Saffold Virus, a Novel Human Cardiovirus with Unknown Pathogenicity

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Although cardioviruses have been thought to mainly infect rodents, a novel human cardiovirus, designated Saffold virus (SAFV), was identified in 2007. SAFV is grouped with Theiler-like rat virus and Theiler’s murine encephalomyelitis virus (TMEV) in the species Theilovirus of the genus Cardiovirus of the family Picornaviridae. Eight genotypes of SAFV have now been identified. SAFV has been isolated from nasal and stool specimens from infants presenting with respiratory and gastrointestinal symptoms as well as from children with nonpolio acute flaccid paralysis; however, the relationship of SAFV to this symptomatology remains unclear. Of note, the virus has also been isolated from the cerebrospinal fluid specimens of patients with aseptic meningitis. This finding is of interest since TMEV is known to cause a multiple sclerosis-like syndrome in mice. The involvement of SAFV in various diseases (e.g., respiratory illness, gastrointestinal illness, neurological diseases, and type I diabetes) is presently under investigation. In order to clarify the pathogenicity of SAFV, additional epidemiological studies are required. Furthermore, identification of the SAFV cellular receptor will help establish an animal model for SAFV infection and help clarify the pathogenesis of SAFV-related diseases. In addition, investigation of the tissue-specific expression of the receptor may facilitate development of a novel picornavirus vector, which could be a useful tool in gene therapy for humans. The study of viral factors involved in viral pathogenicity using a reverse genetics technique will also be important.

Members of the genus Cardiovirus are positive, single-stranded RNA viruses that belong to the family Picornaviridae. Cardioviruses have been thought to mainly infect rodents; however, the possible existence of authentic human cardioviruses has been debated over the years. In 2007, a novel human cardiovirus, designated Saffold virus (SAFV), was isolated from the stool sample of a girl presenting with a fever of unknown origin (31).

The nucleotide sequence of the SAFV isolate showed a strong similarity to that of Theiler-like rat virus (TRV), which had been previously isolated from rats in Japan (41). Phylogenetic analysis grouped SAFV with TRV, Theiler’s murine encephalomyelitis virus (TMEV), and Vilyuisk human encephalomyelitis virus (VHEV) in the species Theilovirus (34). SAFV was subsequently isolated from nasal and stool specimens of infants presenting with respiratory or gastrointestinal symptoms and from children with nonpolio acute flaccid paralysis. The virus has also been identified in specimens from asymptomatic patients (1, 2, 13). Seroepidemiologic studies have shown that SAFV is a common and widespread virus that causes infection in early childhood (9, 66). In this review, we will discuss findings related to this novel human cardiovirus and focus on its potential pathogenicity for humans.

DISCOVERY OF A NOVEL HUMAN CARDIOVIRUS

In the 1990s, virology textbooks noted that the Cardiovirus genus included two species: encephalomyocarditis virus (EMCV) and TMEV (55). The natural hosts for both species are mainly mice, although EMCV has been isolated from over 30 host species, including various mammals, birds, and invertebrates (65).

In 2003, a virus was isolated from sentinel rats exposed to cage bedding previously used by TMEV-seropositive adult rats in Japan (41). The nucleotide sequence showed a strong similarity to that of TMEV. Therefore, the virus was designated TRV.

For over 100 years, a form of human encephalomyelitis called Vilyuisk encephalitis (VE) has been known to affect the Yakut people who inhabit the Vilyuy Valley in Siberia (22, 35). Between 1954 and 1957, viral isolates thought to be linked to the disease were recovered from human clinical specimens. The virus isolates, VHEV, cross-reacted fully with TMEV and weakly with EMCV (6). It was unclear whether the virus was the human pathogen causing VE, a TMEV inadvertently recovered during isolation and passage in mice, or a recombinant between these two (49). A similar scenario occurred in the case of the isolation of hemagglutinating virus of Japan (HVJ), Sendai virus. HVJ was recovered from mice inoculated with an autopsy specimen from an infant with pneumonia, which was epidemic in Sendai in the early 1950s (7). The agent was later shown to be indigenous to mice, and therefore it remains unclear whether it is the pathogen that caused the pneumonia.

In 1981, an 8-month-old girl presented with a fever of unknown origin. A virus from a stool sample grew well in human fetal diploid kidney cells but failed to grow in primary monkey kidney, A-549, BSC, and RD cells. The virus also grew in suckling mice. Further characterization was carried out at the State of California Viral and Rickettsial Disease Laboratory. As determined by electron microscopy, the agent had a diameter of 28 to 30 nm and appeared to be a typical picornavirus. It was acid stable and temperature sensitive (with no growth at 33°C) (31). The virus was designated SAFV after the middle name of Morris Saffold Jones, the senior author of the corresponding report in 2007. A Saffold-like virus was subsequently isolated from a nasopharyngeal sample collected from a 23-month-old child in 2008 (1). These viruses were subsequently designated SAFV-1 and SAFV-2, respectively (34). SAFV-3 was later isolated from a stool sample obtained from...
a 13-month-old boy who presented with vomiting (66). Eight SAFV genotypes have now been identified (2, 3).

SAFV is a member of the Cardiovirus genus, which consists of two species: Encephalomyelitis virus and Theilovirus. The species Theilovirus includes SAFV as well as TMEV, TRV, and VHEV (34).

PROPERTIES AND GENOME STRUCTURE OF SAFV
Since SAFV belongs to the genus Cardiovirus of the family Picornaviridae, its properties and genome structure are similar to those of other cardioviruses. The genome is approximately 8,050-nucleotides (nt) long. The 5’ untranslated region (UTR) is approximately 1,040-nt long, with a type II internal ribosome entry site (IRES) similar to that of the murine cardioviruses, TMEV and EMCV (14, 38, 46, 47). The 3’ UTR is approximately 120-nt long with a poly(A) tract. The poly(A) tail of cardiovirus is reported to be short (35 nt) (44); however, a 3’-RACE experiment demonstrated that one of the clones derived from the JPN08-404 strain (HQ902242) had a 124-nt-long poly(A) tract (data not shown).

The genome of picornaviruses contains a higher-order RNA structure, the cis-acting replication element (CRE), used as a template for 3D polymerase-mediated Vpg uridylation. In the case of the murine cardioviruses (TMEV and EMCV), CRE is located in the VP2-coding region and consists of a stem-loop structure (38). SAFV also contains a conserved sequence of CRE in the VP2-coding region (66).

The 2A protein of cardioviruses lacks protease activity. Instead, cardioviruses have an asparagine-proline-glycine-proline (NPG/P) amino acid sequence at the 2A-2B junction. Instead, cardioviruses have an asparagine-proline-glycine-proline (NPG/P) amino acid sequence at the 2A-2B junction.

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L PROTEIN
Viruses in the Aphthovirus, Cardiovirus, Erbovirus, Kobuvirus, Teschovirus, and Sapelovirus genera of the Picornaviridae family contain an L-coding region at the most 5’-terminus of the open reading frame (ORF) (43). Only the L proteins of the aphthoviruses and cardioviruses have been studied in any detail.

In foot-and-mouth disease virus (FMDV), an aphthovirus, L protein is a papain-related thiol protease (23). FMDV L autocatalytically cleaves the viral polyprotein between its C terminus and the N terminus of VP4 (45, 58). It also cleaves the translation initiation factor eIF4G; the latter cleavage inhibits cellular translation, resulting in a shut-off of host protein synthesis (11).

In contrast to the L protein of aphthoviruses, the L protein of cardioviruses has a very different sequence that has an N-terminal zinc finger and a C-terminal acidic domain (38). Cardiovirus L is not a protease but plays a key role in the regulation of virus transcription (15), the phosphorylation of nucleoporins (48), and the inhibition of transcription of alpha/beta interferon (IFN-α/β) (24). L protein of EMCV has been reported to have an antipoptotic activity related to a mitochondrial-dependent pathway (52).

Special attention has been directed to the L protein of TMEV, a member of the Theilovirus species. TMEV L is a multifunctional protein that is important in neurovirulence, viral RNA encapsidation, anti-IFN activity, and viral persistence (29). In contrast to EMCV L protein, L protein of TMEV has a proapoptotic activity (16, 42) or an antiapoptotic activity, depending on the TMEV strain and the particular cell type (57). TMEV L is thought to be an important factor in inducing the pathology found after infection of the mouse. The special properties of TMEV L may be a result of the presence of an S/T-rich domain present in the C terminus, in addition to the zinc finger and acidic domains found in the case of EMCV (21, 29).

Of note, SAFV has a hybrid L protein with features of both the Theilovirus and EMCV species; SAFV has a partially deleted S/T-rich domain along with a zinc finger and an acidic domain (34). Studies of the function of SAFV L are likely to be important in order to clarify the virus’s pathogenicity.

L* PROTEIN
Attenuated Theiler’s original (TO) subgroup strains of TMEV have an alternative translation initiation site at nt 1079, just downstream from the authentic initiation site for the polyprotein at nt 1066. From this alternative translation initiation site, a small 17- to 18-kDa protein, referred to as L*, is synthesized out-of-frame with the polyprotein. The synthesis of L* is specific to the TO subgroup strains of TMEV because its initiating AUG is present only in these strains and not in neurovirulent GDVII subgroup strains (where the L* AUG is replaced by an ACG) (33, 39). Because L* protein is synthesized only in TO subgroup strains, it is thought to play a role as a determinant for viral persistence and demyelination, which are seen after infection of mice with TO subgroup strains. L* protein is also important for virus growth in macrophages and exhibits antipoptotic activity in certain cell types (26, 27). Of note, SAFV lacks an AUG initiating codon at the position used by TMEV to translate L* protein; however, there is an ACG in this region. If a non-AUG-initiated start codon were used to synthesize SAFV L*, as has been reported in the case of TMEV (61), the presence of stop codons predict that the L* protein would be only 57 amino acids (SAFV-1, California/81) or 34 amino acids (SAFV-2, Can112051-06, and SAFV-3, JPN08-404) (28, 34) in length. At this time, it remains unclear whether an L* protein of SAFV is expressed and, if so, whether it has functional activity (2).

SAFV-RELATED VIRUSES
The Theilovirus species of cardioviruses includes TMEV, TRV, and VHEV. Among them, TMEV is the most extensively studied because of its unusual phenotype. TMEV is divided into two subgroups of strains. GDVII subgroup strains are highly virulent. Intracerebral and peripheral routes of inoculation cause an acute fatal polioencephalomyelitis in mice. Infected mice show progressive flaccid paralysis, and almost all infected mice die within 2 weeks. Neither virus persistence nor demyelination is observed in the few surviving mice (4, 29, 54). On the other hand, TO subgroup strains cause a milder encephalomyelitis 1 to 2 weeks post-inoculation (p.i.). Mice recover and then develop a chronic, progressive demyelinating disease with spastic paralysis 1 to 2 months p.i. Since its pathological findings are reminiscent of multiple sclerosis (MS), TO subgroup strain-induced demyelinating disease serves as an excellent animal model for this disease.

An understanding of the mechanisms of TMEV persistence and demyelination remains incomplete. Infectious cDNAs constructed in the late 1980s to the early 1990s (5, 18, 37, 53, 59) have been used to prepare recombinant viruses from the GDVII and DA (or BeAn) strains in order to clarify the region(s) responsible for TMEV biological activities. Although the precise region(s) responsible for virus persistence and demyelination is unclear, several regions have been highlighted. The capsid proteins, especially
VP1 and VP2, were found important for some of the biological activities (29). In addition to these structural proteins, the two nonstructural viral proteins, L and L*, play an important role in TMEV disease pathogenesis (4, 29, 54).

Investigation of TMEV indicates that the role(s) of the capsid proteins (VP1 to VP4) as well as the nonstructural proteins (L and L*) will need to be clarified to better understand the pathogenesis of SAFV-induced disease. An infectious SAFV cDNA which was recently constructed by Himeda et al. (28) will be a most useful tool for a reverse genetics study.

THE PATHOGENICITY OF SAFV IN HUMANS

The data that follow indicate that SAFV-1 is a worldwide infection that occurs early in life and involves the respiratory and gastrointestinal systems. SAFV-2 and SAFV-3 have been isolated in North and South America, Europe, and Asia (1, 2, 8, 9, 13, 31, 30, 51, 66), while SAFV genotypes 4 to 8 have been isolated in South Asia (2). Zoll et al. showed by virus neutralization studies that SAFV-3 infection occurs early in life (>75% seropositivity at 24 months) and that the seroprevalence reaches >90% in older children and adults in several countries in Europe, Africa, and Asia (66). Chiu et al. reported that 91% of U.S. adults carry antibodies to SAFV-2, of which 80% generate neutralizing antibodies (9).

In Japan between 2009 and 2010, 1,525 nasopharyngeal swab specimens were obtained from patients younger than 18 years with acute respiratory illness. SAFV-2 sequences were detected by nested reverse transcription (RT)-PCR in 3.5% of patients (30). In addition, SAFV-3 sequences were detected by nested RT-PCR in 1.4% of 423 nasopharyngeal swab specimens from patients with acute respiratory illness (60). Of note, however, Chiu et al. examined 719 respiratory specimens (89% from patients with acute respiratory illness) for SAFV by real-time quantitative RT-PCR (qRT-PCR) and found all were negative (8).

Screening of 751 stool specimens from 498 individuals in a gastrointestinal cohort found 1.2% of 498 individuals were positive for SAFV (genotypes 2 and 3). All positive stool specimens were from children (<2 years old) (8). Of note, SAFV was implicated as a cause of enteric disease in Minnesota in 2008 (19).

Attention has been directed to whether SAFV is a possible cause of central nervous system (CNS) disease, since the closely related TMEV causes a demyelinating CNS disease that resembles MS (29). The isolation of SAFV-3 (JPN08–404) from the cerebrospinal fluid (CSF) of a 9-year-old boy with aseptic meningitis (28) is noteworthy and suggests that SAFV may have CNS tropism and pathogenicity. However, Chiu et al. examined 400 CSF specimens from patients with neurological diseases (aseptic meningitis, encephalitis, and 40 cases of MS) and found that all were negative for SAFV by qRT-PCR (8).

In summary, epidemiological studies have failed to provide a clear picture of the relationship between SAFV infection and actual disease in humans. Further studies that include a control group of healthy persons will help clarify this issue.

THE PATHOGENICITY OF SAFV IN EXPERIMENTAL ANIMALS

Animal experiments have been carried out in order to study the tropism and pathogenicity of SAFV. Hertzler et al. found that high doses of SAFV-2 intracerebrally inoculated into FVB/n mice produced paralysis with neuropathological changes consistent with acute encephalomyelitis, particularly in the limbic system (25). Of special note was the presence of inflammation in the spinal cord white matter. Sorgeloos et al. presented data describing the results of experiments involving intraperitoneal inoculation of SAFV-2 and SAFV-3 into 129/Sv mice. Both of these SAFVs infect the heart and the CNS; however, the major viral load was in the pancreas. SAFV-3 is more neurotropic than SAFV-2, and intracerebral inoculation of SAFV-3 into FVB/n mice caused acute encephalitis (56). These data suggest that SAFV is neurotropic in mice.

The finding that the major viral load following SAFV-2 and SAFV-3 intraperitoneal inoculation was in the pancreas is noteworthy. Several viruses in the Picornaviridae family, particularly Coxsackie B viruses (members of the Human enterovirus B species), have been implicated in the etiology of type I diabetes (17, 20, 32, 63, 64), a disease characterized by the destruction of insulin-producing β cells in the pancreas. In addition, EMCV is known to induce pancreatitis and type I diabetes in rodents. The fact that EMCV infection can also lead to encephalomyelitis, myocarditis, orchitis, and sialodacryoadenitis in rodents (12) and that it has been isolated from two febrile patients (10, 40) will help guide further investigations of the relationship of SAFV to disease. Furthermore, epidemiological studies will be important to investigate the relationship between SAFV and type I diabetes.

FUTURE PROSPECTS

The relationship of SAFV to disease in humans remains unclear. Data suggest that this virus may have diverse potential pathogenicity (e.g., respiratory illness, gastrointestinal illness, neurological diseases, and/or type I diabetes). In order to clarify the pathogenicity of SAFV, further epidemiological studies are needed. In addition, the establishment of an animal model of SAFV infection will be useful in investigating the pathogenesis(es) of SAFV-induced diseases. An infectious full-length cDNA clone of SAFV (28) will be a powerful tool in reverse genetic studies in order to identify viral factors involved in pathogenicity. The preparation of a chimeric virus expressing green fluorescent protein may make it possible to identify the receptor for SAFV infection, as has been carried out in the case of enterovirus 71 infection (62). The identification of the receptor for virus infection would enable the preparation of transgenic mice as a novel animal model for SAFV infection. Furthermore, a clarification of the tissue-specific expression pattern of the receptor in human tissues will facilitate studies on SAFV tropism and pathogenicity. If SAFV is not found to be pathogenic in humans, it may still be a useful tool in gene therapy as a novel picornavirus vector. Therefore, further studies to clarify the pathogenicity of SAFV are needed.

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