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TITLE: Role of the Human Polyomavirus, BKV, in Prostate Cancer

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designated by other documentation.
BKV is a human polyomavirus that establishes a lifelong, persistent infection of the urinary tract. The virus encodes oncoproteins that induce tumors in animal models, and BKV DNA has been detected in human urinary tract tumors, including prostate. Prostate tumors have a relatively low frequency of mutations in the \( p53 \) and \( Rb1 \) genes, indicating that an agent such as a virus may be inactivating their functions. The aims of this proposal are to determine if BKV is present in prostate tumors and, if so, whether viral oncogenes are expressed. To accomplish this, normal and tumor tissue from individual patients will be analyzed. PCR, in situ PCR, and in situ hybridization will be performed to determine the presence of viral sequences, and RT-PCR and immunohistochemistry will be used to examine gene expression. Viral sequences from patients will be cloned and their function compared to wild type virus. This past year, we optimized most of these assays and began to analyze samples. We detect the presence and expression of the virus in a subset of normal and cancer cells. At this time, firm conclusions cannot be drawn as more samples need to be analyzed. If BKV is associated with some prostate cancers, our knowledge of the virus will be useful in designing drugs and vaccines for treatment.
# Table of Contents

Cover...........................................................................................................1

SF 298.........................................................................................................2

Table of Contents.......................................................................................3

Introduction.................................................................................................4

Body............................................................................................................4

Key Research Accomplishments.................................................................6

Reportable Outcomes..................................................................................6

Conclusions.................................................................................................6

References....................................................................................................7

Appendices..................................................................................................7
INTRODUCTION

BK virus (BKV) is a human polyomavirus that establishes a lifelong, persistent infection of the urinary tract. The virus encodes two oncoproteins, the large T and small t antigens, that have been shown to cause cancer in animal models, and recently BKV DNA has been detected in various urinary tract tumors including tumors of the prostate. Among the spectrum of human cancers, prostate tumors have a relatively low frequency of mutations in the \textit{p53} and \textit{RB1} genes, indicating that an outside agent such as a virus may be inactivating the function of their gene products. Both large T and small t antigens are capable of doing so. The aims of this proposal are to determine if BKV is indeed present in these tumors and, if so, whether these oncogenic proteins are expressed and function to deregulate cell growth control.

BODY

During the third year of this project we have made significant progress on our analysis of tumor samples. Our progress is described below after the appropriate items from the approved Statement of Work. Please note that rather than reproducing figures from our manuscript in press in \textit{Oncogene} in the body of this report, I have referred to them instead. The manuscript is attached as an Appendix.

Task 1. To examine prostate tumors and matched normal tissue for the presence of BKV sequences (months 1-30).

- isolate DNA and RNA from microdissected normal and tumor tissue (months 1-6)
- perform PCR analysis of DNA (months 3-18)
- perform IS-PCR and ISH analysis of DNA (months 3-24)

This Task is almost complete in that we are now assaying tissue microarrays to boost the number of samples which we have analyzed. In the tissues that we have examined, however, we now have clear evidence for the presence of BKV in both normal ducts and in atrophic epithelium. This has been shown both by solution PCR and by in situ hybridization, both using probes that are highly specific and sensitive for BKV. Thus, the extra time it took us to develop these assays at the beginning of this project has paid off in that our results are very clean and unambiguous. These results are shown in Figures 2 and 4 of the \textit{Oncogene} manuscript, and are also included in the summary (Table 1).

- perform sequence analysis of DNA (months 6-24)

The DNA sequence analysis is shown in Figure 3 of the manuscript. The analysis of the T antigen coding region (early region) proves that the virus we are detecting is BKV and not SV40 or JCV, which are highly homologous to BKV. In addition, the sequence analysis of the regulatory regions indicates that at least three different strains of virus are present in the samples. Indeed, since submitting this manuscript we have performed additional analyses which indicate the presence of at least one other strain in prostate tumors. Of interest is that we do not detect the archetype strain, which is the strain that is thought to remain latent in the urinary tract. Rather, we find strains that are associated with acute disease. Moreover, the fact that we find strains that
we do not study in the laboratory argues that we have not inadvertently contaminated our samples with laboratory virus.

- perform RT-PCR and IS-RT-PCR analysis of RNA (months 12-30)

Having obtained a good handle on the status and sequence of the viral DNA, during the past few months we have turned our attention to the RNA. Again, we have taken our time to carefully determine the best conditions for isolation of the RNA and its amplification for sequencing. We initially have concentrated on the p53 gene since we would predict that it would not be mutant in the BKV-positive cells. This is because the presence of T antigen is equivalent to mutations due to its inactivation of the p53 protein. We began by trying to amplify the entire p53 cDNA in a single reaction. However, this was not possible, probably due to the nature of the RNA extracted from paraffin-embedded tissue. Therefore, we are now amplifying subregions of the mRNA, concentrating on exons 5-9, in which most p53 mutations are found. Rather than sequence the products from RNA isolated from bulk sections, however, we have decided to use laser capture microdissection to purify the cells that are virus-positive, as judged by in situ hybridization and immunohistochemistry (see below). This is because we desire to only analyze the p53 from the affected cells (i.e., cells in the normal and abnormal ducts) and not the total p53 mRNA, which would include undoubtedly wildtype, no matter what the pathology, derived from the stroma. We believe that the sensitivity of our RT-PCR reaction has reached the point where we can now perform it on the small number of cells we will obtain by laser capture.

As we discussed in the past two years' reports, we have added immunohistochemical assays for T antigen to this task. All samples have been analyzed by this method and a fraction of the samples are positive for T antigen expression. Interestingly, while both normal and atrophic epithelia are positive for viral DNA, only the atrophic lesions express T antigen (Figure 5 of the manuscript). Also of interest is the finding that the T antigen is cytoplasmic. This indicates that we are not detecting viral replication, which would require T antigen to be present in the nucleus. We have also stained for p53 protein and in the T antigen positive cells, the p53 co-localizes to the cytoplasm (Figure 6 and 7), indicating that its function has been compromised: as a transcription factor, it must be in the nucleus to function. In those samples that are T antigen negative, the p53 is nuclear. As discussed above, we are very interested in examining the sequence of the p53 gene in these different samples.

Task 2. To determine how alterations in viral sequences in tumors affect the replication and transformation properties of the virus (months 9-36)

- construct genomic viral clones containing patient-derived sequences (months 9-24)
- determine the ability of these clones to replicate in permissive host cells (months 12-36)
- construct early region expression vectors containing patient-derived sequences (months 9-24)
- determine the ability of these clones to interfere with normal growth control pathways involving pRb and p53 (months 18-36)
We have begun to clone the regulatory regions from the patient derived materials. We have also developed an in vitro cell culture system, using primary renal proximal tubule epithelial cells, to study BKV replication in its natural host cell (Virology manuscript by Low, et al., see Appendix). While this was not originally proposed in the application, we found it necessary to develop this system as we found other cell lines suboptimal for studying the viral life cycle. We will begin examining the function of the patient isolates very shortly, which is one of the reasons we have requested and been granted a no-cost time extension for the project.

KEY RESEARCH ACCOMPLISHMENTS

- detection of BKV in normal and atrophic prostatic epithelia
- detection of BKV T antigen in atrophic cells
- co-localization of p53 with T antigen in cells
- sequence analysis of BKV isolates from prostate
- development of in vitro system for assaying BKV replication in its natural host cell

REPORTABLE OUTCOMES

Two abstracts were presented at international meetings this year:

1. Oral presentation by Dr. Das:


2. Poster presentation by Ms. Abend:


Two manuscripts resulted from this work:


CONCLUSIONS

BKV is associated with atrophic lesions of the prostate, which are thought to be the first step in the progression from normal to cancerous epithelium. Viral sequences are present in both normal and atrophic epithelial cells, and viral T antigen, the transforming protein of the virus, is expressed in the latter cells. Moreover, the T antigen co-localizes in the cytoplasm with p53, indicating that the virus is not replicating and the p53 cannot function as a tumor suppressor. The
future goals of this work are to further analyze the BKV-positive cells in an attempt to collect additional evidence that the virus is indeed contributing to neoplastic progression.

REFERENCES

none

APPENDICES

Copies of abstracts and manuscripts reported above in the Outcomes section.
We have previously described human proximal tubule epithelial (HPTE) cells as a model system to study BKV in an environment similar to that which the virus encounters in an immunocompromised host. HPTE cells regulate blood pressure and volume, return soluble molecules to the bloodstream, secrete vitamin precursors, and mediate immune responses to the kidney through the secretion of cytokines and chemokines. The HPTE cells used in our assays are able to maintain their differentiated structure and function for up to six passages in vitro. BKV undergoes a lytic infection in these cells, while SV40 cannot enter them. SV40 is capable of lytic infection after the addition of the ganglioside GM1, recently shown to be an SV40 receptor. We have now elucidated additional interactions between the virus and the cell. Upon infection, BKV downregulates the expression of a set of inflammatory molecules expressed by the HPTE cells. We also find that infecting HPTE cells in the presence of IFN-γ downmodulates the expression of T antigen. These studies suggest that inflammatory molecules produced by HPTE cells may play a role in keeping BKV in a subclinical state either through direct suppression of viral genes, or through the recruitment of immune cells to the site of BKV infection. Upon infecting HPTE cells with SV40 in the presence of GM1, we observe that while the majority of cells are destroyed due to a lytic infection, a subset of cells continues to grow past the point at which uninfected HPTE cells senesce. These cells undergo changes in morphology and produce infectious SV40.
BKV and SV40 Infection of Human Kidney Tubular Epithelial Cells

In Vitro

Jonathan Low, David Humes, Mark Szczypka, and Michael Imperiale, Department of Microbiology and Immunology University of Michigan Medical School, Michigan, USA, 48108

Infection with BK virus (BKV) is nearly ubiquitous in the human population. Primary infection with BKV most often occurs as an asymptomatic infection during childhood followed by a persistent or latent infection of the kidney that can be reactivated upon immunosuppression, often leading to hemorrhagic cystitis or nephritis. Previous studies have found BKV to be tumorigenic in rodents, and there have been reports of the presence of BKV sequences in various human tumors. Little is known about the interaction of BKV with its natural target cells, human kidney epithelial cells. In vitro infections of human primary kidney epithelial cells were performed to simulate a BKV infection in its natural host cell. BKV goes through a lytic replication cycle in this system: high levels of T antigen expression were first detected at 36 hours post-infection, while viral DNA replication and capsid protein expression were observed at 48 hours post-infection. A corresponding production of infectious BKV was also seen at 48 hours post-infection. Levels of p53 in the infected cells increased, and hyperphosphorylated forms of pRb appeared. SV40 causes a similar disease in monkeys and is believed to be able to infect humans. It was observed that SV40 is incapable of infecting human kidney epithelium except in the presence of the GM1 ganglioside, recently reported to be an SV40 receptor.

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Jonathan Low, (734) 936-0876, X5
Detection and Expression of Human BK Virus Sequences in Neoplastic Prostate Tissues

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BK virus is ubiquitous in the human population and establishes a lifelong, subclinical persistent infection in the urinary tract. When the immune system is compromised, it can cause severe disease in the kidney and bladder. Detection of BKV sequences in urinary tract neoplasms has led to the postulation that this virus may induce human oncogenesis through the function of its large T antigen (TAg). In this study, examination of prostate tumor tissue sections using in situ hybridization shows the presence of BKV sequences in benign and atrophic epithelium. Solution PCR on DNA extracted from the tissues and sequence analysis of the products reveal the presence of BKV regulatory and early region sequences. Sequence analysis of the regulatory region reveals the presence of the TU strain in most of the samples, along with the wt-501 and Dunlop strains to a lesser extent. Some of the regulatory regions showed patchy homology to the Gardner, renal and urinary tract isolates of BKV. Additionally, immunohistochemical analysis using monoclonal antibodies specific to TAg or p53 shows the expression of TAg in some of the samples and p53 staining which can be correlated to TAg expression. Though the normal cellular localization of TAg and p53 is nuclear, double immunofluorescence labeling with p53 and TAg antibodies indicates co-localization of p53 and TAg to the cytoplasm in the glandular epithelial cells of the sections. Although BKV DNA was found in benign and atrophic lesions, TAg and p53 co-expression was observed only in atrophic lesions. The implications of these results as they relate to a possible role for BKV in prostate cancer will be discussed.
Detection and expression of human BK virus sequences in neoplastic prostate tissues

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BK virus (BKV) is ubiquitous in the human population and establishes a lifelong, subclinical persistent infection in the urinary tract. When the immune system is compromised, it can cause severe disease in the kidney and bladder. Detection of BKV sequences in urinary tract neoplasms has led to the postulate that this virus may induce human oncogenesis through the function of its large tumor antigen (TAg). In this study, examination of prostate tumor tissue sections using in situ hybridization shows the presence of BKV sequences in atrophic epithelium. Solution polymerase chain reaction on DNA extracted from the tissues and sequence analysis of the products reveal the presence of BKV regulatory and early region sequences. In addition, immunohistochemical analysis using monoclonal antibodies specific to TAg or p53 shows the expression of TAg in some of the samples and p53 staining that can be correlated to TAg expression. Although the normal cellular localization of TAg and p53 is nuclear, double immunofluorescence labeling with anti-p53 and TAg antibodies indicates colocalization of p53 and TAg to the cytoplasm in the glandular epithelial cells of the sections. Although BKV DNA was found in benign and atrophic lesions, TAg and p53 coexpression was observed only in atrophic lesions.

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Keywords: polyomavirus BK; large T antigen; p53; postatrophic hyperplasia; proliferative inflammatory atrophy; in situ hybridization; immunohistochemistry; immunofluorescence; high-grade prostate intraepithelial neoplasia

Introduction

Prostate cancer (PCA) is the second leading cause of cancer deaths in older men in the US (Gonzalgo and Isaacs, 2003; Jemal et al., 2003). In spite of the availability of biomarkers for PCA, the basic molecular mechanisms regulating its development and progression are still very poorly understood (Nupponen and Visakorpi, 1999; Jiang et al., 2001; Kuefer et al., 2002; Rubin et al., 2002; Varambally et al., 2002; Yang et al., 2002). Recognition of the precursor lesions of PCA has become a primary focus in recent years. High-grade prostate intraepithelial neoplasm (HGPIN), characterized by proliferation of epithelial cells in ducts and acini (Bostwick and Brawer, 1987), has been reported to be the most likely precursor lesion for PCA in the US (Myers et al., 1994; Bostwick, 1995; reviewed in Gonzalgo and Isaacs, 2003). Postatrophic hyperplasia (PAH), also referred to as proliferative inflammatory atrophy (PIA), is a proliferative atrophic lesion characterized by a decrease of cytoplasmic mass as compared to benign glands. Atrophic epithelium in PAH has recently been proposed to be a precursor to PIN and PCA (De Marzo et al., 1999; Putzi and De Marzo, 2000; Shah et al., 2001; reviewed in De Marzo et al., 2003; Nelson et al., 2003). Several studies have identified focal areas of epithelial atrophy and have suggested their importance in prostatic carcinogenesis (Franks, 1954; Gardner and Bennett, 1992; De Marzo et al., 2003; Nelson et al., 2003). It has also been reported that atrophy of prostatic glands is common in middle-aged and elderly men (Gardner and Culberson, 1987; McNeal, 1988).

The role of tumor suppressor genes (p53 and Rb) in PCA is not very clear. Mutations in the Rb1 gene are infrequent in PCA (Bookstein et al., 1990; Sarkar et al., 1992; Melamed et al., 1997; reviewed in Abate-Shen and Shen, 2000). Although the mutation rate of the p53 gene in PCA is also low, p53 inactivation has been implicated in PCA progression (Bookstein et al., 1993; Bauer et al., 1995; Brewster et al., 1999; Osman et al., 1999; reviewed in Abate-Shen and Shen, 2000). These observations have led researchers to speculate that an infectious agent might play a role in PCA (McNicol and Dodd, 1991; Ibrahim et al., 1992; Rotola et al., 1992; Cuzik, 1995; Noda et al., 1998). Polyomaviruses are candidates for this agent, although the etiological role of polyomaviruses in human tumors remains debated despite several reports demonstrating the presence and expression of sequences from these viruses (Imperiale, 2000, 2001; Reploeg et al., 2001; Tognon et al., 2003).

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BKV and prostate cancer
D Das et al

BK virus (BKV), a polyomavirus, was first isolated from the urine of a renal transplant patient (Gardner et al., 1971). It persistently infects almost 100% of the human population by early childhood (Gardner, 1973; Padgett, 1981; Dörries et al., 1994). It resides in the kidneys in a latent or persistent state, but can be reactivated upon immunosuppression of the host and is associated with hemorrhagic cystitis and polyomavirus nephropathy (Arthur et al., 1986; Hiraoka et al., 1991; Pappo et al., 1996; Randhawa et al., 1999; Li et al., 2002). BKV oncogenically transforms rodent cells in culture, causes kidney tumors in transgenic mice, and transforms primary human cells in culture when coexpressed with an activated ras oncogene (Portolani et al., 1975; Pater and Pater, 1986; Small et al., 1986; Dalrymple and Beemon, 1990). BKV has been reported to be detected in a number of human tumors, including those in the urinary tract, brain, pancreatic islet, lung, eye, liver, Kaposi's sarcoma, and bone (reviewed in Imperiale, 2000, 2001; Reploeg et al., 2001; Tognon et al., 2003).

The BKV genome is divided into regulatory, early, and late regions, and encodes five major proteins (Figure 1; Seif et al., 1979; Frisque et al., 1984; Pipas, 1992). The early proteins, encoded by alternatively spliced mRNAs, are the large tumor antigen (TAg), the main regulatory protein, and the supporting small tumor antigen. TAg aids in the oncogenic transformation of cells through its ability to bind and inactivate the products of tumor suppressor genes, including p53 and

![Figure 1](image-url)
the pRb family (Nakashatri et al., 1988; Dyson et al., 1989, 1990; Kang and Folk, 1992; Simmons, 1995; Staib et al., 1996; Shivakumar and Das, 1996; Harris et al., 1996, 1998a, b). TAg also regulates viral DNA replication and gene expression, and coregulates activated and basal cellular transcription factors (Moens and Rekvig, 2001). Small tumor antigen is also involved in transformation and viral and cellular DNA replication (Moens and Rekvig, 2001). The late proteins, the capsid proteins VP1, VP2, and VP3, are also expressed from alternatively spliced mRNAs, after viral DNA replication. The regulatory region contains promoter/enhancer elements of the early and late genes and the origin of DNA replication (Imperiale, 2001; Replog et al., 2001; Hirsch and Steiger, 2003). Different BKV strains show sequence variability in their regulatory regions. These strains derive from a common archetype strain, which infects the general population, due to deletions, duplications, and rearrangements in the regulatory region (Rubinstein et al., 1987; Shah, 1996).

The role of BKV in human neoplasia is not clear, due to conflicting reports. In the urinary tract, Monini et al. (1995) demonstrated the presence of BKV sequences by polymerase chain reaction (PCR) in more than 50% of both normal and tumor tissues, including prostate. Viral DNA could be detected in some tumors by Southern blotting, whereas PCR amplification was always required to find BKV in normal tissues, and further analysis revealed that viral sequences were integrated into the host chromosome in the six tumors that were positive by Southern blotting. Sequence analysis of the viral origin of replication discovered the presence of point mutations in one of the samples, defining it as a new strain of BKV, designated as URO1. It has been shown in cell culture that similar mutations impair the replication ability of the virus, leading to an increased transformation potential (Shinhara et al., 1993). Hence, it was speculated that mutant genomes impaired for replication might integrate into the host genome and eventually lead to cancer. Another report found high levels of BKV TAg expression in primary and metastatic bladder carcinoma but not in non-neoplastic urothelium (Geetha et al., 2002). In these studies, BKV TAg and p53 were colocalized to the nuclei of tumor cells. Given the results obtained with prostate and bladder carcinoma, along with kidney being the main site for BKV persistence, tumors of the urinary tract are the most logical target sites for an etiological association with BKV. Since p53 and Rb1 mutations are not ubiquitous in PCA, we reasoned that TAg might inactivate their function in a subset of tumors, similar to the role of the human papillomavirus E6 and E7 gene products in cervical carcinomas (Scheffner et al., 1991; Ibrahim et al., 1992; Iwasaka et al., 1993; Cuzik, 1995). Hence, we were interested in screening prostatic carcinomas for BKV sequences and, if present, in determining if DNA could be correlated to protein expression.

In this report, we demonstrate the presence of BKV sequences in the epithelium of benign ducts and atrophic lesions of neoplastic prostate tissues using in situ and solution analysis. The primers and probe used in our studies are BKV specific and do not crossreact with JCV or SV40. Using immunohistochemistry, we also detected the expression of TAg in the atrophic lesions. Moreover, TAg expression could be correlated to p53 expression in this set of samples. Interestingly, double immunofluorescence labeling studies demonstrate that TAg and p53 colocalize to the cytoplasm, but in the TAg-negative tumors, p53 is nuclear.

Results

Before beginning to analyze resection specimens (patient samples) for the presence of BKV, we developed PCR and in situ hybridization (ISH) conditions and reagents that would have a high degree of specificity and sensitivity for BKV and not the related viruses, SV40 and JCV, which share about 69–75% sequence identity (Seif et al., 1979; Frisque et al., 1984; Pipas, 1992). To demonstrate this specificity, PCR amplification of plasmids containing SV40, JCV, and BKV sequences was performed (Figure 2a). Using the early region primers, BKVf o r (4935–4907) and BKV r e v (4358–4382), a product of 577 base pair (bp) was observed with the BKV plasmid and no product was detected with SV40 or JCV. A Southern blot of the products using the BKV2 (4474–4479) probe, which was also used for ISH studies, confirmed the sequence-specific amplification of the BKV early region. In these reactions, we could detect as little as 0.1 fg of BKV DNA (data not shown). Similarly, both the nested primers to the early region and the regulatory region were determined to be sequence specific, although the regulatory region assay is less sensitive (data not shown). A direct Southern blot of BKV, JCV, and SV40 plasmids with the BKV2 probe confirms its specificity (Figure 2b).

Having developed specific assays, we began analysing patient samples. In all, 21 neoplastic adenocarcinoma prostate tissue samples were selected for evaluation (Table 1). To test for the presence of BKV sequences, DNA extracted from formalin-fixed paraffin-embedded samples was analysed by solution PCR using regulatory and early region primers. Extreme care was taken to avoid sample contamination: plasmid controls were not analysed with the tissue samples, different sets of pipettors were used for the tissues and plasmids, and high-quality DNA grade water was used. The integrity of the DNA was evaluated by amplification of the β-globin gene. Nested amplification was employed for the regulatory and early regions since it was possible that the copy number of the viral genome would be low. First-round amplification was performed for 30 cycles for the early region, after which a product was detectable only by Southern blot (data not shown); after a second round (30 cycles) of amplification with nested primers, enough product resulted for direct detection by ethidium bromide staining. For the regulatory region, two rounds of 40 cycles were required to detect the PCR product due to the lower sensitivity of this assay. Representative EtBr-stained agarose gels of
the PCR amplification products of DNA are shown in Figures 2c and d and the results from an average of at least three independent amplifications are summarized in Table 1. A correct size early region product of 452 bp was obtained in 12/16 of the samples every time they were tested. Some samples, such as 4, 5, and 13, were positive for the early region all but one time. Of the samples, 13 yielded PCR products of approximately 300 bp with the regulatory region primers, of which 11 were positive each time they were assayed. The product size for the regulatory region is variable due to possible rearrangements. Seven of the regulatory regions were
confirmed to be BKV by sequence analysis (see below). Sample 9 was positive for the early region in three out of six amplifications and was positive for the regulatory region.

To confirm the identity of the PCR products, they were sequenced (Figure 3). Identical results were obtained from all the early region samples and the sequences were identical to the published BKV genomic sequence (Figure 3a). The majority of the regulatory regions showed identity or similarity to the TU strain (Sundsfjord et al., 1990), while sample 9 appears to be most similar to Dunlop (Figure 3b). Interestingly, one sample (#5) appeared to have two different regulatory regions, one identical to TU and the other most similar to wt-501 (Watanabe and Yoshiike, 1989). We were unable to obtain clean sequencing data from some of the regulatory region products. Products that were not able to be sequenced directly were cloned into a plasmid vector and sequenced, but these sequences did not appear to be homologous to BKV. Thus, 44% of the samples contained regulatory regions that could be confirmed to be BKV.

We wished to use an independent means of detecting BKV DNA in these samples. We chose ISH using an independent probe as compared to scrambled probe, which has the same G+C content as the BKV2 probe. The signal was localized specifically to the nuclei of epithelial cells in the breast samples (panel G) and stromal lesions (panels D and E). We did not detect any signal in stromal cells or in invasive tumor cells. Breast carcinoma tissue sections processed in the same manner as the prostate tissues did not react with this probe (panel H). The ISH studies revealed BKV sequences in 15/21 of the prostate samples.

We next examined the expression of TAg in these samples using immunohistochemical (IHC) labeling. The absence of TAg would be a strong evidence against a role for BKV in oncogenesis. We first examined BSC-1 and BSC-BKT cells as controls (Figure 5). TAg was detected only in the nucleus in BSC-BKT cells, whereas BSC-1 cells were negative for TAg. IHC labeling of prostate samples revealed the expression of TAg in the epithelial cells of atrophic lesions but not in tumor or normal epithelium, or stromal cells (panels D and E). Surprisingly, the TAg appeared to be localized to the cytoplasm/perinuclear compartment. A serial section probed with a monoclonal antibody to a 90 kDa tumor-associated glycoprotein expressed in mammary carcinomas gave nuclear staining in the breast samples (data not shown) (Colcher et al., 1981). The IHC labeling results reveal that 9/21 of the prostate tissue sections are positive for TAg expression, that the TAg is mostly cytoplasmic, and that it is expressed in atrophic lesions.

Next, we were interested in examining the prostate samples for the expression of p53, which becomes stabilized and inactivated on association with viral oncoproteins including TAg (Lane and Crawford, 1979; reviewed in Pipas and Levine, 2001). Anti-p53 staining results fall into three categories (Figure 6): TAg and BKV DNA negative (nuclear p53); p53 and TAg positive (cytoplasmic/perinuclear localization of both proteins in atrophic epithelium, panels D and E); and TAg negative but BKV DNA positive (nuclear p53 in atrophic prostate gland; panels F and G). TAg-negative BSC-1 cells stain weakly for p53, whereas the TAg-positive BSC-BKT cells show nuclear staining (panels A and B). Breast carcinoma tissue sections had robust p53 staining (panel H) (Cattoretti et al., 1988; Moll et al., 1992), and isotype-matched control antibodies did not stain the prostate (panel C). We did not detect any p53 staining in stromal cells or normal epithelium. Since p53 and TAg expression could be correlated, with what appeared to be identical staining patterns, it was of interest to examine p53 and TAg colocalization using double immunofluorescence labeling. The results show staining in the epithelial cells of the atrophic lesions, with cytoplasmic colocalization of both proteins (Figure 7).

### Table 1  Data summary

<table>
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The samples have been listed in groups based on staining patterns. Strain homology is indicated after + symbol. ND, sequence not determined; N, negative; +, positive and cytoplasmic; + (N), positive and nuclear. Sample was PCR positive with early region primers four out of five times. Sample was PCR positive three out of four times. Sample was PCR positive with early region primers four out of five times. Sample was PCR positive three out of four times.

Listed are the results of early region PCR and ISH on prostate tissues. Samples 18 and 19 were excluded from the analysis because they did not react with the BKV2 probe. The signal was localized specifically to the nuclei of epithelial cells in the benign ducts (panel G) and stromal lesions (panels D and E). We did not detect any signal in stromal cells or in invasive tumor cells. Breast carcinoma tissue sections processed in the same manner as the prostate tissues did not react with this probe (panel H). The ISH studies revealed BKV sequences in 15/21 of the prostate samples.

The absence of TAg would be a strong evidence against a role for BKV in oncogenesis. We first examined BSC-1 and BSC-BKT cells as controls (Figure 5). TAg was detected only in the nucleus in BSC-BKT cells, whereas BSC-1 cells were negative for TAg. IHC labeling of prostate samples revealed the expression of TAg in the epithelial cells of atrophic lesions but not in tumor or normal epithelium, or stromal cells (panels D and E). Surprisingly, the TAg appeared to be localized to the cytoplasm/perinuclear compartment. A serial section probed with a monoclonal antibody to a 90 kDa tumor-associated glycoprotein expressed in mammary carcinomas gave nuclear staining in the breast samples (data not shown) (Colcher et al., 1981). The IHC labeling results reveal that 9/21 of the prostate tissue sections are positive for TAg expression, that the TAg is mostly cytoplasmic, and that it is expressed in atrophic lesions.

Next, we were interested in examining the prostate samples for the expression of p53, which becomes stabilized and inactivated on association with viral oncoproteins including TAg (Lane and Crawford, 1979; reviewed in Pipas and Levine, 2001). Anti-p53 staining results fall into three categories (Figure 6): TAg and BKV DNA negative (nuclear p53); p53 and TAg positive (cytoplasmic/perinuclear localization of both proteins in atrophic epithelium, panels D and E); and TAg negative but BKV DNA positive (nuclear p53 in atrophic prostate gland; panels F and G). TAg-negative BSC-1 cells stain weakly for p53, whereas the TAg-positive BSC-BKT cells show nuclear staining (panels A and B). Breast carcinoma tissue sections had robust p53 staining (panel H) (Cattoretti et al., 1988; Moll et al., 1992), and isotype-matched control antibodies did not stain the prostate (panel C). We did not detect any p53 staining in stromal cells or normal epithelium. Since p53 and TAg expression could be correlated, with what appeared to be identical staining patterns, it was of interest to examine p53 and TAg colocalization using double immunofluorescence labeling. The results show staining in the epithelial cells of the atrophic lesions, with cytoplasmic colocalization of both proteins (Figure 7).
BKV Tag in neuroblastomas has been reported. PCR has been viewed with skepticism by some, due to examine Tag expression, however. The presence of Detection of viral sequences in tumors using solution with archival specimens. These investigators did not detect BKV sequences in neoplastic prostate specimens.

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the presence of BKV DNA in urinary tract tumors: We analysed PCA specimens for BKV because of concerning the oncogenic potential of the virus (Im-
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determine if there was a correlation. Since BKV is bladder carcinoma found high expression of BKV Tag
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The experiments in this study were designed to that the antibody used by these authors is not specific

Discussion
The experiments in this study were designed to investigate if BKV sequences are present in neoplastic prostatic tissues. In addition, we wanted to examine viral oncoprotein expression and cellular p53 levels, and determine if there was a correlation. Since BKV is ubiquitous in the human population, there is speculation concerning the oncogenic potential of the virus (Imperiale, 2000, 2001; Tognon el al., 2003). The presence of BKV sequences in tumors has been reported in several studies (De Mattei el al., 1995; Monini et al., 1995, 1996; Flaugstad el al., 1999; Jorgensen et al., 2000; Geetha el al., 2002). There are contradictory reports on the presence of BKV DNA in urinary tract tumors: Monini et al. (1995) detected BKV DNA using PCR in 31/52 samples, whereas Völter et al. (1997) were unable to find it (0/15). Zambrano et al. (2002) detected BKV in 4/50 fresh tissue prostate samples and were unsuccessful with archival specimens. These investigators did not examine Tag expression, however. The presence of BKV Tag in neuroblastomas has been reported (Flaugstad et al., 1999), but these studies have been called into question by a report which demonstrated that the antibody used by these authors is not specific for Tag (Zambrano and Villarreal, 2002). The antibody we used is specific for Tag and does not cross react with any cellular proteins (Harlow et al., 1981). A report on bladder carcinoma found high expression of BKV Tag in primary or metastatic tumors but not in normal epithelium (Geetha et al., 2002). In these studies, p53 was colocalized with Tag to the nuclei of tumor cells, suggesting stabilization and inhibition of p53 function by Tag. This report by Geetha et al. (2002) is the first in which BKV localization to tumor cells was demonstrated, suggesting a causal role for BKV in oncogenesis.

We analysed PCA specimens for BKV because of these previous reports along with the facts that the urinary tract is the main site for BKV persistence and that prostate carcinomas have a low frequency of p53 and Rbl mutations. Using ISH and solution PCR, we detect BKV sequences in neoplastic prostate specimens. Detection of viral sequences in tumors using solution PCR has been viewed with skepticism by some, due to

**Figure 3** Sequence alignment of BKV isolates from prostate samples. (a) Alignment of three patient samples with early regions of BKV, JCV, and SV40. Identical sequences are in bold. (b) Alignment of four patient samples with BKV regulatory regions of archetype, MM, Dun, TU, UROI, wt-501, and Gardner strains (Seif et al., 1979; Yang and Wu, 1979; Berg et al., 1981). There are contradictory reports on stratified, suggesting a causal role for BKV in oncogenesis.
after both rounds of PCR amplification, indicating that region in 81% of the archival specimens, although we controls without any DNA gave no PCR product even amplified the BKV early region in 94% and regulatory

Figure 3

Archetype

Gardner TG-CGAGC--C
Dunlop CGACAAGCT TCAGTG AAAGTTGGTAAAAC

Sample 5

TG-CGAOC--CTAGG AATCTTGGCA----

Sample 9

G--------- ---- -----CCAA--AGOC

wt-501

GACATGTTTTGCGAG CCTAGGAATCTTGGC ----

Sample 12

AAAGAAGCTGCTGT9AG CTGGCGGCTAATAGG AGCCAGCAGTCTGCTG----TG GCAGCAG----TGCA

Figure 3 Continued

the possibility of false positives caused by contamination of reagents with laboratory viral sequences. Our controls without any DNA gave no PCR product even after both rounds of PCR amplification, indicating that the positive signals were due to authentic amplification of DNA from the tissues. Moreover, our ISH results confirm the presence of viral DNA in the samples. We amplified the BKV early region in 94% and regulatory region in 81% of the archival specimens, although we could only confirm the sequence of the regulatory region

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Figure 4 Detection of BKV DNA in human PCA using ISH. (a and b) BSC-I and BSC-BKT cells, respectively, stained with BKV specific probe. (c-e) Atrophic regions of the prostate tissue section stained with scrambled probe (c) and BKV-specific probe (d and e). (f and g) Benign regions of the prostate tissue section stained with scrambled and BKV specific probe, respectively. (h) Breast carcinoma stained with BKV probe. Magnification: × 400
Figure 5  Expression of TAg in prostate samples. (a and b) BSC-1 and BSC-BKT cells, respectively, immunostained with anti-TAg antibody. (c–e) Atrophic regions of prostate tissue sections stained with IgG<sub>1</sub>, isotype control (c) or anti-TAg (d and e). (f) Prostate tissue section stained with anti-AR. (G) Breast carcinoma stained with anti-TAg. Magnification: ×400 (a–c, g, and f); ×100 (d); and ×1000 (e)
Figure 6  Expression of p53 in prostate samples. (a and b) BSC-1 and BSC-BKT cells, respectively, immunostained with anti-p53 antibody. (c-e) Atrophic regions of prostate sections stained with IgG2 isotype control (c) or anti-p53 (d and e). (f and g) TAg-negative prostate tissue samples stained with anti-p53 (f) or anti-TAg (g). (h) Breast carcinoma stained with anti-p53. Magnification: × 400 (a–c, f, and g); × 100 (d); and ×1000 (e).
BKV and prostate cancer
D Das et al

Figure 7 Colocalization of TAg and p53 expression in prostate samples. (a–d) BSC-I cells. (a) DAPI staining; (b) anti-p53- and FITC-conjugated secondary; (c) anti-TAg- and TR-conjugated secondary; (d) merged image of (b and c). (e–h) BSC-BKT cells. (e) DAPI staining; (f) anti-p53- and FITC-conjugated secondary; (g) anti-TAg- and TR-conjugated secondary; (h) merged image of (f and g). (i–l) Atrophic regions of the prostate. (i) Anti-p53- and FITC-conjugated secondary; (j) anti-TAg- and TR-conjugated secondary; (k) merged image of (i and j); (l) image (k) merged with DAPI stain to show nuclei in 44% of the samples. Some samples were positive for the early region but not the regulatory region. Since the regulatory region has been shown to be prone to rearrangement, it is possible that rearrangements led to loss of recognition by the primers. In addition, the regulatory region PCR assay is less sensitive than that used for the early region. Our DNA sequencing data indicate that the early regions are identical to the early region of BKV. We found a number of regulatory region sequences, which were homologous to the TU, Dunlop, and wt-501 strains. There is no obvious correlation between the regulatory region sequences and the histopathology of the samples. Owing to the heterogeneity of the cells in each sample, drawing such a correlation will require sequence analysis of DNA isolated from specific cells in each section rather than from bulk DNA isolated from an entire section. We have not determined if BKV is present episomally or is integrated into the genome. Our ISH studies detected BKV sequences in 71% of the samples, and in all cases, the signal localized to the benign and proliferative atrophic lesions.

IHC analysis detected TAg expression in 43% of the samples. TAg was expressed in focal atrophic lesions (simple atrophy) and in hyper-PIA, but was undetectable in either benign or tumor areas of the tissues. Since our ISH and PCR analyses specifically detect BKV DNA that can be correlated to TAg expression in the same location, we are confident that we are detecting BKV TAg and not that of JCV or SV40, which are also recognized by this antibody. We speculate that BKV infects normal cells, induces the cells to PAH or PIA, and these eventually transform to tumor cells, since atrophic lesions are proposed to be precursors to HGPIN. It is unclear why BKV might be lost upon progression to frank carcinoma, but it is possible that the reported proapoptotic effects of TAg, mediated through interactions with pRB family proteins, or chromosomal damaging effects of TAg, might be incompatible with growth at that stage of carcinogenesis (Theile and Grabowski, 1990; Tognon et al., 1996; Trabanelli et al., 1998).

TAG expression could be correlated to elevated p53 expression in the atrophic lesions and both proteins were localized to the cytoplasm of the secretory cells. In the absence of TAg, p53, where detectable, was nuclear. It is unclear why these two proteins, particularly TAg, are not entering the nucleus, although this might be related to the function of the cellular protein, Parc, which has previously been shown to sequester p53 in the cytoplasm (Nikolaev et al., 2003). In the cytoplasm, TAg would not be able to support replication of the virus. We propose that TAg drives proliferation of these cells through its interactions with pRB and, by sequestering p53 in the
cytoplasm, inhibits p53-mediated cell cycle arrest or apoptosis (Knippschild et al., 1996; Moll et al., 1996). Two groups have observed cytoplasmic BKV and SV40 TAg in neuroblastoma and thyroid tumors, respectively (Jorgensen et al., 2000; Vivaldi et al., 2003). There is also precedence for the occurrence of cytoplasmic p53 in human tumors, including inflammatory breast cancer, neuroblastoma, colon carcinoma, and malignant melanoma (Moll et al., 1992; Castresana et al., 1993; Bosari et al., 1994, 1995; Moll et al., 1995; Weiss et al., 1995). To our knowledge, there is no report of cytoplasmic p53 in PCA. Since the cytoplasmic staining of p53 is mostly in atrophic lesions, it is possible that others might not have classified these lesions as cancer, however. Together, these results suggest that a defect in p53 nuclear import could contribute to tumorigenesis.

Our present findings support the hypothesis that PIA lesions are precursors to HGPIN or PCA, as suggested previously. Distinct histopathological relationships between cells found in PIA, HGPIN, and PCA have been reported, suggesting that PIA develops into HGPIN or PCA (De Marzo et al., 1999). PIA is frequently associated with acute and chronic inflammatory processes and is highly proliferative when compared with normal epithelium (Ruska et al., 1998). Atrophic regions containing proliferative epithelial cells, which are unable to differentiate into columnar secretory cells, are usually located in the periphery of the prostate, where PCAs most often occur (Nelson et al., 2003). Although there is evidence to support the association between inflammation and PCA, the exact mechanism still remains elusive. We speculate that BKV infects benign glands, transforms them to atrophic lesions through TAg expression, and this ultimately leads to HGPIN and cancer.

The results in this report are in agreement with an earlier report of the presence of BKV DNA in prostate tumors (Monini et al., 1995). Our data extend this finding by demonstrating expression of TAg in atrophic lesions in the tumors, where it colocalizes with p53. The presence of the BKV genome and, more importantly, expression of its early protein TAg, is suggestive of a role in the development of PCA. These observations emphasize the need for rigorous and more detailed investigations of the association of this virus with PCA and the elucidation of pathways that may be regulated by BKV.

Materials and methods

Human tissue specimens

Paraffin-embedded adenocarcinoma prostate resection specimens from radical prostatectomies, staged using standard TNM (primary tumor, regional nodes, metastasis staging) criteria, were obtained from the Tissue Procurement Core at the University of Michigan Comprehensive Cancer Center (UMCCC). One section from each of the tissue specimens was stained with hematoxylin and eosin to confirm diagnosis. The samples were a mixture of three different histological types: benign, atrophic, and tumor. Each case was evaluated for benign, atrophic, or tumors by the pathologist. Paraffin-embedded breast tissue blocks were obtained from the breast tissue procurement core at the UMCCC. The Gleason scores of the tissue specimens are listed in Table 1.

DNA extraction

Eight serial paraffin-embedded 5-um tissue sections were used for DNA extraction. The sections were treated with 100% xylene for 2 x 10 min at room temperature to remove the paraffin, washed with 100% ethanol, and dried in a Savant SpeedVac using medium heat. The EX-WAX™ extraction kit (Serological Corp.) was used to extract DNA as indicated by the manufacturer, with minor modification. DNA was precipitated from the tissues by incubation at -80°C for 2 h and dissolved in 50 µl of resuspension solution from the manufacturer. The DNA was subsequently stored at -20°C. A 2-µl aliquot was used in the PCR assays. The extraction process was performed in an area that was BKV free and care was taken to avoid crosscontamination of the samples by frequent changing of gloves between samples.

Oligonucleotide primers and probes

The sequence of oligonucleotide primers and probes used for these studies are shown in Figure 1. The human β-hemoglobin gene primers, KM38 and PC03, amplify a 167 bp fragment (Vilchez et al., 2002).

The first-round BKV early region PCR was performed using primers BKVforf(4935-4907) and BKVrevf4878) to generate a 577 bp PCR product. The second, nested round was performed using BKVnesfrevf4985-4871) and BKVnesfrevf4443-4467), which amplify a 452 bp product. The first round BKV regulatory region PCR was performed using primers BKRRLTf1599-101) and BKRRLT(1599-400 (Monini et al., 1995), yielding a 379 bp product. The nested, second round was performed using primers BRP163-101) and BRP2338-339, resulting in a 276 bp product if there is no rearrangement of the regulatory region.

The BKV early region oligonucleotide probe for ISH and Southern blotting, BKV2(4443-4478), was 45 bases, and is modified from a previous report (Bersagel et al., 1992). A scrambled probe with the same length and G + C content was used as a control in the ISH studies. The sequence of the scrambled probe is: 5'TGTTGATGTAGGATATGCGTCTG TTCTTCTTACCCGTGCACAA3'.

Plasmids

The plasmids used to determine the specificity of the PCR and probe were pBK-GEM, containing the BKV early region (Harris et al., 1996); pJC, containing the complete JCV genome, strain Mad1 (ATCC #45027); and pJYM, containing the complete SV40 genome, strain 776 (Lusky and Botchan, 1981).

PCR amplification

Extracted DNA was amplified using Titanium™ Taq DNA polymerase (Clontech) in a ThermoHybaid thermocycler (PX2). All reactions were performed in a final volume of 100 µl containing 200 nM each primer, 200 µM dNTPs, 2 µl template, and 1 U polymerase in 0.5 x buffer (20 mM Tricine-KOH, pH 8.0, 8 mM KCl, 1.75 mM MgCl₂, and 1.87 µg/ml bovine serum albumin (BSA)). The quality of the DNA was first ascertained by PCR (45 cycles) with the β-globin primers. The program used for β-globin amplification consisted of initial denaturation at 94°C for 5 min followed by denaturation at 94°C for 30 s, annealing at 43°C for 30 s, and elongation at
72°C for 1 min, followed by final elongation at 72°C for 7 min. For early region amplification, two rounds of 30 cycles each were used. The template for second-round amplification consisted of 30 μl product from the first-round amplification. The program consisted of initial denaturation for 5 min at 94°C followed by denaturation at 94°C for 30 s, annealing at 47°C for 30 s, and elongation at 72°C for 1 min, followed by final elongation at 72°C for 7 min. For regulatory region amplification, each reaction was increased to 40 cycles and 1 min for each reaction. The program consisted of initial denaturation for 5 min at 94°C followed by denaturation at 94°C for 30 s, annealing for 30 s, and elongation at 72°C for 1 min, followed by final elongation at 72°C for 7 min. The annealing temperature for primers BKRR/LTI/BKRR/LT6 was 52°C and BRP1/BRP2 was 47°C. The negative control tube contained all components except DNA template. Plasmid DNA controls were not amplified at the same time as tissue samples, to avoid cross contamination.

Sequence analysis

PCR products were separated by agarose-gel electrophoresis, extracted (Qiagen gel extraction kit, Qiagen), and sent for sequencing by the DNA Sequencing Core at the University of Michigan. Samples that did not yield interpretable results were cloned into pGEM-T easy (Promega) and sequenced. The sequences were analysed using the ClustalW server alignment (NCBI Server).

Cell fixation for in situ studies

Cells in culture were fixed using a slightly modified published protocol (Berger, 1986). BSC-1 (African green monkey kidney cells) and BSC-BKT cells were grown on sterilized charged Silane prep™ slides (Sigma) in Dulbecco's modified Eagle's medium (GIBCO), supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum, at 37°C in a 5% CO₂ incubator, and fixed by 20 min incubation in 4% paraformaldehyde solution (16% stock obtained from Electron Microscopy Sciences) at room temperature. After fixation, the cells were rinsed in phosphate-buffered saline (PBS), first with 3 x PBS (5 min) and then 1 x PBS (3 x 2 min). The slides were dehydrated using increasing concentrations of ethanol (50, 70, and 95%; 5 min each at room temperature), air dried, and stored at −80°C.

In situ DNA hybridization (ISH)

ISH was performed using a modified protocol (Singer et al., 1986; Nuovo, 1996; Wiedorn et al., 1999), with digoxigenin (DIG)-labeled 45 bp probes. The probes were labeled with terminal transferase using the DIG oligonucleotide tailing kit (Roche). Formalin-fixed paraffin-embedded sections (4 μm), mounted on AES charged slides, were deparaffinized by immersing in xylene solution (2 x 5 min) and then rehydrated by incubation with decreasing concentration of ethanol (95, 75, and 50%; 1 min each). The sections were incubated in Tris-buffered saline (TBS; 0.1 M Tris-HCl, pH 7.4, and 0.15 M NaCl) for 10 min before being treated with proteinase K (4.5 μg/ml in TBS) at 37°C for 25 min for permeabilization. The proteinase K treatment was stopped by washing in TBS rigorously for 10 min, with a change of buffer after 5 min. This was followed by two 10 min washes, first with ddH₂O then with ethanol, and air-drying. For hybridization, the sections were prehybridized in 10% formamide, 10% dextran sulfate, and 2 x SSC for 15 min at 45°C. The probe (100 pmol/ml) was added to the prehybridization mixture and denatured for 10 min at 92°C after application on the sections, and hybridization was performed for 2 h at 45°C in a humidified chamber. The sections were then washed with 2 x SSC containing 0.2% BSA fraction V (Roche) at room temperature for 15 min followed by 0.1 x SSC containing 0.2% BSA fraction V for 15 min at 54°C. All subsequent incubations were at room temperature. The sections were blocked with BSA fraction V (50 mg/ml) in TBS for 30 min then incubated with alkaline phosphatase-conjugated anti-DIG antibody (Roche) for 30 min in a humidified chamber. The anti-DIG antibody was diluted 1:65 in conjugate dilution buffer (0.1 M Tris-HCl pH 7.3, 0.15 M MgCl₂, 10 mg/ml BSA fraction V). After two 10 min washes with TBS, the sections were incubated with BCIP/NBT substrate, mounted with crystal mount (Biomeda Corp), and visualized using an Olympus BX41 microscope.

Immunohistochemistry (IHC)

IHC was performed using a modified protocol (Brown et al., 1996; Shi et al., 2001). Paraffin-embedded 4 μm sections were deparaffinized and rehydrated as above. Nonenzymatic antigen retrieval was performed by treatment with 0.01 M sodium citrate, pH 6.0, at 92°C for 50 min with a change of buffer after 20 min. The sections were cooled for 25 min, rinsed with PBS (3 x 5 min), treated with 3% H₂O₂ for 10 min at room temperature to quench endogenous peroxidase, and rinsed in PBS for 2 x 5 min. The sections were blocked with 1.5% horse serum (Vector Labs) in PBS for 45 min and incubated with primary antibody in PBS containing 1.5% horse serum for 45 min at room temperature in a humidified chamber. We tried five different antigen retrieval
techniques and the results were identical. The anti-TAg antibody was a mouse monoclonal antibody originally raised against SV40 TAg (Harlow et al., 1981), which crossreacts with BKV TAg (PAb416; 1:200 dilution in blocking buffer; isotype IgG2a). The anti-p53 was a mouse monoclonal antibody, p53Ab-6 ((Vojeesk et al., 1992) (Clone DO-1; 1:20 dilution in blocking buffer; isotype IgG2a, Lab Vision)). The anti-androgen receptor was a mouse monoclonal antibody, ARAB-1 ((Ruizeveld de Winter et al., 1991) (Clone AR 441; 1:10 dilution in blocking buffer; Lab Vision)), and the anti-breast carcinoma 90kDa glycoprotein was a mouse monoclonal antibody, ((Colcher et al., 1981) (Clone B6.2; 1:50 dilution in blocking buffer; BioGenex)). Purified mouse IgG2a was used as a negative control (Sigma; 1:20 dilution in blocking buffer). After incubation with primary antibody, sections were washed with PBS for 3 x 5 min and incubated with biotinylated secondary anti-mouse immunoglobulin antibody (ABC kit; Vector labs) at 1:200 dilution in blocking buffer (ABC kit; Vector labs) for 45 min at room temperature. Sections were subsequently washed with PBS for 3 x 10 min and incubated with avidin/biotin-conjugated peroxidase (1: 50 dilution in PBS) for 45 min at room temperature. The sections were subsequently washed with PBS for 10 min, developed with diaminobenzidine tetrahydro-chloride substrate (Sigma), counterstained with hematoxylin, mounted and crystal mount (Biomebra Corp), and visualized.

Double-labeling immunofluorescence

For double-labeling immunofluorescence, deparaffinization and antigen retrieval were performed as described above. (DAMD 17-01-1-0076).

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Rapid Communication

BKV and SV40 infection of human kidney tubular epithelial cells in vitro

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Abstract

The interaction of BKV with its natural target cells, human kidney epithelial cells, has not been studied. In vitro infections of human primary kidney epithelial cells were performed to investigate a BKV infection in its natural host cell. BKV undergoes a lytic replication cycle in this system: high levels of T antigen expression were first detected at 36 h postinfection, while viral DNA replication, capsid protein expression, and progeny virus were observed at 48 h postinfection. It was observed that the related polyomavirus SV40 is incapable of infecting human kidney epithelium except in the presence of the GM1 ganglioside, recently reported to be an SV40 receptor.

Keywords: BKV; SV40; Kidney; Proximal tubule

Introduction

The process by which BKV infects its host, completes an acute replication cycle, and then remains subclinical for the lifetime of the host or becomes active upon immunosuppressive conditions is currently poorly understood. Therefore, we wanted to develop a model system in which BKV could be studied in vitro under conditions similar to those it might encounter in vivo. Early studies on BKV were performed using monkey-derived Vero cells, human embryonic kidney cells, or human embryonic fibroblast cells, which are not the normal target cells for the virus in vivo (Maraldi et al., 1975; Seehafer et al., 1975). We have chosen to characterize a BKV infection in human kidney epithelial cells due to the known ability of BKV to infect tubular epithelium in humans (Randhawa et al., 1999). Human proximal tubule epithelial cells (HPTE cells) are an important part of the kidney nephron. These cells recover essential non-waste blood products and return them to circulation, produce vitamin precursors, maintain blood pressure and blood volume, and communicate with the host immune system through the release of cytokines when they sense infections or toxic molecules (Briggs et al., 2001; Daha and van Kooten, 2000). A system has been developed in which HPTE cells are able to maintain their differentiated state in vitro and function in a renal assist device. This device was developed to perform the nutrient recovery, blood pressure, and blood volume functions for patients with failing kidneys (Humes et al., 2002). The HPTE cells used in the renal assist device are capable of being passaged in culture up to six times. Using these cells, we were able to observe viral protein expression, DNA replication, and growth over the course of infection of a known target cell type for BKV. We find that BKV is capable of replicating efficiently and producing infectious virus particles within these cells. Interestingly, SV40 cannot infect these cells except in the presence of the GM1 ganglioside. The characterization of BKV infection of HPTE cells sets the groundwork for further studies to better understand how BKV is able to persist within the human host and reactivate under immunosuppressed conditions.
Results

We began these studies by asking whether BKV could infect HPTE cells in vitro, and what the outcome of an infection would be. We first examined expression of large T antigen (TAg) as a marker for early gene expression. The expression of TAg and its interaction with p53 and the retinoblastoma family of tumor suppressors are critical for a successful infection by polyomaviruses. We infected HPTE cells with BKV at an MOI of 5 fluorescence forming units (FFU)/cell, isolated total protein over the course of 11 days, and assayed TAg expression by immunoblotting (Fig. 1A). We first detected TAg expression between 12 and 24 h postinfection. Much higher levels of TAg expression were detected between 24 and 36 h postinfection. The expression of TAg peaked at 7 days postinfection, and remained high throughout the rest of the time course.

The next step in the BKV life cycle is replication of its DNA. To assess this step, we isolated low molecular weight DNA from HPTE cells infected with BKV and digested with EcoRI to linearize the BKV genome. We analyzed the amount of viral DNA present by Southern blotting using the BKV genome as a probe. We detected the presence of low levels of viral DNA starting at 0 h postinfection (Fig. 1B). Due to the low and constant level of viral DNA seen during the first 36 h of the infection, we believe that this is DNA from input virus. The first increase in viral DNA occurred at 36 h postinfection, which is expected due to the onset of expression of TAg 12 h earlier. The level of viral DNA increased throughout the course of infection, peaking at 9 days postinfection. Metabolic labeling experiments using tritiated thymidine confirmed that viral DNA synthesis did not begin until 48 h postinfection (data not shown).

After determining when early viral proteins were expressed and viral DNA was replicated, we went on to establish when the major late viral protein VP1 was expressed. Total cellular protein was isolated from HPTE cells infected with BKV over the course of 11 days. Immunoblot analysis revealed the presence of VP1 at all time points from 0 h postinfection to 11 days postinfection (Fig. 1A). As was the case for viral DNA, the VP1 present at 0 h postinfection is probably from the input virus. The first time that VP1 was expressed de novo was at 36 h postinfection, when there was a marked increase in its steady-state levels. High levels of VP1 expression were seen 12 h later. The amount of VP1 increased throughout the remainder of the infection and was at its highest level when the time course ended at 11 days postinfection.

When BKV infects a human kidney, it establishes a subclinical infection unless the immune system has been compromised. Under immunocompromised conditions or immunosuppression, usually resulting from organ or bone marrow transplantation, pregnancy, or HIV infection, high levels of BKV replication are observed in the kidneys and urinary tract, which can lead to hemorrhagic cystitis and polyomavirus nephropathy (PVN) (Binet et al., 1999). Three of the morphologic hallmarks of PVN are intranuclear viral inclusions, focal necrosis of tubular cells, and cells rounding up from the epithelial layer and extruding into the tubular lumen (Nickeleit et al., 1999). We examined the morphology of BKV-infected HPTE cells. As early as 4 days postinfection, we detected morphological characteristics in HPTE cells that are also commonly observed in PVN. The HPTE cells rounded up from the cell monolayer, likely due to cell death, and contained enlarged nuclei and what appeared to be inclusions (Fig. 2). These characteristics were more evident at 9 days postinfection. Thus, BKV apparently undergoes a lytic infection in HPTE cells, producing morphologic changes similar to those seen in PVN.

The key event to which all other viral events lead is progeny virus production. We determined the viral yield over the course of an 11-day infection (Fig. 3). At 24 h postinfection, we detected the eclipse phase of infection, when fewer mature virions were present. We then observed

![Fig. 1. Expression of BKV early and late proteins, and DNA replication. (A) Total cellular protein (25 µg) isolated at the indicated times postinfection was analyzed as described in Materials and methods. (B) EcoRI-digested low molecular weight DNA was separated on an agarose gel, transferred to PVDF, and probed for BKV sequences.]
a relatively rapid rise in the amount of virus that peaked at 4 days postinfection, concomitant with robust viral DNA and late protein synthesis. After this early peak, the release of progeny virus remained relatively constant for the remainder of the infection.

We were also interested in whether SV40 can infect HPTE cells. SV40 is the best studied of the polyomavirus family and a close relative of BKV. An examination of SV40 infection of human kidney cells is particularly interesting because of accumulating evidence that SV40 infects humans (Butel and Lednicky, 1999; Garcea and Imperiale, 2003). SV40 is known to infect proximal tubule cells in the monkey kidney, causing a disease similar to that seen in humans (Sheffield et al., 1980). Upon infecting HPTE cells and BSC-1 cells, an established line of monkey kidney cells, with SV40 we expected similar morphologies to those seen in BKV-infected HPTE cells. However, at 11 days postinfection, cytopathic effects were evident in SV40-infected BSC-1 cells, but not in HPTE cells. We looked for SV40 TAg as a marker of early protein expression. We detected high levels of TAg expression in BSC-1 cells, but none at all in HPTE cells, even at 13 days postinfection (Fig. 4A). To determine if SV40 had entered the HPTE cells, we infected HPTE and BSC-1 cells, extracted low molecular weight DNA, and probed for the presence of SV40 DNA using a Southern blot. In BSC-1 cells, the level of SV40 DNA began to increase at 24 h postinfection, but in HPTE cells no SV40 DNA was detected even at 4 days postinfection (Fig. 4B). These results suggest that SV40 is unable to infect HPTE cells.

It has been reported that the cellular receptor for SV40 is the major histocompatibility complex (MHC) class I molecule (Atwood and Norkin, 1989). Immunoblotting of protein lysates from the HPTE cells revealed that these cells express this molecule at levels similar to human fibroblasts susceptible to SV40 infection (data not shown). More recently, however, Tsai et al. (2003) have reported that the ganglioside GM1 is required for entry of SV40 into mammalian cells. We considered the possibility that a lack of GM1 on the proximal tubule cells was the cause for the lack of

Fig. 2. Morphology of BKV-infected HPTE cells. (A and B) HPTE cells were mock infected or infected with BKV at an MOI of 5 FFU/cell. Cells were fixed and stained with hematoxylin at 4 days postinfection. (C) Kidney biopsy from a patient with PVN stained with hematoxylin. Arrows identify enlarged nuclei and inclusion bodies in proximal tubule cells. Magnification is x400.

Fig. 3. Growth of BKV in HPTE cells. Triplicate wells of HPTE cells were infected with BKV at an MOI of 5 FFU/cell. Viral lysates were prepared at the indicated days pi, and the titer determined. The mean and standard deviation are shown.
Fig. 4. SV40 infection of HPTE and BSC-1 cells. Cells were infected with SV40 at an MOI of 5 PFU/cell. Total cellular protein (25 μg; A) or equal amounts of DNA (B) isolated at the indicated days pi were analyzed as in Fig. 1, except that an SV40 probe was used for the Southern blot. (C) Cells were incubated with the indicated ganglioside for 18 h before infection with BKV or SV40. Protein lysates were prepared at 0 and 96 h postinfection and analyzed as in Fig. 1A.

Discussion

The experiments described in this paper define a system in which BKV can be studied in vitro using a cell type that it infects in vivo. While others have examined BKV infections in cell culture, none of these previous studies have been performed at a molecular level in a cell type infected in vivo (Flägstad and Traavik, 1987; Maraldi et al., 1975). We have shown that BKV causes morphological changes in vitro which are similar to those seen in BKV-induced nephropathy in vivo and that a productive infection occurs. While descriptive, the characterization of BKV infection of these cells is a critical first step to understanding how BKV causes disease in the human host.

We first observed expression of high levels of TAg at 36 h postinfection. This 36-h delay from infection to expression of abundant TAg corresponds to work by others who observed that in human fibroblasts, nuclear uncoating of BKV occurs as late as between 24 and 30 h postinfection (Maraldi et al., 1975). We also observed high levels of VP1 expression and DNA replication beginning at 48 h postinfection, 12 h after TAg was strongly expressed. Two days after the components of the mature virion were first synthesized, we observed a large peak in mature virion production.

We do not have any indication that BKV can establish a latent or persistent infection in tubular epithelial cells in vitro. No cells survived the infection despite repeated attempts to passage them. One major difference between HPTE cells infected in vitro and in vivo is the lack of an immune system. Therefore, our system reproduces what likely occurs during a BKV infection in an immunosuppressed individual. Because BKV is reactivated upon immunosuppression, it is clear that the body keeps viral replication in check without destroying the entire population of infected cells. A component of the immune system controlling BKV infection may be chemokines or cytokines.
For example, it has been demonstrated that cytokines are capable of inhibiting promoter activity of other viruses: IFN-α inhibits murine CMV immediate early gene expression and IFN-γ inhibits gene expression of constructs under control of the human CMV or SV40 promoters (Gribaudo et al., 1995; Harms and Splitter, 1995; Qin et al., 1997). Further study is required to determine under which conditions BKV gene expression and replication might be repressed. The immune system may not be the only factor playing a role in activation of BKV from its latent or persistent state. In studies of Py, it has been reported that mouse kidneys become permissive to lytic infection upon cellular damage and subsequent tissue regeneration (Atencio et al., 1993). It may be that a similar situation exists in the transplant setting, in which BKV replication is stimulated due to either ischemia or kidney damage during handling. It is also possible that a change in hormone levels or stress within an individual could cause the reactivation of BKV, such as during pregnancy. PCR analysis of DNA isolated from uninfected HPTE cells revealed that these cells did not harbor any detectable BKV sequences before infection (data not shown). Because it is known that BKV commonly infects the proximal tubule in vivo, it may be slightly surprising that we did not detect any viral sequences in our cell lines. It is possible that during an in vivo infection, only a few cells are infected, with this infection being kept in check by the immune system and localized to certain areas of the kidney (Chesters et al., 1983). Upon immunosuppression, the infection may become lytic and infect a much larger population of kidney cells.

We have also demonstrated that SV40 does not infect HPTE cells. Because SV40 infects tubular epithelial cells in monkeys, (Sheffield et al., 1980) we expected that our proximal tubule lines would be infected by SV40. The virus apparently does not enter HPTE cells without the addition of the GM1 ganglioside. It remains possible that in the absence of GM1, SV40 is internalized into HPTE cells through a different mechanism, but is unable to traffic to the nucleus and begin lytic replication. Overexposures of our Southern blots demonstrate the presence of input SV40 DNA in HPTE infections in the absence of added gangliosides, but we are unable to determine if the virus has been internalized or remains on the cell surface. It is also possible that in humans SV40 infects a different type of cell within the kidney, retaining its organ tropism, or infects a different tissue entirely. Two groups have reported the detection of SV40 sequences in diseased human kidneys (Butel et al., 1999; Li et al., 2002), so it is possible that SV40 can lytically infect kidney cells under some circumstances. Further study is required to determine why SV40 cannot infect proximal tubule epithelium and which human cells, if any, might act as reservoirs for the virus in vivo.

We have developed a system in which we can study lytic BKV replication in phenotypically normal human kidney epithelial cells. Given the prevalence of BKV in the human population and its known associations with human disease, we believe that this system is an important first step towards better understanding how BKV interacts with the host. We are left with at least two major areas of study to pursue. First, there are likely many cellular proteins and processes that are influenced during infection. Second, we wish to develop a model of BKV persistent infection and determine what causes BKV to reactivate from its subclinical state.

Materials and methods

Cell culture

Human kidney proximal tubule epithelial cells, isolated from cadaveric tissue, were cultured as previously described (Humes et al., 2002). The experiments described in this report were performed on cells isolated from two separate donors with no difference in the results.

Viruses

BKV stocks were initially propagated in 293 cells from virus obtained from the American Type Culture Collection (VR-837). Viral lysates were made through three cycles of freezing the infected cells at −80 °C and thawing at 37 °C. Once we determined that HPTE cells could be lytically infected, one subsequent passage was performed on HPTE cells. The regulatory region of these stocks, as determined by sequencing of PCR products, was identical to the TU strain (Sundsfjord et al., 1990). For the SV40 studies we used strain 776.

Viral titers

Viral titer was determined using a fluorescent focus assay, rather than hemagglutination inhibition, due to its ability to detect infectious virus. Seventy percent confluent HPTE cells in 12-well dishes were infected with 10-fold dilutions of BKV for 4 days at 37 °C. The cells were fixed for 30 min using 70% ethanol/30% PBS (Liu and Atwood, 2001) PAB416 (Harlow et al., 1981) followed by a fluorescein-conjugated anti-mouse antibody (Sigma) used to detect TAg. Titer, measured in fluorescence forming units (FFU), was determined by counting five random fields. The titer of SV40 was determined on BSC-1 monolayers by the agar overlay method (Brockman et al., 1973).

Infections

Cells were grown in 6-well dishes to 70% confluence, and infected with BKV or SV40 at an MOI of 5 FFU/cell. To stain with hematoxylin (Gill III formula), cells were fixed in 4% paraformaldehyde, washed with PBS, and rehydrated with ethanol. For infections to which gangliosides were added, cells were preincubated with either media, 3.2 μM GM1, or 2.7 μM GD1a (gift of B. Tsai) for 18 h at 37 °C,
then washed three times with media before infection (Tsai et al., 2003).

**Immunoblotting**

Total cell protein was extracted using E1A lysis buffer (Harlow et al., 1986) containing 0.05 M NaF and Complete EDTA-free protein inhibitors (Roche). Protein concentration was determined using the Bio-Rad assay, and equal amounts of protein were electrophoresed on an 8% SDS–polyacrylamide gel. PAb416 was used to detect TAg expression, P5G6 (gift of D. Galloway) for VP1 expression, and GAPDH Ab8245 (Abcam) for glyceraldehyde-3-phosphate-dehydrogenase expression. After washing, blots were probed with a horseradish peroxidase-conjugated secondary antibody (Sigma). Antibody complexes were detected using ECL+ reagent (Amersham) and exposure to film.

**DNA replication assays**

Low molecular weight DNA was isolated from infected cells using the protocol described by Hirt (1967). One quarter of the DNA isolated from each sample was digested with EcoRI, electrophoresed, and transferred to a PVDF membrane. A random-labeled probe was synthesized from a plasmid containing either the BKV or SV40 genome. The probe was allowed to hybridize for 16 h at 65 °C, after which the membrane was washed twice with 2× SSC and once with 2× SSC and 1%. The membrane was then exposed to film.

**Virus growth assay**

Viral lysates, prepared by three freeze–thaw cycles as described above (Knowles, 2001), were collected in triplicate from HPTE cells infected with BKV at an MOI of 5 FFU/cell. The viral lysates were prepared by three freeze–thaw cycles as described above (Knowles, 2001), were collected in triplicate from HPTE cells infected with BKV at an MOI of 5 FFU/cell. The titer of the virus in these lysates was determined using the fluorescent focus assay described above.

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