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MINIREVIEW

The role of sialic acid in human polyomavirus infections

Gretchen V. Gee · Aisling S. Dugan · Natia Tsomaia · Dale F. Mierke · Walter J. Atwood

Abstract JC virus (JCV) and BK virus (BKV) are human polyomaviruses that infect approximately 85% of the population worldwide [1,2]. JCV is the underlying cause of the fatal demyelinating disease, progressive multifocal leukoencephalopathy (PML), a condition resulting from JCV induced lytic destruction of myelin producing oligodendrocytes in the brain [3]. BKV infection of kidneys in renal transplant recipients results in a gradual loss of graft function known as polyomavirus associated nephropathy (PVN) [4]. Following the identification of these viruses as the etiological agents of disease, there has been greater interest in understanding the basic biology of these human pathogens [5,6]. Recent advances in the field have shown that viral entry of both JCV and BKV is dependent on the ability to interact with sialic acid. This review focuses on what is known about the human polyomaviruses and the role that sialic acid plays in determining viral tropism.

Keywords JCV · BKV · Sialic acid · Polyomavirus

G.V. Gee
Department of Molecular Biology, Cell Biology and Biochemistry, Brown University, Providence, RI 02912

A.S. Dugan
Graduate Program in Pathobiology, Brown University, Providence, RI 02912

N. Tsomaia · D.F. Mierke
Department of Molecular Pharmacology, Physiology and Biotechnology, Brown University, Providence, RI 02912

W.J. Atwood (✉)
Department of Molecular Biology, Cell Biology and Biochemistry and Graduate Program in Pathobiology, Brown University, Box G-E434, 70 Ship Street, Providence, RI 02903
e-mail: Walter_Atwood@Brown.edu
Tel.: 401-863-3116
Fax: 401-863-9653

Introduction

The human pathogens, JCV and BKV, are members of the family, *Polyomaviridae* and are closely related to simian virus 40 (SV40) and murine polyomavirus (mPyV). These viruses are small and non-enveloped, measuring about 40–45 nm in diameter [7]. Polyomaviruses contain double stranded DNA minichromosomes of about 5000 base pairs that code for six or seven proteins. The closed-circular DNA is packaged with the cellular histones H2A, H2B, H3 and H4 [8,9].

The viral capsids are made up of 360 copies of the major coat protein, VP1. The VP1 molecules are arranged in 72 pentamers that form an icosahedral viral capsid [10]. The VP1 pentamers are associated with a single minor coat protein, either VP2 or VP3 [7,11,12]. Although the capsids of polyomaviruses all have similar structures, they each use unique receptors [13,7] (Table 1). SV40 uses MHC class I molecules as cell surface receptors [14]. MPyV uses $\alpha 4\beta 1$ integrin as a cellular receptor although initial attachment is mediated by sialic acid [15–19]. JCV uses the serotonergic receptor $5HT_{2A}$ to infect cells, but infection is also dependent on $\alpha 2$-6-linked sialic acid [20,21]. Gangliosides seem to play a role in BK infection of Vero cells and recently, these molecules have been implicated as receptors for SV40 and mPyV [22–25]. Recent evidence suggests that BKV requires an N-linked glycoprotein with $\alpha 2$-linked sialic acid [26].

Sialic acid is the generic name for the 9-carbon sugars based on neuraminic acid. These sugars typically occupy the distal end of a glycan, often making these molecules the first part of a cell to come into contact with a ligand. These molecules vary by substitutions of the hydroxyl groups at carbon positions 4, 5, 7, 8 and 9, which add specificity to the molecule. This specificity is evolutionarily determined, often with different modifications occurring specifically in eukaryotes, archaea or bacteria. Another level of diversity is

Table 1 Cellular components associated with viral entry

Virus	Protein component	Sialic acid	Associated gangliosides
JCV	5-HT_{2A}R	Terminal α2-6-linked sialic acid	GT1b
BKV	Unknown	Terminal α2-3-linked sialic acid	Type II gangliosides
SV40	MHC class I molecules	No requirement	GM1
mPyV	$\alpha 4\beta 1$ integrin	α2-3-linked sialic acid α2-6-linked sialic acid	GD1a, GT1b

added based on the sugar to which they are linked and whether that linkage that occurs between the 2-carbon of the sialic acid and either the 3, 6 or 8-carbon of the penultimate sugar [27]. These linkages of sialic acid are often tissue specific based on the expression of the sialyltransferases, the molecules that attach sialic acid to glycans [28].

The role of sialic acid in the entry of other viruses has been well documented. The ability of the low neurovirulent form of Theiler's murine encephalomyelitis viruses (TMEV) to cause a persistent CNS infection has been mapped to the amino acids on the capsid that interact with sialic acid [29]. Likewise, the ability of mPyV to spread and cause tumors in mice has been linked to a single amino acid substitution in the VP1 molecule [30]. More recent examination of the crystal structure of the mPyV VP1 pentamer in complex with sialic acid provided a molecular basis behind the ability of single amino acid substitutions to alter receptor usage [31–33]. It has been shown that amino acid substitutions in the receptor binding hemagglutinin (HA) glycoproteins of influenza virus can alter receptor binding based on their contact with sialic acid. Mutations of amino acids that were directly involved in sialic acid binding prevented the virus from binding human erythrocytes and mutations that increased hydrogen bonding to sialic acid increased binding to the cells. Interestingly, other substitutions of amino acids that had indirect interactions had an intermediate effect on cellular binding [34].

Research has also shown that sialic acid binding may also play other roles in addition to initial binding. One group has shown that binding of sialic acid to the viral capsid may trigger a conformational change, possibly exposing a second receptor-binding site [35]. It is clear that sialic acid can play a crucial role in the tropism of many viruses and recent advances have implicated a role for sialic acid in the tropism of the human polyomaviruses. This review focuses on the biology of these viruses and the role of sialic acid in the viral lifecycle.

JC virus

Initial infection with JCV is thought to occur during childhood but is not linked to symptomatic illness [36,37]. The mode of transmission is not entirely clear as the virus can be detected in urban sewage, suggesting a fecal-oral route, as well as in the tonsil, suggesting a respiratory route of transmission [38–41]. The current hypothesis holds that JCV establishes a lifelong persistent infection in the kidneys, but under immunosuppressive conditions, it can gain access to the brain in B-lymphocytes to result in disease [42].

The underlying risk factor for Progressive Multifocal Leukoencephalopathy (PML) is immunosuppression [43,44] and prior to the acquired immunodeficiency syndrome (AIDS) epidemic, PML was considered a rare illness and was typically observed only in patients with lymphoproliferative diseases. The first diagnosis of PML in an AIDS patient was made in 1982 [45]. Since the early 1980's, there has been a dramatic increase in the incidence of the disease and current estimates find that 1–10% of AIDS patients develop PML [46].

Today, the highest risk factor for developing PML is AIDS, and the number of deaths due to PML has been increasing with the AIDS epidemic [47]. There are multiple reasons for the high incidence of PML in HIV positive individuals. One reason may be that the changes in the blood-brain barrier induced by HIV allow trafficking of JCV infected B-lymphocytes to the brain, where the virus can infect oligodendrocytes [48,49]. It has also been shown that the HIV tat protein can stimulate protein expression from the late JCV promoter [50] and that HIV infected microglia secrete extracellular factors such as interleukin-1 beta (IL-1β) and other inflammatory cytokines that can upregulate transcription factors that stimulate the JCV promoter [51]. Loss of JCV specific $CD4^+$ cytotoxic lymphocytes in AIDS patients has also been correlated to the development of PML [52,53]. Recent evidence has confirmed three cases of PML in HIV negative patients treated with the anti-inflammatory drug natalizumab, a monoclonal antibody against $\alpha_4$integrins, indicating that immune surveillance of the CNS plays a strong role in preventing this disease [54–56].

The cellular host range that can support productive infection of JCV is much more restricted than that of the more promiscuous SV40 and mPyV. There are multiple steps in the viral lifecycle that may be cell specific and cells may produce positive and negative regulators of these processes [57–59]. Chen and Atwood used a hybrid JCV/SV40 virus to show that the restricted tropism of JCV is partially controlled by the virus-receptor interactions. The hybrid virus, JC/SV, contained the structural proteins of JCV and the regulatory regions and early genes of SV40. Infection studies showed

that the new virus maintained the more restricted host cell range of JCV, indicating the viral capsid limits the tropism of the virus [60]. An understanding of the interaction of the virus with the receptor may hold the key to how the virus causes disease.

In vivo, JCV infects glial cells and infection of oligodendrocytes leads to PML. JCV has been shown to infect lymphoid cells from the bone marrow and spleen of PML patients as well as peripheral blood lymphocytes of both PML patients and healthy individuals [61–63]. The virus can also be isolated from the urine of healthy, pregnant and immunosuppressed individuals indicating the kidney as a site of viral persistence [64–68].

In vitro, JCV can also infect hematopoietic progenitor cells, B-lymphocytes and tonsillar stromal cells [40]. Human cell lines that are susceptible to JCV include primary human fetal glial cells (PHFG), a human neuroblastoma cell line (IMR-32), SV40 T Ag transformed PHFG cells (SVG) and certain B cell lines [2,6,69,70,71,72]. The expression of SV40 T Ag in non-permissive cell lines also allows JCV DNA replication in non-human cells [73].

Receptors for JC virus

The abundance of gangliosides in the brain, coupled with their roles in SV40, BKV and mPyV infection makes these molecules attractive candidates for JCV receptors [22–25], although the role of gangliosides in JCV entry is unclear. Overlay studies done with virus like particles (VLP) made up of only VP1 molecules have shown that VLPs can bind glycolipids and glycoproteins containing sialic acid. This, in addition to the fact that the pretreatment of virus with the ganglioside GT1b inhibited infection of glial cells, was used to draw the conclusion that the virus can use these glycolipids as receptors [74]. Gangliosides have not been shown to block entry of viral particles and may actually interfere with other steps in the viral lifecycle. Although these molecules contain sialic acid and may have a role in initial attachment, they are not required for entry and may in fact act as second messengers or by inhibiting the primary receptor [75]. Experiments using agents that prevent glycolipid biosynthesis may determine an exact role for gangliosides in the JCV lifecycle.

Recent evidence has shown that JCV requires the serotonin receptor $5HT_{2A}$, in addition to terminal α2-6-linked sialic acid, in order to infect cells [20,21]. Identification of the $5HT_{2A}$ receptor as the proteinaceous component of the receptor should lead to a better understanding of viral entry into the cell and complement work done with the initial binding steps mediated by the sialic acid component of the receptor.

α2-6-linked sialic acid was first shown to act as a receptor for JCV based on the fact that crude neuraminidase could inhibit hemagglutination of red blood cells and viral binding to and infection of glial cells, whereas an α2-3 specific neuraminidase could not. Binding of JCV to glial cells also inhibits binding of *Sambucus nigra* lectin (SNA), the straight chain α2-6-linked sialic acid specific lectin, demonstrating the preferential binding of JCV to α2-6 linked sialic acid rather than α2-3-linked sialic acids. The ability of an N-linked glycosylation inhibitor, but not an O-linked glycosylation inhibitor, to inhibit viral infection of glial cells led the authors to conclude that JCV uses an N-linked glycoprotein with terminal α2-6 linked sialic acid as a receptor [21].

As the exact linkage of sialic acid has been shown to determine receptor usage and disease progression of mPyV [30,31,76,77], the importance of the interaction of JCV with the sialic acid component of the receptor becomes more apparent. The distribution of sialic acid in various human tissues supports a role for these molecules in determining the tropism of JCV. Using SNA, Eash and colleagues were able to characterize the distribution of α2-6-linked sialic acid on B lymphocytes in the tonsil and spleen, as well as on oligodendrocytes and astrocytes, correlating with cell types that have been shown to be susceptible to infection by JCV [6,40,41,78]. They were also able to identify the receptor type sialic acid in the lungs and kidneys, which may play a role in the mode of transmission and site of persistence in the viral lifecycle [39,41,78].

Obstruction of the initial virus-receptor interaction is an ideal target for drug design to prevent the onset of PML. In order to develop drugs that can block the interaction of JCV with sialic acid, a better understanding of the nature of the interaction is necessary. X-ray crystallography studies have provided details on the molecular level of the interaction between mPyV and sialic acid fragments [32,33]. Tropism can be altered by even the slightest changes in the molecular interaction between a virus and its receptor. Because manipulation of single amino acids can alter receptor usage, mutations that have an effect on cellular binding can be used to identify a rough binding pocket for the receptor. This binding pocket can then be further refined by molecular modeling, in order to visualize the interaction between the virus and the receptor, without the use of crystallography. This approach was used to model the interaction between JCV and α2-6-linked sialic acid using site-directed mutagenesis to identify key VP1 amino acids that were involved in binding. Mutagenesis was combined with molecular modeling to create an energetically stable model of the interaction between the VP1 monomer and terminal α2-6-linked sialic acid to create a tool that can be useful in future studies [79].

Although polyomaviruses all share significant sequence homology, the most significant differences lie in the regions forming the extracellular loops and these differences result in the structural variations of the binding pockets [7,80]. A comparison of the current models for the interaction of JCV

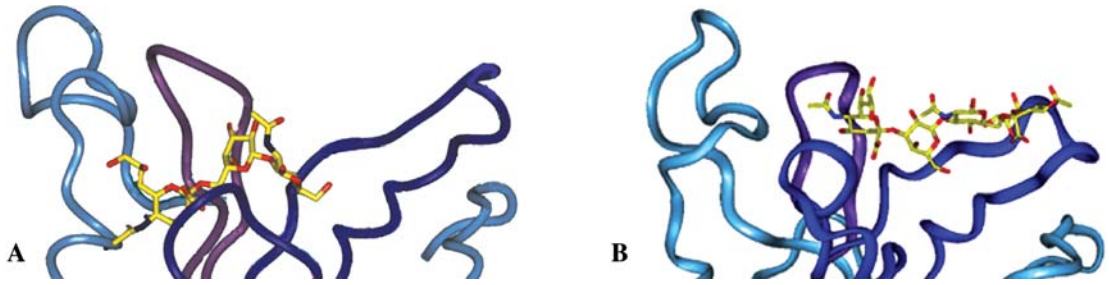

Fig. 1 Close up views of VP1 molecules bound to sialic acid receptor fragments. The purple ribbon represents the HI loop and the dark blue represents the BC loops. (A) Modeled view of JCV VP1 exterior loops that interact with NeuNAcα2-6-Galβ1-3-GlcNAc. Adapted from [96]. (B) View of mPyV VP1 exterior loops that interact with NeuNAcα2-3-Gal-β1-3-[α2-6-NeuNAc-α2-6]-NAcβ1-3-Gal-β1-4-Glc. Adapted from [32] PDB ID: 1VPS

VP1 with α2-6-linked sialic acid and mPyV with a disialylated receptor fragment reveals the intrinsic differences between these two receptor binding events [32,79]. The shape of the binding pocket determines the linkage of sialic acid that can act as the receptor. Modeling experiments reveal a much deeper binding site for sialic acid within the groove formed by the BC and HI loops of JCV VP1 than the more superficial binding of mPyV VP1 to a disialylated receptor fragment shown by X-ray crystallography [32,79] (Fig. 1) The apparent inaccessibility of the JCV VP1 sialic acid binding site may play a role in the limited tropism of the virus.

BK virus

BKV rarely causes clinical pathology in its human host; although recently, the dramatic rise in renal transplant recipients undergoing immunosuppressive therapy has led to the increased incidence of polyomavirus associated nephropathy (PVN). PVN affects as many as 8% of kidney recipients [81]. The rise in PVN is postulated to be a complication of the potent immunosuppressive drugs used to prevent organ rejections, as well as a better awareness of the condition by clinicians [82,83]. This disease is associated with high BKV load with a mean of 6000 virions per infected cell [84]. The cytopathic nature of the virus induces lytic cell death in the transplanted kidney as well as necrosis and inflammation that can ultimately lead to organ dysfunction. Some studies have shown loss of kidney function in as high as 45–60% of organ recipients who developed PVN [85,86].

Initial infection of BKV at an average of 4-5 years of age is independent of JCV infection and almost always asymptomatic [36,37,87,88]. The mode of transmission is currently unknown although it is speculated to occur via a respiratory or oral route [105,89]. The virus persists in the kidney and urinary tract, and periodic reactivation characterized by viral shedding into urine is usually subclinical [1].

Very little is known about the receptor or receptor complex that BKV utilizes to bind to and enter a human cell. There are four antigenic variants of BKV in human populations, subtypes I-IV, with subtype I being most prevalent [90,91]. There is no clear link between any of these strains and increased pathogenicity although subtype III has been associated with HIV infected individuals and pregnant women [92]. The determinants of these subtypes are mapped to a region in the VP1 sequence between amino acids 61 and 83. This area encompasses a hydrophilic region that aligns with the BC-loop of SV40 [93,94]. Whether each subtype uses the same receptor has not been investigated; however as with mPyV, a single amino acid substitution in the BC-loop of mPyV can alter sialic acid recognition, plaque size, hemagglutination activity (HA) and pathogenesis [19,30].

Receptors for BK virus

In vitro studies have helped to elucidate what little is known about the BKV receptor. BKV is capable of hemagglutinating human type O erythrocytes. This binding is unaffected by pH change, or high salt concentrations [95]. Proteolytic enzymes such as trypsin, papain, and α-chymotyrpsin do not greatly influence the ability of BKV to hemagglutinate erythrocytes. Inhibition of hemagglutination can be obtained at high concentrations and is likely due to the loss of carbohydrates resulting from protein cleavage. KIO_4 a compound that oxidizes vicinal alcohol groups on glycoconjugates and interacts with specific amino acids, has been shown to inhibit hemagglutination as well. In contrast, treating erythrocytes with glutaraldehyde, which stabilizes components of the cell surface, results in a four-fold increase in BKV induced hemagglutination. Most interestingly, neuraminidase-treated red blood cells (RBC) fail to hemagglutinate in the presence of BKV [96]. This suggests terminal sialic acids have an important role in initial BKV cellular binding.

Sialic acids are also necessary for BKV to infect Vero cells. Neuraminidase-treated Vero cells show a significant reduction in BKV infection [23]. VLPs generated in a baculovirus expression system by overexpression of BKV subtype I VP1 molecules, show a reduction in gene delivery to neuraminidase-treated host cells as compared to untreated

cells [97]. Moreover, when VP1 from strains II and III are expressed in a yeast system, the pseudovirions that are formed are able to hemagglutinate red blood cells [98]. These data confirm the requirement of sialic acids for attachment of BKV and implicate VP1 as the viral protein that binds to the sialic acid [97,98].

The human kidney and epithelial derived Vero cells express terminal sialic acids linked to C3 and C6 of galactose (α2-3 and α2-6 linkages) [26,78]. The length of the *N*-acyl side chain conjugated to carbon 5 of sialic acid has been shown to influence the efficiency of BKV infection. Using *N*-substituted D-mannosamine, one group was able to synthetically alter the length of approximately half of the cell surface sialic acid side chains on of Vero cells. By forming *N*-propanoyl and *N*-butanoyl sialic acids that contain one or two additional carbons in the *N*-acyl group of sialic acid, the authors were able to significantly enhance infection. Kinetic studies suggested that this was due to an increase in receptor affinity. Interestingly, the addition of one more carbon, to form *N*-pentanoyl-D-mannosamine completely, renders Vero cell resistant to BKV [99].

Recent evidence has confirmed the requirement of an N-linked glycoprotein and α2-3 linked sialic acid in BKV infection. This was shown by inhibition of infection upon removal of sialic acid with neuraminidase which could be restored by replacing α2-3-linked sialic acid with an α2-3-(N)-sialyltransferase, but not with an α2-6-specific sialyltransferase or a α2-3 sialyl transferase that is specific for O-linked proteins. This data is further supported with the use of metabolic inhibitors of glycosylation [26].

As with SV40 and mPyV, early literature suggests a role for gangliosides, in the BKV life cycle. Experiments demonstrated certain types of exogenous gangliosides could block the virus from binding to the surface of red blood cells. The glycolipids that most significantly inhibited hemagglutination were the Type II variety, which are polysialyled gangliosides. Commercial preparations of GD1a combined with GT as well as erythrocyte purified fractions that contained GM3, GD1a, and GD3 significantly reduced hemagglutination. Conversely, monosialylated gangliosides in the GM category showed no influence over HA. Neuraminidase treated RBC showed a dramatic reduction in hemagglutination that could be completely restored by pre-treating these asialo cells with either Type II gangliosides or a crude ganglioside preparation derived from red blood cells. Together this implicates di- and polysialo-gangliosides as the primary target for BKV hemagglutination [100].

Gangliosides seem to have a role in infection as well as binding. When Type II gangliosides and crude gangliosides purified from Vero cells are present during initial infection, there is a dose-dependent reduction in the percentage of cells infected. The presence of gangliosides GM1 and GD1a inhibited infection although less significantly [23]. Other studies show that human kidney tubular cells (HPTE), which lack GM1, are permissive to BKV infection, indicating that unlike SV40, GM1 is not required for BKV infection [101]. Furthermore, re-coating cells, stripped of sialic acid and galactose, with a mixture of all gangliosides derived from Vero cells restored the capacity of BKV to infect Vero cells. [23]. Gangliosides appear to have a role in BKV attachment and infection; however, the exact mechanism has not been clearly defined.

In addition to glycolipids, two early studies suggest that the cellular phospholipid bilayer has a role in BKV induced hemagglutination and infection. Pre-incubating BKV with phospholipids decreases the infection of Vero cells, presumably, because the virus binds the exogenous lipid instead of the host lipid. Treating cells with either phospholipase A2 or D, thereby cleaving fatty acids, reduces the susceptibility of the cell to infection with BKV. Adding back various preparations of phospholipids such as, L-α phosphatidylcholine and phospholipids derived from Vero cells, to these phospholipase-treated cells can restore susceptibility to infection and induced hemagglutination of RBC back to untreated titers [96,102]. Strangely, phospholipase C treated RBCs have an elevated HA titer compared to control [96]. The reason for this difference is unknown, although the authors have suggested this is due to lost polarity of the membrane and binding of phospholipids could be enhanced by removing those phospholipids not involved in BKV HA.

Sialic acids and gangliosides are critical for BKV both to bind to RBCs and to infect cells. Altering expression of these molecules could be a determinant of tropism and impact viral spread in humans. Additionally, the variations of BKV subtypes on the BC-loop may change receptor specificity, host range, and infectivity.

Summary

Much work has been done to identify the cellular receptor components required for entry by SV40 and mPyV (Table 1). SV40 uses MHC class I molecules to enter cells but, unlike other polyomaviruses, it has not been shown to require sialic acid [13,103]. MPyV uses $\alpha 4\beta 1$ integrin to enter cells but also requires sialic acid [17,18]. It is well documented that mPyV requires α2-3-linked sialic acid to bind functionally to cells, although it is also clear that the virus can bind α2-6-linked sialic acid which acts as a pseudoreceptor [31]. These classical findings have been somewhat confounded by emerging evidence that both viruses can use gangliosides as cellular receptors [22,25,104].

JCV has been the focus of much of this research and its requirement for terminal α2-6-linked sialic acid has been intensely studied [21,78,79]. The recent discovery that this virus also requires the serotonergic receptor 5-HT_{2A} to infect cells promises to enhance our understanding of the pathway the virus must follow in order to establish a productively

infected cell [20]. Future work will focus on understanding a number of unanswered questions about JCV, including, if 5-HT_{2A} must be sialylated or if the virus interacts with another sialylated protein, how the virus physically interacts with both 5-HT_{2A} and α2-6-linked sialic acid, as well as what role gangliosides play in viral entry.

Recently, the early events in the lifecycle of the other human polyomavirus, BKV, have been explored. Although it has long been known that the virus requires terminal sialic acid to bind to cells, the exact nature of the receptor and specific linkage that determines entry has only recently been elucidated as an N-linked glycoprotein with α2-3-linked sialic acid [26,97,98]. Polysialylated gangliosides also seem to be important for viral infection but it has not yet been determined if these molecules can function in the absence of the N-linked glycoprotein component of the cellular receptor [23,100]. As more information is learned about the early steps in human polyomavirus infection, future work will focus on developing therapy strategies that target the steps necessary to establishing productive viral infection.

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