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Stable Pediatric Kidney Transplant Recipients Run Higher Urine Indoleamine 2, 3 Dioxygenase (IDO) Levels than Healthy Children

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Abstract

Immune cells utilize the indoleamine 2,3 dioxygenase (IDO) enzymatic conversion of tryptophan (trp) to kynurenines (kyn) to determine T cell activation versus anergy/apoptosis. In prior studies, urine IDO levels were higher in rejecting renal allografts than in stable state. However, urine IDO levels in healthy subjects or children are unknown.

As a corollary to a larger longitudinal and prospective study of serum and urine IDO levels for transplant immune monitoring, here we analyzed the difference between urine IDO levels in stable post-transplant versus healthy children. IDO levels were measured by tandem mass spectrometry and expressed as kyn/trp ratios.

We compared one-time urine samples, from 34 well children at general pediatric clinics, to the first month post-transplant urine samples from 18 children, while in stable state (no acute rejection or major infection event in next 30 days). Urine kyn/trp ratios were significantly higher in stable children in first month post-kidney transplant (median 16.6, range 3.9-44.0) versus healthy children (median 9.2, range 3.51-17.0; p value = 0.0057 by non-parametric Mann-Whitney test).

Higher urine IDO levels even with stable transplant suggests a continuous ongoing low-grade allorecognition/inflammatory process. Our data also provide baseline urine IDO levels in healthy subjects for use in future studies. (word count = 200)

Keywords

Indoleamine 2, 3 dioxygenase; pediatric; transplant; urine

Introduction

The enzyme indoleamine 2, 3 dioxygenase (IDO) catabolizes tryptophan (trp) to further intermediate metabolites, collectively known as the kynurenines (1). The enzyme is coded for by the IDO gene located on chromosome 8p12 in humans. Immune cells and the semi-

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allogeneic fetus (2) utilize this IDO pathway and trp depletion to determine immune unresponsiveness, either through anergy or regulatory cell development. In addition, metabolites of tryptophan including kynurenine, quinolinic acid, and picolinic acid are directly toxic to CD8 and CD4 Th1 cells (3). Immune cell IDO enzyme activity is thus a part of a complex milieu of factors that promote peripheral immune tolerance, along with FOXP3, IL-10 and Fas ligand (4, 5). The activity of IDO, as conventionally expressed by the ratio of L-kynurenine (kyn) to trp, has also been used as a biomarker in transplantation (6, 7).

Prior studies have evaluated the value of serum or urine IDO activity levels as biomarkers of acute rejection in kidney transplant recipients. In a prospective study in the first three weeks post kidney transplant in adults, Brandacher et al (8) demonstrated that blood and urinary kyn/trp ratios were elevated above baseline during acute rejection episodes. In another study of retrospectively collected plasma samples from subjects out to 6 months post-kidney transplant, both kyn and trp levels increased prior to acute rejection (9). This study also evaluated serum kyn and trp levels in end-stage renal disease patients, who were found to have a higher kyn/trp ratio than healthy adults. Both studies utilized an HPLC platform for kyn and trp assays. Utilizing a more sensitive and precise tandem mass spectrometry assay for kyn and trp (10), we have shown that serum, but not urine, kyn/trp levels are elevated prior to acute rejection in subjects out to 1 year post-transplant (11).

The knowledge gap that exists relates to urine kyn/trp levels in healthy subjects, both adults and children. Another question for which data are lacking are whether kidney transplant recipients in a stable state run the same levels as healthy children. In a separate corollary to our recently published longitudinal transplant study (11), we also sought to determine the urine kyn/trp ratios in healthy children and compare with kidney transplant recipients in a stable state, the objective of this report.

Methods

Subjects

For group 1, we collected random urine samples from 34 healthy subjects between the age of 6 and 19 years old, who were being seen for routine physical exam in general pediatric outpatient clinics. All subjects were healthy, had no active infection or recent infection or fever during previous two weeks, and were not on immune modulating medications. Subjects provided a one-time urine sample by clean-catch method. We chose not to use diaper or catheterization-obtained urine samples.

As part of the larger longitudinal transplant biomarkers study we also collected monthly urine samples from pediatric kidney transplant subjects during months 1 through 12 post-transplant. The urine samples utilized for the main analyses were collected when the transplant subjects were in the first month post-transplant and in stable condition; i.e., had no infection or rejection prior, or in the next 30 days. The 18 first-month urine samples from these subjects formed group 2.

As depicted in figure 2B of our prior report (11), we have shown that urine kyn/trp ratio did not change over the course of the first year post-transplant in those patients who remained stable, without infection or rejection events. Thus, the subsequent month urine kyn/trp results in stable patients are not likely to be different from those in first month. However, as an *additional* sensitivity analysis, we also then compared the kyn/trp ratios in the healthy subjects to the kyn/trp ratios from first time point collected urine samples in subjects who enrolled in the study when already past their first month post-transplant. For these 10 additional samples, we confirmed that the subjects were in stable state, with no acute

rejection or infection in the 30 days prior or subsequent to sample collection. In the transplant subjects, to assess if renal function affected the urine kyn/trp ratio, we further analyzed for association between serum creatinine or estimated GFR (eGFR) with urinary kyn/trp ratios at same visit (even after first month) by non-parametric Spearman correlation. While we routinely screen for dipstick proteinuria, we did not collect data on urinary protein to creatinine ratio or HLA-direct antibodies as we do not routinely screen for them at our center.

All study procedures and forms were approved by the University of Florida Institutional Review Board. All subjects provided informed consent.

Sample analysis

IDO activity is expressed as the ratio of kyn/trp X100. Urine levels of trp and kyn (Sigma, St Louis, MO, USA) were measured from batched samples stored at -80°C by HPLC tandem mass spectrometry using a Thermo TSQ Quantum Ultra spectrometer (Thermo, San Jose, CA, USA). Detailed procedures have been published by us previously (10, 11).

Data Analysis

We compared the kyn/trp ratio (a continuous variable and the measure of IDO activity) in the urine of healthy subjects to the kyn/trp ratio in stable transplant subjects by using the non-parametric Mann-Whitney test, via GraphPad software 6.0 (San Diego, CA, USA). The more rigorous non-parametric test was chosen since normal values for urine kyn/trp in healthy children were not known and our scatter plot showed that the equal variance assumption between groups for a t-test was violated. A two-tailed p value < 0.05 was considered significant.

Results

We initially analyzed 34 urine samples from 34 healthy subjects and 18 urine samples from 18 transplant subjects who were in stable state in first month post-transplant. An additional 10 subjects enrolled at the beginning of our transplant study were already past their first month post-transplant but within their first year. In these patients, our additional sensitivity analysis also includes their first collected urine samples in stable state, no infection/rejection in prior or subsequent 30 days. Our larger longitudinal study of both serum and urine transplant biomarkers had 29 total subjects enrolled (11), but one subject had an open vesicostomy that continuously drained to exterior and adequate volume of clean urine collection was not possible in this subject.

The demographic characteristics of both groups are summarized in table 1. The two groups were similar in basic characteristics including median age, gender proportion and racial/ethnic group proportion. Since we only used clean catch samples from healthy subjects, the age range for this group went down to 6 years only. The 18 transplant recipients exhibited the following additional transplant characteristics: deceased donor transplants in 14 (77%), mean HLA mismatch 4.5, primary renal disease breakdown as follows: hypoplasia/dysplasia in 4, obstructive uropathy in 4, glomeronephritides in 5, other in 5.

As shown in figure 1, we found a statistically significant difference in IDO enzyme activity between the two groups. The urine kyn/trp ratio was significantly higher in the transplant subjects in stable state in first month post-transplant (median 16.61, range 3.99 to 44.0) compared to the healthy subjects group (median 9.22, range 3.51 to 17.08) with Mann-Whitney non-parametric p value 0.0057. When we excluded the two highest kyn/trp ratios from the stable transplant population, the differences in urinary kyn/trp ratio were less but

remained significant, median ratio 15.86 in stable transplant versus 9.22 in healthy subjects, Mann Whitney p value = 0.02.

When we added the 10 first collected urine samples from patients enrolled when already beyond their first month, we obtained very similar results: median 17.24, range 3.99 to 44 in the transplant subjects, Mann Whitney p value = 0.003 compared to healthy subjects.

In the transplant patients, we further compared trend in urinary kyn/trp ratio at each visit to serum creatinine and estimated GFR (eGFR) at same visit. The urinary kyn/trp ratio did not show an association with serum creatinine at same visit (Spearman correlation coefficient -0.041 , p value 0.619) or with eGFR (Spearman correlation coefficient -0.014 , p value 0.889).

Among the 18 transplant subjects, 6 had primary non-function or delayed graft function. The median first month urinary kyn/trp ratio in the six with delayed graft function was 12.16 versus a median ratio of 20.09 in the 12 without delayed graft function, p = 0.1223 by Mann Whitney test.

Discussion

In adults, serum or plasma kyn/trp ratios have been measured across a spectrum of disease conditions by Lahdou et al. In their study, healthy adult subjects had a kyn/trp ratio range of 0.03 to 0.07 (when both kyn and trp were measured as $\mu\text{mol/L}$, rendering the ratio as unitless; we and others multiply this ratio by 100 to make it easier to read). In comparison to healthy subjects, adults with ESRD in the Lahdou et al study ran three-fold higher plasma kyn/trp ratios of 0.11 to 0.20. Post-transplant, adults without acute rejection in the first 6 months post-transplant showed a drop in plasma kyn/trp ratios down to a range of 0.05 to 0.10.

Brandacher et al replicated the findings in serum in the post-transplant population. In their study, serum kyn/trp ratios started around 120 $\mu\text{mol/mmol}$ (equivalent to 0.12 by the Lahdou et al study units or 12 in our study units) and dropped rapidly within a few days to 30 $\mu\text{mol/mmol}$ (equivalent to 0.03 by Lahdou et al study units) in subjects who did not experience acute rejection in the first 3 weeks. When the results of these two studies are combined, the data suggest that serum IDO levels in stable transplant recipients return to levels seen in healthy subjects and rise again in acute rejection.

In contrast to serum or plasma, urinary kyn/trp ratios tend to run higher in the stable state in the prior studies. Brandacher et al found the mean ratios of kyn/trp in urine to be around 100 $\mu\text{mol/mmol}$ (equivalent to 0.1 by Lahdou study units or 10 in our study units) in stable subjects without acute rejection, in contrast to 30 $\mu\text{mol/mmol}$ in serum. To our knowledge, the urine kyn/trp ratio in ESRD adults has not been tested, perhaps since these ESRD subjects are likely to be anuric if on dialysis. However, we are also not aware of any studies measuring the urine kyn/trp ratios in healthy adults. What is not known is whether stable transplant recipients ever have urine kyn/trp levels return to healthy subject levels, in a manner similar to serum levels.

To the best of our knowledge, ours is the first study to provide data about urinary IDO enzyme activity and kyn/trp ratios in healthy pediatric subjects. Our study demonstrated a significant difference in IDO activity between healthy children and stable pediatric transplant patients. Since IDO activity has been postulated to be a marker of immune activation, the increased IDO activity in stable transplant subjects suggests an ongoing inflammatory or low level allo-recognition immune activation process even in the absence of infection and/or rejection. This concept is not new, but has not been emphasized in data

from studies of other biomarkers. From our review of the literature, urinary IP-10 and fractalkine levels, both biomarkers of acute rejection, run higher in stable transplant patients than in healthy controls (12, 13) and then rise substantially prior to acute rejection. The renal handling of kyn and trp is unknown. We are not aware of any association between urinary kyn/trp ratio elevation and reduced GFR, nor did we find that in our study.

Our study also provides for the first time, the normative data for urinary kyn/trp, which can be used as a reference value in future studies. It is not known if healthy adults would run the same urinary kyn/trp ratios as in children. When comparing our kyn/trp ratios in children with stable transplant to the data from adult transplant recipients in the Brandacher et al study, the children in our group ran a higher median urinary kyn/trp ratio of 17 (converts to 170 per units when using $\mu\text{mol}/\text{mmol}$), versus 100 $\mu\text{mol}/\text{mmol}$ in stable adults. It is therefore possible that healthy children may run higher mean levels than healthy adults. However, the Brandacher et al study did not exclude patients with active infection. Holmes et al have previously shown that infections may cause elevations in serum kyn and trp (14), hence we considered a stable state to be the absence of both acute rejection and major infection.

The limitation of this report is the small number of subjects that did not allow for analyzing subgroups based on gender, age and race. In the future, a larger study will be helpful to create a reference of normal urine IDO enzyme activity in pediatric or adult subjects classified based on gender, race and age group.

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Figure. Scatter plot graph depicting the urinary kyn/trp ratios between healthy subjects (n =34; circles) versus children in first month and stable state post-kidney transplants (n =18; triangles). Horizontal line represents the median value.

Table 1

Demographic data for our study groups

	Control (n = 34 subjects)	Transplant (n = 18 subjects)
Age range (median)	6–19 (12.8)	1.5–18.7 (12.5)
Female Gender (%)	16 (47%)	8 (44%)
Race		
Caucasian	17 (50%)	10 (55%)
African American	11 (32%)	6 (33%)
Hispanic	4 (12%)	1 (6%)
Others	2 (1 Asian, 1AA/C) (7%)	1 (6%)