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Rhesus Macaque Rhadinovirus-Associated Disease

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Abstract

Rhesus macaque rhadinovirus (RRV) is a gamma-2 herpesvirus that naturally infects rhesus macaque (RM) monkeys and is closely related to human herpesvirus-8 (HHV-8)/Kaposi's sarcoma-associated herpesvirus (KSHV). Infection of immunodeficient RM induces disease in infected RM that resembles KSHV-associated pathologies. Importantly, RRV possesses homologues of KSHV ORFs that are postulated to play a role in disease development. As such, RRV has emerged as a prominent *in vivo* model system for examining mechanisms of infection and disease of these pathogenic herpesviruses, and has provided unique insight into how these viruses cause disease.

INTRODUCTION

Rhesus macaque rhadinovirus (RRV) is a natural pathogen of rhesus macaques (RM) that is genetically similar to Kaposi sarcoma-associated herpesvirus (KSHV), and has been demonstrated to induce similar diseases *in vivo* [1,2]. In recent years, RRV has become a prominent animal model for better understanding KSHV infection and disease development, as well as a tool for deciphering the molecular mechanisms of pathogenesis of oncogenic rhadinoviruses.

Rhadinoviruses

The *Herpesviridae* family is divided into the alpha, beta, and gamma subfamilies, based on biological properties and genomic sequence similarities amongst viruses. The *Gamma-herpesvirinae* subfamily includes lymphotropic viruses that infect and replicate mainly in lymphoid cells, and are also capable of causing cellular transformation and cancer. Based on genomic organization and sequence homology, the *Gamma-herpesvirinae* subfamily can also be divided into the *lymphocryptovirus* (or γ -1) genus and the *rhadinovirus* (or γ -2) genus. In humans, the γ -1 genus is represented by Epstein-Barr virus (EBV), the causative agent of infectious mononucleosis, while the γ -2 genus is represented by KSHV. Viruses

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belonging to the *rhadinovirus* genus, which includes RRV, have been identified in species ranging from mice to humans, with primate rhadinoviruses having been identified in chimpanzees, spider monkeys, African green monkeys, gorillas, mandrill and drill monkeys, pig tail macaques, squirrel monkeys, and RM [3–9].

Identification and Classification of RRV

Two independent strains of RRV have been identified; strain H26–95 was isolated and identified in RM at the New England Primate Research Center [10,11], while strain 17577 (RRV₁₇₅₇₇) was isolated and identified in a RM at the Oregon National Primate Research Center in a simian immunodeficiency virus (SIV)-infected RM that had developed B cell LPD [1]. Upon complete sequencing and comparison of both viral genomes, it was found that RRV H26–95 and RRV₁₇₅₇₇ are genetically similar. Nevertheless, although RRV₁₇₅₇₇ is associated with disease development in infected RM, no similar pathogenic properties have yet been attributed to RRV H26–95 [12], suggesting differences between these two strains exist in regard to their ability to induce disease. Thus, RRV₁₇₅₇₇ is currently the only virus capable of serving as a disease model for KSHV in Old World monkeys.

Examination of the RRV₁₇₅₇₇ genome indicates that it is co-linear with KSHV, retaining many of the open reading frames (ORFs) believed to be involved in the pathogenesis of KSHV [13]. Specifically, 67 of 79 RRV ORFs share similarity to those found in KSHV. Overall, the organization of the RRV₁₇₅₇₇ genome is similar to that of other gamma-herpesviruses, consisting of a single long unique region (LUR) of approximately 131 kb in length, interspersed with several internal repeat regions, and possessing terminal repeat sequences at both ends of the genome.

RRV-associated disease development

Screening of captive RM for prior exposure to RRV revealed that RRV is a natural pathogen of RM, with up to 90% of animals held in captivity testing positive for RRV [10]. Although RRV is highly prevalent in captive RM, the development of RRV-associated disease is rare. In fact, only in the context of immunodeficiency, such as that induced by SIV infection, is disease presented [1,2]. This is similar to the development of KSHV-related diseases in HIV patients who are also infected with KSHV.

Experimental inoculation of RM with RRV₁₇₅₇₇ that have previously been infected with SIV_{mac239} indicates that the virus establishes a persistent infection in B lymphocytes, and promotes the development of B cell LPD [1,14]. The hyperplastic B cell LPD observed in RRV-infected RM closely resembles the plasma cell variant of MCD seen in humans infected with KSHV. More recently, RRV infection has also been associated with lymphomagenesis, as well as the development of a mesenchymal cell proliferative lesion observed in SIV-infected RM referred to as retroperitoneal fibromatosis (RF), a disease that shares histopathological similarities with KS [2] (Figure 1). Thus, RRV is capable of inducing abnormalities in immunodeficient hosts similar to those seen in KSHV/HIV-infected humans.

Given that KSHV infection of primates has been limited, and due to the high genetic similarity and pathogenic properties to KSHV, RRV infection of RM has become an animal model to study KSHV disease development *in vivo*, particularly in the setting of SIV-induced immunodeficiency. Further, the availability of a molecular bacterial artificial chromosome (BAC) clone of RRV allows for the genetic manipulation of the RRV genome for the purposes of producing recombinant viruses that can be analyzed both *in vitro* and *in vivo* to define the function of specific ORFs in pathogenesis [15]. Thus, RRV is an invaluable model system for examining KSHV-associated disease development, and

provides the unique ability to dissect the mechanisms of viral pathogenesis and determine potential targets for vaccine development and therapeutics against KSHV.

Mechanisms of RRV pathogenesis

In the case of KSHV, numerous ORFs have been directly implicated in pathogenesis (see Table I), and importantly, a majority of these ORFs are also conserved in RRV [11,13]. Therefore, dissecting the role of individual RRV ORFs that possess similarity to KSHV ORFs involved in pathogenesis is of particular interest, since the RRV-RM infection model allows for the study of the function of these ORFs in disease development.

Many of the viral ORFs implicated in pathogenesis and contained in the KSHV and RRV genomes are homologues of cellular genes, and are thought to have been pirated from their hosts during evolution. As such, the products of many of these ORFs are capable of affecting numerous processes, such as cellular proliferation, resistance to apoptosis, and regulation of the host immune response. By expressing these products, these viruses are capable of altering the growth properties of infected cells, and also manipulating the host immune response to prevent viral clearance.

The purpose of these viral ORFs is thought to allow establishment of a favorable environment for virus replication and persistence in the host, ultimately promoting survival and further propagation of the virus. However, due to the fact that many of these viral ORFs are capable of dysregulating normal cellular function and immune controls, their expression may also ultimately lead to disease in an infected host, including cellular transformation and development of virus-associated cancers. The expression of a single ORF alone is likely not responsible for pathogenesis; instead, a combination of effects induced by numerous ORF products presumed to be involved, as are interactions with host factors, timing of gene expression, and other variables.

RRV-encoded factors implicated in pathogenesis

Cellular proliferation is affected by both RRV and KSHV infection, and several ORFs in both viruses have been implicated as regulators of cell growth. One of the major factors thought to be involved in both KSHV and RRV-related disease is viral interleukin 6 (vIL-6), a cytokine homologue encoded by both viruses that directly promotes B cell proliferation. KSHV vIL-6 has been shown to be a functional homologue of cellular IL-6, and expression of this protein has been associated with KS, PEL, and MCD [16]. The RRV-encoded vIL-6 homologue shares similarity to KSHV vIL-6, and studies indicate that RRV vIL-6 is also capable of promoting B cell proliferation *in vitro* [17]. Further, expression of RRV vIL-6 has been found to be directly associated with the B cell LPD and RF in RRV-infected RM [2], demonstrating that this factor plays a role in disease *in vivo* (Figure 2).

Another RRV ORF, ORF74, encodes a viral G protein-coupled receptor (GPCR) that shares sequence similarity to the KSHV vGPCR, a protein that has been hypothesized to play a major role in KSHV oncogenesis [18–21]. The vGPCRs of KSHV and RRV are homologues of the cellular chemokine receptor CXCR2 (Interleukin-8 receptor), and are capable of regulating cellular signaling pathways in cells in which they are expressed. Like the KSHV vGPCR, the RRV vGPCR has been shown to possess constitutive and ligand-dependent signaling abilities, promote cytokine secretion, and induce transformation of cells in which it is expressed [22]. Therefore, like its KSHV counterpart, expression of the RRV vGPCR has the potential to induce transformation *in vivo*, and may play a role in the development of virus-associated malignancies.

Immunoregulation during infection is critical for allowing viruses to establish and maintain an infection and prevent clearance by the host immune system. One of the first steps critical

to the establishment of KSHV and RRV infection is regulation of the innate immune response, as evidenced by the fact that both viruses encode proteins that can inhibit innate immune-modulators. For example, KSHV and RRV both encode viral interferon regulatory factors (vIRFs), proteins that share sequence similarity to cellular IRF transcription factors that are directly involved in regulating the expression of IFN. KSHV encodes four vIRFs that display the ability to inhibit IFN expression *in vitro* by altering the normal function of cellular IRFs [23,24]. RRV encodes eight vIRF proteins, and in-depth analysis indicates they possess similar functions to their KSHV counterparts. Specifically, using a recombinant form of RRV devoid of vIRF expression, it was found that their absence during infection *in vitro* results in earlier and higher levels of IFN production [25]. Analysis of this same virus *in vivo* in infected RM indicated that lack of vIRF expression results in an earlier and sustained production of proinflammatory cytokines, an earlier induction of T cell responses, lower viral loads, and decreased levels of B cell hyperplasia [26]. These studies directly demonstrate that vIRFs have a major impact on immune responses against RRV.

Other RRV-encoded molecules are involved in manipulating the adaptive arm of the immune response. An example is the viral CD200 (vCD200) molecule, a homologue of KSHV vCD200. Both KSHV and RRV vCD200 molecules are themselves homologues of cellular CD200, a membrane protein involved in negatively regulating the activation of target cells through interaction with its cognate receptor CD200R, which is mainly expressed on the surface of myeloid lineage cells and some lymphocytes. KSHV and RRV vCD200 have been shown to function similarly to cellular CD200 *in vitro*, demonstrating the ability to inhibit the activation of myeloid cells and the production of pro-inflammatory cytokines [27–31]. Expression of vCD200 *in vivo* is thought to inhibit immune responses against RRV to prevent its clearance, ultimately leading to enhanced viral persistence. Due to unique transcript splicing events, RRV is also capable of producing a secreted form of vCD200 [28]. Interestingly, a soluble form of cellular CD200 was recently identified in human plasma, and has been suggested to be involved in chronic lymphocytic leukemia [32]. The exact role that the secreted form of vCD200 plays in RRV disease remains unknown.

RRV also encodes non-coding RNAs, such as micro RNAs (miRNAs) postulated to be involved in the pathogenesis of many viruses [33–35]. Expression of the miRNAs is thought to be directly involved in disease development. The complete function and extent of the regulatory mechanisms affected of these miRNAs is not yet fully understood, but it is likely they regulate numerous targets within infected cells, and as such have widespread effects that can directly affect pathogenesis. Importantly, RRV-encoded miRNAs are expressed in B cell lymphoma and RF tissues derived from RRV-infected RM, suggesting these molecules may in fact be important to the development of virus-induced cancers *in vivo* [36]. Table 1 summarizes viral ORFs implicated in pathogenesis.

CONCLUSIONS

RRV has become a widely used model for the study of KSHV pathogenesis *in vivo*. RRV shares a high level of genetic similarity to KSHV, encodes homologues of factors believed to play a role in KSHV pathogenesis, and induces similar disease in infected RM as is seen in KSHV-infected humans. Due to the ability to readily propagate RRV *in vitro*, and the availability of a nonhuman primate model system for the study of RRV-associated pathogenesis, RRV serves as an ideal model system for understanding pathogenesis of KSHV infection. Further, the availability of methods to create recombinant forms of RRV with mutated or deleted ORFs allows for the examination of the contributions of specific viral ORFs to viral pathogenesis. The use of the RRV/RM infection model will therefore continue to provide unique insight into the mechanisms of KSHV disease development, and

allow for the development of novel anti-viral therapeutics and vaccines that may also help prevent and treat human diseases associated with KSHV infection.

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Highlights

1. RRV-associated disease
2. RRV genome is highly similar to Kaposi sarcoma-associated herpesvirus genome
3. RRV encodes similar open reading frames as KSHV that are postulated to be associated with disease
4. Recombinant RRV clone is pathogenic and can be manipulated to interrogate whether viral ORFs are in fact determinants of viral pathogenesis

Figure 1. Lymphoma and retroperitoneal fibromatosis (RF) in RRV-infected rhesus macaques (A) Hematoxylin and eosin (H&E) stain of lymphoma isolated at biopsy from the breast of animal 19185, original magnification $\times 1000$. (B) Combined in situ hybridization of lymphoma with RRV cosmid probe (purple) and immunohistochemistry with mouse anti-CD20 (brown) and anti-CD3+ (gray) demonstrates that RRV is present in CD20+ cells comprising the lymphoma, original magnification $\times 1000$. (C) Same as B, except cosmid vector control replaces the RRV cosmid probe. (D) H&E stain of lymphoma isolated from liver of animal 19286 during necropsy, original magnification $\times 200$. (E) Same as B except RRV cosmid probe detected by immunofluorescence (purple) with no evidence of CD20 staining. (F) Similar to C, this is the control for panel E. (G) H&E stain of RF attached to the stomach of animal 18483, original magnification $\times 400$. (H) Combined in situ hybridization with RRV cosmid probe (purple) and immunohistochemistry with anti-CD20 (brown) and anti-CD3 (gray) shows that RRV is not present in the CD20- or CD3-positive cells, original magnification $\times 1000$. Arrows point to RRV-positive cells that have spindle-like morphology. (I) Identical to H, except vector control replaces the RRV cosmid, original magnification $\times 1000$. "This research was originally published in *Blood*. Orzechowska et al., Rhesus macaque rhadinovirus-associated non-Hodgkin lymphoma: animal model for KSHV-associated malignancies. *Blood*. 2008; 112:4227–4234. © the American Society of Hematology."

Figure 2. Detection of vIL-6 in RRV-associated lymphomas, RF and MCD

Lymphoma from RM 19185 was stained with murine monoclonal antibodies specific for RRV vIL-6 and CD20 (A), lymphoma from animal 19286 was stained with murine monoclonal antibodies specific for RRV vIL-6 and goat anti-human IgM, and nuclei (C). B and D are isotype controls, original magnification $\times 630$. Panel A, vIL-6 (green) and CD20 (red); panel B, isotype controls for vIL-6 and CD20; panel C, vIL-6 (green) and IgM (red), and nuclei (blue); and panel D, isotype controls for vIL-6 and IgM. RF from animal 18483 was stained for vIL-6 (green), vimentin (red) and nuclei (blue) (Panel E), original magnification $\times 630$. MCD lesion from animal 19455 was stained for vIL-6 (green), CD20 (red) and nuclei (blue) (Panel F), original magnification $\times 630$. "This research was originally

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TABLE 1

Selected RRV-encoded factors implicated in pathogenesis

Name	Description	Activities
vIL-6	interleukin 6 homologue	B cell proliferation
vIRFs	interferon regulatory factor homologues	Inhibition of innate interferon response in infected cells
vGPCR	CXCR2 chemokine receptor homologue	Activation of cellular signaling pathways, transformation, cytokine secretion
vCD200	CD200 homologue	Regulation of adaptive immune response
miRNAs	Viral microRNAs	Modulation of target transcripts