The neuropathogenicity of the Saffold virus in mouse models

Summary of Doctoral Thesis

Osamu Kotani
(Supervised by Professor Fumihiro Taguchi)

Graduate School of Veterinary Medicine and life Science
Nippon Veterinary and Life Science University

The Saffold virus (SAFV) belongs to the genus *Cardiovirus* in the family *Picornaviridae*. In 2007, SAFV was isolated from a stool sample obtained in 1981 from an infant with a fever of unknown origin. Since its identification, SAFV has been considered to be mainly associated with acute gastroenteritis and acute upper respiratory symptoms in children. Although SAFV is occasionally detected in cases of aseptic meningitis and non-polio acute flaccid paralysis, its role in neurological diseases remains unknown. Hence, this study aimed to elucidate the neuropathogenicity of SAFV in mouse models. Chapter 1 describes an immunohistochemical analysis method we established for identifying the virus in paraffin-embedded tissue samples, using a polyclonal antibody raised against SAFV type 3 (SAFV-3). Chapter 2 describes the determination of the pathogenicity of two clinical isolates of SAFV-3 in mice, along with the isolates' tropism and neurovirulence in the cerebellum. Chapter 3 describes passage of SAFV in the cerebellum of neonatal mice, and the virological, pathological, and immunological characterization of the resultant strain.

Chapter 1: Establishment of a histopathological diagnosis of Saffold virus infection.

The detection of pathogen antigens in tissues by immunohistochemistry is necessary for accurate pathological diagnoses and identification of the relationship between a pathogen and an illness. An immunohistochemical analysis on paraffin-embedded tissues using a polyclonal antibody raised against SAFV-3 was established. The antibody against SAFV-3 was tested

for specificity to and cross-reactivity with enteroviruses (EV), which are a major cause of aseptic meningitis.

Anti-SAFV polyclonal antibody was produced by hyper-immunizing rabbits with an isolate strain of SAFV-3, the JPN08-404 strain. Three anti-EV polyclonal antibodies were raised against two strains of EV71 and poliovirus type 1 (PV1), with each virus being first denatured with sodium dodecyl sulfate and heat. Other antibodies were also used: one against recombinant viral capsid protein VP1 of Coxsackievirus B3 (CVB3), one against PV1, and one against peptide 2C of PV1.

The specificity and cross-reactivity of the polyclonal antibodies were determined by immunohistochemistry using formalin-fixed, paraffin-embedded cultured cells, and mouse tissues infected with SAFV, EV-A, -B, or EV-C. Degenerated cells within the SAFV-infected cultured cells and mouse tissues tested positive with an anti-SAFV antibody. The SAFV antigen–positive cells were positive for viral RNA, as demonstrated by *in situ* hybridization. Although the specificity of the anti-EV antibodies was low, and they were not able to distinguish between different EV serotypes, they were specific for the EV genus and did not recognize other genera.

In summary, an immunohistochemical method for detecting SAFV-3 viral antigens in paraffin-embedded tissues was established. The anti–SAFV-3 antibody had high specificity for SAFV and was able to distinguish it from the EV agents that cause aseptic meningitis. These antibodies are useful for the diagnosis of not only SAFV infection, but also picornavirus infections.

Chapter 2: Neuropathogenicity of two Saffold virus type 3 isolates in mouse models

SAFV is occasionally detected in children with acute flaccid paralysis, meningitis, and cerebellitis; however, the neuropathogenesis of the virus is still unknown. In this chapter, to elucidate the neuropathogenesis of SAFV, two clinical isolates of SAFV-3 were analyzed pathologically, virologically, and immunologically in neonatal ddY mice and 6-week-old BALB/c mice. One clinical isolate was obtained from the cerebrospinal fluid of an aseptic meningitis patient (JPN 08-404, referred to here as the AM strain), and the other was from a throat swab of a patient with upper respiratory tract inflammation (Gunma/176/2008, referred to here as the UR strain); both samples were obtained in 2008. Both viruses were inoculated intracerebrally into neonatal ddY mice.

After inoculation, the AM-inoculated mice developed a mild neurological sign suggesting ataxia, but rapidly recovered. During the observational period, the rate of body weight gain was significantly lower for the UR-inoculated mice than that of the MEM-inoculated control mice. However, the difference was not fatal. While both AM- and UR-infected mice had viral antigens in the cerebellum and around the ventricle, as detected by immunohistochemistry, the viral antigen–positive cells were mainly in the Bergmann glia of the cerebellum in AM-inoculated mice and in the neuroepithelial cells surrounding the lateral ventricle in UR-inoculated mice. In addition, the UR-inoculated mice also had viral antigen–positive epithelial cells in the oral mucosa and tooth germ.

In the blood, viral RNA levels peaked at 3 to 5 days post-inoculation (p.i.) in both SAFV-3 inoculated mice, though UR-inoculated mice had higher levels. In addition, viral RNA levels in the brain of both groups increased after day 3 p.i. and were correlated with the level of type I interferon (IFN). The UR-inoculated mice had significantly more viral RNA in the cerebrum and brain stem on days 3 and 5 p.i. than did the AM-inoculated mice, though the UR-inoculated mice had less viral RNA in the cerebellum on day 5 p.i. In addition, the UR-inoculated mice had high expression of type I IFN and inflammatory infiltrations into regions of the brain, including the cerebellum. Next, the neuroinvasiveness of SAFV-3 was evaluated by intraperitoneal inoculation. Histopathological and immunohistochemical examinations revealed that both strains invaded and infected the cerebellum and the region around the ventricle. Inflammatory infiltration was also observed in the brain of the UR-inoculated mice on day 21 p.i.

After examining these phenotypes in neonatal ddY mice, I next examined the neurovirulence of SAFV-3 in 6-week-old BALB/c mice using intracerebral inoculation. Both SAFV-3 strains caused significant body weight loss, compared to MEM, during the first 3 days p.i.; however, the mice exhibited no obvious neurological signs during the observation period. With both strains, the degenerated and necrotic nerve cells around the ventricle were positive for viral antigens on day 3 p.i., whereas only the AM strain had tropism to the cerebellum and triggered expression of type I IFN. Moreover, on day 8 p.i., the AM-inoculated mice exhibited mild inflammation in the cerebellar cortex. The neuroinvasiveness of SAFV-3 in 6-week-old BALB/c mice was also assessed with intraperitoneal, intravenous, intranasal, and

per oral inoculation, but there was no evidence for neuroinvasiveness. Notably, oral and intranasal inoculations of the UR strain induced seroconversion in the BALB/c mice.

In summary, two clinical isolates of SAFV-3 had neurotropism and mild neuropathogenesis in neonatal and young mice. In addition, both viral strains were also neuroinvasive in neonatal ddY mice. The two strains had different cell tropism and neurovirulence in mouse models. These results suggest that SAFV-3 is a candidate neurotropic pathogen.

Chapter 3: Infection with Saffold virus passaged in mouse cerebellum affected cerebellar development in neonatal mice.

SAFV-3 has tropism to the Bergmann glia of the cerebellum in neonatal mice, and no other picornaviruses have a similar reported tropism. Therefore, I focused on the tropism of SAFV to the cerebellum. The AM strain was passaged in the cerebellum of neonatal mice, and the passaged strain was characterized virologically, pathologically, and immunologically.

The AM strain was serially passaged five times in the cerebellum of neonatal BALB/c mice. Three days after intracerebral inoculation, the cerebellums were collected and homogenized. The supernatant was then serially inoculated five times into neonatal mice. After five passages, the virus was propagated in rhesus monkey kidney epithelial (LLC-MK_2) cells, and is referred to here as the AM-5Cb strain.

During the serial passages, the AM strain obtained two amino-acid replacements at VP2 and VP3, sequentially. According to the prediction of the viral capsid protein structure, the site of the VP2 mutation is adjacent to

the virus receptor binding site. Moreover, the AM-5Cb strain has a hydrogen bond between the two mutation sites. Its replication was elevated in LLC-MK_2 cells and human rhabdomyosarcoma (RD) cells, but reduced in baby hamster kidney (BHK) cells. The murine aneuploid fibrosarcoma cell line L929, the mouse neuroblastoma cell line Neuro2A, and the murine astrocyte cell line KT-5 were not susceptible to either AM or AM-5Cb strains.

The neurovirulence of the AM-5Cb strain in neonatal ddY mice was examined using intracerebral inoculations and the methods of Chapter 2. AM-5Cb-inoculated neonatal mice had noticeable ataxia in the early phase of the infection (days 2–4), and after, some mice developed hydrocephalus. Thus, the virulence of the AM strain increased in neonatal mice after five serial passages. Histopathological and immunohistochemical analyses revealed numerous degenerated/necrotic nerve cells and microglia infiltrations in the cerebrum, brainstem, cerebellum, and spinal cord of the AM-5Cb-inoculated mice. Viral antigen–positive cells were present in the lesions at a high frequency. The number of viral antigen–positive cells in AM-5Cb-inoculated mice was higher than that of AM-inoculated mice, but the cell tropisms were similar. Viral titers and the amount of viral RNA in the cerebrum/brainstem and cerebellum of the AM-5Cb-inoculated mice were significantly higher than those of the AM-inoculated mice on day 3 p.i. This result indicates that the replication of the AM strain in the brains of neonatal mice increased after five serial passages. In addition, high levels of type I IFN and massive inflammatory infiltrations were detected in the AM-5Cb–inoculated mice. Thus, the neuropathogenicity of the AM strain in neonatal mice increased after the passages.

To evaluate the effect of the SAFV infection on cerebellum development in neonatal mice, cerebellum gene expression was investigated. On day 3 p.i., cerebellar expression of the astroglia-specific glutamate transporter *GLAST* (related to the maintenance and differentiation of Bergmann glia) and *Hes5* (related to the induction of astroglia differentiation), was higher in AM-5Cb–inoculated mice than in AM-inoculated mice. In addition, Delta/Notch-like EGF-related receptor (*DNER*) and *Calbindin*, both of which are involved in differentiation and growth of Purkinje cells, were expressed in cerebellum at higher levels in AM-5Cb-inoculated mice than in AM-inoculated mice on day 21 p.i. Histopathologically, the axonal neurites of Purkinje cells seemed to be elongated in both AM- and AM-5Cb–inoculated mice, compared to those of the MEM-inoculated mice. These results suggest that the association of Bergmann glial cells and Purkinje cells during the differentiation and growth of the cerebellar cortex was uncontrolled after the infection.

To confirm the effect on the development of the cerebellar cortex, neonatal mice were intracerebrally inoculated with a high viral load of AM-5Cb. The inoculated mice exhibited cerebellar hypoplasia, with only a rudimentary layer on day 19 p.i. The mice had massive necrosis in the cerebrum and also exhibited cerebellar cortex dysplasia, indicating that AM-5Cb was highly virulent and infective. Because SAFV-3 has the potential to affect cerebellum development in human infants, a mouse model using the cerebellum-passaged strain of SAFV-3 might be useful for studying the neuropathogenesis of SAFV infection.

In conclusion, this is the first study of the neuropathogenesis of SAFV-3 using mouse models and virological, histopathological, and immunological methods. This study revealed that SAFV-3 is a potential neurotropic pathogen. This mouse model using SAFV-3 may be useful for studying the mechanisms controlling the severity of SAFV infection, for identifying antiviral factors, and for developing novel vaccines.