patients and in controls, respectively, $P < 0.05$ between groups; and $P < 0.001$ vs the normal standard value of the assay = 2.1±0.2 pg/ml). After dDAVP, urine osmolality did not change significantly in either group, and it still was markedly lower in the SCD patients than in controls (416.6±6.6 vs 894.0±42.3 mosm/kg H$_2$O) ($P < 0.001$, Figure 2).

The impairment of the kidney’s ability to concentrate urine was associated with significant weight loss in the SCD individuals (−0.83±0.13 kg, $P < 0.0001$), while normal subjects could maintain stable body weight (−0.34±0.19 kg, NS). This finding indicates that young adults with SCD are more prone to dehydration than healthy matched individuals.
Regulation of urinary ET-1

SCD individuals excreted much more ET-1 in their urine than controls (ANOVA, \( P < 0.001 \)) under both the test conditions: on day 0, prior to the overnight fast (33.44 ± 16.15 vs 7.22 ± 2.17 pg/μmol of creatinine, \( P < 0.05 \)) and on day 1, after overnight water deprivation, both before dDAVP (13.20 ± 4.22 vs 3.61 ± 1.40 pg/μmol creatinine; \( P < 0.05 \)) and after dDAVP (14.53 ± 3.58 vs 3.09 ± 0.75 pg/μmol creatinine, \( P < 0.05 \), Figure 3).

Since urinary ET-1 excretion is mainly tubular, we also compared raw ET-1 urinary excretion rates without normalization against urine creatinine. This comparison showed more than a 2-fold higher active ET-1 excretion (3.86 ± 0.95 vs 1.65 ± 0.68 pmol/h on day 1, \( P < 0.01 \)) in SCD patients and controls respectively.

Interestingly, overnight fast induced an appreciable decrease in renal ET-1 production in both groups. This additional novel observation suggests that water deprivation may inhibit the release of ET-1 by renal tubular cells. These results are in accordance with those of Kohan and Padilla [18], who demonstrated that hypertonic media suppressed ET-1 synthesis in cultured inner medullary collecting duct cells and that, in vivo, urinary ET-1 excretion decreased in volume-depleted rats. In our clinical study, this physiological mechanism occurred both in healthy controls and in SCD patients; it only affected the renal compartment, for plasma ET-1 did not change significantly.

There was no linear correlation between urinary osmolality and ET-1 excretion rate when the two groups were analysed separately. Because the very limited dispersion of the rate of urinary excretion of ET-1 and of urinary osmolality in both groups did not

Microalbuminuria and renal ET-1

Mean albuminuria was significant [3.1 ± 0.7 g/mol of creatinine (SI) or 35.2 ± 8.1 mg/g of creatinine] in the SCD group. Of 17 SCD patients, eight had abnormal microalbuminuria of between 30 and 300 mg/g of creatinine [mean albuminuria: 5.1 ± 0.7 g/mol of creatinine (SI) or 57.4 ± 8.3 mg/g of creatinine in the morning of day 1]. Urinary ET-1 outputs during 3 h in the morning on day 1 and the ratios of urinary albumin to creatinine were plotted for each individual SCD subject. A significant correlation was observed between these two parameters (\( P < 0.01 \)) (Figure 4).

Discussion

Sickle cell disease is a frequent chronic disease that affects kidney function and structure [2,3]. Although the role of circulating plasma ET-1 in the pathophysiology of vascular and acute renal disease is still controversial, a large body of evidence points to the participation of this peptide in the progression of chronic renal failure [10,11]. Urine and plasma levels of ET-1 were elevated in young adult patients with SCD, compared with African-French and African controls. Furthermore, urinary ET-1 excretion was associated with marked urine-concentrating defect and microalbuminuria.

Overnight fast induced a marked decrease in renal ET-1 production in both groups. This additional novel observation suggests that water deprivation may inhibit the release of ET-1 by renal tubular cells. These results are in accordance with those of Kohan and Padilla [18], who demonstrated that hypertonic media suppressed ET-1 synthesis in cultured inner medullary collecting duct cells and that, in vivo, urinary ET-1 excretion decreased in volume-depleted rats. In our clinical study, this physiological mechanism occurred both in healthy controls and in SCD patients; it only affected the renal compartment, for plasma ET-1 did not change significantly.

There was no linear correlation between urinary osmolality and ET-1 excretion rate when the two groups were analysed separately. Because the very limited dispersion of the rate of urinary excretion of ET-1 and of urinary osmolality in both groups did not
allow confirmation of such a correlation, we pooled the data from both groups for a higher analytic power to study the relationship between urinary ET-1 output and urine osmolality, and we then observed a significant negative correlation between these two variables.

Since ET-1 promotes increased free-water clearance and counters the effects of ADH on tubular functions [12,13], the marked increase in urinary ET-1 excretion that we report during SCD at steady state is likely to favour diabetes insipidus and subsequent dehydration, a potential trigger of an acute crisis. In addition, increased renal ET-1 production may promote renal hypoperfusion and progressive tissue scarring [11]. Interestingly, about half of the variance of ET-1 excretion seemed to be a function of microalbuminuria ($R^2=0.5$, Figure 4). Besides hypoxia (favoured by SCD), albumin has been shown to be a potent in vitro stimulant of ET-1 synthesis by the tubule [19]. Although it is beyond the scope of the present study, it would be interesting to determine if active reduction of microalbuminuria (with angiotensin converting enzyme inhibitor therapy for instance) can blunt the output of urinary ET-1—which could provide indirect evidence that albuminuria might stimulate renal ET-1 in vivo. Meanwhile, pharmacological interventions that reduce microalbuminuria are likely to exert significant changes in renal haemodynamics, and consequent changes in tubular perfusion. Our clinical data and available experimental facts together strongly suggest a pathophysiological link between abnormal glomerular haemodynamics or structural damage and renal tubular synthesis of ET-1.

Another target of the high ET-1 concentration in the kidney could be red blood cells (RBC). The stimulation of endothelin receptor type B subtypes favours RBC dehydration through the activation of the Gardos channel [20], causing cellular volume to decrease and cellular haemoglobin concentration to increase. This increase in erythrocytic haemoglobin concentration leads to an increased rate of HbS polymerization under hypoxic and hypertonic conditions, for example, in the renal medulla [1,2]. Therefore, in view of the potential action of ET-1 on renal vascularity and tubules and on red blood cells, our data suggest that elevated endothelin synthesis is implied in the pathophysiology of chronic renal damage in SCD.

In conclusion, we report that the production of renal ET-1 is elevated in SCD at steady state—providing a clinical example where this peptide could be a marker of renal medullary ischaemia, and that it probably promotes nephrogenic diabetes insipidus and kidney fibrosis. We also observed a strong association between the rate of excretion of urinary ET-1 and microalbuminuria. In addition, we provide, for the first time, clinical evidence that the renal production of ET-1 is decreased by water deprivation.

These findings support the need for further prospective studies and testing of endothelin receptor antagonists in the setting of nephrogenic diabetes insipidus and renal damage in sickle cell disease.

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Conflict of Interest Statement. None declared.

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