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Rare subtypes of BK virus are viable and frequently detected in renal transplant recipients with BK virus-associated nephropathy

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ABSTRACT

BK virus-associated nephropathy (BKVN) occurs in up to 5% of kidney transplants and is a significant cause of graft loss. Four major subtypes of BKV have been described, with the vast majority of individuals persistently infected with BKV Type I (>80% of the population). Sequencing of BKV isolates subcloned from BKVN patients revealed a high percentage of variants in the urine (40%) in the VP1 subtyping region. In vitro analysis of several viral variants revealed that all variants recovered from the urine of BKVN patients produced infectious viral particles and were replication competent in cell culture while some of the variants induced cytopathic changes in infected cells when compared to the major BKV subtype, VP1 Type I. These results suggest that rare BKV VP1 variants are more frequently associated with disease and that some variants could be more cytopathic than others in renal transplant recipients.

Introduction

Serological studies indicate that more than 80% of the population worldwide is infected with the polyomavirus, BKV (Egli et al., 2009; Gardner, 1973; Portolani et al., 1974). Rates of seropositivity are significantly related to age with BKV seroprevalence reaching 91% at 5–9 years of age (Knowles et al., 2003) and falling to 71% at age 50–59 years (Egli et al., 2009). Primary infection with BKV is usually unapparent, although in some instances viral infection has been accompanied by mild respiratory illness or urinary tract disease (Goudsmit et al., 1981; Padgett et al., 1983). During primary infection, viremia occurs and the virus spreads to several organs of the infected individual, where it remains in a latent state. Results from virion isolation studies have established that the kidney is the main site of BKV latency in healthy individuals (Chesters et al., 1983; McCance, 1983). Occasionally BKV can reactivate in healthy individuals and virus becomes readily detectable in the urine by PCR (Polo et al., 2004). In the context of immunosuppression, reactivation of latent BKV in the kidney causes renal stenosis and interstitial nephritis in approximately five percent of kidney transplant patients (Ramos et al., 2009; Trofe et al., 2004).

The latter disease, which is known as BKV-associated nephropathy (BKVN) or polyomavirus-associated nephropathy (PVAN), is a serious inflammatory complication in renal transplant recipients that can progress to allograft failure. The factors that control the balance between the state of viral latency and reactivation in the human host are closely tied to the individual's immune status. While most adults have antibodies against BKV, this humoral immune response is apparently unable to prevent virus reactivation and development of disease. It is not clear whether immunosuppressive regimens used by renal transplant patients to prevent rejection have a direct effect on viral reactivation and the course of BKV lytic infection or whether they act indirectly by affecting the host cell or by their effect on the immune system. Many risk factors for BKVN have been proposed including male gender, older age, negative serostatus of the patient before transplantation, donor positive serostatus, prior acute rejection episodes, HLA mismatches, and rearrangement in the non-coding control region (NCCR) of BKV (for reviews of risk factors evaluated, see Ramos et al., 2009; Trofe et al., 2006). BKVN is a complex disease that most likely results from the interaction of multiple donor, organ and recipient risk factors.

Currently, there are no FDA approved non-nephrotoxic agents for the treatment of BKVN and no drugs with anti-viral activity specifically directed at the polyomavirus life cycle are available for prophylaxis or therapy. Reduction in immunosuppression has been successful for control of BKVN in a handful of centers (Brennan et al.,

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2005; Ginevri et al., 2007), but the risk of acute graft rejection may prohibit such a reduction in many patients. The anti-viral effect of anecdotal treatments which have been attempted has been difficult to assess due to the concomitant reduction in immunosuppression, their broad inclusion criteria, small sample sizes and lack of data in randomized trials. As such, optimal prophylaxis or therapy for BKVN has not been established to date (Ramos et al., 2009).

The human polyomavirus BK belongs to the Polyomaviridae, a family of small, non-enveloped DNA viruses with an icosahedral capsid of 45 nm in diameter that contains a circular double-stranded genome of approximately 5 kb (Imperiale and Major, 2007). The capsid is comprised of the late proteins, VP1, VP2 and VP3, which are produced by alternative splicing of the viral late gene transcript. VP1, which is the major capsid protein, is present on the surface of the capsid and is responsible for receptor binding to host cells. In addition, VP1 is highly immunogenic, is the target of neutralizing antibody and is required for virion assembly and hemagglutination of red blood cells. Four distinct serotypes of BKV have been previously determined by hemagglutination inhibition, named Subtypes I–IV by Jin et al (1993). Genetic analyses of VP1 sequences have determined that the serotyping region contains a variable region of the BKV genome between nucleotides 1744 and 1812 (amino acids 61 to 83). Antibodies to this region of the virus capsid are capable of neutralization and prevent the virus from interacting with host cells. A homology model of BKV VP1 built using SWISS-MODEL and predicted structures of other polyomavirus proteins present in public databases suggests that three major loop structures within the BC loop are likely responsible for interaction of the virus capsid with sialic acid containing receptors on host cells (Dugan et al., 2005). Interestingly, the BC loop corresponds to the serotyping region of BKV VP1 responsible for the diversity between the four subtypes. Closer examination of the four BKV subtypes reveals amino acids varying in charge, and while some of the amino acid substitutions between Types I and IV are conserved changes in charge, there are several non-conserved substitutions as well. Genotype I is the most frequent in the normal human population worldwide (80%) with Type IV is the second most frequent (15%), while genotypes II and III are infrequently detected in normal adults (Takasaka et al., 2004). Several evolutionary studies using phylogenic analysis of the four subtypes have determined that the BK virus has co-evolved with humans (Krumbholz et al., 2008; Luo et al., 2009; Yogo et al., 2009; Zheng et al., 2007).

In addition to Types I–IV, other rearrangements or variants in the VP1 subtying region have been reported in BKVN patients (Baksh et al., 2001). DNA sequencing of the VP1 genotyping region from kidney biopsies derived from patients affected by BKV interstitial nephritis underlined the existence of “hot spots” of nucleotide polymorphisms, and in most of the cases the follow-up of the patients showed that this region was not stable over time. It has been suggested that these “hot spots” for mutations could represent an advantage for the virus in escaping the host immune surveillance (Randhawa et al., 2002). In some cases, the variability predicted changes in the amino acid sequence with potential consequences for the secondary and tertiary structure of the protein. Recent in vitro studies on variant subtypes show amino acid substitutions can alter the ability of virus to interact with host cells suggesting that the variants may have functional relevance (Dugan et al., 2007). However, two recent studies in which the BKV subtyping was performed by PCR amplification found no correlation between BKV subtypes and the development of BKV infection in renal transplant recipients (Krautk-tkrämer et al., 2009; Tremolada et al., 2010).

In an attempt to investigate the role of BKV genotypes in the pathogenesis of BKVN, we examined their distribution in renal transplant recipients at the time of diagnosis with BKVN through the subcloning and sequencing of the subtyping region of the VP1 gene (nt.1633-2009). Analysis of the sequences obtained revealed a number of BK virus VP1 polymorphisms in BKVN patients which resulted in amino acid substitutions not found in the four established BKV genotypes. These polymorphic genotypes, together with the uncommon genotype IV, were much more frequent in BKVN patients in comparison to their distribution in the normal population. Analysis of selected variants in a cell culture system suggests they are replication competent and could contribute to the cytopathic effects seen in BKVN.

Results

All 20 KTX patients were diagnosed with BKVN by renal transplant biopsy and a positive ISH for BK virus as described in Materials and methods within a mean of 12.9 months after renal transplantation (range 3 to 38 months), which is typical for most transplant centers (Ramos et al., 2009). As summarized in Table 1, patients who developed BKVN had a range of underlying disease responsible for loss of renal function in their native kidneys. All patients had serum BKV loads $>1\times10^3$ and 19 (95%) had urine BKV loads $>1\times10^6$ with the exception of one patient who had a urine BKV load of 5.15×10^5 at diagnosis though subsequent urine viral loads exceeded 1×10^6. These viral loads are consistent with reported diagnostic thresholds for BKVN (Hirsch et al., 2002, 2005).

Compared to United Network for Organ Sharing national transplant data as well as center-specific data from the University of Cincinnati and The Christ Hospital (based on OPTN data, October 10, 2007), the demographics of the patient population developing BKVN were similar to the national profile in terms of recipient age, gender, race, type of transplant (deceased donor, living related or unrelated donor), transplant number and chronic kidney disease etiology with the exception of a higher percentage of male BKVN patients (80% compared to national average of 60% of kidney transplant recipients during this same time period). Patients typically received induction therapy with anti-thymocyte globulin, basiliximab or daclizumab and began double or triple immunosuppressive regimens at the time of transplantation that were a combination of mycophenolate plus tacrolimus or sirolimus, with or without prednisone at the time of BKVN diagnosis.

It is unclear whether a particular subtype or a variant of BKV plays a role in the development or the pathogenesis of BKVN. To address this question, the VP1 polymorphic serotyping region was amplified, subcloned and sequenced from the urine of renal transplant patients at the time of diagnosis with BKVN. A minimum of three individual subclones were sequenced from each patient sample. The prototypical BKV Dunlop strain, Type I, represents the predominant subtype detected in the human populations and accounts for greater than 80% of all circulating virus while Type IV is found in approximately 15% of the normal human population, and Types II and III are relatively rare (Knowles, 2001; Takasaka et al., 2004; Yogo et al., 2009). In contrast, in the patient population with BKVN, only 11/20 (55%) had Type I alone in urine and only 1/20 (5%) had Type IV alone (Table 1). The remaining patients, 8/20 (40%), had variant subtypes of virus that had amino acid substitutions not corresponding to Type I–IV sequence as the only detected subtype(s) in the urine (2/20) or present in conjunction with either Type I (4/20) or Type IV (2/20). This indicates that BKVN patients often have rare subtypes of BK virus present in urine. No correlation was seen between viral subtype and viral load in the serum or urine nor could any correlation be made between the viral subtype and clinical outcome.

In order to determine the potential contribution of different subtypes and polymorphisms observed in our patients toward the pathogenesis of BKVN, subtypes I, II, III and IV as well as several viral variants were subcloned. Only variants which harbored changes that resulted in amino acid substitutions were selected. In addition, BK virus amino acid substitutions that appeared in more than one patient were selected in order to ensure that any findings might have a broader implication. In order to ensure that no sequences outside of the viral subtyping region could be responsible for observed

Table 1
BKV subtypes and amino acid substitutions detected in urine of patients with polyomavirus-associated nephropathy at time of diagnosis.

Patient	Viral subtype	No. clones/ total sequence	Underlying disease	Months to PVAN	SCr (mg/dL)	BKV viral load	
						Serum	Urine
1	I	3/3	Type I diabetes	7	1.8	5.33×10^{6}	1.86×10^{8}
2	I	3/3	Unknown	11	2.1	4.43×10^{4}	4.28×10^{6}
3	I	2/3	Type I diabetes	8	1.6	2.59×10^{6}	7.08×10^{8}
	I/K69R*	1/3					
4	I	1/3	Membranous	15	2.2	1.02×10^{7}	9.82×10^{8}
	I/D75N*	2/3	Glomerulonephritis				
5	I	3/3	Type 2 diabetes	7	1.9	9.94×10^{5}	9.71×10^{8}
6	IV	1/3	Focal segmental	14	3.6	1.65×10^{5}	1.70×10^{8}
	IV/E77Q	1/3	Glomerulosclerosis				
	IV/A72V/E77Q	1/3					
7	I	3/3	Unknown	9	1.5	5.88×10^{5}	1.03×10^{9}
8	I	1/5	Type I diabetes	3	1.6	2.73×10^{5}	1.91×10^{7}
	III	1/5					
	II/III/N62H*	3/5					
9	IV	3/3	Glomerulonephritis	4	2.4	4.08×10^{4}	3.17×10^{6}
10	I	3/3	Systemic lupus erythematosus	13	1.8	1.26×10^{4}	4.35×10^{8}
11	I/K69R*	3/3	Unknown	31	1.5	3.93×10^{6}	1.46×10^{9}
12	I	1/5	Polycystic kidney disease	19	2.2	3.73×10^{4}	5.15×10^{5}
	I/R83G	1/5					
	I/E73D/E82N*	2/5					
	IV	1/5					
13	I	3/3	Infectious glomerulonephritis	6	2.6	1.99×10^{6}	4.53×10^{9}
14	I	3/3	Hypertension	6	1.9	1.28×10^{6}	1.68×10^{10}
15	I	3/3	IgA nephropathy	9	2.0	4.81×10^{6}	3.42×10^{8}
16	I/K69N	1/3	Hypertension	18	2.6	6.49×10^{5}	7.78×10^{9}
	I/A72/V	1/3					
	I/P81H	1/3					
17	IV	1/3	Type I diabetes	38	2.0	1.81×10^{4}	1.08×10^{9}
	IV/N62H*	2/3					
18	I	3/3	Unknown	6	1.8	1.53×10^{5}	7.22×10^{9}
19	I	3/3	Glomerulonephritis	11	1.9	8.07×10^{5}	4.59×10^{9}
20	I	3/3	IgA nephropathy	23	1.9	4.71×10^{4}	7.11×10^{9}

differences, each of the subtypes was cloned into a plasmid vector containing the entire genome of the Dunlop subtype (Type I) such that they replaced the analogous region of the VP1 sequence. The unique *Bam*HI and *Eco*RI sites flanking the VP1 subtyping region were used in this procedure so that except for the introduced subtyping changes, the sequence of viral genome remained intact and the coding region of VP1 was unaltered. The amino acid sequences of the subtyping region (amino acids 61–83 of VP1) of the BKV subtypes I–IV and variants used in this study are shown in Table 2. The overall percentages of patients with clones of Types I through IV and variants in this study are shown in Table 3.

Virus stocks were prepared from these cloned isolates by transfection/infection with the linear BK viral genome excised from the plasmids by digestion with *Bam*HI. The digested genomic DNA was then directly transfected into Cos-7 cells using liopofectamine reagent (InVitrogen). Viral stocks were generated in Cos-7 cells because of their high-level expression of SV40 T-antigen, which acts in *trans* at the BKV non-coding control region (NCCR) to upregulate viral DNA replication, and perhaps other steps in the virus life cycle, for efficient virus production. Cultures were then expanded when confluent and harvested when signs of cytopathic effects (CPE) were evident in the cultures. The VP1 subtyping regions of all the viral stocks were sequenced to ensure no substitutions or mutations had occurred. Subtypes II, III and IV and the selected variants were cultured in vitro and viral stocks of each virus strain were prepared. No difficulties were experienced in culturing of the variants that would suggest defective packaging or assembly of viral particles during preparation of the viral stocks.

Since the VP1 subtyping region is responsible for the hemagglutinating activity of BKV, it is possible that variations in the amino acid sequence in this region might lead to differences in the efficiency of hemagglutination. This might lead to changes in the apparent titer of viral stocks measured by hemagglutination when comparing different subtypes of BKV. In order to address this possibility, we measured the

Table 2
BKV subtypes I–IV and mutants used in the present study.

Amino acid (VP1)	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83
consensus	D	N	L	R	G	Y	S	L	K	L	S/T	A	E	N	A	F	E	S	D	S	P	D	R
I	E					F					S				D		S					E	
II											T						D						K
III								Q	H		S												
IV	N	D							R		T			T									
I/K69R	E					F			R[a]		S				D		S					E	
I/K69M/D75N	E					F			M[b]		S				N[b]		S					E	
I/E73D/E82N	E					F					S		D[c]		D		S					N[b]	
II/III/N62H		H[b]						Q	H		T						D						K

[a] Amino acid substitution at a subtying position with an amino acid encoded by another subtype.
[b] Amino acid substitution at a subtyping position resulting in a mutation not seen in subtypes I through IV.
[c] Amino acid substitution at a non-subtyping position resulting in a mutation in a region conserved within subtypes I through IV.

Table 3
Percentage of patients with clones of Types I through IV and mutants in this study of 20 patients and 64 clones.

Predominant type	Position	Substitution	Charge	No. patients	Clones	% Total
Type I				15	38	59.4
Type II				0	0	0
Type III				1	1	1.5
Type IV				4	6	9.4
K69R[a]	Subtyping	Type IV	conserved	5	4	6.3
K69N	Subtyping	Mutant	+ to neutral	1	1	1.5
A72V	Conserved	Mutant	conserved	2	2	3.2
D75N[a]	Subtyping	Mutant	− to non-polar	1	2	3.2
P81H	Conserved	Mutant	non-polar to +	1	1	1.5
R83G	Subtyping	Mutant	+ to −	1	1	1.5
E73D[a]	Conserved	Mutant	conserved	1	2	3.2
E82N[a]	Subtyping	Mutant	− to no charge	1	2	3.2
N62H[a]	Subtyping	Mutant	no charge to +	2	5	7.8
E77Q	Subtyping	Mutant	− to no charge	1	2	3.2

[a] Amino acid mutations examined in this study.

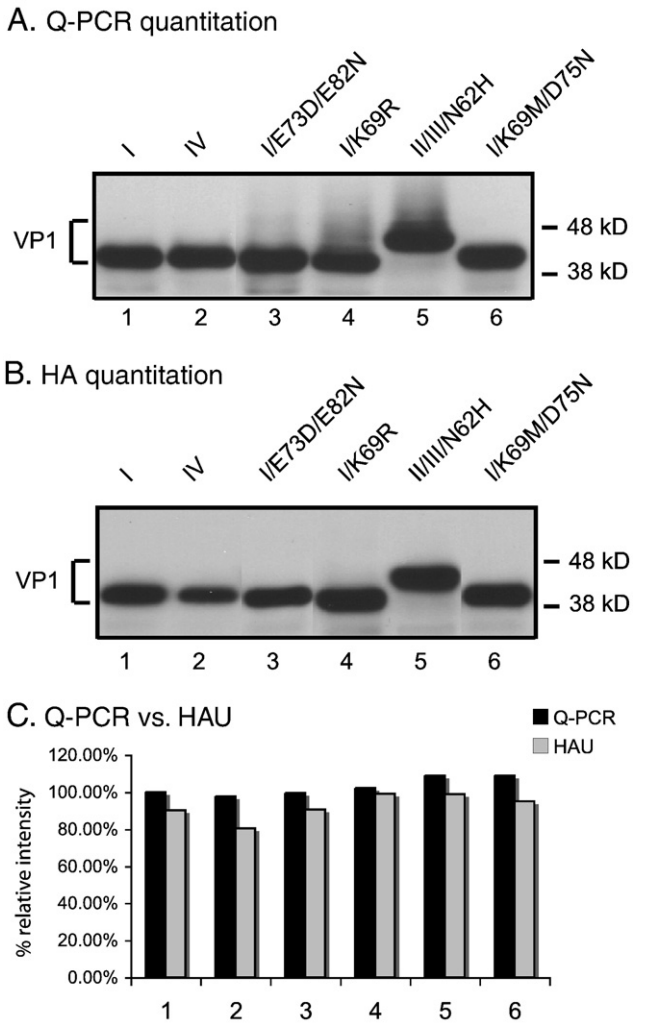

Fig. 1. Comparison of BK virus variants by Q-PCR, HA and Western blotting. BK virus Types I–IV and several variants with amino acid substitutions detected in patients with BKVN were cloned, viral stocks produced in vitro and independently titered by Q-PCR and HA assay. The 1×10^8 viral particles were subjected to Western blot analysis for the major capsid protein, VP1. (A) Immunoblotting of 1×10^8 BK virus particles as determined by Q-PCR shows production of equivalent amounts of VP1 in the major subtype, Type I, in comparison with BK virus Type IV and BKVN variants. (B) Immunoblotting of 1×10^8 particles as determined by HA assay shows similar levels of VP1 in all viral subtypes analyzed. (C) Quantitation of VP1 levels determined in Panel A (Q-PCR) versus Panel B (HA units or HAU) presented as percent relative intensity when compared to the level of VP1 detected in BK virus Type I by Q-PCR, i.e., level of VP1 in Panel A, Lane 1 set at 100%.

titer of viral stocks by both Q-PCR and by hemagglutination and then quantitated the amount of VP1 by immunoblotting (Fig. 1). As shown in the Western blot in Fig. 1A, one million BK virus particles as determined by Q-PCR shows production of equivalent amounts of VP1 for Type I BKV when compared to Type IV BKV and the BKVN variants. Similarly, one million BK virus particles as determined by hemagglutination assay using the conversion factor of 1 HA units = 10,000 plaque forming units (pfu) (as determined previously by others, Miller et al, 1983; Flaegstad and Traavik, 1987) showed similar levels of VP1 by Western blot for the viral stocks of all subtypes analyzed (Fig. 1B). Quantitation of the levels of VP1 in Figs. 1A and B was performed by densitometry, and the results are presented as percent relative intensity when compared to the levels of VP1 detected by Q-PCR for Type I BKV, i.e., all values are normalized to Fig. 1A, lane 1 being set as 100% (Fig. 1C). Good agreement was found in the levels of VP1 measured by Q-PCR (black columns) as compared the levels of VP1 measured by hemagglutination assay (grey columns).

The viruses were compared in parallel using the Vero cell line which is derived from African green monkey kidney cells. Vero cells were chosen for this study since they are able to support productive replication of BKV and have been widely used for basic research of the virology of BKV (Acott et al., 2006). In particular, the morphology and growth pattern of the of Vero cells enabled clear detection of cytopathic effects (CPE) during the course of infection. Vero cell cultures were infected with the different viral stocks as described in Materials and methods. Cultures were infected with comparable numbers of infectious units of each variant per cell, and signs of CPE and cell death (detached, floating cells) were scored and supernatant was collected for Q-PCR analysis over time. Cytopathic effects (CPE) were not observed in any of the cultures before 15 days but some CPE was observed at 26 days in cultures infected with BKV Type I (Fig. 2B) and a significant CPE for BKV Type IV (Fig. 2C). For the variants I/E73D/E82N (Fig. 2D) and I/K69R (Fig. 2H), the CPE was much more pronounced at 26 days when compared to BKV Type I (Fig. 2B). The time course of development of CPE in I/K69R-infected cells is shown in Figs. 2E–H. Some cells showed vacuolization that increased over time. It is interesting that Type I, which is the most prominent subtype, showed the least signs of CPE or cell death. Q-PCR results on supernatants from the infected cells (Fig. 2I) showed that all viral stocks replicated in Vero cells. Interestingly, some of the rare isolates and rare variants (Type IV, I/E73D/E82N and I/K69R) were able to replicate as efficiently as Type I, and yet they caused more CPE even though they replicated at the same level as Type I. These data suggest that virus DNA production may not be a comprehensive way to measure viral fitness and pathogenic potential. Similar results were obtained with the CV-1 monkey kidney cell line and human renal proximal tubule epithelial cells (data not shown).

Discussion

Four subtypes of BKV exist in the human population. Our data indicate distinct differences in viral subtype distribution in virus isolated from BKVN patients versus that found in the normal population. The vast majority of the population worldwide (>80%) is persistently infected with the Type I subtype of BKV, with Type IV being the second most frequent (15%), and genotypes II and III being

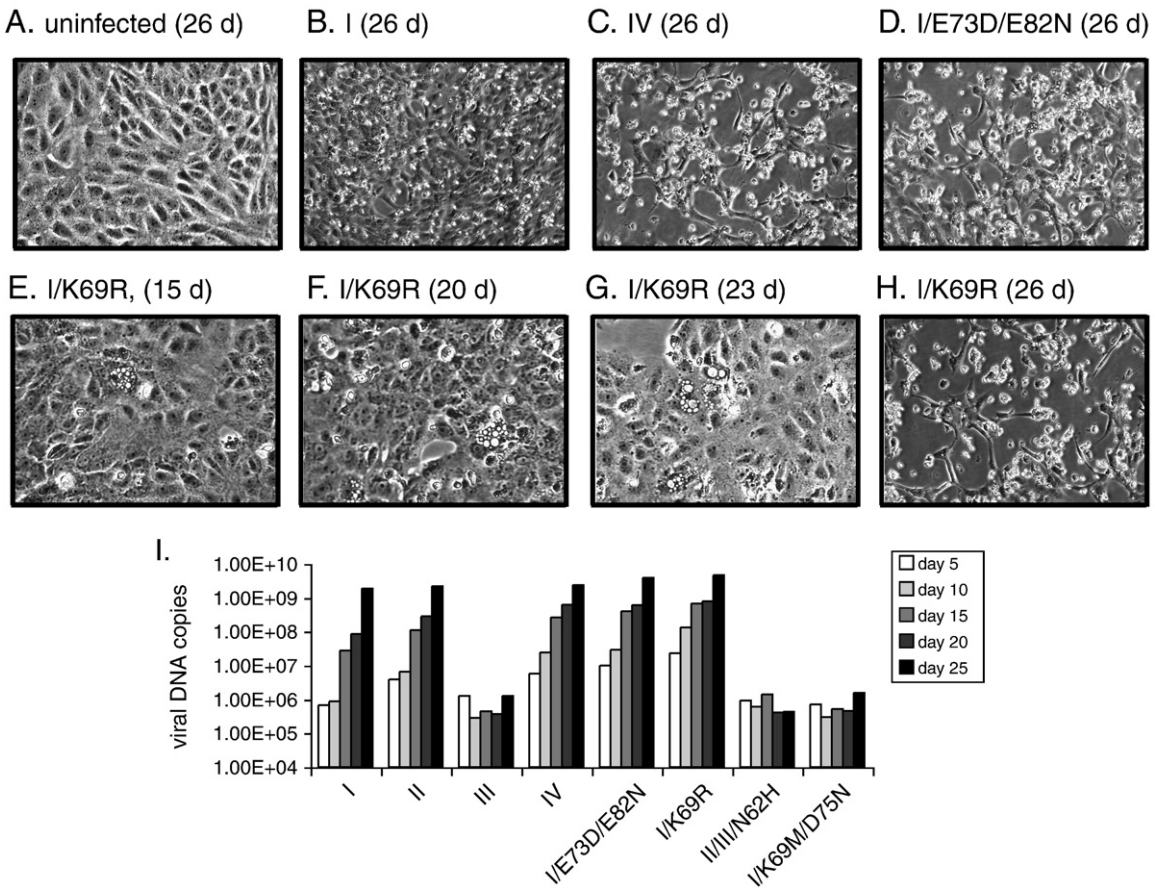

Fig. 2. In vitro replication of BK virus variants in CV-1 and Vero monkey kidney cell lines demonstrates viability of all variants despite differences in replication profile and cytopathic effects. Representative phase-contrast images of Vero cells uninfected (Panel A) versus infected with BK virus Type I (Panel B), Type IV (Panel C) and mutant Type I/E73D/E82N on day 26 post-infection demonstrates significant cytopathic effects in subtypes IV and I/E73D/E82N when compared with Type I. Representative images of Vero cells infected with Type I/K69R on days 15 (Panel E), 20 (Panel F), 23 (Panel G) and 26 (Panel H) post-infection demonstrate progression in cytopathic effects over the course of infection. (I) Replication of BK virus Types I–IV and BKVN variants in Vero monkey kidney cells as determined by Q-PCR.

only infrequently detected in normal adults (Knowles, 2001; Takasaka et al., 2004; Yogo et al., 2009). Here we report that, in urine samples from a patient population with BKVN, Type I was relatively underrepresented and 40% showed the presence of variant subtypes of virus that had amino acid substitutions not corresponding to Type I–IV sequence, i.e., BKVN patients often have rare subtypes of BKV present in their urine. Since the subtype region is involved in hemagglutination and the binding of virions to the viral receptors present on the surface of host cells, this immediately suggested that the differences might be of pathological importance. Our understanding of the importance of these differences is hampered by a lack of fundamental understanding of the molecular events that may be mediated by subtype differences. Therefore, we assessed the virological and molecular biological characteristics of some VP1 subtypes from patient samples in vitro and in a cell culture model.

The subtyping region lies within the major VP1 capsid protein which is prominently displayed on the surface of BKV virions. Differences in VP1 within the subtyping region could thus lead to important differences in infectivity between the subtypes. Additionally, differences in VP1 may be important at late stages of the lytic cycle infection when viral capsids are being assembled. Indeed, we have found that the VP1 subtypes migrate with different mobilities on polyacrylamide gels, even in the presence of SDS, suggesting major structural dissimilarities between these VP1 isoforms which may affect capsid assembly and structure. However, sequence analysis of substitutions in this region do not predict differences in common post-translational modifications such as phosphorylation. In addition, all viruses were viable in our cell culture assay system and quantitation of viral particles by Q-PCR was highly similar among variants as well as in comparison with HA assay results suggesting that capsid assembly and virion formation is not significantly affected.

Analysis of the four BKV subtypes and four variant subtypes, after cloning the subtype region into the same BKV genomic background (Dunlop), revealed that they are all capable of infecting and replicating in a cell culture model system (Vero cells). Two of the Type I variants (I/E73D/E82N and I/K69R) replicated strongly while the other two variants (II/III/N62H and I/K69M/D75N) replicated more slowly. Interestingly, the I/E73D/E82N and I/K69R variants produced a significantly greater CPE than the Type I subtype, suggesting that these variations may be an important correlate of pathogenesis in BKVN. Further studies are needed to address the relationship of subtype variations and BKVN pathogenesis.

Conclusions

In conclusion, our data show that polymorphisms in the VP1 subtype region of virus are frequently found in the urine of patients with BKVN. These polymorphisms strongly influenced the growth of the virus in cell culture and the ability of the virus to induce cytopathic effects. This suggests the possibility that one or more subtype variants may contribute to BKV pathogenesis and that variants may contribute renal allograft damage and subsequent inflammatory response. In future studies, it may be possible to delineate amino acid residues that are crucial for viral growth and pathogenesis, which might facilitate the development of new therapeutic options for BKVN.

Materials and methods

Patient population and accessioning of clinical samples

All patients receiving renal transplants performed at The University of Cincinnati and The Christ Hospitals were offered enrollment in an IRB-approved prospective BKVN study at time of BKVN diagnosis from June 2001 until December 2005. All patients were followed until June 1, 2007. Renal biopsies were performed in patients presenting with an unexplained rise in serum creatinine of at least 20% (SCr). BKVN was diagnosed by light microscopic viral cytopathic changes consistent with BKVN and confirmed by in situ hybridization (ISH) for BK virus on paraffin embedded sections of renal allograft biopsies (Drachenberg et al., 2001). Samples of blood and urine were collected in parallel within 1 week of BKVN diagnosis, stored at 4 °C and accessioned within 24 h of collection. Serum was separated from whole blood by centrifugation, aliquotted and stored at −70 °C until use. Unspun urine was resuspended, aliquotted and stored at −70 °C until use.

Quantitative PCR (Q-PCR) of clinical samples and viral cultures

BK virus loads in patient serum and urine were quantitated by real-time PCR for the presence of BKV DNA essentially as described (Leung et al., 2002; Radhakrishnan et al., 2004). DNA extracted from 40 µl of serum or plasma using the QiaAmp DNA mini kit (Qiagen) or 5 µl of unspun urine was analyzed in triplicate. For virus replication assays, DNA extracted from cell culture pellets using the QiaAmp DNA mini kit was analyzed in triplicate. Q-PCR was performed using primers which represent nt 2511-2531 and 2605-2586 of the VP1 sequence of the Dunlop strain (Type I) of BK virus. Each 50 µl Q-PCR reaction contained 200 and 400 nM of forward (5′-AGTGGATGGGCAGCCTATGTA-3′) and reverse primers (5′-TCATATCTGGGTCCCCTGGA-3′) and 200 nM of BKV-specific probe (nt 2546-2578) (5-FAM-AGGTAGAAGAGGT-TAGGGTGTTTCATGGCACA-BHQ1-3′) in 1× Taqman Universal Master Mix (Perkin Elmer). Cycling conditions were 45 cycles of annealing at 60 °C for 60 s and denaturation at 95 °C for 15 s. Plasmid DNA containing the BK virus Type I (Dunlop) genome served to generate a standard curve against which samples were analyzed using iCycler software (BioRad). All Q-PCR results were calculated as copies per milliliter.

Sequence amplification and analysis

BKV isolates were amplified and cloned from free virus present in urine collected from patients by DNA extraction using the QiaAmp mini-DNA extraction kit. Five microliters of eluate from urine samples was amplified by traditional PCR using primers flanking the subtyping region of VP1 from nt 1630 to nt 1956 to generate a 327-bp PCR product, i.e., 5′-CAAGTGCCAAAACTACTAAT (327-1) and 5′-TGCATGAAGGTTAAG-CATGC (327-2) (Jin et al., 1993), which was subcloned into the vector pCR2.1 using the TA Cloning Kit (Invitrogen, Carlsbad, CA) in order to increase efficiency of the ligation. Individual colonies were propagated, inserts were verified by restriction enzyme digestion with *Xba*I and *Hin*dIII and samples were individually sequenced using T7 and T3 primers.

Cloning of plasmids used in this study

The entire genome of BKV Type I was cloned from pBKV (ATCC), which contains Dunlop strain (Type I) in the *Bam*HI site of pBR322. The BKV Type I genomic DNA was isolated from pBKV by digestion with *Bam*HI, purification by gel electrophoresis, re-ligation, then digestion with *Pst*I. BKV Type I was then ligated into the *Pst*I site of pUC19 after first removing the pUC19 *Bam*HI and *Eco*RI sites by restriction digestion with *Bam*HI and *Eco*RI followed by modification with Klenow. The resulting plasmid, pBKV-DUN, was used as a backbone for replacing the *Bam*HI/*Eco*RI Type I VP1 fragment with VP1 sequences isolated from TA cloning vectors containing *Bam*HI/*Eco*RI genome fragments amplified from patient samples as described above. All constructs were verified by direct sequencing.

Virus production

Cos-7 cells that express the SV40 T-antigen were used for production of viral stocks. Plasmid DNA was digested with *Bam*HI to release the entire genome in linear form, then Cos-7 cells were transfected with the linearized DNA to initiate infection. After transfection, cells were washed and re-fed with DMEM containing 2% FBS. Cells were subcultured for approximately 3 to 4 weeks until CPE were observed, then adherent and non-adherent cells were collected and pelleted. The cell pellet was then freeze–thawed and treated with 0.25% deoxycholic acid to help release the virus. The cellular debris was then pelleted and the virus-containing supernatant was aliquotted and stored at −80 °C until needed. In the case of low-titer stocks, the virus-containing supernatant was used to re-infect a fresh monolayer of Cos-7 cells and the process was then repeated. Viral titer was determined by Q-PCR and HA assay and integrity of the VP1 subtyping region was verified by direct sequencing of denatured viral DNA.

Virus replication assay

Vero or CV-1 African green monkey kidney cells were obtained from the American Type Cell Culture (ATCC). Cells were infected with BKV stocks at a multiplicity of infection of 1 based on Q-PCR results, i.e., one plaque forming unit (pfu) equivalent. Infection was performed in serum-free medium for 3 h at 37 °C. After infection, cells were washed and re-fed with DMEM supplemented with 5% fetal calf serum. Supernatants were collected from uninfected and infected cells at 5, 10, 15, 20 or 26 days post-infection for DNA extraction and cells were subcultured in parallel. Viral DNA was quantitated by Q-PCR.

Hemagglutination assay

Virus was titered by hemagglutination (HA) assay by serial dilution in PBS and incubation with 100 µl of 0.5% human type O erythrocytes in Alsever's buffer after which the samples were allowed to settle for 4 h at 4 °C before reading. The BK virus titers were expressed as the reciprocal of the final dilution in which hemagglutination of the red blood cells was observed (Hamilton et al., 2000).

Western blot analysis

Immunoblotting was performed on isolated viral particles resuspended in TNN buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl and 0.5% NP-40. Viral copies were determined by Q-PCR or HA assay. One million viral particles of BKV subtypes I–IV and four variants were denatured by boiling at 95 °C in SDS–polyacrylamide gel electrophoresis (PAGE) sample buffer, and viral particles were separated by SDS–10% PAGE and transferred to nylon-supported nitrocellulose (Hybond-P; Amersham) overnight at 4 °C. Membranes were blocked in Tris-buffered saline (TBS)–0.1% Tween 20 containing 10% non-fat dry milk for 1 h and incubated for 2 h or overnight in 1x TBS–0.1% Tween 20 containing 0.5% dry milk and antibody to SV40 VP1, which cross-reacts with BK virus VP1 (pAb597; kindly provided by Walter Atwood, Brown University). Proteins were visualized by using horseradish peroxidase-conjugated secondary antibodies and the ECL-Plus system (Amersham) followed by exposure to X-ray film. Quantitation of blots was performed by densitometry calculated as relative intensity of regions of interest (ROI) using Photoshop. Results were confirmed a minimum of twice per viral isolate using at least two different preparations of viral stocks.

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