

Original Article

Urinary HLA-DR and CD54 expression—indicators for inflammatory activity in decoy cell shedding patients

Se Hoon Kim¹, Hyung Joon Ahn², Yu Seun Kim², Soon Il Kim², Hyun Sook Kim³ and Hyeon Joo Jeong¹

¹Department of Pathology, ²Department of Surgery and ³Department of Laboratory Medicine, Institute of Renal Disease, Yonsei University College of Medicine, Seoul 120-752, Korea

Abstract

Background. Polyomavirus (PV) nephropathy may coexist with or follow acute renal transplant rejection. The aim of this study was to evaluate whether HLA-DR and CD54 are useful cellular markers for surveillance of acute rejection in PV-infected patients.

Methods. A prospective study was conducted using 205 renal transplant patients. Urine samples were collected at a regular interval post-transplantation for routine cytology and immunocytochemistry. Urinary levels of tumour necrosis factor α , soluble interleukin-2 receptor and interleukin-6 were used as adjunctive markers for acute rejection.

Results. Of the 699 total samples, decoy cells were identified in 100 samples of 50 patients. Patients with decoy cell-positive (DCP) samples had higher serum creatinine levels than decoy cell-negative (DCN) samples (1.55 vs 1.41 mg/dl, respectively; $P=0.006$). DCP samples were also more likely to be HLA-DR positive (50.0 vs 32.4%; $P=0.029$), as well as CD54 positive (17.4 vs 6.9%; $P=0.038$). However, serum creatinine levels did not correlate with HLA-DR or CD54 positivity among DCP samples. Instead, CD54 positivity correlated with decoy cell grades. Immunosuppression decreased in 11 DCP patients, and HLA-DR was negatively converted in three of them. None of the patients developed acute clinical rejection. Urinary cytokine levels did not correlate with serum creatinine levels, nor did they correlate with HLA-DR or CD54 status among DCP patients.

Conclusions. Urinary tubular HLA-DR and CD54 expression increased in decoy cell shedding patients but did not indicate a concomitant acute rejection. These markers may instead indicate renal inflammatory activity associated with viral reactivation, which has the potential to progress to PV interstitial nephritis.

Keywords: CD54; decoy cell; HLA-DR; polyomavirus; pro-inflammatory cytokine; transplantation

Introduction

Polyomavirus (PV) nephropathy has emerged as a major cause of acute graft dysfunction and failure. Graft dysfunction is largely related to tubular necrosis and interstitial nephritis [1,2]. However, the inflammation may be evoked or aggravated by acute graft rejection either preceding or concomitantly with PV interstitial nephritis [3,4]. Detecting acute rejection in the setting of PV nephritis is important when determining treatment strategies [5]. Renal biopsy can be used as a diagnostic tool to confirm the diagnosis. However, it is an invasive procedure, and it cannot be repeated serially in patients suspected of having PV nephritis.

Urine cytology has been successfully used as a screening test for PV reactivation. It has also been used for the diagnosis of acute rejection. Urinary decoy cells correlate with renal tissue pathology. Likewise, increased HLA-DR and CD54 expressions in detached urinary epithelial cells [6–9] have been used to detect acute rejection [8,10]. We could find only one promising report regarding the predictive value of increased tubular HLA-DR expression in renal allograft biopsies for concomitant acute rejection/PV nephropathy [4]. This study indicated that HLA-DR was a good surrogate marker for the diagnosis of overlapping acute rejection. However, there have been no further reports supporting or disputing this notion. This prospective study explores HLA-DR and CD54 expression in urinary epithelial cells in an attempt to test the validity of immunohistochemistry in the surveillance of acute rejection PV-infected renal allograft patients. Urinary levels of tumour necrosis factor α (TNF α), soluble interleukin-2 receptor (sIL-2R) and interleukin-6 (IL-6) were used as adjunctive indicators for acute rejection.

Correspondence and offprint requests to: Hyeon Joo Jeong, MD, Department of Pathology, Yonsei University College of Medicine, 134 Shinchon-dong Seodaemun-gu, Seoul 120-752, Korea. Email: jeong10@yumc.yonsei.ac.kr

Subjects and methods

Patients

Patients enrolled in the study received renal allografts at Yonsei University Medical Center, from January 2003 to December 2004.

Urine and blood collection and renal biopsy

Morning urine was routinely collected at 1, 3, 6, 9 and 12 months post-transplantation on a regular basis. In patients with urinary decoy cells or renal allograft dysfunction, urine collection was repeated more frequently and continued beyond the first year. Urine was centrifuged at 1000 rpm for 5 min. A cytospin smear was used for Papanicolaou stain and immunocytochemistry. Urine supernatant was divided into aliquots and kept at -70°C until use. Serum collection and measurement of creatinine levels were performed on the same day as the urine collection.

The indication for renal biopsy is as follows: elevated serum creatinine (0.3 mg/dl or more above baseline), proteinuria of greater than 1 g/24 h, and persistent haematuria. No protocol biopsy was performed.

Urine cytology and decoy cell counting

A Papanicolaou stain was done using a cytospin smear. After hydration with graded alcohol, the smears were stained with Harris Haematoxylin for 3 min. After washing with distilled water, cells were quenched with 1% HCl for 30 s, washed and then partially dehydrated. Cells were stained with Orange G6 for 2 min, followed by an Eosin azure 50 stain for 2 min. After dehydration with graded alcohol, cells were cleared with xylene. Decoy cells were counted and subdivided into four grades: (1) Negative, no decoy cells; (2) Grade I, 1–3 decoy cells/10 high power field; (3) Grade II, 4–9 decoy cells/10 high power field and (4) Grade III, 10 or more decoy cells/10 high power field.

Immunocytochemistry

Cytospin-fixed cells were used for immunocytochemistry. Cells were fixed for 10 min in cold acetone, air-dried for 30 min and then rehydrated in graded alcohol. After treatment with 3% H_2O_2 for 20 min in order to abolish the endogenous peroxidase activity, the cells were placed in a citrate buffer (pH 6.0), exposed to microwaves for 10 min, placed in a TRIS buffer (pH 7.6) for 10 min and then exposed to microwaves again for another 10 min. After blocking endogenous biotin, the cells were incubated with primary antibodies against CD54 (DakoCytomation, Glostrup, Denmark, in 1:200 dilution) and HLA-DR (DakoCytomation, in 1:40 dilution) overnight at 4°C . They were then washed with a phosphate-buffered saline buffer. Link antibodies and streptavidin-biotin peroxidase was sequentially applied using the LSABR2 kit (DakoCytomation). The reactions were coloured using 3,3-diaminobenzidine (DAB). The samples containing 50 or more tubular cells per slide were evaluated. Positively stained tubular epithelial cells were counted and expressed as the percentage of total tubular cells.

Enzyme linked immunosorbent assay

Cytokine concentrations were measured using enzyme-linked immunosorbent assay (ELISA). A 96-well plate was coated with polyclonal rabbit antibody against $\text{TNF}\alpha$, sIL-2R and IL-6 (Bender MedSystems GmbH, Campus Vienna Biocenter 2, Vienna, Austria). Examination was done in duplicate. After thawing the urine samples at room temperature, $100\ \mu\text{l}$ of undiluted urine was added to each well. The same amount of sample diluent was added in the control wells. After adding $50\ \mu\text{l}$ of diluted biotin-conjugate in each well, the plate was incubated for 2 h at room temperature. They were then washed with wash buffer to remove unbound biotin-conjugated cytokine. Next, $100\ \mu\text{l}$ of Streptavidin-HRP was added to each well. The plate was incubated for 1 h at room temperature and then washed with wash buffer. After adding $100\ \mu\text{l}$ of tetramethyl benzidine substrate solution, the plate was incubated for 10–20 min at room temperature. The reaction was stopped by adding $100\ \mu\text{l}$ of stop solution. The colour intensity was measured using a spectrophotometer at 450 nm. Cytokine concentrations in each sample were calculated using a standard curve. Urinary cytokine levels were expressed as urinary cytokine concentration:urine creatinine ratios to standardize samples.

Serum and urine creatinine measurement

Using an automatic analyser (Hitachi 7600-210, Japan), serum and urine creatinine levels were measured according to the kinetic alkaline picrate method (Jaffe reaction).

Statistical analysis

Data were analysed using SPSS software, version 11.0 (Chicago, IL, USA). The Student *t*-test was applied to the serum creatinine levels. A chi-square or Fisher's exact test was applied in order to test the relationship between urinary HLA-DR and CD54 expression and decoy cell shedding. One-way ANOVA with Scheffe's *post hoc* test was used to compare urinary cytokine secretion among decoy cell shedding groups. Statistical significance was defined as *P*-values <0.05 .

Results

Urinary decoy cell shedding and correlation with clinical findings

A total of 699 urine samples were collected from 205 patients. Urine samples were collected once from 39 patients, twice from 28 patients, three and four times respectively from 2 groups of 33 patients each, five times from 63 patients and more than five times from nine patients. Urinary smear showed a mixture of tubular cells, urothelial cells and inflammatory cells. It was sometimes difficult or impossible to distinguish between urothelial and tubular cells. Urothelial cells are, however, generally larger than tubular cells, have plump cytoplasm, and constitute a minor proportion in decoy cell-positive (DCP) samples. Tubular cells were readily identified mostly singly or rarely in small

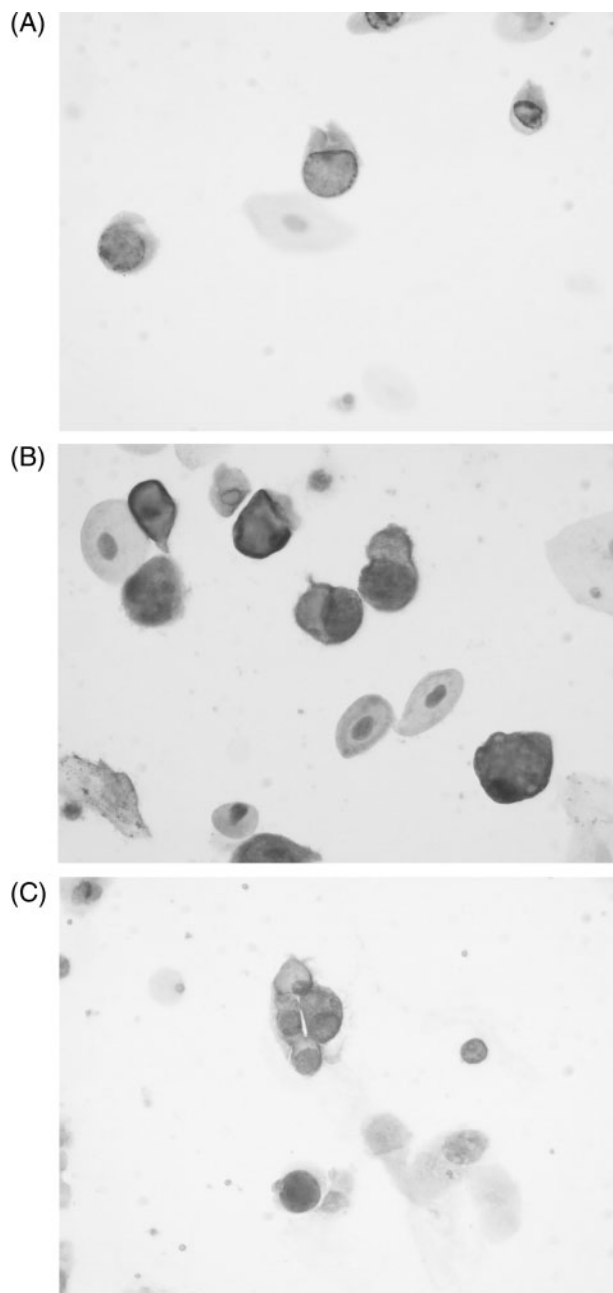


Fig. 1. HLA-DR and CD54 immunoreactivity in a decoy cell-positive case. Tubular nuclei are enlarged and show a ground glass appearance [(A), Papanicolaou stain, 400 \times]. Tubular cytoplasm of infected cells is diffusely stained with both HLA-DR [(B), 400 \times] and CD54 antibodies [(C), 400 \times].

clusters in DCP samples, probably because viral replication in tubular epithelial cells is often associated with epithelial necrosis and acute tubular injury. Nuclei of viral infected decoy cells showed characteristic cytopathic changes with intranuclear inclusions (Figure 1A); however, immunohistochemistry against SV40 large T antigen demonstrated positive nuclear staining in enlarged as well as normal appearing tubular and urothelial cells.

Decoy cells were positive in 14.3% of the samples. It was once in 33 patients, twice in 14, three times in 5,

Table 1. Urinary decoy cells and serum creatinine levels

Urinary decoy cells	No. of patients	No. of samples	Serum creatinine (mg/dl)
Negative	155 (75.6%)	599 (85.7%)	1.41 ^a
Positive	50 (24.4%)	100 (14.3%)	1.55 ^a
Grade I	34 (16.6%)	50 (7.2%)	1.56
Grade II	12 (5.9%)	16 (2.3%)	1.46
Grade III	26 (12.7%)	34 (4.9%)	1.58
Total	205 (100%)	699 (100%)	1.43

^a $P=0.006$.

four times in 7 and five times in 1 patient. The degree of decoy cells fluctuated frequently from sample to sample, even in the same patient. Of the 138 patients who had performed urinalysis three times or more, decoy cells were persistently negative in 96 (69.6%), and decoy cells of grade III occurred two or more times in seven patients (5.1%). The average serum creatinine level was 1.43 mg/dl for all samples. Serum creatinine levels were significantly higher in decoy-positive (DCP) samples than in the samples without decoy cells ($P=0.006$); however, serum creatinine level did not differ according to degrees of decoy cells (Table 1).

Immunosuppression was modulated in 11 patients, secreting grade III decoy cells at least once, or grade II at least twice. In six patients, the number of decoy cells decreased within 6 months without significant changes in serum creatinine level. In the remaining five patients, follow-up data were not included since immunomodulation occurred after the last urinalysis during the study period. Clinical episodes of acute rejection were observed in 34 patients in the first year post-transplantation. Six of those 34 patients had decoy cells that became positive after rescue therapy (grade I in four patients, and grade III in two patients).

Correlation of urinary decoy cells with renal biopsy findings

Renal biopsy was performed in only 18 of 205 patients enrolled in this study. Excluding one unsatisfactory sample, twenty-one renal biopsy samples were available for evaluation. Decoy cells were present in four of the 18 patients, grades I and III in two patients each. Two patients who secreted decoy cells of grade III at the time of biopsy were diagnosed as PV nephropathy. In two patients with grade I decoy cells, one patient was diagnosed as IgA nephropathy and the other as having mild tubular atrophy and interstitial fibrosis. Of the remaining 14 decoy cell-negative (DCN) patients, nine patients had acute rejection (five as tubulointerstitial, four as vascular rejection). Decoy cell shedding was not observed in any of the nine patients before or after the diagnosis of acute rejection. Two patients had chronic rejection. IgA nephropathy and membranoproliferative glomerulonephritis was diagnosed in one patient each. Biopsy findings were non-specific in one patient.

Urinary HLA-DR and CD54 expression and its relationship to decoy cell shedding

Immunocytochemistry against HLA-DR and CD54 was done on 692 urine samples. Of these, 308 samples contained at least 50 tubular cells per slide, with a mean value of 110 cells (range 50–303) stained for HLA-DR and 95 cells (range 50–523) stained for CD54, and were the subject of the study (Figure 1B and C). Forty-six samples contained decoy cells. HLA-DR and/or CD54 were stained in tubular cells as well as acute and chronic inflammatory cells. To distinguish inflammatory cells positive for HLA-DR and/or CD54 from tubular cells, staining against cytokeratin antibody was performed using extra-unstained smears having prepared in samples with a sufficient amount of urine volume.

The percentages of HLA-DR-positive tubular cells per slide were higher in DCP samples than DCN samples (36.2 vs 25.6%), as were CD54-positive tubular cells (12.4 vs 8.4%). However, only the latter was statistically significant ($P=0.123$ vs $P=0.008$). When the cut-off value was set at 30%, HLA-DR was positive in 50% of DCP samples, which was higher than the samples without decoy cells (32.4%, $P=0.029$). The same held true for CD54 positive cells (17.4 vs 6.9%, $P=0.038$). CD54-positive samples were also HLA-DR-positive (Figure 1). HLA-DR positivity did not differ according to decoy cell grades, whereas CD54 positivity was significantly different between grades I, II and III ($P=0.047$) (Table 2). When the DCP samples were divided according to HLA-DR reactivity, the serum creatinine levels of HLA-DR-positive samples ($n=22$, 1.56 mg/dl) did not differ from the HLA-DR-negative samples

Table 2. Urinary HLA-DR and CD54 expression according to decoy cell excretion

Urinary decoy cells	No. of samples	No. of HLA-DR-positive samples	No. of CD54-positive samples	Serum creatinine (mg/dl)
Negative	262	85 (32.4%) ^a	18 (6.9%) ^b	1.45
Positive	46	23 (50.0%) ^a	8 (17.4%) ^b	1.62
Grades I and II	23	10 (43.5%)	1 (4.3%) ^c	1.60
Grade III	23	13 (56.5%)	7 (30.4%) ^c	1.64
Total	308	108 (35.1%)	26 (64.9%)	1.48

^a $P=0.029$, ^b $P=0.038$, ^c $P=0.047$.

Table 3. Urinary cytokine levels

Urinary cytokine level	Healthy controls (pg/mg)	Transplant		<i>P</i> -value
		Without decoy cell (pg/mg)	With decoy cell (pg/mg)	
TNF- α	0.16 ($n=5$)	2.84 ($n=8$)	1.40 ($n=62$)	0.055
IL-6/Ucr	0.01 ($n=10$)	0.10 ($n=25$)	0.07 ($n=78$)	0.18
sIL-2R/Ucr	0.02 ($n=10$)	0.01 ($n=20$)	0.006 ($n=85$)	0.001

*Ucr: urine creatinine level.

($n=23$, 1.67 mg/dl). Two patients who were diagnosed with PV nephropathy 2 months prior to and at the time of urinalysis, respectively, demonstrated HLA-DR and CD54 positivities, along with increased serum creatinine levels (1.9 and 2.7 mg/dl, respectively). Both pre- and post-immunomodulation HLA-DR staining was performed in five patients. Three patients who were initially HLA-DR-positive showed negative conversion with decreased immunosuppression, whereas two HLA-DR-negative patients remained negative.

Six patients with acute rejection (three with the tubulointerstitial type and three with the vascular type) showed HLA-DR positivity of 83.3% and CD54 positivity of 33.3% at the time of biopsy. The serum creatinine level (2.43 mg/dl) was significantly higher in these patients as compared with the remaining patients (1.46 mg/dl).

Urinary cytokine secretion and its relationship to decoy cell shedding

Cytokine levels were tested in 85 DCP samples, 32 transplant urine samples without decoy cells and 10 samples from healthy controls. TNF α and IL-6 levels were higher in the transplant samples than in the controls. However, there was no statistical difference. The sIL-2R levels were lower in transplant samples than in controls. This proved to be statistically significant (Table 3). No relationship was observed between urinary cytokine and serum creatinine levels.

Discussion

Several studies have demonstrated the usefulness of HLA-DR and CD54 in the diagnosis of acute renal allograft rejection [6–10]. HLA-DR has been reported to be more sensitive, whereas CD54 has been reported to be more specific. In the study, HLA-DR and CD54 expression was significantly increased in urinary epithelial cells in patients with biopsy-confirmed acute rejection (83.3 and 33.3%, respectively). This confirms the usefulness of these two markers in the detection of immune activation. Tubular HLA-DR and CD54 expressions were 50 and 17.4%, respectively, in DCP samples. This was also significantly higher than that of DCN samples.

We speculated that increased HLA-DR and CD54 expression in DCP samples might be the result of incorporating samples of patients with coexisting or ongoing acute rejection. However, none of our data

supported this hypothesis. There were no differences in serum creatinine levels or clinical features between HLA-DR-positive and -negative samples at the time of urinalysis. No patients with HLA-DR positivity developed acute rejection, irrespective of decreased immunosuppression. On the contrary, HLA-DR negatively converted, with a decrease of decoy cells in three DCP patients without significant changes in follow-up serum creatinine levels. CD54-positive rates correlated with decoy cell grades, but did not correlate with serum creatinine level. Furthermore, urinary HLA-DR and CD54 were increased at the time of biopsy in two of the three patients with PV nephropathy who also did not have histological features of a coexisting acute rejection. Finally, urinary markers for acute rejection such as TNF α , sIL-2R and IL-6 [11] were not helpful in distinguishing the two conditions. Their levels were no different in samples with and without decoy cells. These unexpected results made us consider other conditions that might be responsible for the increased immunoreactivities.

Conditions in which tubular HLA-DR and CD54 may be elevated include renal ischaemia [12–14], urinary tract infection [15], obstructive uropathy [16], aging [17] and tubulointerstitial nephritis [18]. These diverse conditions have a common feature that they may accompany interstitial inflammation [11,19]. It may also apply to PV nephritis since reactivation of PV leads to decoy cell shedding in urine and progresses to interstitial nephritis. A higher serum creatinine level in patients with urinary decoy cells compared with that in patients without decoy cells in our study may indicate that an inflammatory response was present in a substantial proportion of DCP patients. Since the history of acute rejection episode was a risk factor for PV replication, persistent inflammation from a previous acute rejection may also cause a temporary increase in the HLA-DR expression in several patients. A recent report that proinflammatory genes equal in character to that seen in acute rejection can be transcribed in PV nephritis [20] also supports our observation. Taken together, increased HLA-DR and CD54 expressions in decoy cell shedding patients may reveal renal inflammatory activity, driven either via proinflammatory response from the renal parenchyma to viral replication or as a result of non-specific tubular damage and necrosis.

The major drawback of this study was the limited number of biopsies. The lack of biopsies limited the extent to which we could correlate to cytology samples. Although urine cytology is helpful in the diagnosis of acute cellular rejection, it cannot provide any information about the presence of glomerulitis, vasculitis or peritubular capillary C4d deposition, which are crucial in the diagnosis of concurrent acute rejection in PV nephropathy patients [5]. Inflammatory response can be minimal to severe, both in acute rejection and PV nephropathy according to the stages, and 30–40% of PV nephropathy was concomitant with acute rejection [21]. Therefore, the interpretation of urinary immunocytology should be cautious without

biopsy findings or other supportive clinical findings and PV replication data before exclusion of concurrent acute rejection. Despite the limitations, our study demonstrated that an increase in HLA-DR and CD54 expressions in patients secreting numerous decoy cells may be at risk or already have some degree of inflammation related to PV replication. In addition, negative conversion of decoy cells with newly developed HLA-DR positivity after immunomodulation may raise a reasonable suspicion for ongoing immune activation. These patients should therefore undergo careful follow-up.

Conflict of interest statement. None declared.

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